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Review

Fagopyrum esculentum Moench: A crop with many purposes in agriculture and human nutrition

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Buckwheat is a dicotyledonous crop that quickly grow at high altitudes. It presents high tolerance to acidity and ability to grow in poor soil. This study aimed to identify different buckwheat forms of use and benefits in agriculture and human nutrition. It can be used as green manure, being an option as soil cover plant and recycling of nutrients, as well as an alternative grain and forage. At the plant flowering, it can provide a food source to the predators of common insects' pests, increasing their populations. In livestock, it can feed cattle, sheep, pigs, goats and poultry because it features similar quality to millet forage, but with higher concentration of protein. Buckwheat could act as a functional forage to manipulate ruminal fermentation. The presence of tannins in plants can positively influence the health of small ruminants because these represent a promising alternative control of gastrointestinal nematodes. For human nutrition, the buckwheat is an important food that contain a well-balanced amino acid profile with a high quantity of lysine, limiting amino acid in grasses such wheat, relatively high fibre content, zinc (Zn), copper (Cu), manganese (Mn) and selenium (Se). Furthermore, this flour is gluten-free and it can be used as a supplement for patients with celiac disease. Therefore, it is a culture that should be best explored in different regions of the world.

Key words: Buckwheat, celiac sprue, green manuring, high protein, tanniferous plants, recycling of soil nutrients.

INTRODUCTION

Buckwheat (*Fagopyrum esculentum* Moench) is a dicotyledonous crop belonging to Polygonaceae family taxonomically unrelated to wheat (Heffler et al., 2014). It is popular in the mountainous regions of China and in

Table 1. World's largest countries producers of buckwheat in 2013 (FAOSTAT, 2013).

Countries	Production (tonnes)	Area (ha)	Yeld (t ha^{-1})
RussianFederation	833936	905911	0,921
China	733000*	705000*	1,040
Kazakhstan	276840	202008	1,370
Ukraine	179020	168400	1,063
France	154800	44500	3,479
Poland	90874	70384	1,291
USA	81000†	77500†	1,045
Brazil	62000†	48000†	1,292
Japan	33400	61400	0,544
Belarus	30353	31403	0,967
World Total	2547014	2386212	1,067

* Aggregate, may include official, semi-official or estimated data. †FAO estimate.

other countries at the northern hemisphere. It can be grown at high altitudes and has a short growing span (Zhou et al., 2012).Buckwheat has been a culture of secondary importance, however it is produced in almost all countries where cereals are grown (Campbell, 1997).

The crop is not a cereal, but the seeds (achenes) are usually classified among the cereals grains because of its similar usage. The grain is generally used as human food and as animal feed. The dehulled groats can be cooked as porridge and the flour can be used in the preparation of pancakes, biscuits, noodles, cereals, among others (Campbell, 1997). Buckwheat contains proteins with high biological value and balanced amino acid composition, presenting relatively high content of fibre, Zn, Cu, Mn and Se (Ahmed et al., 2014).

Currently, China, Russian Federation, Kazakhstan and Ukraine are the leading producers of buckwheat, with production in other countries of different continents, according to Table 1, which shows the current leading producers of buckwheat, area harvested and yield in 2013. The leading continents producers are Europe and Asia (FAOSTAT, 2013). So, the aim of this study was to identify different buckwheat forms of use and benefits in agriculture and human nutrition.

DESCRIPTION OF BUCKWHEAT

Fagopyrum esculentum Moench is common buckwheat, widely cultivated over the Northern and to some extent the Southern hemisphere. There are many cultivars or landraces in this species and their achene forms can vary, some of them being winged on the angles. It is an annual crop, branched, glabrous, and reaching up to 1 m tall (Campbell, 1997).

The leaves are petiolate, blades are ovate-triangular to triangular, 2 to 8 cm long, with acuminate tips, bases are cordate or approximately hastate; upper leaves are smaller, sessile. The inflorescences are terminal and auxiliary, branch in dense corymbose or paniculate cyme. Flowers are white or pink, 6 mm in diameter; pedicel is 2 to 3 mm long, articulate; perianths are 3 mm long; 8 nectaries are yellow, alternating with stamens; being heterostyly, capitate stigma (Campbell, 1997). The achenes are triquetrous, acute angle, longer than 5 mm, more than twice the persistent perianths lenght, brown or black-brown, lucid (Campbell, 1997). Details of buckwheat are presented in Figure 1.

USES IN AGRICULTURE

Buckwheat can be used as a cover crop (green manure) by having high tolerance to acidity and good ability to grow in poor soils. It can reach 30 Mg ha^{-1} of green mass and dry mass up to 7 Mg ha^{-1} with a height up to 1.30 m at 72 days after sowing (DAS). Klein et al. (2010) found higher concentration of potassium and nitrogen and micronutrients zinc, manganese and iron, showing good ability to recycle nutrients from the soil (Table 2). In alfafa (*Medicago sativa*), green manure crops (like buckwheat) may provide benefits in production systems by increasing pathogen antagonists (Samac et al., 2013). Other benefits of using green manures include reduction on the dependence on mineral fertilizers, maintenance of organic matter in the soil providing nutrients for plant growth (Yadav et al., 2000) and increase of size and activity of soil microbial communities (Kautz et al., 2004; Manici et al., 2004; Tejada et al., 2008). However, the positive effects of green manure are affected by the crops chosen for this purpose (Mancinelli et al., 2013).

The crop is known to increase beneficial insects which are predators of common insects pests and can help to reduce their populations. The increase in population is due to the food source provided to the insect in the plant flowering. As examples, can be mentioned hover flies, predatory wasps, minute pirate bugs, insidious flower bugs, tachinid flies, and lady beetles (Valenzuela and
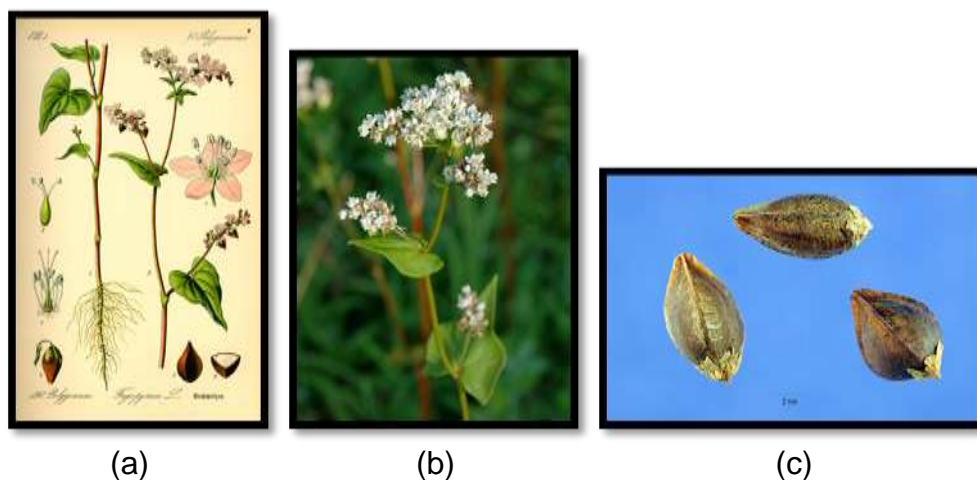
Figure 1. Details of buckwheat: (a) Structures (Thomé, 1903); (b) Buckwheat specimen (Smith, 2007); (c) Seeds (Hurst, 2015).

Table 2. Dry mass production and mass nutrients recycled per hectare and the carbon/nitrogen ratio of plants of an early and a late cultivar (Klein et al., 2010).

Sample	Drymass	N	P	K	C/N ratio
	kg ha^{-1}				
Late	5633	113.22	17.46	208.99	20.17
Early	6870	111.30	18.55	220.53	23.97

Smith, 2002).

Buckwheat should not be grown in fields with presence of root lesion nematodes (*Pratylenchus penetrans*) and root-knot nematodes, because the crop is suscetible to these nematodes (Valenzuela and Smith, 2002). In a previous trial work, buckwheat showed promising as a nematode suppressant, significantly reducing *Pratylenchuszeae* numbers in comparison to sugarcane (Berry and Rhodes, 2006). In addition, may have ability to increase the population of beneficial soil nematodes to crops, like *Helicotylenchus* (Rhodes et al., 2014). The use of buckwheat can be an option to reduce the use of chemical control, because in general, nematode control has traditionally relied to this kind of control (e.g. aldicarb in South African sugar industry) (Berry and Ramouthar, 2012).

Though producing low amounts of biomass, buckwheat grows and flowers in a short time period. For sugarcane growers, it can be ideal due to pressing circumstances, may need to include only a short fallow in their sugarcane cycle (Rhodes et al., 2014).

USES IN LIVESTOCK

Grains, hay or silage buckwheat can be fed to cattle,

sheep, pigs, goats and poultry (Goepfert, 1968). It features similar quality to millet forage (Gorgen, 2013), but with a higher concentration of protein.

Buckwheat could act as a functional forage to manipulate ruminal fermentation (Amelchanka et al., 2010; Leiber et al., 2012) and, subsequently, milk quality and specially milk fatty acids profile (Kälber et al., 2011) for producing high levels of various secondary compounds (Wijngaard and Arendt, 2006). Additionally, can happen a certain mitigation of methane emission without a concomitant severe decline of rumen microbial productivity (Leiber et al., 2012). The main phenolic constituent in buckwheat which occur in substancial amounts is the flavonoid rutin and also hyperoside and chlorogenic acid (Hinneburg and Neubert, 2005; Kalinova et al., 2006). An important property of rutin appears to be the partial protection of dietary proteins from ruminal degradation (Leiber et al., 2012).

The presence of tannins in plants can positively influence the health of small ruminants because they represent a promising alternative control of gastro-intestinal nematodes of these animals. However, their effects depend on the type and concentration of these metabolites (Oliveira et al., 2011). Karamac´ (2010) found that the total phenolic content of the tannin fraction from buckwheat seeds was higher than that from buckwheat

Table 3. Total phenolic content, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity and Trolox equivalent antioxidant capacity (TEAC) of buckwheat tannin fractions (Karamac´, 2010).

Buckwheat tannin fraction	Total phenolics (mg catechinequivg^{-1})*	DPPH scavenging activity EC$_{50}$ (mg)	TEAC (nmolTroloxequivg^{-1})*
Seeds	477 ± 11[a]	0.019	4.06 ± 0.14[a]
Groats	371 ± 10[b]	0.021	3.55 ± 0.09[b]

Data expressed as means ± standard deviations (n = 3). In the same column, means with different letter (a, b) differ significantly (P<0.05).* Results are expressed as equivalents (equiv) of standard per g of tannin fraction.

Table 4. Comparison of buckwheat flour composition with wheatflour (m g^{-1} DW*).

Nutrient	Buckwheat flour	Wheat flour	References
Carbohydrates	737	835	
Crudeash	22	12	
Crudefat	28	26	Quin et al., 2010; Lin et al., 2009
Crudefibre	23	20	
Crudeprotein	103	106	
Protein	110	115	
Ash	26	17	
Lipid	34	10	Bonafaccia and Fabjan, 2003
Solublefibre	12	10	
Insolublefibre	53	15	
Total fibre	65	24	

*DW, dry weight.

groats. Constituents of tannin fractions, which reacted with Folin-Ciocalteu's reagent (FCR) expressed as catechin equivalents amounted to 477 and 371 mg g^{-1} of fraction (Table 3). The comparison of antioxidant activity of tannin fractions from buckwheat with the literature data concerning the antioxidant activity of fractions isolated from other plants, leads to the conclusion that buckwheat fractions are strong antioxidants.

With regard of nutritional value for animals, crude protein is particularly concentrated in leaves, followed by flowers, whereas stems are characteristically high in fiber. Leiber et al. (2012) found 620 g of non-NDF carbohydrates/kg DM in buckwheat grains, and leaves also were rich in non-NDF carbohydrates. In comparison with ryegrass (*Lolium multiflorum*), the entire aerial part of the buckwheat herb contained less crude protein, ether extract and fibre, but more non-fibre carbohydrates (e.g. starch, oligosaccharides and sugars) and almost six times more total extractable phenols (Leiber et al., 2012).

USE IN HUMAN NUTRITION

Buckwheat is an important ingredient of traditional dishes of Asia (e.g. Japanese and Korean noodles and jellies), Russia (e.g. pancakes or a sort of porridge called "kasha"), and Europa (e.g. French pancakes, Dutch "poffertjes" and Northern Italian hot porridge and pasta). Hulls are also used to fill pillows. It is used to obtain dark gluten-free flour which can be used as supplement for patients with celiac (or coeliac) disease (also known as celiac sprue and gluten-sensitive enteropathy), one of the most common food intolerances in the world (Heffler et al., 2014). Therefore, buckwheat has the potential to be used as natural means of fortification and enrichment in gluten-free, allergen-free foods and to benefit these individuals (Omary et al., 2014).

The composition of buckwheat is similar to other cereals and pseudo-cereals consumed around the world. The comparison of buckwheat and wheat flour is shown in Table 4. The bran contains fagopyritols and rutin, compounds which may be useful medicinally. However, it also contains large amounts of phytic acid, a major anti-nutritional factor in common wheat (*Triticum aestivum*) (Steadman et al., 2001).

Buckwheat grains hulls have some components with biological activity, e.g. flavonoids and flavones, phenolic acids, condensed tannins, phytosterols, fagopyrins, RS, dietary fibre, lignans, plant sterols, vitamins and minerals (Ahmed et al., 2014).

Table 5. Essential amino acid composition (mg g^{-1} protein) of buckwheat and wheat and comparison of mineral composition (mg 100 g^{-1} flour) of its flours.

Amino acid (mg g^{-1}protein)	Buckwheat	Wheat	References
Lysine	51	25	
Methionine	19	18	
Cystine	22	18	
Threonine	35	28	
Valine	47	45	Ahmed et al., 2014
Isoleucine	35	34	
Leucine	61	68	
Phenylalanine	42	44	
Histidine	22	23	
Tryptophan	16	10	
Mineral composition (mg 100 g^{-1})	**Buckwheat Flour**	**Wheat Flour**	
Ca	12.4	14.8	
Cu	0.52	0.16	
Fe	2.86	0.79	
K	450	96	Ikeda et al., 2006
Mg	375	35	
Mn	1.61	0.43	
P	394	124	
Zn	2.51	0.80	

Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; P, phosphorus; Zn, zinc.

Table 6. Vitamin composition of buckwheat (Wijngaard and Arendt, 2006).

Vitamins	Level (mg g^{-1})
A (carotenoids)	2.1
B1 (thiamine)	4.6
B2 (riboflavin)	1.4
B3 (niacin)	18.0
B5 (pantothenicacid)	10.5
B6 (pyridoxine)	7.3
C (ascorbicacid)	50.0
E (tocopherols)	54.6

The protein content in buckwheat is significantly higher than important grasses such rice, wheat, sorghum, millet and maize, being the second highest after oat flour. Buckwheat has a well-balanced amino acid profile with a high quality of lysine, limiting amino acid in grasses such wheat (Table 5). The crop have one of the highest amino acid scores among plant sources (Ikeda et al., 2002). The buckwheat flour is a good source of many essential minerals, contains higher levels of Zn, Cu and Mn (Ikeda et al., 1999; Steadman et al., 2001) (Table 6). The content of these essential minerals is higher in comparison with other cereal flours (Ikeda et al., 2006). Buckwheat

grains contain higher levels of vitamin B1 (thiamine), B2 (riboflavin), E (tocopherol) and B3 (niacin and niacinamide) compared with most cereals. The vitamin content of buckwheat are presented in Table 6 (Wijngaard and Arendt, 2006).

In breads supplemented with 40% seed mixture of buckwheat and quinoa, it was shown potential to improve nutritional characteristics with 2.5% higher protein, 2% higher fat as well as two-fold higher fiber content and higher Ca and P contents. The sensory characteristics of evaluated breads were excellent even at the level of 40% supplementation level and the addition of quinoa and buckwheat seeds also influenced the rheological characteristics of dough. The inclusion of such high levels of seed in bread was possible by modification in technological procedure of seed preparation, and it could enable the development of a range of new baking products with enhanced nutritive value (Demin et al., 2013).

Buckwheat allergy

Buckwheat allergy is already seen in Asia, Europe and USA. In Europe, since the crop was introduced in popular food sectors. Failure to recognize buckwheat allergy can expose people to a risk to health. Allergy to buckwheat is

typically IgE mediated and it is often associated to severe anaphylaxis (Wieslander and Norbäck, 2001). Although various buckwheat allergens have been identified, the proteins 24 kDa (Fag e 1), 26 kDa and 67-70 kDa have been suggested as important (Tohgi et al., 2011). In all patients with allergies to buckwheat, the protein Fag e 1, which is homologous to 11S or 12S globulin, has reacted with all of the serum IgE. The protein 16 KDa is resistant to digestion and has been identified as a major buckwheat allergen in Japanese and Korean patients with allergy (Park et al., 2000).

Over the past decades, many studies on buckwheat allergy has been published (Smith, 1909; Wieslander and Norbäck, 2001; Heffler et al., 2014). The first study was published in 1909, a case about patients who suffered from dyspnoea, acute rhinitis, urticaria and mucosal angioedema after the ingestion of buckwheat flour (Smith, 1909). Failure to recognize buckwheat allergy can expose people to a risk to health. It is recommended to clinician's suspect and test allergy to buckwheat in patients with symptoms of food allergy, when have the consumption of food produced with this plant in the composition (Heffler et al., 2014).

CONCLUSION

Buckwheat is a crop with potential in agriculture, livestock and human nutrition. In agriculture, it can be used as green manure, to increase predators of common insects pests helping reduce their populations etc. In livestock, it can be used to feed cattle, sheep, pigs, goats and poultry. The plant could act as a functional forage to manipulate ruminal fermentation and the presence of tannins can positively influence the health of small ruminants. In human nutrition, buckwheat is an important food, which contains balanced amino acid composition, relatively high fiber content, high contents of available Zn, Cu and Mn and dietary Se. Therefore, it is a culture that should be further explored, as it promotes many benefits and is easily adaptable to various areas, which can be grown in different regions of the world.

Conflict of Interests

The authors have not declared any conflict of interests.

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The authors have not declared any conflict of interests.

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Review

Evolution of methodology for the study of adaptability and stability in cultivated species

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The GxE interaction only became widely discussed from evolutionary studies and evaluations of the causes of behavioral changes of species cultivated in environments. In the last 60 years, several methodologies for the study of adaptability and stability of genotypes in multiple environments trials were developed in order to assist the breeder's choice regarding which genotypes are more stable and which are the most suitable for the crops in the most diverse environments. The methods that use linear regression analysis were the first to be used in a general way by breeders, followed by multivariate analysis methods and mixed models. The need to identify the genetic and environmental causes that are behind the GxE interaction led to the development of new models that include the use of covariates and which can also include both multivariate methods and mixed modeling. However, further studies are needed to identify the causes of GxE interaction as well as for the more accurate measurement of its effects on phenotypic expression of varieties in competition trials carried out in genetic breeding programs.

Key words: Adaptability, stability, GxE interaction, genetic breeding, covariates.

INTRODUCTION

For genetic breeding programs, there is the inherent difficulty in identifying varieties with superior performance in various environments because, even when isolating from the space factor, that is, when such genotypes are planted in similar sites (usually resulting from a subclass of places obtained via stratification), they have accentuated interaction with different crops both within the same year and with different years (Eberhart and Russell, 1966).

The ability that certain genotypes have to grow well in a wide range of environmental conditions is, therefore, of major importance for Agronomy, especially in places where such conditions are extremely variable and, until mid-1950's, the effects of the interaction genotypes *x* environments were estimated only via general mean, according to the mean performance of varieties in various

locations and years (Finlay and Wilkinson, 1963).

Sprague and Federer (1951) were pioneers by showing how the variance components can be used to separate the effects of genotypes, environments, and the interaction between them, equaling the mean square obtained by analysis of variance (ANOVA) to their respective mathematical expectations. Then, Plaisted and Peterson (1959) proposed a new methodology for evaluating the influence of this interaction, which consists of applying a combined analysis of variance, that is, an analysis considering all varieties at all locations in a given year, also known as "two-factor" analysis.

The variation observed between varieties is dynamic in some cases (Finlay and Wilkinson, 1963), and the breeders find themselves faced with the choice between selecting varieties adapted to a particular range of environments (or specific sites), or obtaining varieties with broad adaptability and which, therefore, have a good performance in a range of larger environments. Varieties having site adaptability can be very useful, especially when it comes to environments with unusual conditions, of difficult cultivation, or even extreme conditions.

Several authors, such as Salmon (1951), Horner and Frey (1957), and Sandison and Bartlett (1958), discussed the theme using techniques that consider the interaction genotypes *x* sites, or genotypes x years (or crop) as an adaptability measure. Such techniques are of low precision when it comes to many environments or genotypes to be evaluated.

Meanwhile, and in a non-integrated way, experiments evaluating the nature of phenotypic stability gave experimental support for the understanding of the interaction genotypes *x* environments (Lewis, 1954; Dobzhansky and Levene, 1955; Williams, 1960). Gripping and Langridge (1963), for instance, conducted a study on the influence of heterosis on phenotypic plasticity in *Arabidopsis thaliana* and concluded that the hybrids of this species showed greater stability than homozygous individuals.

From the 1960's, several methodologies for the evaluation of adaptability and stability of genotypes in multi-environments trials have been developed, most of them still used nowadays in breeding programs for plant species cultivated worldwide. Among these, the most widely used have been based on simple linear regression (Yates and Cochran, 1938; Finlay and Wilkinson, 1963; Eberhart and Russell, 1966), multivariate analysis (Mandel, 1971; Kempton, 1984; Gauch, 1988; Zobel et al., 1988; Crossa, 1990; Yan et al., 2000), and mixed models (Piepho, 1997; Resende and Thompson, 2004).

Given this, the study proposes to discuss the technical and practical aspects of the main methodologies available in literature and used to evaluate the adaptability and stability of genotypes, as well as to trace an overview on methods that have been proposed more recently and the challenges for the evaluation of genotypes in trials of multiple environments by genetic breeding programs at the present time.

METHODOLOGIES BASED ON ANOVA COMPONENTS

In addition to the method of Plaisted and Peterson (1959), until the beginning of the 1960s, some methodologies to evaluate the phenotypic stability of genotypes were based only on ANOVA components, among which outstands the Wricke methodology (1962), popularly known as "Ecovalence". Such parameter is estimated by the decomposition of the sum of squares of the GxE interaction (quite similar to the model of Plaisted and Peterson, which in turn proposes the decomposition of variance of the GxE interaction) in parts related to genotypes in an isolated manner, which is given by the expression:

$$\omega_i = r \sum_j \left(Y_{ij} - Y_{i.} - Y_{.j} + Y \right)^2$$

In which: Y_{ij} is the mean of genotype *i* in the environment *j*; $Y_{i.}$ and $Y_{.j}$ correspond to the mean of genotype *i* and to the mean of environment *j*, respectively; and Y is the general mean.

The parameter ω_i measures, as a stability factor, the contribution of each genotype for the GxE interaction component, in which the genotypes that contribute less to the interaction are considered the most stable.

METHODOLOGIES BASED ON REGRESSION METHODS

Finlay and Wilkinson (1963), based on Yates and Cochran (1938), proposed a methodology using linear regression models to compare the performance of a set of varieties evaluated in multiple sites and years in which, for each variety, a regression of their mean was obtained regarding the overall mean of all varieties in each site per year. In addition, each environment was classified as favorable or unfavorable according to the mean of all varieties in that environment.

These authors have modeled environmental factors, simply in terms of the productivity response of genotypes. Thus, the varieties that have regression coefficients equal or close to 1(one) are considered varieties of mean stability. Among these, those that are associated with a high productivity have broad adaptation, and those associated with low productivity are weakly adapted to all environments. Varieties with coefficient significantly greater than 1.0 are considered especially adapted to favorable environments, but have low stability, and those which have coefficient lesser than 1, or tending to 0, are considered more stable and with adaptability to unfavorable environments. Therefore, the optimal variety would be the one with a good performance in all environments and with high stability (that is, regression coefficient close to 0).

Biologically, the interpretation of this factor is that such varieties are so stable that they are unable to respond to any improvement in environmental conditions. According to Eberhart and Russell (1966), the use of the regression coefficient and the deviations from the straight line as parameters of stability aimed at helping to solve this problem. Being i the environmental index for the regression of each variety, in each environment j, defined by:

$$I_j = [(\sum_{i=1}^{n} Y_{ij}) / v] - [(\sum_{j=1}\sum_{i=1}^{n} Y_{ij}) / vn]$$

Where Y_{ij} is the mean of the i-th variety within the j-th environment, v is the number of varieties, and n the number of environments.

Thus, the estimation of the two mentioned parameters is usually defined by the following model:

$$Y_{ij} = \mu_i + \beta_i I_j + \delta_{ij},$$

where the first parameter presented, the regression coefficient (β_i), is the same proposed by Finlay and Wilkinson (1963), defined as: $b_i = \sum_j Y_{ij} I_j / \sum_j I_j^2$, and the second parameter (δ_{ij}), is estimated via the sum of squares of regression deviations, as follows: $s^2_{di} = [\sum_j^n \delta^2_{ij} / (n - 2)] - [s^2_e / r]$, in which s^2_e/r is the estimate of the error set. This procedure thus decomposes the sum of squares of the GxE interaction in two parts: the variation due to the response of each variety regarding the environmental index, and the regression deviations regarding such index. The optimal genotype then becomes the one that features high productivity, associated with a regression coefficient as close as possible to 1, and regression deviations close to 0.

Some authors claim that the approaches that only comprise regression techniques are useful only as preliminary evaluations, for they present, most of the time, large linearity deviations, making the selection of genotypes biased and applied exclusively to the set of the evaluated varieties, being seen as a very distant simplification of the reality presented in genetic breeding experiments (Witcombe and Whittington, 1971). Schlichting (1986) States that there are two important issues in methodologies that are used in the regression analysis: (1) The means and the coefficients assigned to genotypes tend to be positively correlated, that is, stable genotypes tend to have lower expression of the character in question, and (2) the assumption of linearity is not usually met.

NONPARAMETRIC METHODOLOGIES

When data do not meet the assumptions of regression analyses, an alternative is to use nonparametric analyses. In genetic breeding, more precisely in the context of the evaluation of genotypes in MET's, there are some proposals for adaptability and stability evaluation of genotypes based on nonparametric statistics. Studies, such as those of Hühn (1979), Nassar and Hühn (1987), Kang (1988), Lin and Binns (1988), Fox et al. (1990), and Thennarasu (1995), are among the most cited regarding this aspect.

Nassar and Hühn (1987) developed methodologies that have as a fundamental characteristic the interpretation of several measures based on the ranking of genotypes, although they are independent of each other. The most widely used, according to literature, are: $S^{(1)}$ (mean of the absolute rank differences of a genotype over the n environments), $S^{(2)}$ (variance among the ranks over the k environments), $S^{(3)}$ (sum of the absolute deviations), and $S^{(6)}$ (relative sum of squares of rank for each genotype). Such measures can be mathematically described as follows:

$$S_i^{(1)} = 2\sum_j^{q-1}\sum_{j'=j-1}^{q}|r_{ij} - r_{ij'}|/[q(q-1)]$$

$$S_i^{(2)} = \sum_{j=1}^{q}(r_{ij} - \bar{r}_{i.})^2/(q-1)$$

$$S_i^{(3)} = \sum_{j=1}^{q}(r_{ij} - \bar{r}_{i.})^2/\bar{r}_{i.}$$

$$S_i^{(6)} = \sum_{j=1}^{q}|r_{ij} - \bar{r}_{i.}|/\bar{r}_{i.}$$

In which: r_{ij} is the rank of ith genotype in the jth environment (for q environments), and $\bar{r}_{i.}$ is the mean rank across all environments for the ith genotype.

Kang (1988) and Fox et al. (1990) proposed other nonparametric methods, which, in turn, calculate only one statistic and classify the most stable genotypes. Kang nonparametric stability (Rank-sum) uses both "trait single value" and stability variance (Shukla, 1972), and the genotype with the lowest ranksum is commonly the most favorable one (both the highest yielding genotype and the genotype with the lowest stability variance are ranked 1). Fox et al. is based, in many cases, in the "TOP third" statistical ranking, where a stratified ranking of the genotypes at each environment separately is done. The proportion of sites at which the genotype occurred in the top third are expressed in TOP ranking.

Thennarasu (1995) proposed four statistics ($NP^{(1)}$, $NP^{(2)}$, $NP^{(3)}$, and $NP^{(4)}$) for stability measures. These measures are based on ranks of adjusted means of the genotypes in each environment, and stable genotypes as those whose position in relation to the others remained unaltered in the set of environments. Thennarasu measures are defined as:

$$NP_i^{(1)} = \frac{1}{n}\sum_{j=1}^{n}|r_{ij} - M'_{di}|$$

$$NP_i^{(2)} = \frac{1}{n}\left(\sum_{j=1}^{n} |r_{ij} - M'_{di}| \Big/ M_{di}\right)$$

$$NP_i^{(3)} = \frac{\sqrt{\sum(r_{ij} - \bar{r}'_i)^2 / n}}{\bar{r}_i}$$

$$NP_i^{(4)} = \frac{2}{n(n-1)}\left[\sum_{j=1}^{n-1}\sum_{(j'=j+1)}^{n} |r_{ij} - r_{ij'}| \Big/ \bar{r}_i\right]$$

In which: r_{ij} is the rank of adjusted values ($x_{ij}{}^* = x_{ij} - x_i$), $\bar{r}'_i$ and M'_{di} are the mean and median ranks for adjusted values, and $\bar{r}_i$ and M_{di} are the same parameters computed from the phenotypic unadjusted values.

The methodology proposed by Lin and Binn (1988) somewhat differs from the cited ones, since it is not explicitly based on values obtained from the ranking of genotypes, considering that this methodology is based on absolute values obtained for the rated character and not on the numbering of the ranking of genotypes. Lin and Binns proposed obtaining the stability statistics Pi, which is given by the expression:

$$Pi = \sum_{j=1}^{n}(X_{ij} - M_j)^2 / 2n$$

In which: Pi = superiority index of i-th cultivar; X_{ij} = productivity of i-th cultivar planted in the j-th site; M_j = maximum response obtained among all the cultivars in the j-th site; n = number of sites. This expression can be developed into:

$$Pi = \left[n(\overline{X}_i - \overline{M})^2 + \sum_{j=1}^{n}(X_{ij} - \overline{X}_i - M_j + \overline{M}^2\right] / 2n$$

In which: X_i is the mean of the character obtained in n environments; and $\overline{M}$ the mean of the maximum responses of genotypes in all environments.

Such methodologies, despite being considered as alternatives, especially for their lack of robustness, may be considered preferable when applications of regression methods are not possible, or when there is a need for analyses of easier implementation and of most objective interpretation. This fact is clearly observed, for instance, in the methodologies of Kang (1988), Lin and Binns (1988), and Fox et al. (1990), which feature a single value as a stability measure.

METHODOLOGIES BASED ON MULTIVARIATE METHODS

With the advent of more sophisticated computational resources and computers with greater processing capacity, other methodologies, such as those based on multivariate analysis, became more accessible and are preferably used to the extent that most statistical packages were made available. One of the bases for conducting adaptability and stability analyses via multivariate models is the principal component analysis (PCA), which has as its essence the application of the *SVD* (*Singular Value Decomposition*) method, which, in turn, performs the linear decomposition of variables contained in a data array in an iterative manner, in order to summarize the information contained in a smaller number of explanatory vectors.

Among the most used methodologies in adaptability genetic studies, the following models outstands: AMMI (*Additive Main Effect and Multiplicative Interaction*) and GGE Biplot (*Genotype plus Genotype by Environment*) (Kempton, 1984; Zobel et al., 1988; Crossa, 1990; Gauch, 1992; Yan et al., 2000). In the AMMI models, developed by Mandel (1971) and popularized by Zobel et al. (1988) and Gauch (1992), the magnitude of the GxE interaction is estimated according to the response of each variable (here considered as environments) in a rather original approach, by the combination in a single model between ANOVA and the principal component analysis (*PCA*). The idea is to consider the effect of the GxE interaction as multiplicative component (more realistic in biological terms), and other effects (genotypes and environments) as purely additive effect components (Duarte and Vencovsky, 1999). Thus, the AMMI statistical model can be expressed as:

$$Y_{ij} = \mu + g_i + a_j + \sum_{k=1}^{n}\lambda_k\gamma_{ik}\alpha_{jk} + e_{ij}$$

Where: Y_{ij} is the mean response of the genotype *i* in the environment *j*; μ is the general mean of the trials; g_i is the fixed effect of genotype *i*; a_j is the fixed effect of the environment *j*; λ_k is the k-th singular value (scalar) of the original interaction array; γ_{ik} is the element corresponding to the i-th genotype in the k-th singular vector of the column of the interaction array; α_{jk} is the element corresponding to the j-th environment in the k-th singular vector in the line of the array; and e_{ij} is the residual effect.

The AMMI methodology uses *SVD* multivariate technique to reduce the information contained in a data array $n \times m$ (genotypes and environments, respectively) in vectors that accumulate, in a systematic manner (in order of importance), the greater part of variation contained in the data and, consequently, in GxE interaction. Since its disclosure, this methodology has been widely used for studies on adaptability in several important cultivated species such as wheat (Kempton, 1984; Crossa et al., 1999; Paderewski et al., 2011), Corn (Hirotsu, 1983; Ndhlela et al., 2014), soybeans (Gauch, 1988; Zobel et al., 1988; Yokomizo et al., 2013), sugar cane (Silveira et al., 2013), and rice (Samonte et al., 2005).

Another method based on the same principle that has

been increasingly used in recent years is the GGE Biplot, proposed by Yan et al. (2000), which, in general terms, is similar to the AMMI model, with the key difference that, in the multiplicative component for the decomposition via *SVD*, only the effect on the environment is excluded, consequently considering the effects of genotypes and of the interaction together. Therefore, models that consider the effect of the interaction as multiplicative, besides capitalizing the GxE interaction more efficiently (Zobel et al., 1988), have advantages, such as quantification of each genotype and environment to the sum of squares of the interaction, and provide an easy interpretation of results by *Biplot* graphs (Gabriel, 1971; Kempton, 1984).

Both the AMMI methodology and GGE Biplot have the additional advantages of generating information on the genotypes with broad adaptability (combination of phenotypic mean and stability information on the same graph), and aiding in the delineation of agronomic areas via identification of mega-environments (defined as the group of environments with similar GxE interaction standard, and consequently with little change in the ranking of the genotypes evaluated), which may indicate the most representative environments of each site and genotypes with specific adaptation to each region.

METHODOLOGIES BASED ON MIXED MODELS

The usual hypothesis test of the variance analysis assumes independence of the main effects of the model; when such assumption is met, the effects can be tested using the mean square of the residue. Thus, within the context of the trials in multiple environments, any differences found between the effects of genotypes should, theoretically, be the same for any environment tested. However, if there is an interaction between the components of the model (in the specific case, between the effects of genotypes and environments), the hypothesis test is reformulated, leading then to decision making regarding the nature of such effects, that is, the *a priori* definition about which effects must be considered as fixed and which as random.

According to Freeman (1973), if the same set of genotypes is tested in several environments, the hypothesis test for the significance of the effects of genotypes must be performed in relation to the mean square of the interaction rather than the residue, as previously mentioned. When, for instance, such environments are considered as a random sample of all possible environments, it is assumed the use of a mixed model, or even of a completely random one, which means that, for instance, deviations from the normal range should be fully taken into consideration in order to assume the validity of the inferences made from the model.

By assuming the effects of genotypes as random, the BLUP's (*Best Linear Unbiased Predictors*) can be

obtained, which is not possible by the methods of adaptability and stability aforementioned. The BLUP's of the effects of genotypes and of GxE interaction eliminate their noises through the deliberation of such effects by a regressor factor, which is usually referred to as "repeatability" (which, in practical terms, is usually the character's heritability), leading, therefore, to the *Shrinkage* estimates of such effects and to the prediction of genetic values (Searle et al., 1992; Piepho, 1997; Resende, 2007).

Overall, regarding studies on plant genetics, the studies using mixed models were very scarce until the beginning of the last decade; however, their use in the evaluation of the most diverse cultures is increasing, in view of the advantages that this approach offers regarding difficulties (loss of experimental plots, heterogeneity of environmental variances, etc.) routinely found in agronomic experiments, especially in studies requiring many trials, such as the GxE interaction (Bastos et al., 2007; Carbonel et al., 2007; Mathews et al., 2007; Verardi et al., 2009; Borges et al., 2010; Mendes et al., 2012; Silva et al., 2012; Farias Neto et al., 2013; Gouvêa et al., 2013; Rodrigues et al., 2013; Gomez et al., 2014; Torricelli et al., 2014).

The basic model for the application of the methodology of mixed models was initially presented by Henderson (1973), and Resende (2007) defines it in a matricial way for the analysis of trials in the multi-environments, such as: Y = Xb + Zg + Tga + e. Where, according to this model, the relationship of the arrays in terms of means and variances is given by:

$$E\begin{bmatrix} y \\ g \\ ga \\ e \end{bmatrix} = \begin{bmatrix} Xb \\ 0 \\ 0 \\ 0 \end{bmatrix} ; Var\begin{bmatrix} g \\ ga \\ e \end{bmatrix} = \begin{bmatrix} I\sigma_g^2 & 0 & 0 \\ 0 & I\sigma_{ga}^2 & 0 \\ 0 & 0 & I\sigma_e^2 \end{bmatrix}$$

Where: y is the phenotypic data vector, b is the vector of effects of the combination repetition-site (previously determined as fixed), g is the vector of genotypic effects (random), ga is the vector of the dual interaction GxE (consequently random), and e is the vector of residues (naturally random). X, Z, and T are the incidence arrays of these effects, respectively. Thus, the prediction of genetic values via BLUP regarding a particular character, considering the effects of genotypes and GxE interaction as random, can be described by:

$$w_{ij} = y_{.j} + (\sigma_{gl}^2 + N\sigma_g^2 / \sigma_{gl}^2 + N\sigma_g^2 + \sigma^2)y_{i.} - y_{..} + (\sigma_{gl}^2 / \sigma_{gl}^2 + \sigma^2)y_{ij} - y_{i.} - y_{.j} + y_{...}$$

A simple statistic, based on the mixed modeling, is the one proposed by Resende (2007), which is a Harmonic Mean of the Relative Performance of Genotypic Values (HMRPGV):

$$\frac{n}{\sum_{j=1}^{n} 1/Vg_{ij}}$$

Where: *n* is the number of environments evaluated and Vg_{ij} is the genotypic value corresponding to genotype *i* in the environment *j*.

For the estimation of variance components, the *REML* (Maximum Residual Likelihood) method has been used, developed by Patterson and Thompson (1971), in which the values are estimated by the maximization of the likelihood function of residues instead of the observed data. Therefore, in trials that use the mixed models approach, especially in the case of unbalanced experiments, REML/BLUP analysis has been the most indicated (Resende and Thompson, 2004; Schaeffer, 2004).

METHODOLOGIES USING COVARIATES

Eberhart and Russell (1966) highlight that although the genetic variability is notorious in terms of adaptability, it is difficult to explore it to its fullest, both because of the difficulty in evaluating (or even conceptualizing) adaptability, and the evident problem in quantifying the complexity of factors that influence natural environments.

The same authors state that the use of the general mean of the varieties in each environment shows that planting seasons (which cause differences mainly caused by unforeseen factors such as rainfall) are much more influential in the response of the varieties than the differences inherent in environments such as soil type. In addition, the breeders are inclined to disregard the importance of the results obtained in unfavorable environments, therefore leading to a successive loss of varieties which can justify huge adaptability.

Therefore, the use of an environmental index linked to means of varieties in each environment, such as those used by Finlay and Wilkinson (1963), Eberhart and Russell (1966), and Perkins and Jinks (1968), as the only factor of environmental information is not optimal, and the mathematical relationship between other environmental factors, such as rainfall, temperature, soil type, and the variable response, might be able to generate indexes less biased and more independent of the effect of variety within the analysis. Hardwick and Wood (1972) go further and claim that the fact that the deviations from the regression are not independent of the environmental mean also invalidates the use of the second parameter proposed by Eberhart and Russell (1966).

Freeman (1973) States that, in the face of great difficulty in efficiently capitalizing the GxE interactions to find which environments can maximize genotypes of interest, the use of other variables can be useful to find the factors that are behind the real difference between the genotypes. Freeman and Perkins (1971) reiterate that the use of a regression index would need to be based on measures independent of the environment, whether of physical or biological quality. Fripp and Caten (1971), therefore, through this type of approach, compared the

use of physical and biological variables and found that when the number of genotypes evaluated is large, this approach provides a value similar to that which uses the environmental mean.

However, Perkins (1972) found differences in genotype groups by the use of multiple regression based on climatic factors. Shukla (1972) and Wood (1976) used similar approaches, in which a correlation between a linear combination of genotypes and a linear combination of environmental factors were performed. According to Wood (1976), such an approach, when compared to others, provided a more logical explanation for the genotypic variation in different environments.

Overall, information about environmental measures are hardly available, but considering that the performance of a genotype can considerably vary from one environment to another, it is extremely important that the environmental cause of such change of behavior is measured in order to determine whether such differences can be due to factors inherent to climate or soil, or even due to management strategies. However, it has been noted that even when the experimental sites representing each region are fully selected, the management factors, soil characteristics, and climatic factors are usually not taken into consideration (Schlichting and Levin, 1986).

Some studies, such as those of Beckett (1982), aimed to quantify the environmental factors responsible for the interaction. This author performed a linear regression of each environmental variable in relation to productivity, aiming to identify the predominant factor and possibly the most influential one on the component of the interaction. Nevertheless, according to Weisberg (2005), when there are several factors in equal magnitude influencing the interaction, or when these factors present a certain degree of correlation between them, the simple linear regression analysis can be inappropriate.

From the 1980's, the use of environmental variables and the prediction of their influence on the productivity of some species have been widely applied in the studies on the GxE interaction, and currently several authors have been inserting environmental information, whether as characterization factors and environmental stratification as covariates in the analysis models of GxE interaction (Haun, 1982; Denis, 1988; Van Eeuwijk et al., 1996; Vargas et al., 1998; Crossa et al., 1999; Van Eeuwijk et al., 2005; Voltas et al., 2005; Thomason and Phillips, 2006; Vargas et al., 2006; Boer et al., 2007; Ramburan et al., 2011; Heslot et al., 2014).

Van Eeuwijk et al. (1996), in a seminal study, summarizes some methods based on factor analysis for the insertion of information about environmental covariates for the explanation of the GxE interaction, and, according to the author, such models are just an extension of the most general case:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \rho_i z_j + e_{ij}$$

Where: ρ_i is a coefficient that reflects the sensitivity of the

genotype i, and z_j is the measure of the environmental variable z in the environment j. According to what was expressed, this strategy can be useful for the inclusion of a single environmental covariate such as "rainfall".

However, the idea of factorial regression can be generalized for the inclusion of other covariates, as follows:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \rho_{i1}z_{j1} + \rho_{i2}z_{j2} \ldots + \rho_{im}z_{jm} + E_{ij},$$

where $\rho_{im}z_{jm}$ corresponds to the effect of a variable m in genotype i within the environment j. The successive addition of many environmental variables can reduce the accuracy of the prediction, considering that these variables may be modeling only the non-addictive part of the GxE interaction, that is, to the extent that more variables are added, the same can be inflated with the residue.

Thus, one can resort to the use of a reduction index of covariates of the model by the expression: $\zeta_j = \sum_{h=1}^{H}\lambda_h z_{jh}$, then becoming that which incorporates the synthetic covariate λ_h, with an initially unknown value, which is the more likely linear combination (via least-squares criterion) that can be generated from the available variables, therefore obtained via data set. The model becomes more thrifty (with reduced degrees of freedom) and can be written as:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \rho_i(\sum_{h=1}^{H}\lambda hz_{jh}) + E_{ij}$$

Where: H is the number of environmental covariates (Van Eeuwijk et al., 1996; Vargas et al., 1998; Crossa et al., 1999).

Some studies have been using explanatory covariates in the most variable way possible. Voltas et al. (2005) used the factorial regression and GGE Biplot methodologies for cultivation zoning and subsequent selection of superior genotypes, coupled with detection of main environmental factors that influenced the GxE interaction in 21 wheat genotypes evaluated in 8 environments. On the other hand, Yan and Tinker (2006), in a job evaluating 145 barley genotypes in 25 environments, use the combination of the two approaches aforementioned, by the integration of both into a single mathematical model. However, these authors only used genotype covariates (21 productivity components characters) for the explanation of the interaction regarding the productivity character.

Vargas et al. (2006) used more completed factorial regression models described by Van Eeuwijk et al. (1996) to decompose the GxE interaction effect on corn, with the aid of both genotype variables (*QTL's* predicted via molecular markers) and environmental variables, estimating what the authors called *QTL* x environment interaction. Ramburan et al. (2011), studying varieties of sugar cane, used 14 environmental covariates (mean temperature per day, daily regimen of rain, mean daily

evaporation, soil moisture, among others) combined with the principal component analysis (PCA) to characterize the relative influence of each of the variables on the different environments. The authors then modeled the GxE interaction via the AMMI model, and verified through correlation analysis the relationship between the main components of such analysis with the most significant environmental variables.

According Resende (2007), using regression methods, as well as their combination in multivariate models, are disadvantageous when there are experimental evidences of experimental unbalance factors or heterogeneity of variances between sites. Considering that such strategies take the effect of genotypes as fixed, their use becomes incoherent when you want to estimate variance components and other genetic parameters based on these experiments. Thus, only when a prediction of the genotypic values (as opposed to the use of phenotypic means) is made, actual values regarding the cultivation and use of a variety can be obtained.

COMBINED APPROACHES

An advantageous approach can be the combination of multiplicative and mixed models. Piepho (1998), Resende and Thompson (2004), and Resende (2007), describe in detail the methods named as Analysis of Factors under Mixed Models (*FAMM*) and Principal Component Analysis under Mixed Models (*PCAM*). In the latter, rather than the data array with purely phenotypic values, values previously predicted are used considering random effects (both genotypes and environments, or both). Thus, for a PCA analysis under mixed models, you can adopt the equations relating to:

$$Y = Xb + Z(Q \otimes Ig)(Q^{-1} \otimes Ig)a + \bar{e}$$

Where: $Q = V_m$ is the matrix of eigenvectors associated with m covariates.

More research is necessary before the complete use of environmental variables in the evaluation of adaptability and stability of genotypes (both in cultivated species and in natural populations), considering the question of how to properly analyze the environmental information is still not well established (Schlichting, 1986; Ramburan et al., 2011).

RESULTS AND DISCUSSION

Although the development of methodologies specifically applied to the adaptability and stability studies on cultivated species only occurred in the last 50 years, considerable advances that have occurred in the fields of statistics (mainly regarding multivariate methods), computer science, as well as in the concept of GxE

interaction, enable a leap for such methodologies, which is quite important considering the current context, in which trials of evaluation of genotypes in a number of environments are carried out.

The methodologies based on linear regression only feature important limitations. Among them outstands the use of the mean of all the cultivars in each environment, such as environmental index, which may not occur in this case; the independence between variables, especially when the number of genotypes evaluated is small, which is a restriction of the use of regression, especially considering the current scenario where there is a need for an increasingly amount of tests for the adaptability and stability evaluation (Table 1).

The Ecovalence methodology has the advantage of easy interpretation, considering that it is based on the interpretation of a single numeric value (Wi), however, it does not provide information about environments. In addition, the use of a single value to determine adaptability and stability can be difficult to apply to different objectives, such as the simultaneously broad recommendation and local recommendation of genotypes. In such situations, it may be preferable the use of methodologies, such as those based on multivariate analysis, that have the ability to reduce the data in order to provide a simple interpretation of the results. However, the methodologies based on linear regression can still be applied in a situation with smaller number of MET's.

Among the advantages of methodologies based on multivariate methods are the possibility of application of a biologically more realistic concept of GxE interactions, the ease of interpretation of results – provided by the use of Biplots charts – and the information level generated by the analysis (Table 1). To obtain information about each genotype evaluated and each environment is very useful to the breeders, since it enables a better separation of the concepts of adaptability (wide and local) and stability. Therefore, the use of methodologies such as AMMI and GGE Biplot is encouraged for most cases.

Nonparametric methods, such as those of Lin and Binns (1988), Hühn (1979), Nassar and Hühn (1987), Kang (1988), Fox (1990), and Thennarasu (1995), may be an alternative when the prerequisites of other methodologies are not understood (for instance, when the data do not clearly follow any probability distribution). However, such methodologies are less robust than the others because they are based on the ranking of genotypes only. In addition, the ambiguity caused sometimes by obtaining more than one ranking, such as the methods of Thennarasu (N1, N2, N3, and N4) and Nassar and Hühn (S^1, S^2, S^3, and S^6), can hinder the decision of plant breeders when such decision is based only on one of these methodologies. It is worth mentioning that the methods of Lin and Binns avoid this last factor, for it provides a single measure (Pi) for the interpretation of adaptability and stability (Table 1).

Considering that the imbalance of data and the consequent loss of information are more common, or at least more likely to occur, in the current context, in which the number of environments and genotypes tested is increasing, methodologies that need to meet assumptions, such as the normality of data and absence of residual correlation, may not be the most suitable ones. For these cases, we recommend the use of more robust methodologies that include the use of mixed models, since these methodologies consider phenotypic values for obtaining predictors of real genotypic values. In the current context, in which the processing power of computers is huge, there are no more practical limitations to the application of this approach.

The inclusion of environmental variables in the adaptability and stability evaluation of genotypes is advantageous for dividing physical environments (sites) in generalized environmental factors. This type of approach can be advantageous for the ability to deal with unexpected environmental factors that often decisively influence the performance of genotypes. When including environmental effects separately, the problem of temporal variation of environments (variation between crops and years in the same site) is more elegantly approached. However, some care must be taken into consideration: Do the listed variables really affect the species in any meaningful way? How many environmental covariates must be used in order to obtain the most parsimonious model?. However, the main limitation for the use of such methodologies seems to be the difficulty in obtaining environmental data *in loco* during the exact period of performing experiments, whether by lack of interest of the plant breeder, whether by technological limitations.

The direct comparison between the several methodologies available is not an easy task, and is often inconsistent, mainly due to the fact that many are based on statistical principles quite distinct. A smart approach can be to base the choice of methodology according to the profile and characteristics of the data set to be analyzed. It is not very surprising that it is becoming costly to obtain a more detailed knowledge about the GxE interaction, considering that, in the context of evaluation of multi-environments trials, most of the efforts is usually focused on measuring the performance of the genotypes, while little or no attention is more accurately given to the evaluation of the environments. There is a need for better researches dedicated both to the study on the nature of the GxE interaction, and to the development of statistical genetic models able to comprise greater number of information related to genotypes and environments evaluated.

Conflict of Interests

The authors have not declared any conflict of interest.

Table 1. Comparison between the main characteristics of the methodologies used the most for the evaluation of adaptability and stability in multi-environments trials.

Methodologies	Based Model	No. of parameters/measures	Advantages	Disadvantages/Limitations
Wricke (1962)	ANOVA	1 (ω_i)	Simple interpretation; Simple calculations.	Data need to be balanced and meet the assumptions of a regression analysis.
Finlay and Wilkinson (1963)	Regression	1 (β_{1i})	Simple interpretation; Simple calculations.	Assumes/requires that regression deviations are insignificant; Data need to be balanced and meet the assumptions of a linear regression analysis; Use of environmental index dependent on the mean of the evaluated genotypes.
Eberhart and Russell (1966)	Regression	2 (β_{1i} ; δ_{ij})	Simple interpretation; Simple calculations.	Data need to be balanced and meet the assumptions of a linear regression analysis; Use of environmental index dependent on the mean of the evaluated genotypes.
Nassar and Hühn (1987)	Non-parametric	4 ($S_i^{(1)}$; $S_i^{(2)}$; $S_i^{(3)}$; $S_i^{(6)}$)	Simple calculations; Requires no assumptions.	Less robust model; absence of inferences about environments; 4 independent measures can hinder the conclusion.
Kang (1988)	Non-parametric	1 (Rank-sum)	Simple interpretation; Simple calculations; Requires no assumptions.	Less robust model; absence of inferences about environments.
Lin and Binns (1988)	Non-parametric	1 (P_i)	Simple interpretation; Simple calculations; Requires no assumptions.	Less robust model; absence of inferences about environments.
Fox et al. (1990)	Non-parametric	1 (TOP)	Simple interpretation; Simple calculations; Requires no assumptions.	Less robust model; absence of inferences about environments.
Thennarasu (1995)	Non-parametric	4 ($NP^{(1)}$, $NP^{(2)}$, $NP^{(3)}$, $NP^{(4)}$)	Simple calculations; Requires no assumptions.	Less robust model; absence of inferences about environments; 4 independent measures can hinder the conclusion.
AMMI (Zobel et al., 1988; Gauch, 1992)	Aditive/Multiplicative	---	Biologically realistic; Simple and Graphical interpretation; Inferences about environments and genotypes.	Limited when applied to unbalanced data or with significant residual correlations; Graphical interpretation is disadvantageous when the number of genotypes and environments is very large.
GGE Biplot (Yan et al., 2000)	Multiplicative	---	Biologically realistic; Simple and Graphical interpretation; Inferences about environments and genotypes.	Limited when applied to unbalanced data or with significant residual correlations; Graphical interpretation is disadvantageous when the number of genotypes and environments is very large.
HMRPGV (Mixed Models)	Mixed	1 (HMRPGV)	Simple interpretation; Applicable to unbalanced data; Tolerates residual correlation; Array of kinship can be inserted; Obtains BLUP predictors; Applicable to any size of data file.	Difficulties inherent to the degree of complexity of the approach that considers mixed models.
Covariate based methods	Regression; Multiplicative; Mixed	---	Works with information about types of environments and not with specific environments; Models better the influence of unforeseen factors; Offers the same advantages of Multiplicative and mixed Models, when allied to them.	Data collection for the inclusion of environmental and/or genotypic covariates in the model is still difficult; There are no precise criteria about which covariates should be chosen yet.

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Full Length Research Paper

Effects of low nitrogen on chlorophyll content and dry matter accumulation in maize

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For photosynthesizing plants, studies on the effect of different levels of Nitrogen on chlorophyll could let us know about the overall physiological status of the plant under different environmental conditions. Objective of our study was to understand if there was any difference among the ten recombinant inbred lines (RILs) of IBM population and their parents B73 and Mo17 for chlorophyll content and dry matter accumulation in their response to the N stress. Five IBM genotypes that carry predominantly B73 alleles and five IBM genotypes that carry Mo17 alleles at 5 quantitative trait loci (QTL) regions associated with root traits and N use efficiency from the published Maize Genome Database were evaluated in this study along with their parents. Plants were grown in the Guterman Green House (Cornell University, USA). Two nitrogen treatments (solution with high N contained 65.79 g Ca (NO3)2 4H2O in 100L of 1X solution making 5.0mM NO3 and solution with low N contained 2.63 g Ca (NO3)2 4H2O in 100L of 1X solution making 0.2 mM NO3) were given. Plant traits like leaf dry weight, stalk dry weight and root dry weight were observed. Chlorophyll content was estimated to measure the effect of different N levels on photosynthetic activity. Genotype with high in B73 composition had relative advantage over Mo17 in chlorophyll content, and dry weight of roots under low N condition. The highest root-shoot ratio under low and high N was observed in IBM056 and IBM153 respectively. Except for IBM153 and IBM337, all other genotypes showed reduced LFS at high N. The result showing lower root-shoot ratio and leaf fraction of shoot (LFS) under the high N treatment suggests that shoot growth increases more than root growth in response to increased N application and that within the shoot, stem growth increases more than leaf growth. Results support the conclusion that under low N condition, shoot growth is retarded than root growth.

Keywords: Nitrogen, Maize, Chlorophyll, Dry matter accumulation, Photosynthesis.

INTRODUCTION

Nitrogen supply has substantial effects on plant growth and development, as it is one of the main constituents of leaf cell components, particularly those associated with the photosynthetic apparatus, including carboxylating enzymes and membranes proteins (Pandey et al., 2000). N deficiency inhibits plant growth and development, especially in the older leaves near the base of the plant and ultimately they turn yellow and fall off under severe N

stress condition. Yield is also severely affected by lower grain number and less grain weight which is caused by fewer fertilized ovum, kernel abortion and other changes at physiological and biochemical level (Uhart and Andrade, 1995 a). On the other hand, problems of eutrophication and ground water contamination occur when high amount of N fertilizers are applied to the soil. MacDonald et al., (1997) reported that unfavorable environmental conditions decreased crop yield, but it was not due to less N content in the soil. Plants could perform considerably better even under limited N condition provided that partitioning of N within the plant and efficient utilization of N at the cellular level (Sattelmacher et al., 1994). This emphasizes the fact that plant breeders need to develop varieties and hybrids with high N use efficiency (NUE). For annual crops (like rice, wheat, maize, sorghum etc.), N uptake is regulated by the crop dry mass accumulation under non-limiting nitrogen supply within species and across environments (Lemaire et al., 2007). Tollenaar and Wu (1999) indicated that increased leaf longevity, increased water and nutrient uptake, and greater assimilate supply during grain filling were related to increased low N tolerance in Canadian maize hybrids. N stress cause reduction in leaf area, enhances leaf senescence, and decreases radiation use efficiency (RUE) (Uhart and Andrade, 1995 b). Results of Subedi and Ma (2005) indicated that hybrids with greater yield or NUE were accompanied by greater dry matter production and more N uptake during the grain-filling period. Comparative study between early-senescing maize hybrid and late-senescing maize hybrid, both grown under field conditions with a high fertilisation input involving large quantities of fertilizer showed that leaf senescence occurs independently of the source-to-sink transition at the high level of fertilisation used involving large quantities of fertilizer (Martin et al., 2005). Gastal and Nelson (1994) found that synthesis of Rubisco and other chloroplastic proteins occur largely from recycling of N that was previously incorporated into proteins during cell production and N content was the highest in cell production zone. The relationship between plant photosynthetic capacities, chlorophyll degradation during leaf senescence, and the shift from N assimilation to N remobilization has been investigated in a number of crops by studying the impact of prolonged green leaf area duration on yield of maize (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999 a, b) and other major crops (Thomas and Smart, 1993; Borrell et al., 2001; Spano et al., 2003). Research is also carried out to identify some of the components responsible for the physiological control of the 'stay-green' phenotype particularly in relation to NUE. For example, in both sorghum and maize, delayed leaf senescence allowed photosynthetic activity to be prolonged, which had a positive effect on the N uptake capacity of the plant. In sorghum this enabled the plant to assimilate more carbon and use more N for biomass production (Borrel et al., 2001), whilst in maize yields were higher (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999 a, b). However, further investigation is required to characterize better the physiological and molecular basis of the stay-green phenotype (Verma et al., 2004) in relation to N supply, root N uptake capacity, root architecture, and leaf structure, and to determine whether such a phenotype can be beneficial when N fertilization is reduced (Borrell et al., 2000).

MATERIALS AND METHODS

A list of 260 IBM Recombinant Inbred Lines (RILs) was obtained from the Maize Genome Database (Maize GDB, 2004) (http://maizegdb.org/). The RILs were classified into 2 groups based on the constitution of genotype (abundance of B73 and Mo17) in 5 key regions of the genome that had been previously associated with root traits on Chromosome 1 (Liu et al., 2008) and N use efficiency (NUE). The IBM genotypes which have more of B73 in these 5 target regions are classified as IBM RILs with high B73. The IBM genotypes which have more of Mo17 in these regions are classified as IBM RILs with high Mo17. Five genotypes from each group (high B73 and high Mo17) were selected for the experiment along with parental inbreeds B73 and Mo17 to compare the difference among the genotypes and the parents.

Fine, medium and coarse sand were mixed well and used to fill the 120 black plastic cylindrical pots to equal depth. The 10 IBM RILs and their parents (B73 and Mo17) were planted in the Guterman Laboratory (greenhouse) (total of 120 plants). Two seeds were sown in each pot and seedlings were thinned to one per pot shortly after germination. Day temperature was about 21.1°C (70°F) and night temperature 15.6°C (60°F). Along with normal sunlight, artificial lights (1000 Watt metal halide lamps) were provided to supplement enough light for plant growth.

Three stock solutions following Engels and Kirby (2001) were prepared: solution A in high and low N versions and solution B. Solution A comprised 10 L of high N and 10 L of low N prepared in 2 different plastic containers (Sen et al., 2015). Iron-EDTA was added in solution A. Solution B contained all other nutrients except Ca $(NO_3)_2$ $4H_2O$ and Iron-EDTA and a total of 20 L was prepared in a plastic container. The stock solution was diluted to 10 X solution. Beginning at 8th day after planting, 30 ml solution was applied to each pot and watering was done every other day. At one month after sowing, 30 ml solution was applied every day and continued for 20 days. The plants were harvested at 50 days after planting.

Measurements of dry matter and chlorophyll content

Dry weight

Plants were separated into leaves, stems, and roots and dry weights of each part were measured after drying them in the oven for five days at 60°C.

Root – shoot ratio

Root-shoot ratio was derived by taking the ratio of root and shoot dry weight. Shoot dry weight of a particular plant was obtained by adding leaf and stem dry weight for that plant.

Leaf fraction of shoot (LFS)

LFS was measured by taking the ratio of leaf dry weight and shoot

Table 1. List of IBM RILs selected for screening for nitrogen use efficiency.

IBM RILs with high B73 (high A)	IBM RILs with high Mo17 (high B)
IBM189	IBM055
IBM280	IBM056
IBM284	IBM153
IBM337	IBM236
IBM346	IBM248

Table 2. Sums of squares from analysis of variance for plant traits.

Source	DF	Chlorophyll	Root/Shoot	Leaf/Shoot
Replication	4	0.26	1.76	0.13
Treatment	1	97.82**	0.13**	0.06*
Treatment×Replication	4	0.24	0.1	0.06
Genotype	11	7.68	1.44	0.44
Genotype×Treatment	11	7.87	0.77	0.17
Error(b)	88	7.56	4.55	0.74

*** Significant at $P<0.05$ or $P<0.01$, respectively.

Table 3. Mean values for plant traits at low and high nitrogen levels, evaluated for 12 genotypes in 5 replications in the greenhouse.

Nitrogen treatment	Chlorophyll (mg g^{-1})	Root/Shoot	Leaf fraction of shoot (LFS)
Low	0.71	0.72	0.44
High	2.52	0.55	0.38
Std. Error	0.04	0.03	0.01

dry weight.

Chlorophyll

Five leaf disks of 0.25 cm were taken from each plant. Chlorophyll was extracted in 1 ml of 95% (v/v) ethanol/water. The absorbance of chlorophyll at 654 nm wavelength was measured in a spectrophotometer. This wavelength was chosen based on the findings of Wintermanns and de Mots (1965) who reported that chlorophyll has an extinction coefficient of 83.4 L/g at this wavelength.

Experimental design and statistics

A split plot design was used for the experiment with 5 replications. Statistical analysis of various observations was done using the JMP software package.

RESULTS

N treatments significantly affected chlorophyll content, root-shoot ratio, and leaf fraction of shoot (LFS) (Tables 1 and 2). Chlorophyll content was higher for the high N treatment (Tables 3 and 4). Under low N conditions, IBM RILs with high Mo17 at the target QTL regions had higher root-shoot ratio and LFS than the high-B73 RILs (Table 5). Chlorophyll content was increased with increased level of N in all 12 tested genotypes (Figure 1). IBM337 showed the highest chlorophyll content under high and low N conditions (Figure 1). Low root-shoot ratio under high N conditions indicates that the increased level of N promotes shoot growth more than root growth (Table 4). All the genotypes showed decreased root-shoot ratio at high N except the genotype IBM337 (Table 4 and Figure 2). IBM056 had the highest root-shoot ratio under low N and IBM153 had the highest root-shoot ratio under high N (Figure 3). Except for IBM153 and IBM337, all other genotypes showed reduced LFS at high N (Figure 4). IBM337 was extreme in its dry weight partitioning at high N, with high root-shoot ratio, high LFS, and high chlorophyll content at high N. The high N treatment increased LFS and root-shoot ratio also in IBM153, but did not affect its chlorophyll content (IBM153 showed the lowest chlorophyll content at high N of all the genotypes). IBM337 is one of the "high B73" RILs whereas IBM153 is a "high Mo17" RIL.

Table 4. Mean performance of plant traits of 12 genotypes in 5 replications with high B73 and Mo17 under low nitrogen evaluated in green house.

Genotype	Chlorophyll (mg g^{-1})	Root/Shoot	Leaf/Shoot
IBM 055	0.69	0.78	0.51
IBM 056	0.74	1.06	0.51
IBM 153	0.77	0.93	0.48
IBM 189	0.60	0.98	0.48
IBM 236	0.75	0.89	0.49
IBM 248	0.63	0.80	0.49
IBM 280	0.85	0.56	0.34
IBM 284	0.80	0.66	0.46
IBM 337	0.88	0.63	0.37
IBM 346	0.51	0.62	0.32
B73	0.89	0.73	0.50
Mo17	0.45	0.85	0.36

Table 5. Mean performance of plant traits in genotypes with high B73 or high Mo17 under low nitrogen.

Genotype	Chlorophyll (mg g^{-1})	Root/Shoot	Leaf fraction of shoot (LFS)
High in B73	0.73	0.63	0.39
High in Mo17	0.72	0.89	0.50
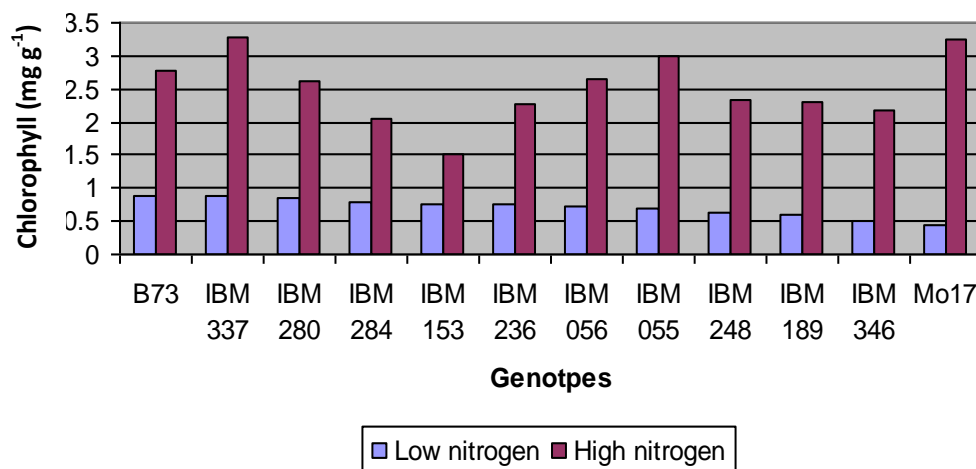
Figure 1. Effect of nitrogen treatment on chlorophyll content of 12 maize genotypes evaluated in 5 replications in the greenhouse.

DISCUSSION

N stress reduces crop photosynthesis by reducing leaf area development and leaf photosynthesis rate and by accelerating leaf senescence (Bänziger et al., 2000; Graham and Vance, 2000). N uptake is positively related to crop growth rate and to biomass accumulation (Gastal and Bemaire, 2002). In this study, the chlorophyll concentration in the IBM RILs was lower than that in the parents at high N (except for IBM337 and IBM055) but fell between Mo17 (lowest chlorophyll) and B73 (highest chlorophyll) at low N. IBM337 was high in chlorophyll at both N levels, which suggests good potential breeding value since delayed senescence is an important criteria for abiotic stress tolerance. Delayed senescence (or stay-green) was proposed as an indirect selection criterion for
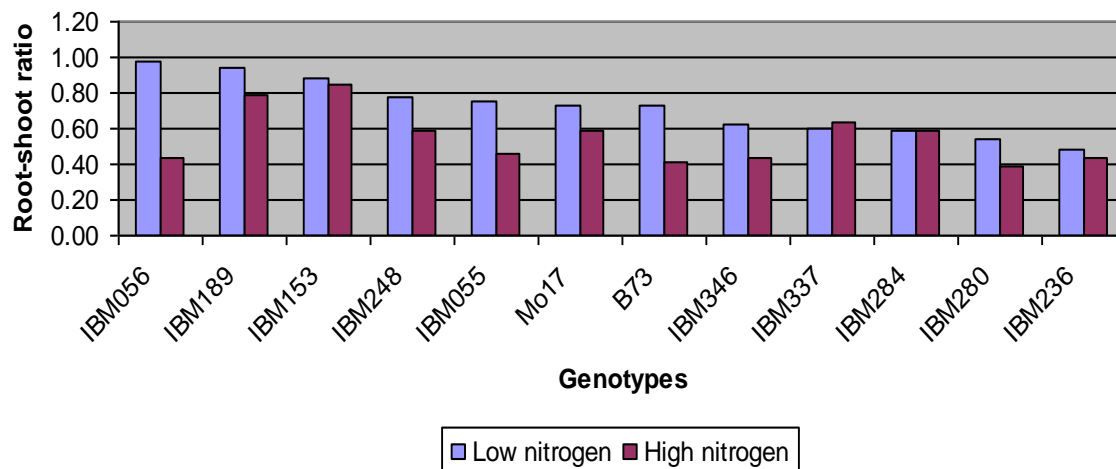
Figure 2. Effect of nitrogen treatment on root-shoot ratio of 12 maize genotypes evaluated in 5 replications in the greenhouse.

Figure 3. Maize genotype IBM236 grown under high nitrogen shows less root growth compared to shoot.

low N tolerance (Moll et al., 1994). Uhart and Andrade (1995a) reported that leaf N decrease has a direct effect on canopy photosynthesis, resulting in more kernel abortion and lower grain number. The IBM RILs with high B73 showed higher chlorophyll content and dry root weight under low N conditions than the IBM RILs with high Mo17. "Stay green" concept under stress conditions is important for better photosynthetic activities and supplying carbohydrates for developing sink organs (young leaves, kernels, grains) and hence genotypes that had higher chlorophyll content could be further studied for future improvement of low N tolerance. Chlorophyll
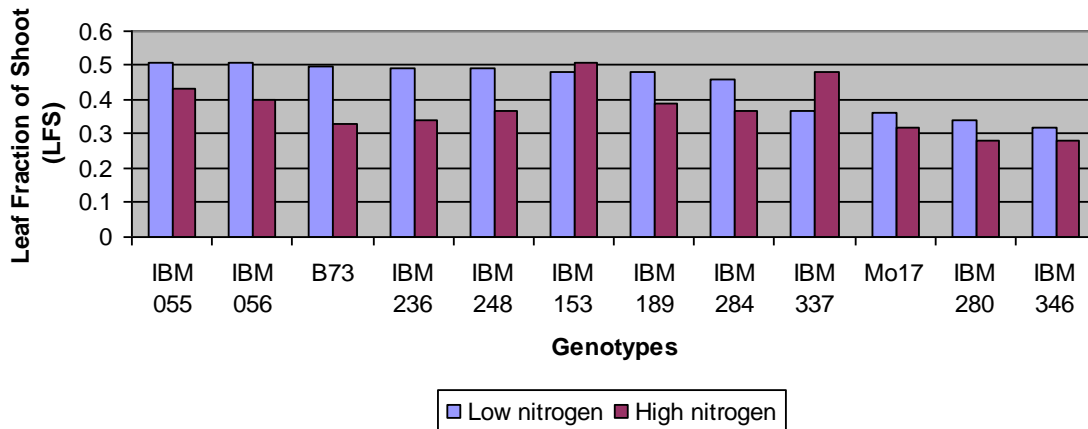
Figure 4. Effect of nitrogen treatment on leaf fraction of shoot of 12 maize genotypes evaluated in 5 replications in the greenhouse.

content of maize leaf can be used effectively in developing recommendations for soil N replenishment (Ványiné et al., 2012).

Costa et al. (2002) and Chun et al. (2005) reported that low N reduced root biomass in all the tested genotypes, which is in accordance with the obtained results showing reduced root dry weight at low N. Root-shoot ratio was higher in N stressed genotypes, which indicates that under low N conditions the shoot growth of the plant is reduced more than the root growth supported by the findings of Amos and Walters (2006). This increased root-shoot ratio at low N may be an adaptive response that conserves plant N while maximizing the ability to acquire more N from the soil and can result from decreased shoot growth with or without increased root biomass (Chun et al., 2005; Yu et al., 2015). The absolute dry root-mass is usually less for plants grown under low N conditions than under normal soil containing sufficient N (Costa et al., 2002; Bänziger et al., 2000), a pattern that is clearly confirmed by root dry weight data from the present study. IBM337 had higher chlorophyll content and high root dry matter content. This establishes the fact that there is direct relationship between higher photosynthetic activity and increase in dry matter content.

Except for the genotypes IBM189 and IBM346 all other genotypes with high B73 composition had higher chlorophyll content. Genotypes with high Mo17 showed more root-shoot ratio than the genotypes with high B73 except the genotype IBM055. The genotypes with high B73 performed better in root dry weight. All the genotypes with high B73 had higher root dry weight than the genotypes with high Mo17 composition. Overall genotypes with high B73 had better performance than the genotypes with high Mo17. Determining the maize chlorophyll content in leaves and maize dry matter content will provide in near future a way to develop maize hybrids with better NUE which can be grown in both resource poor and resource rich environment.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Ecological effects on the flowering phenology of *Cenchrus ciliaris* L. collections from the arid and semiarid lands of Kenya

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Flowering is critical in plant ecology. Through flowering, plants evolve into new plant species that are better adapted to environmental variations. *Cenchrus ciliaris* is an important forage grass in Kenya, which is drought tolerant but is increasingly becoming depleted in grazing areas requiring reseeding. To identify suitable germplasm for such initiatives, collections from varied ecologies were evaluated at Kiboko to assess any adaptive morphological traits, particularly flowering related, that they may have been acquired in their evolution. Significant variation in days to start flowering (DSF) and days to full flowering (DFF) were observed between sites of ecotype origin and among the ecotypes. Ecotypes collected from Kilifi flowered significantly earlier than those from Kiboko while one Magadi ecotype, MGD3, was late flowering despite being collected from an arid zone. DSF was negatively correlated ($p \leq 0.001$) to percent fertile tillers and the number of inflorescence per plant. Inflorescence length was positively correlated ($p \leq 0.05$) to the number of spikelets per inflorescence but negatively correlated ($p \leq 0.001$) to the percent fertile tillers. There was a trade-off between plant size and period to flowering where early flowering ecotypes were smaller in size and vice versa. However, a unique ecotype that defied the trade-off, MGD1 from arid agro-ecological zone VI, with both early flowering and robust traits was identified. Findings from Magadi collections indicate that collections from special niches may not be applied as wide area adaptations, especially with regard to drought tolerance. The early flowering trait of some of the ecotypes matched results from various studies involving a wide range of crops other than grasses. The findings of the study provide opportunities for further selection and breeding work.

Key words: Grasses, *Cenchrus ciliaris*, ecotype, flowering, grass reseeding.

INTRODUCTION

Grass reseeding in arid and semi-arid areas has been promoted for adoption as an option for rehabilitating denuded lands. Among the commonly promoted grasses is *Cenchrus ciliaris*, (African foxtail grass) which is well

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adapted, grazing resistant, persistent and extremely drought tolerant with good response to both small and large rainfall amounts. It is mostly preferred by livestock due to its high nutritive value and herbage yield (FAO, 2012). The species is apomictic resulting in insignificant intra-variety variation. This has led to the selection of true breeding genotypes from ecotypes that are highly variable in traits of both ecological and agronomic importance due to adaptation to multiple environments (Boonman, 1993; Arshad, 2007). The species has been evaluated in various studies for reseeding potential (Mnene, 2006; Kirwa et al., 2010), allelopathic effects (Kirwa et al., 2012), performance in mixtures (Mganga et al.,, 2010a) and seed and herbage yields (M'seddi, 2005; Visser et al., 2008). Studies on morphological characterization of accessions of *C. ciliaris* resulted in various groupings particularly using variation in plant size and flowering time (Pengelly et al., 1992; Jorge et al., 2008). Examples of commonly known cultivars from selections include the American and Gayndah for medium height and early flowering and Biloela and Molopo as tall and late flowering varieties. Despite the progress made in selection and release of varieties of *C. ciliaris*, this benefit is yet to be felt in Kenyan arid and semiarid lands (ASALs). Although the species is widely cultivated in the agro-pastoral parts of the Southern rangelands of the country for both seed and herbage production, this is being done using the wild collections resulting in an informal seed marketing system. This study aimed at evaluating collections of *C. ciliaris* on various traits, particularly variation in flowering phenology.

Flowering time has been used as a trait of importance in the development of cultivars for various plant species. It is critical in plant ecology and may determine the presence or absence of a species in a community (Craine et al., 2011). Variation of flowering time between plant populations is an effective way for their isolation in the speciation process. Flowering phenology is affected by a wide range of genetic and environmental factors (Rathcke and Lacey, 1985). It is variety specific (Hauser and Weidema, 2000), but can also evolve as an adaptation to environmental conditions, such as edaphic properties (Antonovics and Bradshaw, 1970; Rajakaruna, 2004), grazing (Reisch and Poschlod, 2011), pollinator presence or other climate related factors such as temperature and drought. Correlation between flowering time and latitude of plant population origin has also been established (Stinchcombe et al., 2004; Novy et al., 2013). While studying the effect of management on grasslands, Reisch and Poschlod (2011) found that mowing resulted in earlier flowering populations than grazing. In another study by Macnair and Gardiner (1998), edaphic factors resulted in early flowering serpentine endemic species *Mimulus pardalis* and *Mimulus nudatus*, and copper-mine endemic *Mimulus cupriphilus* from *Mimulus guttatus*. On the other hand, the observed global climate change with increasing temperature is resulting in reduced time to start flowering in plants (Cleland et al., 2006; Bloor et al., 2010). The increasing temperatures have been linked to reduction in flowering time by 4.5 days in 385 plants species, studied for 4 years in South Central England (Fitter and Fitter, 2002). Similar findings were recorded by Bloor et al. (2010).

This study evaluated 11 ecotypes of *C. ciliaris* collected from different agro-ecological zones in Kenya for identification of unique important genotypes that could be registered and used in a formal seed system. The main assessment was with regards to flowering time and related traits.

MATERIALS AND METHODS

Ecotypes collection

The study involved collection of grass seeds and tuft splits of *C. ciliaris* from the wild in four agro-ecological zones (AEZ), represented by Kilifi, Taita Taveta, Makueni and Kajiado counties for AEZ III, IV, V and VI, respectively (Mganga et al., 2010b). The targeted collection sites per county were Kilifi, Taveta, Makindu and Magadi districts, respectively. Three sites were purposively selected in each of the target sites of ecotype origin and actual collections done. Samples of 20 plants per species were harvested in each site using randomly stratified technique (Guarino et al., 1995), where seeds were not available or available in small quantities, tuft splits were uprooted as collections. The collections were made in July to September 2012 (Table 1).

Study site

The study was carried out at KALRO's Arid and Rangelands Research Institute (ARLRI) - Kiboko Centre pasture plots located in Makindu Sub-County in the semi-arid County of Makueni, Kenya. Kiboko Research Centre is located 160 km South East of Nairobi on Longitudes 37° 83'E, latitudes 02° 28'S and an altitude of 975 m above sea level. The area receives a bi-modal rainfall pattern with the long rains occurring in March to May and short rains in October to December. The dry seasons come in the months of January to February (short dry season) and June to October (long dry season). The annual mean rainfall and temperature are 534 mm and 23.4°C, respectively (Ndathi et al., 2011).

Planting and field management

Seeds were planted in plastic germination trays in September 2012 and seedlings transplanted after one month to 4 by 4 m un-replicated plots. Individual plants were spaced at 1 and 0.5 m between and within rows, respectively. In cases where tuft splits had been collected, they were directly transplanted to the experimental plots. The target was to have 45 plants per plot per ecotype. The plots were irrigated once a week during the dry seasons. Standardization through cutting of the herbage to 5 cm was done in May 14th 2013 and repeated in September 2013. From then on, data were collected on the regrowth following successive harvest of entire plot herbage. Nitrogen fertilizer at 100 kg N per ha was applied 7 to 10 days after every cutting. The September harvest has been used as the first cut (C1) data in this paper. Cut 2 and 3 (C2 and C3) was a result of 2 other consecutive cuttings (Table 2). C1 data was collected during the dry season while C2

Table 1. Ecotypes of *Cenchrus ciliaris* and description of site of origin.

No.	Collection No/ Ecotype	Site of ecotype origin	GPS point	Altitude (m)	Rainfall (mm)	Temperature (°C)	Management/use
K1	CeciKBK 1	Kiboko	37M 0356997 UTM 9754961	900	575	14 - 35	Controlled grazing land
K 2.	CeciKBK 2	Kiboko	37M 0364664 UTM 9742932	1059	”	”	Riverine, grazing land
K 3.	CeciKBK 3	Kiboko	37M 0358340 UTM 9751011	900	”	”	Controlled grazing land
K 4.	CeciKLF 1	Kilifi	37M 0592230 UTM 9602470	49	1200	20 - 31	Continuously grazed individual land
K 5.	CeciKLF 2	Kilifi	37M 0588462 UTM 9609848	97	”	”	Edge of cultivated land
K 6.	CeciKLF3	Kilifi	37M 0591436 UTM 9583080	32	”	”	Frequently mowed Sisal farm
K 7.	CeciMGD 1	Magadi	37M 0206621 UTM 9799034	822	600	28.6 – 32.9	Open communal grazing land
K 8.	CeciMGD 2*	Magadi	37M 0209795 UTM 9805824	856	”	”	Open communal grazing land
K 9.	CeciMGD 3	Magadi	37M 0206631 UTM 9781498	810	”	”	Controlled communal grazing land
K 10.	CeciTVT 1	Taveta	37M 0360211 UTM 9623156	770	440	20 -30	Edge of cultivated land with controlled grazing
K 11.	CeciTVT 2	Taveta	37M 0361675 UTM 9632568	908	”	”	Open grazing land
K 12.	CeciTVT 3	Taveta	37M 0362386 UTM 9637556	922	”	”	Edge of irrigation canal with minimal grazing

*not included in the study.

and C3 were on a rainy season. C3 received high intensity rainfall, although it was expected to be a short dry season. The time to cut was dependent on the conditions of the subsequent season, following Visser et al. (2008) who also worked with *C. ciliaris* ecotypes. For instance, C2 was done at the beginning of the short rains.

Data collection

Flowering, defined as emergence of an inflorescence per plant was recorded daily from the date of each cut. The total number of plants that had flowered per plot was recorded daily until all the plants in the plot had flowered. The days to the first plant flowering was recorded as the days to start flowering (DSF), days to all plants in the plot flowering as days to full flowering (DFF) and the days from DSF to DFF as the flowering period (FP). Collection of all the other plant growth attributes was done in one season (C2) to reduce variations due to environmental effects in characterization data (Van de Wouw et al., 1999). Measurements were done in ten randomly selected plants per ecotype as also recommended by Van de Wouw et al. (1999) (Table 3). Where measurements targeted parts of a tiller such as leaf or stem thickness then ten observations were done on ten randomly selected plants. Leaf attributes were recorded on the second leaf below the flag leaf.

Data analysis

Data analysis was performed using Genstat 15th edition analysis tools (Payne et al., 2012). Data for the flowering traits (DSF, DFF and FP) for the 3 cuts were subjected to analysis of variance (ANOVA) and their means separated using least significant difference (LSD). These means were then used to generate a principal components analysis and to develop a similarity matrix whose output was used to produce hierarchical cluster analysis. Finally, the means of all the plant measurements for the different ecotypes were subjected to correlation analysis.

RESULTS AND DISCUSSION

Variation in flowering between cuts

There was significant variation (p≤0.05) in DSF among the 3 cuts. The number of DSF reduced with increase in the growth cycle of the ecotypes or change from one cut to the successive one (C1 26.1[a], C2 21.7[b] and C3 16.4[c]; P≤0.001; coefficient of variation (CV)=11.2%). Similar trends are indicated within sites of ecotype origin analysis (Figure 1a) where C3 remained significantly lower than C1 in all the sites. The trends in variation on DFF were similar to DSF. It took significantly fewer days to reach DFF in C3 than in both C1 and C2 (C1 30.9.1[a], C2 29.7[a] and C3 24.4[b] P≤0.001; CV=9.9%). Kiboko collections had significantly (p≤0.05) longer DFF and FP than the rest of the ecotypes in C2 (Figure 1b and c).

Flowering period in C1 was significantly (P≤0.001) shorter (5.9 days) than C2 and C3 with 9.1 and 9.0 days, respectively. This probably indicated uniformity to flowering pattern among the ecotypes than in the other two cuts. Kiboko and Magadi collections had similar trends in FP. Their flowering period in C1 was significantly shorter than C2 and C3 (Figure 1c).

The variation in DSF between cuts could be attributed to differences in temperatures during

Table 2. Cutting dates, rainfall amounts and length of preceding growing periods.

	Cut 1	Cut 2	Cut 3
Cutting date	11-09-2013	6-11-2013	14-02-2014
Number of days between cuts	55	99	67
Rainfall amounts (mm)	Irrigated	172.9	203.6
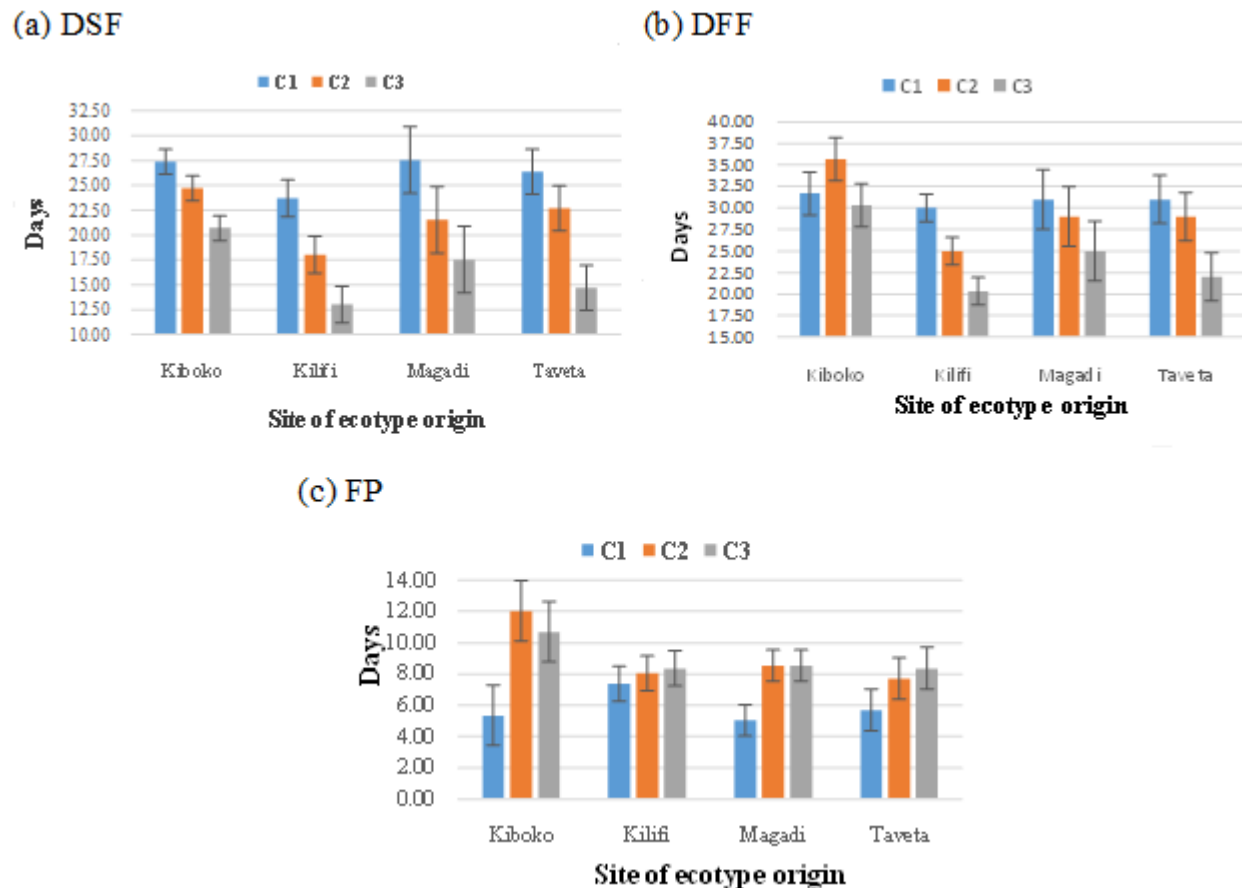
Figure 1. Mean of DSF, DFF and FP and LSD[0.05] for sites of ecotype origin at different cuts (a) DSF, (b) DFF, (c) FP; C1=cut 1, C2=cut 2 and C3 = cut 3.

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The variation in DSF between cuts could be attributed to differences in temperatures during the three periods (C1, C2 and C3) of data collection. These results could be explained by findings of several other workers who worked on different plants species and populations. Mean monthly temperatures in Kiboko are above the annual mean from October to June except December and February to March temperatures are normally the highest (Ndathi et al., 2011). Cong and Brady (2012) noted that temperatures significantly affect the length of the growing

Table 3. List of morphological characteristics used in data collection and their descriptions.

Growth stage	Morphological characteristic	Description	Units	No. of observations
	1. Flag leaf length	From the ground to the tip of the flag leaf	cm	10 Plants
	2. Plant height	From the ground to the tip of inflorescence	cm	10 Plants
	3. Stem thickness	Average culm diameter above the lowest node	mm	10 Observations
Full bloom	4. Number of nodes	Count of all nodes in 1 randomly selected tiller per plant	No.	10 Observations
	5. Leaf length	Ligule to the tip of the leaf	cm	10 Observations
	6. Leaf breadth	Width of leaf at widest point	mm	10 Observations
	7. Days to start and days to full flowering	Daily record of no. of flowering plants per plot from the time of cutting	No.	Whole plot
	8. Total tiller number	Count of all tillers on a plant	No.	10 Plants
	9. Inflorescence count	Count of all panicles on a plant	No.	10 Observations
Seed maturity	10. % Fertile tillers	Tillers with panicles as a percentage of all tillers on the particular plant	%	
	11. Inflorescence length	From the lowest cluster to the top of bristle	cm	10 Observations
	12. Spikelet number	Count of all spikelets on an inflorescence	No.	10 Observations

Table 4. Mean of DSF, DFF and FP in relation to the site of origin of *Cenchrus ciliaris* ecotypes.

SITE	DSF	DFF	FP
Kiboko	24.2[a]*	32.6[a]	9.3
Magadi	22.2[ab]	28.3[ab]	7.3
Taveta	21.2[ab]	27.3[ab]	7.2
Kilifi	18.2[b]	25.1[b]	7.9
P value	<.001	<.001	n.s
LSD	2.46	3.27	1.93
CV (%)	11.2	11.3	23.5

*Column means with different letter superscript significantly different at P value≤0.001.

seasons. The previous month's temperature has the greatest effect on flowering (Fitter and Fitter, 2002). Cleland et al. (2006), in studies on possible effects of global warming, found out that increase in temperature reduces the time to start flowering. Also, while assessing effect of manipulated climatic conditions, Bloor et al. (2010) recorded acceleration in flowering by 3.2 days per degree rise in warming.

Variation in flowering between sites of ecotype origin

Comparison between sites of ecotype origin on DSF indicate that Kilifi (KLF) collections started to flower significantly (P≤0.001) earlier than Kiboko's (KBK) (Table 4). Magadi (MGD) and Taveta (TVT) collections on the other hand were not significantly different in DSF to either KLF or KBK collections. Similar results were recorded with DFF while there were no significant differences in FP between the sites of origin.

However, results within the cuts indicated that

Table 5. Mean and ranges of DSF, DFF and FP for two major clusters developed using Hierarchical cluster analysis.

Group	Ecotypes	DSF	DFF	FP
Early flowering	KLF1, KLF2, KLF3, TVT1, TVT2 and MGD1	17.2 (17 - 20.7)*	25.6 (24 - 26.7)	7.4 (6.3 - 8.7)
Late flowering	KBK1, KBK2, KBK3, MGD3, TVT3	24 (22. - 25.7)	31.6 (29.7 - 34.7)	8.7 (7 - 11.3)
	P value	<.001	<.001	0.106
	Grand mean	21.4	28.3	8.0
	LSD	1.98	2.28	1.69
	CV (%)	6.8	5.9	15.4

*Range of the mean.

Kiboko collections significantly (p≤0.05) took longer (FP=12 days) to reach full plot flowering in C2 than the rest which were at 7.7, 8 and 8.5 for TVT, KLF and MGD collections, respectively. Also, Kiboko (35.7) collections had significantly the highest DFF than KLF (25) in C2. Similar results were recorded with DSF in C2 and C3 (KBK 20.7; KLF 13 days; p≤0.05). The TVT (14.7 days) collections also flowered earlier than Kiboko in C2. From these results, KLF collections are seen to be early flowering ecotypes hence implying that DSF and DFF are related to the site of ecotype origin. This was confirmed by cluster analysis using the three flowering attributes (DSF, DFF and FP) (Table 5). All KLF and KBK collections clustered in Groups 1 and 2, the early flowering and late flowering group, respectively.

Days to start flowering (DSF) is related to the prevailing environmental conditions of the site of ecotype origin (Boonman, 1993) and has a strong relationship with the length of growing season. Late heading implies long growing seasons and vice versa. Plants in long growing seasons would spend more time in exponential growth phase and thus accumulate disproportionately more resources which is also reflected in the reproductive phase (Franks et al., 2006). However, under short seasons, late flowering plants are disadvantaged with seed setting occurring during unfavourable conditions. Such conditions have led to an escape mechanism of flowering early (Franks et al., 2006; Wissman, 2006; Boonman, 1993). This is described as a conservative strategy in "bet-hedging evolutionary theory" (Childs et al., 2010) and plant species do optimize fitness with regards to prevailing environmental conditions. Although the site for origin for KLF1, KLF2 and KLF3 receives the highest rainfall as compared to all the other sites and with potentially longer growing periods, grazing for KLF1 and 2 and mowing for KLF3 could have led to early flowering. KLF1 and KLF2 were collected from unprotected grazing areas adjacent to cultivated areas and KLF3 from a sisal plantation that is periodically mowed.

Early flowering is adaptive in environments with highly variable resource availability (Houle, 2002) and allows successful allocation of resources to reproduction before the onset of the harsh environmental conditions (Latta and McCain, 2009). It is an escape mechanism to a predictable disturbance, such as drought (Franks et al., 2006), grazing (Wissman, 2006) or light in case of other plant's canopy cover. Levin (2009) notes that selective divergence in flowering time is associated with tolerance to marginal habitats.

Early flowering has been observed to occur in certain ecotypes as an adjustment to terminal drought occurrences. For instance, Franks et al. (2006) observed that in an extreme drought event in 2000 to 2004 that resulted in a shortened growing season, descendant ecotypes of *Brassica rapa* significantly shifted to early flowering when compared to their ancestors. Similarly, Craufurd and Wheeler (2009) reported late flowering genotypes of sorghum that reduced their optimum flowering time by about 20 days due to reduced rainfall amounts and consequently the growing seasons. However, caution is made against treating escape from drought as drought tolerance (Boonman, 1993).

It was clear from this study that KLF collections (3.6° S) are from the more southern latitudes and, therefore, more distant from the equator compared to KBK (2.2° S) collections, an observation similar to several other previous findings (Rathcke and Lacey, 1985; Stinchcombe et al., 2004; Novy et al., 2013). These authors reported close relationship between the sites of ecotype origin, especially latitude, with flowering attributes. Our results also agree with those of Stinchcombe et al. (2004) who found ecotypes of *Arabidopsis thaliana* from the more Northern latitudes flowering later than those from less Northern latitudes. On the contrary, Novy et al. (2013) reported that the more northern latitude collections of *Microstegium vimineum,* an annual grass were earlier flowering and small-sized with lower biomass.

Variation in flowering between ecotypes

a) DSF between ecotypes

The ranges for DSF were 17 (for KLF 3) to 25.7 days (for KBK2), with a significant (p≤0.001) variation among the

Table 6. Mean of days to start flowering for each *Cenchrus ciliaris* ecotype in different cuts.

ECOTYPE/ CUT	KLF1	KLF2	KLF3	TVT1	TVT2	TVT3	MGD1	MGD3	KBK1	KBK2	KBK3
Sep-Oct	22	27	22	29	24	26	26	29	27	29	26
Nov-Dec	18	18	18	19	23	26	18	25	25	26	23
Feb-Mar	12	16	11	14	13	17	16	19	21	22	19
Mean*	17.3[bc]	20.3[abc]	17[c]	20.7[abc]	20[abc]	23[ab]	20[abc]	24.3[a]	24.3[a]	25.7[a]	22.7[abc]

CV=9.3%; LSD=3.37 days; *Row means with different letter superscript are significantly different at P value≤0.001.
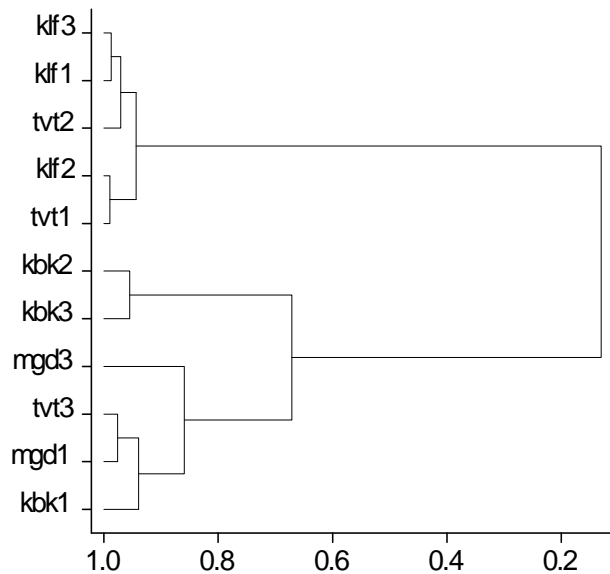
Figure 2. Dendogram of 11 ecotypes of *C. ciliaris* based on robust related characteristics developed.

ecotypes (Table 6). Also, KLF1 and KLF3 flowered significantly (p≤0.001) earlier than MGD3, KBK1 and KBK2. Ecotypes from the same site of origin did not differ in DSF.

Genetic differentiation in flowering time has been established between populations of different plant species (Boonman, 1993; Craine et al., 2011). As expected, long DSF produced most robust plants, that is, tall plants, long and broad leaves, thick stems with many nodes. The results were confirmed by cluster analysis using robust related traits where two cluster groups were formed (Figure 2). All Kiboko and Magadi ecotypes were grouped in the robust cluster together with TVT3 while all Kilifi ecotypes and TVT1 and TVT2 were clustered as small size. All the late flowering ecotypes were in the robust group, except MGD1, which is an early flowering ecotype. Generally, early flowering plants even in other crops such as soybean are associated with inferior yields under long no-stress seasons because they are unable to fully utilize the seasonal potential (Kuol, 2004). The small size and early flowering are described as features for dehydration-avoidant phenotype by Blum (2005) resulting

from a trade-off between allocation of resources to vegetative production and reproduction (Gardner and Latta, 2008). Plants growing under drought conditions have their leaves mature at smaller size than well watered plants (Chaves et al., 2003). Generally, late flowering grasses are associated with superior herbage yields (Boonman, 1993) and also with higher leaf tissue density Craine et al. (2011).

The expected trade-off between plant size and period to flowering (Zopfi, 1995; Colautti et al., 2010) did not occur in MGD1. The ecotype which was collected from the edges of a dry sandy stream in association with short *Acacia spp* and *Aristida spp* about 10 km from Lake Magadi exhibited early flowering but robust related traits. Although, the ecotype seemed to have moisture stress avoidant trait, which is early flowering as well as having been collected from an arid agro-ecological Zone VI, it should not be assumed to be drought tolerant especially because of its special habitat. The early flowering nature could be due to the arid conditions of the site of ecotype origin. High evaporation rates probably results in soil moisture being available only during the short periods of rains and thus an escape through early flowering. On the contrary, MGD3 ecotype collected at the end of a flooding valley in Magadi was late flowering probably because it was grazed late since the area was strictly used as dry season grazing land for the pastoral Maasai community. While selecting ecotypes for *Chloris gayana* at Kitale Research Station in Kenya, Boonman (1993) found a strong relationship between flowering time and rainfall patterns in sites of population origin and not to drought tolerance. Ecotypes from semi-arid zones (*Kapedo*, *Mpwapwa* and *Rongai)* flowered earlier than humid zone collections (*Pokot* and *Masaba)* due to the short rainy seasons in their sites of origin. The former were from Zone III/IV while the latter were from Zone II/III corresponding to semi-arid and humid conditions, respectively. But, he noted that the *Pokot Rhodes*, a robust and very late heading variety was initially thought to be drought tolerant due to arid conditions of collection region. But, it was later discovered to have been collected from the moist cool parts of the region. The MGD1 results indicated that it is possible to select for early and robust ecotypes of *C. ciliaris*. Also, further studies on its extent of tolerance to drought conditions needs to be done. The positive correlation with the

Table 7. Mean of days to full flowering for each *Cenchrus ciliaris* ecotype in different cuts.

ECOTYPE/ CUT	KLF1	KLF2	KLF3	TVT1	TVT2	TVT3	MGD1	MGD3	KBK1	KBK2	KBK3
Sep-Oct	29	31	30	32	29	32	30	32	33	32	30
Nov-Dec	25	25	25	25	29	33	25	33	38	35	34
Feb-Mar	18	24	19	22	18	26	24	26	33	33	25
Mean	24[c]	26.7[abc]	24.7[c]	26.3[bc]	25.3[bc]	30.3[abc]	26.3[bc]	30.3[abc]	34.7[a]	33.3[a]	29.7[abc]

CV=9.9%; LSD=4.771; Column means with different superscript are significantly different at P≤0.001.

number of nodes is similar to results by Zopfi (1995) and Pleines et al. (2013) who worked with Rhinanthus spp. Zopfi (1998) noted that the number of internodes was a very stable trait that is not affected by management regimes.

There was a positive correlation between inflorescence length (p≤0.01) and the number of spikelets per inflorescence (p≤0.05) but negative to the percent fertile tillers (p≤0.001) (Appendix 1). This implies that the late flowering ecotypes produce more spikelets per inflorescence than early flowering ecotypes. This is further an indication of a mechanism by the early flowering to compromise in resource allocation to growth. Faba beans are known to escape droughts through early flowering and short grain filling periods to optimize production under unfavourable conditions (Kuol, 2004). In other studies on flowering time genes in rice, Heading-date 1 (Hd1) and Early heading date 1 (Ehd1) were found to reduce the number of primary branches in a panicle, resulting in reduced spikelet numbers per panicle (Naokuni and Izawa, 2011). These findings could probably explain the low spikelet number results in early flowering given that the two species are in the same Gramineae family.

Number of fertile tillers and spikelet number per fertile tiller are the 2 main components contributing to potential seed yield (Boelt and studer, 2010). Days to start flowering (DSF) was strongly negatively correlated (p≤.0.001) with percent fertile tillers and the number of inflorescence per plant. This means ecotypes that flowered early had higher percent fertile tillers. Although, this could imply that the seed yield potential for the early flowering ecotypes is high, there was no significant correlation between DSF and seed yield in the study. The high number of fertile tillers and thus high inflorescence number in early flowering ecotypes is not in agreement with findings by Zopfi (1995) where there was a trade off on the number of flowers with early flowering in *Rhinanthus glacialis* herb.

Days to full flowering (DFF) between ecotypes

There was a significant difference (p≤0.001) between ecotypes in DFF (Table 7). KLF1 and KLF3 had full plot flowering significantly earlier than KBK1 and KBK2. There

was no variation between ecotypes from the same site of origin. These results are further depicted by the flowering patterns of the ecotypes in C3 (Figure 3). A clear isolation of KBK1 and KBK2 as late flowering in the cuts is shown.

Days to full flowering (DFF) was significantly positively correlated with DSF (p≤0.001) and FP (p≤0.05). This indicates that ecotypes with delayed DSF, took longer to reach DFF and the FP was also longer. This is shown by the curves in Figure 2. As an escape mechanism, early flowering within the shortest time is necessary to avoid an impending disturbance as opposed to flowering with abundant resources to allocate. Variation in individual plant flowering time within a population is positively related to plant vigor (Boonman, 1993). Plants with higher vigor flower early and vice versa. Based on the preceding results, it could be deduced that there was a big variation in plant vigour within the late flowering ecotypes and thus the delay in their DFF. Days to full flowering (DFF) were significantly correlated to robust related traits (stem thickness, plant height, leaf length and breadth), number of nodes and flowering period (Appendix 1). This was similar to the findings on DSF.

Flowering period between ecotypes

There was no significant difference between the ecotypes in FP (Table 8). The mean flowering period ranged from 6.3 days for TVT2 to 11.3 days for KBK1. However, there was a positive correlation between FP and DFF (Appendix 1) and there was a trend of increasing FP with increase in DFF. FP was not correlated with any of the plant attributes measured except DFF.

CONCLUSIONS AND RECOMMENDATIONS

Ecotypic differentiation among the 11 ecotypes of *C. ciliaris* in regard to flowering patterns was observed which could infer genetic differentiation due to environmental variation. Significant variations were observed between sites of ecotype origin in regard to days to start flowering and days to full flowering. Kilifi collection significantly flowered earlier than Kiboko collections.

There was also a strong relationship between plant robustness and flowering time. These findings are
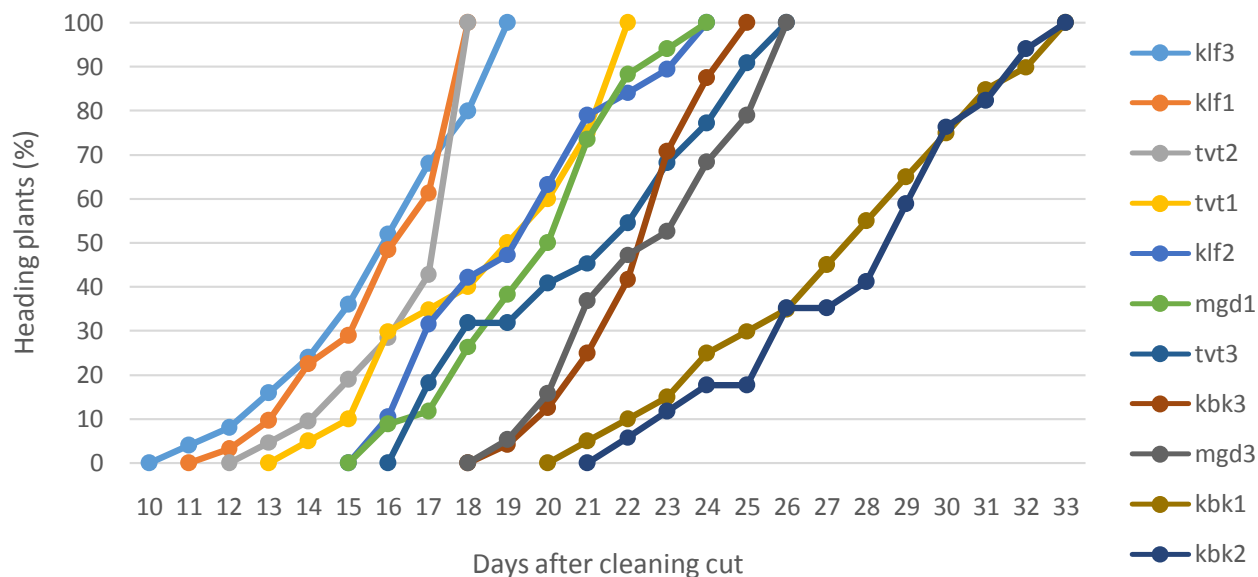
Figure 3. Heading patterns in spaced plants' plots of *Cenchrus ciliaris* ecotypes from the time of cleaning cut in mid Feb. 2014 to DFF.

Table 8. Mean of flowering period for each *Cenchrus ciliaris* ecotype in different cuts.

Ecotype/Cut	KLF1	KLF3	TVT2	KLF2	TVT1	TVT3	MGD1	MGD3	KBK3	KBK1	KBK2
Sep-Oct	8	9	6	5	4	7	6	4	5	7	4
Nov-Dec	8	8	7	8	7	9	8	9	12	14	10
Feb-Mar	7	9	6	9	9	10	9	8	7	13	12
Mean	7.7	8.7	6.3	7.3	6.7	8.7	7.7	7	8	11.3	8.7

P>0.05; CV=22.3%; l.s.d.=3.04.

important in breeding work since selection can be done based on the flowering patterns, which strongly correlate with robustness. Kilifi collections could be selected for the early flowering while Kiboko ones could be selected for late flowering traits. However, further analysis using molecular tools is necessary to ascertain the genetic variability within and among the ecotypes.

Magadi 1 ecotype was unique due to its early flowering and robustness traits. However, caution should be exercised in interpreting special niches as wide area adaptations.

Conflict of interests

The authors have not declared any conflict of interests.

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Appendix

Appendix 1. Correlation matrix between morphological attributes among Cenchrus ciliaris ecotypes

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	%FT	-																				
2	SWT	0.3	-																			
3	AD	0.3	-0.4	-																		
4	BT	-0.1	-0.2	0.0	-																	
5	CNO	-0.1	0.6	-0.6*	0.3	-																
6	CWT	0.1	0.6	-0.5	0.0	0.9***	-															
7	DFF	-0.6*	-0.4	-0.3	-0.1	0.0	-0.1	-														
8	DSF	-0.8***	-0.3	-0.5	-0.1	0.1	0.0	0.9***	-													
9	EDS	-0.1	-0.1	-0.3	0.4	0.4	0.3	0.2	0.2	-												
10	FP	0.0	-0.4	0.2	-0.1	-0.4	-0.3	0.7*	0.4	0.1	-											
11	FLH	-0.6	-0.4	-0.3	0.0	0.1	0.1	0.7**	0.8**	0.6	0.4	-										
12	ITH	-0.5	-0.2	-0.3	-0.1	0.1	0.2	0.8**	0.7**	0.5	0.5	0.9	-									
13	ID	-0.4	-0.9***	0.3	0.5	-0.4	-0.6*	0.2	0.1	0.2	0.2	0.2	-0.1	-								
14	IL	-0.7*	-0.2	-0.7*	0.1	0.3	0.2	0.7**	0.8**	0.6*	0.2	0.8	0.8	0.1	-							
15	IR	-0.1	-0.3	-0.2	0.6*	0.4	0.4	0.0	0.0	0.6*	-0.1	0.4	0.3	0.2	0.4	-						
16	LR	-0.6*	-0.2	-0.5	0.4	0.5	0.2	0.4	0.6	0.5	-0.2	0.7	0.5	0.3	0.7	0.5	-					
17	LB	-0.5	-0.3	-0.3	-0.4	-0.1	0.1	0.8**	0.8**	0.3	0.6	0.8	0.8	0.0	0.7	0.0	0.1	-				
18	LL	-0.7*	-0.3	-0.5	0.0	0.3	0.2	0.8**	0.8**	0.5	0.2	1.0	0.9	0.1	0.9	0.4	0.8	0.7	-			
19	NOD	-0.3	0.4	-0.5	-0.3	0.4	0.4	0.6*	0.6*	0.2	0.4	0.3	0.5	-0.4	0.5	-0.3	0.1	0.6	0.5	-		
20	SNO	-0.6*	-0.6*	-0.1	0.0	-0.2	-0.2	0.7*	0.7*	0.3	0.4	0.9	0.7	0.4	0.7	0.3	0.6	0.7	0.8	0.1	-	
21	ST	-0.7*	-0.4	-0.3	-0.2	0.0	0.0	0.9***	0.9***	0.3	0.4	0.9	0.9	0.1	0.8	0.2	0.5	0.9	0.9	0.5	0.8***	-

***= p≤0.001; **=p≤0.01 and *= p≤0.05.

No.	Abbrev.	Meaning	No	Abbrev.	Meaning	No.	Abbrev.	Meaning	No.	Abbrev.	Meaning
1	%FT	Percent fertile tillers	7	DFF	Days to full flowering	13	ID	Inflorescence density	19	NOD	Number of nodes
2	SWT	Spikelet weight	8	DSF	Days to start flowering	14	IL	Inflorescence length	20	SNO	Spikelet number
3	AD	Awn density	9	EDS	Ease to drop seed	15	IR	Inflorescence ratio	21	ST	Stem thickness
4	BT	Basal tillers	10	FP	Flowering period	16	LR	Leaf ratio			
5	CNO	Caryopsis number	11	FLH	Flag leaf height	17	LB	Leaf breadth			
6	CWT	Caryopsis weight	12	ITH	Inflorescence height	18	LL	Leaf length			

Full Length Research Paper

Gas exchange and carbon metabolism in young plants of muruci (*Byrsonima crassifolia* L.) submitted to water deficit

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The Byrsonima genus covers various fruit species known in the Brazilian Amazon as murucizeiro, which is considered as a species with good nutritional quality and features in its composition a variety of volatile compounds. The adaptation of plants to water stress is a complex physiological and biochemical phenomenon. Depending on the intensity and duration of stress, changes ranges from a rapid change in the flow of ions to improve the osmotic pressure, reduction of gas exchange, stabilization of cell structures by osmotic protection to a more drastic change in plant growth pattern. The aim of this work was to study gas exchange and carbon metabolism in young plants of muruci (*Byrsonima crassifólia* L.) submitted for water suspension. The experimental design was completely randomized with two water conditions: control and drought, with 14 repetitions, totaling 28 experimental units. The parameters analyzed were relative water content, transpiration, stomatal conductance, photosynthetic pigments, concentrations of starch, total soluble carbohydrates and sucrose. The suspension of irrigation for 25 days provided significant differences in all parameters, promoting decreases in the metabolic pathways of plants and reducing the relative water content by 26.92%, transpiration 90%, stomatal conductance 94.79%, photosynthetic pigments (Clrofila (42.1%), Chlorophyll b (50%), Carotenoids (45.1%) and overall (33.3%)) and starch in leaves and roots (73.43 and 63.63%), but increase in the control plants with the total soluble carbohydrates at 63, 87 and 39.5% and sucrose content as 64.73 and 43.99% in the leaves and roots. Therefore, these changes indicated that these plants are susceptible to soils with low water availability.

Key words: Carbohydrates, drought, muruci, photosynthetic pigments, transpiration, sucrose.

INTRODUCTION

The Byrsonima genus covers various fruit species popularly known in the Brazilian Amazon as murucizeiro and in other regions of Brazil as murucizeiro. *Byrsonima crassifolia* (L.) HBK, whose center of origin and diversity

is in the Brazilian Amazon, is widespread in tropical America, constituting the most important species, not only for being the most cultivated but also for having the best quality fruits for consumption (Cavalcante, 2010). It has a fleshy fruit, drupoide type, with a round shape or oblong, from ovarian tricarpellate, with each carpel containing an egg. It is consumed as fresh fruit or used in the preparation of soft drink, ice cream, sweet paste, jam, liqueur and even in savory dishes such as meat stuffing or soups (Carvalho and Nascimento, 2013). The murucizeiro propagation unit is pyrene (core), which contains one to three seeds located in the walls of isolated locules endocarp (Carvalho and Nascimento, 2008).

It is considered a good nutritional food with high quality and has in its composition a variety of volatiles such as ethanol, butyl hexanoate, butanoic acid, hexanoic acid and methyl butyrate, responsible for the distinctive aroma of the fruit (Souza et al., 2012). Besides, it is rich in polyphenols and flavonoids, which gives it great antioxidant capacity and can, therefore, be framed in the group of functional foods (Siguemoto, 2013).

However, although the fruit of murucizeiro have economic, social, cultural importance for small communities harvesting of extractive form for consumption and marketing, little is known about the information on cultivation and its physiological, biochemical and nutritional characteristics. Mainly, these species are subjected to environmental stress conditions that affect their growth and development as a result of the water stress condition in the soil (Almeida et al., 2011).

The adaptation of plants to water stress is a physiological and biochemical complex phenomenon. Depending on the intensity and duration of stress, changes ranging from a rapid change in the flow of ions to improve the osmotic pressure, reduction of gas exchange, stabilization of cell structures by osmotic protectors, to a more drastic change in plant growth pattern can be observed (Alves, 2007).

Water stress can affect multiple morphological and physiological characteristics of plants, photosynthesis being one of the processes more limited by the increase of drought. The intensification of drought caused by environmental changes in anthropogenic and/or natural way can result in limitations to the growth of young plants in the Amazon region since the early stage of development of the field in culture by the plants can be subjected to natural water deficit, especially for presenting a superficial root system (Silva, 2009).

The photosynthetic process can be separated into three stages: the diffusive stage mainly controlled by opening and stomatal closure; photochemical stage, which has a primary function to absorb the incident formation of ATP and reducing power; and biochemistry stage, responsible for carbon fixation (Kreuzwieser and Gessler, 2010). Therefore, this research aimed to study gas exchange and carbon metabolism in young plants of muruci submitted to water suspension.

MATERIALS AND METHODS

Plant materials

The seedlings were from the Association of Exporting Industries of Wood in the state of Pará (AIMEX), 4 months after germination. The seedlings were acclimatized in a greenhouse for a period of three months for ambiance.

Experimental conditions

The study was conducted at the Federal Rural University of Amazonia (UFRA), state of Pará, campus CapitãoPoço, Brazil (Latitude 01° 44 '47' 'and longitude 47 03'34' '). This experiment was conducted in a greenhouse for 4 months, with temperature of air minimum-maximum with values of 24.5/39.1 and 53.3/91% minimum-maximum humidity, respectively.

Substrate, pots and plant nutrition

The substrate used was a mixture in the proportion of 3:1:1 (v/v/v), black earth, chicken manure and earthworm humus, respectively. The polyethylene vessels were used in the dimensions of 0.30 m x 0.30 m (height x diameter), and capacity of 20 kg. Corrections were made in the concentrations of macro and micronutrients from the soil and the pH soil, through the results of the soil chemical analysis realized in the laboratory of soils in Embrapa Eastern Amazon, applying 600 mL of complete nutrient solution (Hoagland and Arnon, 1950), divided in three months, for every month 200 mL of complete nutrient solution was useddbefore the start of the experiment.

Experimental design and treatments

The experimental design was completely randomized with two water conditions (control and drought), with 14 repetitions, totaling 28 experimental units, where each experimental unit consisted of one plant per pot. The experiment was conducted from April, 2013 to July, 2013 in which the water suspension occurred in the 25 days period and the control plants were irrigated daily in an average of 400 ml of water to compensate for the losses by evapotranspiration.

Leaf relative water content

The leaf relative water content was evaluated using leaf disks with 10 mm of diameter and it was carried out in each plant, in which 40 disks were removed and the calculation was done in agreement with the formula proposed by Slavik (1979):

$$LRWC = [(FM1 - DM)/(FM2 - DM)] \times 100$$

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Where: FM_1 is fresh matter, FM_2 is turgid matter evaluated after 24 h and saturation in deionized water at 4°C in dark, and DM is the dry matter determined after 48 h in oven with forced air circulation at 80°C.

Gas exchange

Stomatal conductance and transpiration were evaluated in fully expanded leaves under light, using a steady state porometer (LI-COR Biosciences, model 1600), with the gas change evaluated during the period between 10:00 and 12:00 h in all the plants.

Chlorophyll contents

The determination of the photosynthetic pigments was carried out with 25 mg of leaf tissue, in which the samples were homogenized in the dark and in the presence of 2 mL of acetone at 80% (nuclear). Subsequently, the homogenized was centrifuged at 5.000 g, by 10 min in the temperature of 5°C, in which the supernatant was removed and the chlorophylls a, b, carotenoids and the total were quantified using spectrophotometer SP – 220 from the QUIPAR Company, in agreement with the methodology of Lichthenthaler (1987).

Concentrations of starch

For determination of starch content, 50 mg of milled material was incubated with 5 mL of ethanol at 80°C for 30 min, centrifuged at 2.000 g for 10 min at 25°C, and the supernatant was removed. In addition, a second extraction was carried out with the same milled material incubated with 5 mL of 30% $HClO_4$ at 25°C for 30 min and centrifuged in conditions previously described. The supernatants of the two extractions were mixed. The quantifications of the total soluble carbohydrates and starch were carried out at 490 nm using the method of Dubois et al. (1956), using glucose (Sigma Chemicals) as a standard.

Concentrations of total soluble carbohydrates

The total soluble carbohydrates were determined with 50 mg of leaf dry matter, which was incubated with 5 mL of ultra pure water at 100°C by 30 min, subsequently the homogenized was centrifuged at 2.000 g, within 5 min at 20°C and the supernatant was removed. The quantification of the total soluble carbohydrates were carried out at 490 nm according to the method of Dubois et al. (1956), and glucose (Sigma chemicals) was utilized as a standard.

Concentrations of sucrose

The determination of sucrose was carried out with 50 mg of powder (leaf dry matter), which was incubated with 1.5 mL of MCW solution (methanol, chloroform and water), in the proportion 12:5:3 (v:v^{-1}) at 20°C by 30 min and under agitation, subsequently, the homogenized was centrifuged at 10.000 g by 10 min at 20°C and the supernatant was removed. The sucrose quantification was carried out at 620 nm according the method of Van Handel (1968), and sucrose (Sigma chemicals) was used as a standard.

RESULTS

Relative water content (RWC)

The relative water content was significantly affected in

muruzizeiro plants under water stress during the 25 days of experiment (Figure 1A), with a decrease of 26.92% in this treatment, with a smaller amount of water assimilation when compared with the control plants and the obtained value of 78% in its relative water content.

Plant transpiration rate and stomatal conductance

The low water content changed the transpiration rates, given that plants subjected to water stress (Figure 1B) showed a significant reduction of 90% in their values, 0.27 µmol.m^{-2}.s^{-1} as compared to the control plants which obtained value of 2.83 µmol.m^{-2}.s^{-1}. Consequent to this decrease (Figure 1B), stomatal conductance also decreased (Figure 1C), presenting a significant reduction of 94.79% (0.025 mmol m^{-2}.s^{-1}) in the plants subjected to water stress as compared to the control plants (0.48 mmol.m^{-2}.s^{-1}).

Photosynthetic pigments in leaves

Photosynthetic pigments contents was decreased in plants under water deficit showing significant differences as compared to the control plants (Figure 2), corresponding to a decrease of 42.1% in chlorophyll a (1.1 mmol kg^{-1}MF), 50% in chlorophylls b (0.6 mmol kg^{-1}MF), 45.1% in total chlorophyll (1.7 mmol kg^{-1}MF) and 33.3% in carotenoids (1.2 mmol kg^{-1}MF) as compared to control plants that showed values of 9.0; 1.2; 3.1 and 1.8 mmol kg^{-1}MF, respectively.

Concentrations of starch

The starch concentrations was decreased in plants under water deficit causing significant differences as compared to the control plants (Figure 3A), the values in the starch concentrations in the leaves and roots of murucizeiro plants under water deficit were 0.17 and 0.04 µmol of GLU/gDM representing a decrease of 73.43 and 63.63%, respectively, as compared to leaves of the control plants (0.64 µmol of GLU/gDM) and roots (0.11 µmol of GLU/gDM).

Concentrations of total soluble carbohydrates

The total soluble carbohydrate content was increased in plants under water stress resulting to statistical difference as compared to the control plants (Figure 3B), the values were under water deficit of 11.21 mmol g^{-1}MS leaves for an increase of 63.87% in control plants (4.05 mmol g^{-1}MS) and the roots of 4.48 mmol g^{-1}MS, an increase of 39.5% in control plants (2.71 mmol g^{-1}MS).

Concentrations of sucrose

An increase in sucrose concentrations in the roots and
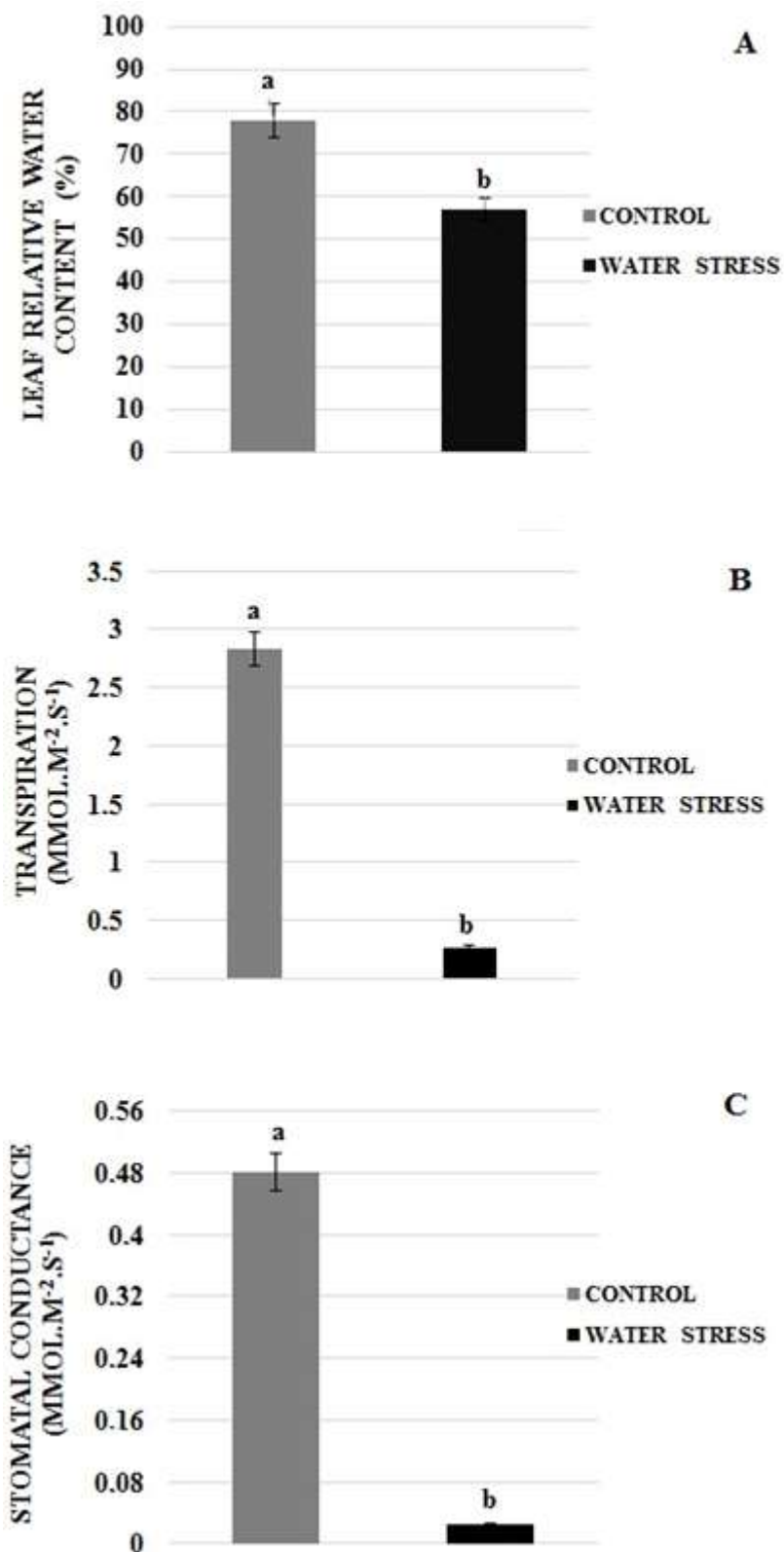
Figure 1. Leaf relative water content (A), transpiration (B) and stomatal conductance (C) in leaves of young plants of murucizeiro submitted during 25 days under water stress. The letters a and b show statistically significant differences between treatments which were compared by Tukey test at 5% probability. The bars represent the standard deviations of the means.
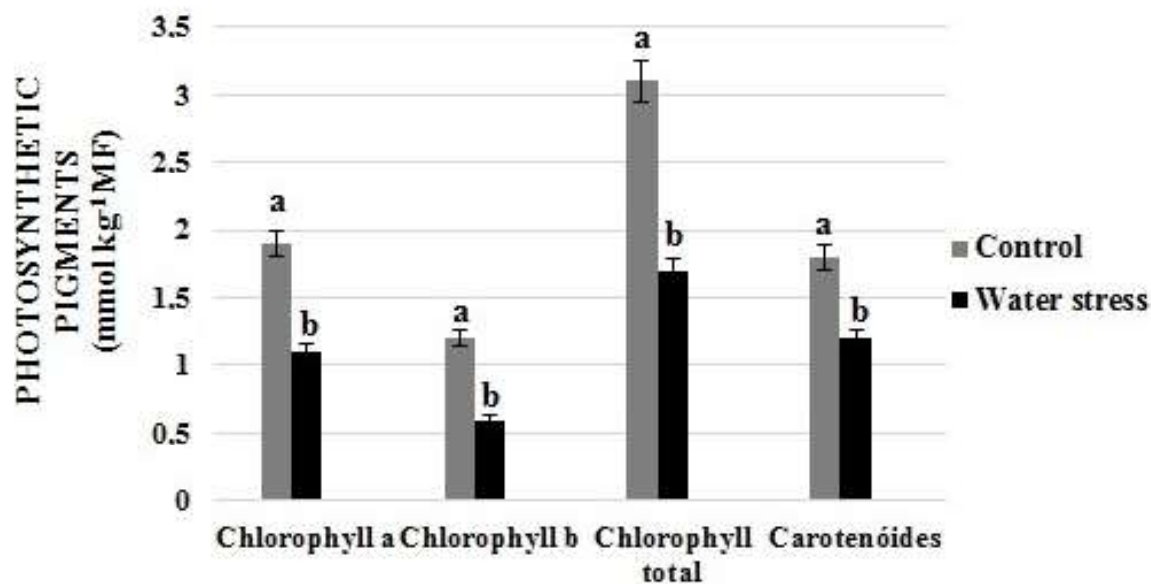
Figure 2. Photosynthetic pigments in leaves of young plants of murucizeiro submitted during 25 days under water stress. The letters a and b show statistically significant differences between treatments which were compared by Tukey test at 5% probability. The bars represent the standard deviations of the means.

leaves of plants subjected to water stress was observed (Figure 3C), the values found in the leaves were 16.98 and 24.45 mg sucrose g^{-1} MS in control plants and water deficit, respectively, with an increase of 64.73%. For the roots, there were 5.87 and 9.67 mg sucrose g^{-1} MS in plant control and drought stress, respectively, meaning an increase of 43.99%.

DISCUSSION

The decrease in the relative water content in leaf tissues (Figure 1A) is associated with water deficit in soil causing a decrease in the water balance of plants, promoting a reduction in cell turgor, reducing the quantity of water in xylem and increasing the tension in xylematic vessels, and making the plant to exerts a force required to absorb the soil water to be transported to the aerial part. With the absence of water in the soil, the hydraulic conductivity of the roots is reduced, leading to an inhibition of metabolic activity and the reduction in ATP production that ends restricting the power supply to the growth of the roots, causing a reduction in development and physiological processes of the plant (Oliveira, 2010; Molle, 2011). After these processes, according to Silva (2013) as the plants defense strategy, possibly increasing osmotically adjust their carbohydrate levels (Figure 3B) and sucrose (Figure 3C) to maintain the water absorption on soil colloids and continue with their metabolic processes. Similar results were found by Wang (2014) in leaves of rubber tree seedlings clone GT1, which showed a continual decrease

with the gravity of the obtained stress, resulting in nine days of water restriction, approximately 20% as compared to the control treatment.

As the water stress increases in the cells of the leaf mesophyll, there is a dehydration of this tissue reducing its water potential, leading to a decrease in gas exchange and consequently reducing photosynthesis. The decrease of transpiration can be linked to stomatal behavior over stress, which is an important mechanism for the survival of plants under water stress situations (Otto et al., 2013).

Similar results were found by Fu et al. (2010) on study of two species of poplar or aspen (*Populus euphratica* and *Populus russkii*), showing a reduction in gas exchange in different volumes of irrigation in a desert area. Increase in drought on the plant of murucizeiro, may have caused a reduction of photosynthesis which is not only a consequence of chloroplastid low levels, but the reduction in stomatal opening and diffusive restriction of CO_2, and also, the mechanisms of photochemical and biochemical steps (Dias and Brüggemann, 2010). The degradation of pigments possibly leads to a decrease in the photochemical step process. The decrease in availability of internal CO_2 and water loss through transpiration directly influence the chlorophyll fluorescence parameters, mainly associated with the ISP (Martinazzo et al., 2012). Another possible cause is leaf dehydration leading to a disruption of the membranes of the thylakoids, resulting in inactivation of electron transfer reactions, reducing the value of photosynthetic rate (Dias and Brüggemann, 2010). In the work performed by
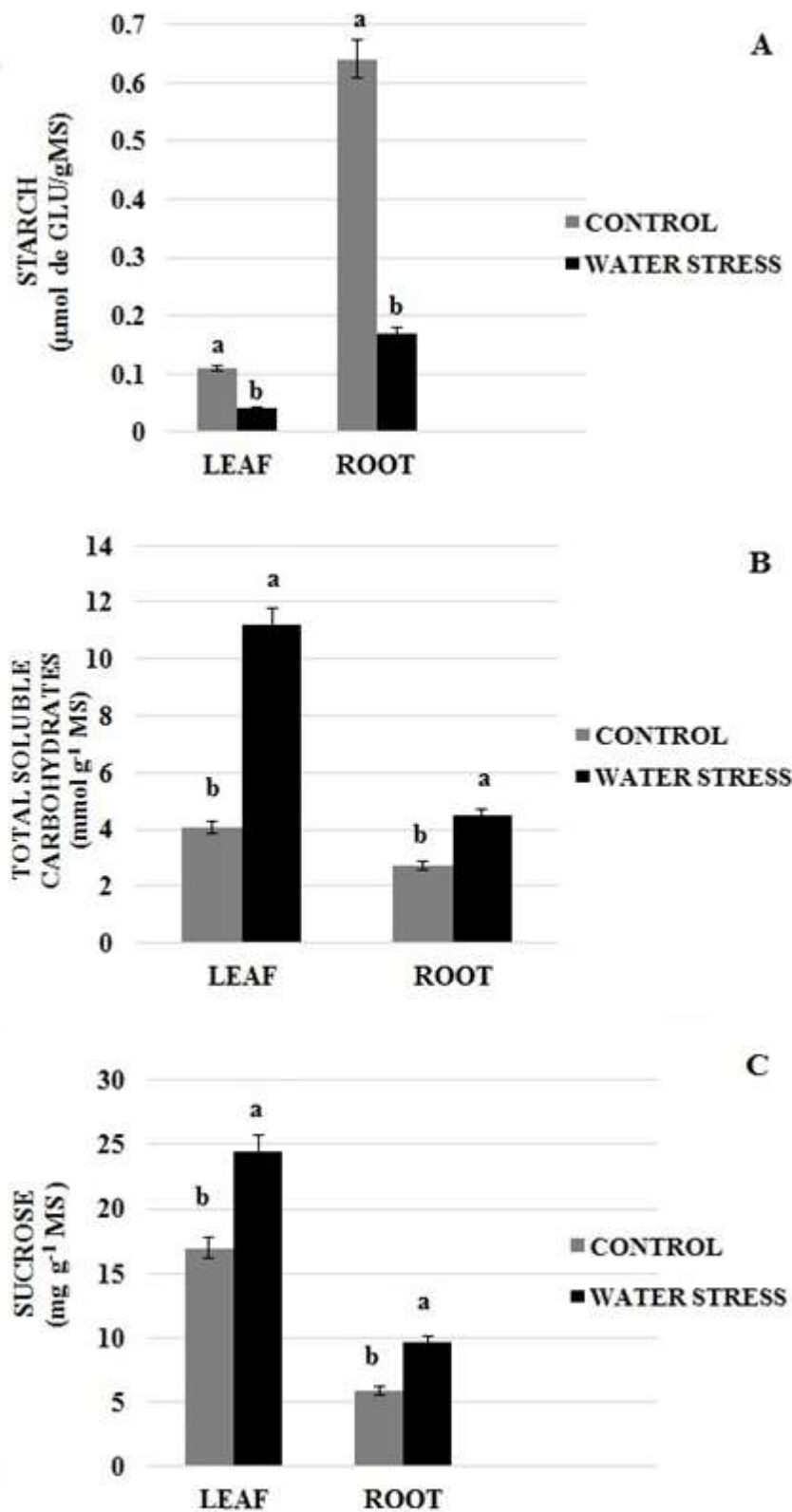
Figure 3. Starch (A), total soluble carbohydrates content (B) and sucrose (C) in leaves of young plants of murucizeiro submitted during 25 days under water stress. The letters a and b show statistically significant differences between treatments which were compared by Tukey test at 5% probability. The bars represent the standard deviations of the means.

Cavalcante (2013) through spectrophotometry analysis, showed that the leaves of *Jatropha curcas* L. under drought, cause a significant reduction in photosynthetic pigments content when compared with irrigated plants.

The decrease in starch levels is related to the function of acting as an osmoprotector, becoming soluble sugars in order to maintain the availability of energy for the plant and especially the influx of water, which occurs at a particular signal and resulting to increase in the synthesis of the amino acid (Silva, 2008). It has been observed in several studies that there is a relationship between the increase in the activity of enzymes responsible for the hydrolysis of starch after stomatal closure and inhibition of photosynthesis; there is an accumulation of sugars in plants subjected to low water availability (Silva et al., 2012). In analysis of physiological changes in coconut, Marinho et al. (2005) found that as a result of water deficit, the starch is degraded in the tissues that accumulate due to the action of the enzyme α and β amylase. The decrease in the quantity of starch is accompanied by some increase in the amount of soluble reducing sugars and it assists in the osmotic adjustment of the plant and consequent reduction in water potential. Similar results were observed by Silva et al. (2010) checking physiological changes and drought tolerance in *Conilon coffee* clones (*Coffea canephora*), which decreased by 70% in starch content. At present, in plant tangerine and acid lime when subjected to water deficit remained constant throughout the stress, and there is a significant reduction when they were irrigated.

The increase of carbohydrates in plants under water stress (Figure 3B) occurred as a form tolerate deficiency, which changed its osmotic adjustment process in metabolism, thus reducing their potential osmotic in order to maintain the hydrated plant, preventing dehydration of the tissues (Souza et al., 2013). According to Nogueira (2015), this increase in carbohydrate concentrations induces a greater protective of biomembranes action that can be degraded in this condition. According to Lima (2015), the increase in the concentration of carbohydrate may possibly be linked to increased abscisic acid in the leaves, and at low water conditions in the soil, increase in the relation root/shoot of the plant together with the effect of inducing ABA the closing of the stomata, helping the plant to face water stress by decreasing their photosynthetic capacity, which possibly reflects a lower accumulation of starch and can signal the need for increased levels of sucrose and carbohydrates. These results corroborate those founded by Castro et al. (2007) who observed an increase of 323.15% in the concentration of soluble carbohydrates in plants of teak (*Tectona grandis* L. f.) subjected to 9 days of water deficit and with the results obtained by Rivas et al. (2013) showing increases in concentration of carbohydrates in *Moringa oleifera* leaves under drought for 10 days.

The possible answer to the increase of sucrose in plants under drought is probably the hydrolysis of sucrose to hexoses release used in the osmotic adjustment processes, which can link to water molecules on the leaf to maintain the water level in leaf organ (Ashraf et al., 2011). Another possible answer is in the activation of enzymesα and β-amylase breaking starch molecules and converting it to sucrose, preventing dehydration and being a source of energy for active cells under water deficit conditions (Gaupels et al., 2011). Silva et al. (2010) observed contrasting results in *Conilon coffee* clones (*Coffea canephora*) in severe water stress condition, where the levels of starch and sucrose decreased.

Conclusion

The suspension of irrigation for 25 days was enough to change and to promote a decrease in the metabolic routes of young plants of muruci, reducing the relative water content, transpiration, stomatal conductance, photosynthetic pigments and starch, however, increasing the total soluble carbohydrate content and sucrose, indicating that this plant can tolerate some periods of water stress. These changes indicate that these plants are susceptible to soils with low water availability.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Carbon dioxide enrichment studies: Current knowledge and trends in plant responses

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Atmospheric CO_2 concentrations are increasing and studies about the impact on plant species' responses are on the rise. Unfortunately the wide range of variations in the published data is a concern when it comes to usefulness and application. Simple descriptive analyses on the published results are needed to make sense of the overall trends in plant responses to CO_2 enrichment. In the present report, 90 articles were the basis of a 395-entry database analyzed for general trends on how terrestrial plant species were reported to have responded to CO_2 fertilization. The CO_2 concentrations that were studied range between 440 ppm and 900 ppm. The results revealed that 238 and 40 entries dealt with C3 and C4 pathways; respectively. A significant regression analysis (P=0.111), between CO_2 levels and average response, was detected for C3 plant species only. This highlights the need for more studies on C4 plants which constitute an important component of primary productivity on terrestrial ecosystems. Of the total entries into the database C4 plants had the highest average magnitude of response (27.1±0.4%). At the functional group level, woody species were reported to have the highest average response (33.5±0.4%). Salinity, nutrient, defoliation and water stresses had average responses of 15.7±0.2, 12.3±0.3, 10.80±0.2 and 7.6±0.2%, respectively. In short, the above simplistic descriptive approach places much of what was studied in relation to plant responses to CO_2 fertilization into a practical perspective. Furthermore, detailed periodic analyses, including meta-analyses, are therefore highly recommended in order to summarize the body of published data, suggest up-to-date interpretations and make it available for practical use.

Key words: Stress, CO_2, floral response.

INTRODUCTION

Atmospheric CO_2 concentrations have increased by more than a third and are expected to rise because of fossil fuel and changes in land usage (Houghton, 2003). Globally as well as locally, this has led to what is currently referred to CO_2 fertilization. The impact of CO_2 enrichment studies has become better understood with the advances in the available technology dealing with field as well as greenhouse experimentations. The

variations within the published data are the consequences of differences in experimental protocols adopted, plant species used, ecosystems covered, biotic and abiotic stresses applied and CO_2 concentrations tested. Added to these in-consistencies, are the complexity of scales, both temporal and spatial, and interpretations of research outcomes. Consequently, data comparisons are becoming challenging and may lead to various, and sometimes contradictory, interpretations and uses. The increase in plant production was reported to be negative (Newman et al., 1999), neutral (Ghannoum et al., 1997) or positive (Kinsman et al., 1997). Carbon dioxide enrichment trials also have a range of setups such as Free Air Carbon Dioxide protocol, also known as FACE sys- tem, (Idso and Idso, 1994), open top chambers (Kimball, 1992), controlled growth chambers (Cave et al., 1981) and even locally made chambers erected within greenhouses (Ksiksi and Youssef, 2010).

The variety of studies relating to CO_2 enrichment and plant responses has led to major challenges facing collective interpretation of results from CO_2 trials. Grass species such as *Agrostis curtisii* have been reported to have no response to CO_2 enrichment (Norton et al., 1999), while other grass species (*Lolium perenne*) were reported to have benefited by about 20% from CO_2 enrichment. A forb like *Hemizonia congesta* has been reported to benefit from CO_2 by about 69.6% (Edwards et al., 2001). Different photosynthetic pathways (eg. C3 and C4) have also been assessed with varied responses (Ebersberger et al., 2003; Mateos-Naranjo et al., 2008).

Ecosystem variations are an added complexity when trying to compare results from the variety of the published body of information on CO_2 en-richment. Studies have been reported about temperate (Kammann et al., 2005), humid (Ebersberger et al., 2003), semi-arid (Xu and Zhou, 2008) and Mediterranean (Roumet et al., 2000) ecosystems, with a wide range of re- sponses. The CO_2 concentrations that have been studied were between 440 and 900 ppm.

In this study, we conducted an analysis of 90 articles published between 1994 and 2010. Analyses included simple descriptive information on proportions of responses as well species functional groups in addition to correlation and regression analyses. The focus was on overall morphological responses of each plant species. The overall aim was to make sense of what was found in order to contribute toward future research directions as well as modeling exercises relating to the field of CO_2 enrichment and plant responses.

MATERIALS AND METHODS

Data sources

A total of 90 published papers was included in this database. The years of publications ranged between 1994 and 2010. Each of the articles included in the database has more than one entry because

of different species, functional groups and/or photosynthetic pathways reported. Therefore, a total of 395 entries was analyzed in the present attempt to understand the practical implications of (CO_2 enrichment studies. Data analyzed in this report were extracted from the published data (tables and graphs) within each of the database articles. Each data entry contained the following variables: photosynthetic pathway (C3, C4 and unknown), plant functional groups (forbs, grasses, legumes, mixed and woody), biotic/abiotic stress (CO_2 (defoliation, nutrient, salinity, temperature and water) and the studied ecosystem (alpine, dry, humid, Mediterranean, spring, temperate and semi-arid). Studies dealing with Cadmium stress were not included in the summary. The magnitude of response was another important variable which was entered as an average plant species response for a specific database entry. Consequently, an average response was calculated for each functional group and photosynthetic pathway based on specific species magnitude of responses. Specific ecosystem differences were not included as the sample data covering many of the ecosystems are not enough to run a robust analysis.

Standard deviations of each group is also included in the graphs (vertical bars). Some low SD values are an indication of the limited number of articles within the studied category, in addition to low variations among the different entries. Magnitude of response above 200% were not included in the analysis. Simply because of the high variability and most of these data points were identified as outliers using Statistical Package for the Social Sciences or SPSS (Norusis, 2010). It is also worth noting that the listed article published by (Wand et al., 1999) is not included in the analysis. It was an article dealing with meta-analysis of published data.

Descriptive and analytical approach

SPSS was also used to perform a correlation analysis using Pearson correlation coefficient between the magnitude of responses and the level of CO_2 under which the study was conducted. Linear regression analyses were performed between CO_2 fertilizing levels and average magnitude of responses within each photosynthetic pathway (that is, C3, C4 and unknown). It was decided to report the exact P values to allow the reader to make their own judgment on the relevance of the statistical significance of the regression tests. The hypothesis was meant to address the question if the increase in the CO_2 content was correlated with the magnitude of plant growth, within each photosynthetic pathway (C3, C4 and unknown). The analyses did not show any significant correlation for all 3 pathways at $P \geq 0.05$.

RESULTS

The database included 90 references with a total of 395 entries. As each article has multiple entries for different species, functional groups and/or photosynthetic path-ways. Averages are reported in this section, including standard deviations. Please refer to the experimental section below for more details on the articles and resulting database included in this analysis.

Out of the total entries, the results revealed 238 and 40 entries for C3 and C4 pathways; respectively (Figure 1). All unidentified photosynthetic pathways were grouped into a class of unknowns with a total of 117 entries grouped under this rubrique. Figure 2 summarizes the magnitude of response of the different photo- synthetic pathways. Of the total entries into the database. C4 plants
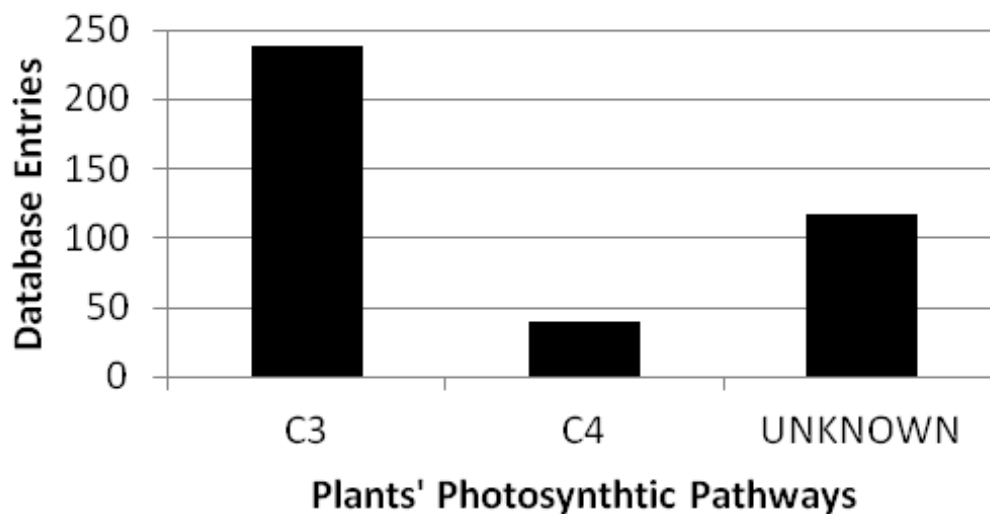
Figure 1. Variations of the different database entries from 90 publications relating to plant responses to $_{CO2}$ fertilization for C3, C4 and unknown photosynthetic pathways.
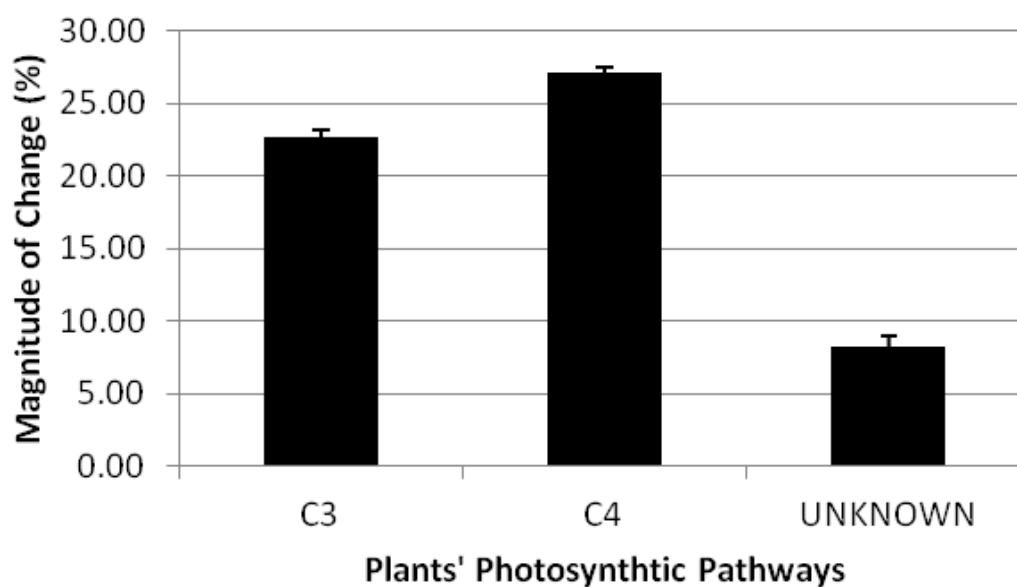
Figure 2. Magnitude of change in in published studies relating to plant responses to CO_2 fertilization for C3, C4 and unknown photosynthetic pathways. Vertical bars show Standard Deviation.

had an average magnitude of response of 27.1±0.4%. C3 plant species had an average growth response of 22.7 ±0.4% while 8.2±0.8% was the response attributed to unknown pathways of the species included in the database. For comparison purposes, the average response across pathways was 19.3 ±0.5%.

Figure 3 presents the average response for each of the studied plant functional groups. Studies with woody species reported the highest magnitude of plant responses (33.5±0.4%). Legumes were reported to have had an average magnitude of plant response of

31.4±0.5%. Forb and grass species were reported to have a growth response of 23.8±0.7% and 16.9±0.5%; respectively. Mixed species functional group had the lowest reported response of -1.25±0.3%.

Figure 4 shows the summary of the plant species responses to CO_2 enrichment for various stress factors. Among the experimental factors or stresses studied in the reviewed articles, CO_2 alone was reported to have the highest magnitude of response of about 20.4±0.6%. Temperature stress, coupled with CO_2 enrichment, had the lowest response of about 5.2 ± 0.1%. Salinity,

Figure 3. Magnitude of change in published studies relating to plant responses to CO_2 fertilization for different plant functional groups. Vertical bars show Standard Deviation.
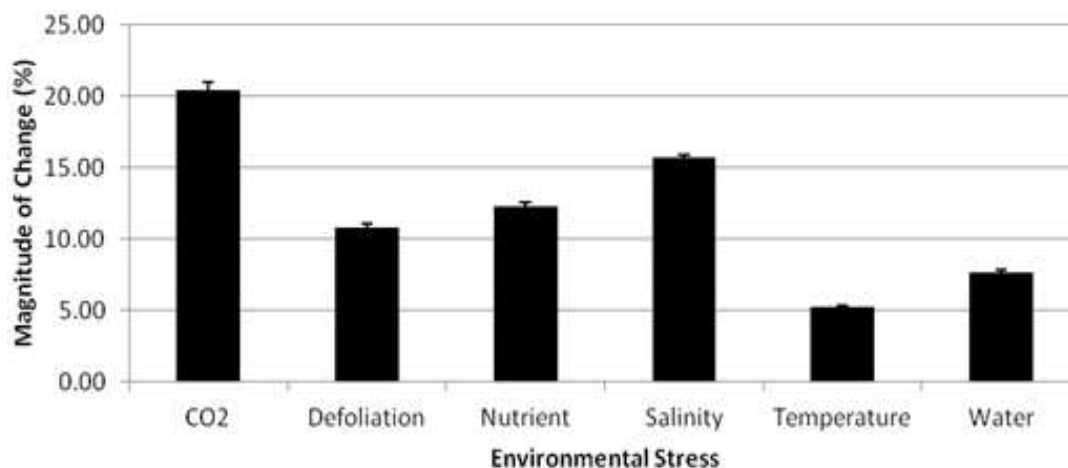
Figure 4. Magnitude of change in published studies relating to plant responses to CO_2 fertilization for C3, C4 and unknown photosynthetic pathways. Vertical bars show Standard Deviation.

nutrient, defoliation and water stresses had average responses of 15.7±0.2%, 12.3±0.3%, 10.80 ±0.2% and 7.6±0.2%; respectively.

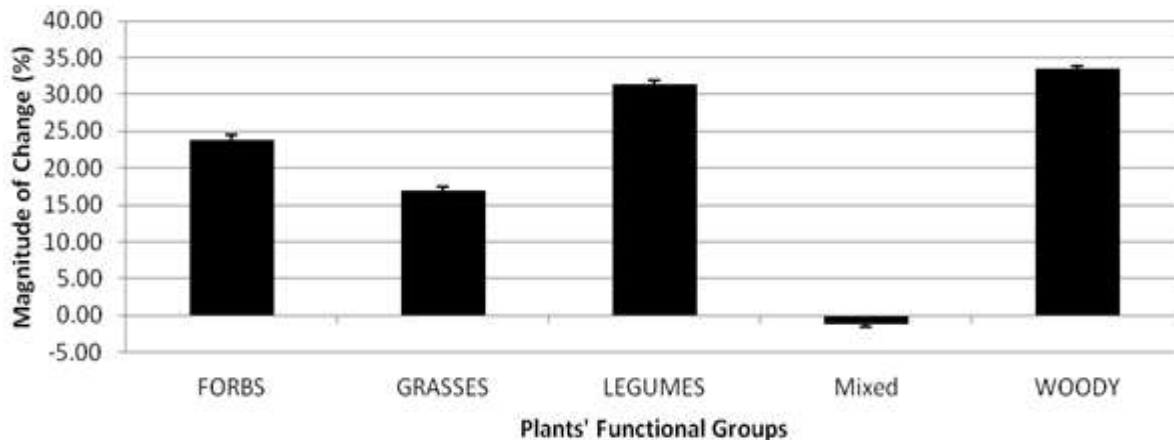Figure 5 presents the average plant responses within the various terrestrial ecosystems for studies dealing with CO_2 fertilization. The highest average response was reported for alpine ecosystems (187 ± 0.2%) while the second highest response was for dry ecosystems (126.3 ± 0.2%). The average magnitude of plant responses within humid and mediterranean ecosystems was 93±0.04% and 71.5±0.1%; respectively. Negative reponses, however, were reported for semi-arid and temperate ecosystems as -32.2 ±0.02% and -141.2 ±1.6%; respectively. Article number 38 (THÜRIG et al., 2003) was not included in the report as it was discovered that it dealt with a spring ecosystem, not considered a terrestrial ecosystem, in Switzerland.

DISCUSSION

Multiple factors interact under CO_2 enrichment, or fertilization, and simple predictions are to be adopted to foresee future projections at regional and global scales. Sophisticated modeling and experimentation techniques have come a long way in studying CO_2 fertilization (Norby and Luo, 2004). Moreover, interactions between CO_2 and biotic/abiotic stresses are to be simplified in order to predict possible outcomes at the species, population and ecosystem levels. Especially that extreme variations in the published data are a concern during application and modeling. Plant species groups responded differently to CO_2 enrichment (Reich et al., 2001). C4 plant species have been reported to benefit from CO_2 fertilization (Ghannoum et al., 1997). While other reports stated reduction in biomass production for C4 plants (Reich et
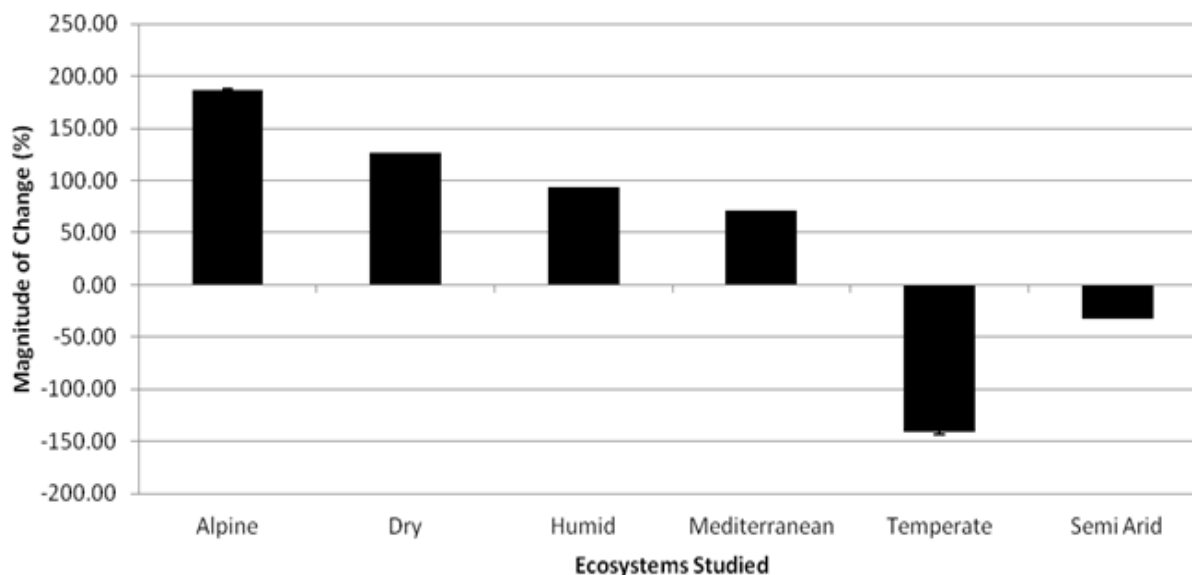
Figure 5. Magnitude of change in published studies relating to plant responses to CO_2 fertilization for various terrestrial ecosystems. Vertical bars show Standard Deviation.

al., 2001). Classifications based on functional groups may be useful but not enough to assess plant and ecosystem responses to CO_2 enrichment (Reich et al., 2001). The present 90-article assessment revealed a high bias for C3 plant species, against those with C4 photosynthetic pathways. This high-lights the need for more studies on C4 plants which constitute an important majority of primary production within terrestrial ecosystems. On average, C4 plants were reported to have growth responses of about 27.1 ±0.4%. At the functional group level, woody species had the highest magnitude of plant responses (33.5 ±0.4%), while salinity as a stress tested under CO_2 enrichment conditions – led to an average response of 15.7±0.2%. The regression analyses revealed a negative predictive power (P=0.111) between CO_2 levels and magnitude of C3 plant responses. The regression results were not significant for C4 plants and for all other plant species grouped as unknown. The above attempt places much of what was studied in relation to plant responses to CO_2 enrichment (1994 to 2010) into an understandable level which can contribute in directing future research endeavours in the field of CO_2 enrichment and plant responses. It is also believed that much of what has been reported here could be incorporated into simplistic predictive modeling of CO_2 enrichment impact on terrestrial ecosystems, functional plant groups and floral species. This does not lessen the importance of deeper and more sophisticated analyses, such as those using meta analyses, to summarize the body of published data and make it useful and practical. But whichever analysis we adopt, periodic assessments, every 12 to 15 years, are to be conducted in order to keep up with the body of research into CO_2 fertilization

and terrestrial plant responses.

In short, there are unbalances in the published data on CO_2 responses of C3 vs C4 species. Stresses such as salinity, nutrient, defoliation and water have not been appropriately studied too.

Conflict of Interests

The authors have not declared any conflict of interests.

Conflict of interests

The author has not declared any conflict of interest.

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Full Length Research Paper

Availability and uptake of P from organic and in organic sources of P in teak (*Tectona grandis*) using radio tracer technique

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Pot culture experiments were conducted on two bench mark soil series of Kerala (varying in available P status) at the Radiotracer Laboratory, Kerala Agricultural University, with the objectives of studying the effect of organic and inorganic sources of P on the growth of teak seedlings, uptake of P, percent P derived from fertilizer (% Pdff), P use efficiency (PUE) and A value using ^{32}P. The treatments consisted of combination of four levels of weed compost (0, 100, 150 and 200 g pot^{-1}) as organic source and three levels of inorganic P (4, 8 and 16 mg P kg^{-1}) in the form of KH_2PO_4. The experiment was laid out by adopting a factorial completely randomized block design. Results revealed that in both soils with varying levels of P, application of inorganic P at the rate of 4 mg kg^{-1} increased % Pdff and P use efficiency compared to higher levels of P application. Combined application of different levels of compost with inorganic P at the rate of 4 mg kg^{-1} resulted in significant improvement in % Pdff and P use efficiency. But shoot biomass, total P uptake and A value increased with increasing levels of inorganic P. Combined application of different levels of compost with inorganic P at 16 mg kg^{-1} also significantly improved shoot biomass, total P uptake and A value. The results in general indicated that combined application of compost with inorganic P fertilizer was more effective than application of inorganic fertilizer alone in both soils for enhanced absorption and use efficiency of P. Thus the integrated use of fertilizer and manure will enhance the productivity of teak plantation.

Key words: Phosphorus, ^{32}P, % Pdff, P use efficiency, teak.

INTRODUCTION

Vigorous teak growth requires fertile, deep, well drained soils (Kollert and Cherubini, 2012). Teak will also grow on degraded sites (Osemeobo, 1989), where it serve to rehabilitate the soil, produce timber and provide other products and services (Roshetko et al., 2013). On infertile and impoverished soil teak will not achieve its upper growth potentials. Plantations of teak have a long history in India, especially in the state of Kerala. The first teak plantation was established in Nilambur as early as 1836. However by second and third rotation the productivity of plantations stated to decrease. This decrease in productivity, to a large extend was attributed to soil

deterioration. (Rugmini and Balagopalan, 2001; Geetha and Balagopalan, 2009). In view of low availability of land to meet the growing demand for timber the only option before teak growers is to increase productivity of per unit of land. The problems of soil degradation can be remedied to a certain extend by application of fertilizers and organic amendments. The importance of application of organic amendments to impart fertility to degrading soils has been realized not only in the agricultural sector but also in plantation forestry. Among the major nutrients, availability and absorption of phosphorus (P) in degraded lateritic soils of teak plantations in Kerala has become a major constraint due to low pH, high P fixation capacity and low to medium available P status (Suresh Kumar, 1999 and Geetha et al., 2010). Phosphatic fertilizers applied to such soils are subjected to large fixation in the soil reducing the availability to the fertilized crop. Integrated use of chemical fertilizers along with organic amendments is found to be a viable option to improve the use efficiency of applied nutrients. Accelerating prices of chemical fertilizers due to withdrawal of subsidies also created a need for alternate P sources and increasing the efficiency of phosphatic fertilizers. Even though, lots of information is available on the combined use of fertilizers and organic amendments for improving crop yields and soil fertility, the direct quantification of P extracted by teak seedlings from applied fertilizer alone and in combination with organic amendments has not yet been studied. Therefore, this study mainly intends to use ^{32}P as tracer to estimate quantitatively the P absorption and P use efficiency from applied inorganic source and the synergistic effect of organic amendment on P availability in two soil series of Kerala differing in available P status.

MATERIALS AND METHODS

In order to achieve this objective, pot culture experiments were conducted using ^{32}P to find out the rate of absorption and nutrient use efficiency in teak seedlings by growing them in two soil series in which teak is widely grown *viz.,* Velappaya and Panikkulam, having low and medium levels of available P respectively. The experiment was conducted in a green house at Radiotracer Laboratory of Kerala Agricultural University during 2007-2008 mainly to study the absorption and nutrient use efficiency of P by teak seedlings using ^{32}P labelled KH_2PO_4.

Collection of soils

Surface soils (0 to 15 cm) were collected from two bench mark soil series of Thrissur District *viz.,* Velappaya and Panikkulam from Killannoor and Panjal panchayaths respectively. Velappaya soil series was with low available P status while Panikkulam was with medium level. Soils were air dried and sieved through 2 mm sieve for laboratory analysis as well as for pot culture studies.

Green house experiment

Air dried soils of the soil series mentioned above were used to fill 36 plastic pots of uniform size and 1 kg capacity. Weed compost

containing 2.3% N, 1.23% P and 1.83% K was air dried, sieved through 2 mm sieve and used as organic amendment in the experiment. The treatments consisted of combination of four levels of weed compost (0, 100, 150 and 200 g pot^{-1}) and three levels of inorganic P (4, 8 and 16 mg P kg^{-1}). The experiment was laid out by adopting a factorial Completely Randomized Block Design and continued up to 30 days.

Organic P: 4 levels
Inorganic P: 3 levels
Total treatment combination : 4 × 3 = 12
Replication: 3
Total no. of pots in one soil series: 12 × 3 = 36
No. of soil series: 2
Total no. of pots in the experiment: 36 × 2 = 72

Weed compost was applied in each pot as per the treatment and mixed with the soil to a uniform consistence, one week prior to the planting of teak seedlings. Nitrogen and potassium were applied in the form of urea and muriate of potash as per Package of Practices Recommendations (KAU, 2007). Teak seedlings were raised in the nursery for three months and then transplanted to the pot. Each pot was planted with two seedlings.

The isotope ^{32}P (t1/2- 14.3 days; E max: 1.71 Mev) obtained as ^{32}P in dilute hydrochloric acid (HCl) medium from the Board of Radiation and Isotope Technology (BRIT), Mumbai was used for the study. The source of inorganic P, KH_2PO_4 was labelled with the above ^{32}P so as to get a specific activity of 2.0 mCi / mg of P. This solution was used as the source of inorganic P. This solution was placed in band around seedlings. Regular watering was done daily to maintain optimum soil moisture.

Seedlings were maintained in the pots for one month. Plant samples collected 30 days after planting were oven dried at 65°C ± 5 to a constant weight, powdered and kept ready for analysis. Samples were then digested with diacid mixture nitric- perchloric acid (2:1) and P in this solution was determined by vanado-molybdate yellow colour method (Piper, 1966) and the intensity was measured in a spectrophotometer.

Radio assay

The radioactive P in the above digest was determined following Cerenkov counting (Wahid et al., 1985). The counts per minute (cpm) of ^{32}P in all the samples were recorded and corrected for back ground and decay. The specific activity in the applied fertilizer and that in plant samples were computed using counts rates (cpm g^{-1}) in the fertilizer and plant samples. The data from radio assay was used to compute percent P derived from fertilizer (% Pdff), percent P derived from soil (% Pdfs), A values and P use efficiency as suggested by Fried and Dean (1952). A value is the availability index of the nutrient from soil. Banded application of fertilizer ensures accurate A values.

$$\% \ Pdff = \frac{\text{Specific activity in plant sample}}{\text{Specific activity in fertilizer}} \times 100$$

$$\% \ Pdfs = 100 - \% \ Pdff$$

$$\text{Uptake of P from fertilizer (mg P pot}^{-1}) = \frac{\% \ Pdff}{100} \times \text{Total P uptake (mg P pot}^{-1})$$

$$\text{A value (mg P 100 g}^{-1} \text{ soil)} = \frac{\% \ Pdfs}{\% \ Pdff} \times \text{P applied (mg P 100 g}^{-1} \text{ soil)}$$

Table 1. Effect of combined application of inorganic P and compost on % Pdff by teak seedlings grown in soils with varying levels of P.

Treatment	Inorganic P (mgkg^{-1})		
Compost (g pot^{-1})	4	8	16
Velappaya series (low P)			
0	9.51^b	8.02^c	5.56^d
100	10.46^a	6.05^d	5.74^d
150	9.33^b	6.13^d	6.39^d
200	7.53^c	5.92^d	5.75^d
F Value		9.838**	
Panikkulam series (medium P)			
0	6.24^{ab}	5.11^{cd}	3.98^{efg}
100	6.67^a	4.35^{def}	3.13^g
150	5.56^{bc}	3.88^{efg}	3.17^g
200	4.66^{cde}	3.90^{efg}	3.46^{fg}
F Value		2.764*	

Means with same letter as super script are homogeneous, ns- nonsignificance, ** - significant at P= 0.01,*-significant at P= 0.05.

$$P \text{ use efficiency (\%) (PUE)} = \frac{\% \text{ Pdff} \times \text{Total P uptake (mg P pot}^{-1})}{\text{Fertilizer P added (mg pot}^{-1})}$$

Statistical analysis

The data obtained in the experiment was subjected to analysis of variance using the statistical package SPSS (Norusis, 1988). Mean comparisons between treatment means were done using Duncan Multiple Range Test (DMRT).

RESULTS

Results obtained were % P derived from fertilizer (% Pdff), 'A' value, P use efficiency, shoot biomass and uptake of P. All results are discussed below and summarized in Tables 1 to 5.

Percent P derived from fertilizer (% Pdff)

Statistical analysis of % Pdff data (Table 1) revealed significant interaction between two factors, inorganic P and compost, as well as significant variation between treatments in the both soils series.

In low P soils, % Pdff varied from 5.56 to 10.46. Application of inorganic P at 4 mg kg^{-1} along with compost at 100 g pot^{-1} resulted in maximum % Pdff in this soil. Data also indicated a general decreasing trend in % Pdff with increase in the rate of inorganic P applied.

In medium P soils, % Pdff varied from 3.13 to 6.67. As seen in low P soil, application of inorganic P at 4 mg kg^{-1}

along with compost at 100 g pot^{-1} resulted in maximum % Pdff and this was on par with the treatment P at 4 mg kg^{-1} alone. Data also indicated a general decreasing trend in % Pdff with increase in the rate of inorganic P applied.

A value

Statistical analysis of 'A' value data (Table 2) revealed significant interaction between two factors inorganic P and compost, as well as significant variation between treatments in both the soil series.

In both low and medium P soils, treatments varied significantly with respect to A value. In low P soil, A value varied from 3.43 to 27.28. Application of inorganic P at 16 mg kg^{-1} without compost resulted in maximum A value and this was on par with the treatment P at 16 mg kg^{-1} with 100 and 200 g pot^{-1} of compost. In general, A value increased with every successive rate of inorganic P applied. In medium P soil, A value ranged from 5.65 to 49.81. Significantly higher A value was recorded in the treatment inorganic P at 16 mg kg^{-1} along with 100 and 150 g compost.

Phosphorus use efficiency

Statistical analysis of the P use efficiency data (Table 3) revealed significant interaction between two factors, inorganic P and compost, as well as significant variation between treatments in both the soil series.

In low P soil, P use efficiency ranged from 0.76 to 2.67. Application of inorganic P at 4 mg kg^{-1} along with compost at 200, 150 and 100 g pot^{-1} recorded significantly

Table 2. Influence of combined application of inorganic P and compost on A value (mg 100 g $^{-1}$ of soil) of soils with varying level of P.

Treatment	Inorganic P (mgkg^{-1})		
Compost (g pot^{-1})	4	8	16
	Velappaya series (low P)		
0	3.82^d	9.24^d	27.28^a
100	3.43^d	12.56^c	26.43^a
150	3.89^d	12.31^c	23.51^b
200	4.92^d	12.71^c	26.23^a
F Value		3.940**	
	Panikkulam series (medium P)		
0	6.10^g	14.90^f	38.61^c
100	5.65^g	17.66^{ef}	49.81^a
150	6.80^g	19.83^d	49.03^a
200	8.58^g	19.70^d	44.71^b
F Value		5.422**	

Means with similar letter as superscript are homogeneous, ns- nonsignificant, ** - significant at P= 0.01,*- significant at P= 0.05.

Table 3. Influence of combined application of inorganic P and compost on phosphorus use efficiency (%) of soils with varying level of P.

Treatment	Inorganic P (mgkg^{-1})		
Compost (g pot^{-1})	4	8	16
	Velappaya series (low P)		
0	1.82^b	1.08^{ef}	0.83^h
100	2.50^a	1.22^{de}	0.76^h
150	2.67^a	1.41^{cd}	0.93^{gh}
200	2.62^a	1.44^c	0.94^{gh}
F Value		8.467**	
	Panikkulam series (medium P)		
0	1.68^b	1.30^c	0.63^f
100	1.95^a	0.86^{de}	0.64^{ef}
150	1.88^{ab}	0.88^d	0.58^f
200	1.91^a	1.18^c	0.70^{def}
F Value		4.988**	

Means with similar letter as superscript are homogeneous, ns-nonsignificant, ** -significant at P= 0.01, *-significant at P= 0.05.

high P use efficiency compared to other treatments. In medium P soil, P use efficiency ranged from 0.58 to 1.95. As seen in low P soil, application of inorganic P at 4 mg pot^{-1} along with compost at 200 and 100 g pot^{-1} resulted in maximum P use efficiency. While, P at 4 mg pot^{-1} along with compost at 150 g pot^{-1} was on par with the above treatment. Similarly, application of higher rates of inorganic P also resulted in a decrease of P use efficiency in this soil.

Shoot biomass

Statistical analysis on shoot biomass data (Table 4) revealed significant interaction between two factors, inorganic P and compost, as well as significant variation between treatments in both the soil series.

In low P soil, significantly higher biomass was obtained due to the application of higher level of inorganic P (16 mg kg^{-1}). Even though shoot biomass significantly

Table 4. Influence of combined application of inorganic P and compost on shoot biomass of teak seedlings (g pot^{-1}).

Treatment	Inorganic P levels (mgkg$^{-1)}$)		
Compost (g pot^{-1})	4	8	16
	Velappaya series (low P)		
0	3.23^{d}	3.40^{cd}	4.44^{ab}
100	3.29^{d}	3.67^{c}	4.11^{b}
150	3.37^{cd}	4.10^{b}	4.15^{ab}
200	3.46^{cd}	4.30^{ab}	4.51^{a}
F Value		4.013**	
	Panikkulam series (medium P)		
0	4.49^{cde}	4.81^{c}	4.56^{cd}
100	4.70^{c}	4.16^{e}	5.81^{a}
150	4.84^{c}	4.29^{de}	5.24^{b}
200	4.62^{cd}	5.36^{b}	5.24^{b}
F Value		20.230**	

Means with similar letter as superscript are homogeneous, ns-nonsignificant, **- significant at P= 0.01, *-significant at P= 0.05.

Table 5. Influence of combined application of inorganic P and compost on uptake of P (mg P kg^{-1}) of teak seedlings.

Treatment	Inorganic P levels (mgkg$^{-1)}$)		
Compost (g pot^{-1})	4	8	16
	Velappaya series (low P)		
0	0.77^{j}	1.08^{i}	2.40^{ab}
100	0.96^{ij}	1.64^{fg}	2.12^{cd}
150	1.15^{hi}	1.84^{ef}	2.34^{bc}
200	1.39^{gh}	1.95^{e}	2.63^{a}
F Value		4.839**	
	Panikkulam series (medium P)		
0	1.09^{h}	2.03^{d}	2.54^{c}
100	1.18^{gh}	1.58^{f}	3.27^{a}
150	1.36^{g}	1.81^{e}	2.95^{b}
200	1.66^{ef}	2.43^{c}	3.25^{a}
F Value		14.138**	

Means with similar letter as superscript are homogeneous, ns- nonsignificant, ** - significant at P= 0.01, *-significant at P= 0.05.

increased with increased rates of compost at lower levels of inorganic P (4 and 8 mg kg^{-1}), the data were on par at higher level of P (16 mg kg^{-1}) irrespective of the quantity of compost applied. The results in general revealed that application of high levels of inorganic P alone and in combination with compost increased shoot biomass.

Compared to low P soils, shoot biomass was significantly higher in medium P soils and the data ranged from 4.49 to 5.81 g pot^{-1}. Here also significantly higher biomass was obtained due to application of higher level of inorganic P (16 mg kg^{-1}). Unlike in the case of medium

P, the compost applied at 100 g pot^{-1} was found significantly superior at higher level of P.

Uptake of P

Statistical analysis of P uptake data by teak plants revealed significant interaction between compost and inorganic P in both the soil series (Table 5). Significant variation between treatments was also observed in both soils.

In low P soil, uptake of P ranged from 0.77 to 2.63 mg kg^{-1}.

Uptake of P increased with higher application rates of inorganic P as well as compost. Maximum uptake of P was observed in the treatment inorganic P at 16 mg kg^{-1} + 200 g pot^{-1} of compost. Application of inorganic P alone at 16 mg kg^{-1} also resulted in higher uptake and this was on par with the treatment (16 mg kg^{-1} + 200 g pot^{-1} of compost) which gave maximum uptake. In medium P soil, uptake of P varied from 1.09 to 3.27 mg kg^{-1}. As observed in low P soil, uptake of P was increasing with increase in the rate of inorganic P as well as compost. Maximum uptake of P was in the treatments with higher rate of inorganic P (16 mg pot^{-1}) along with compost at 100 and 200 g pot^{-1}. The higher shoot biomass and uptake of P in medium P soil could be attributed to the relatively higher soil pH coupled with higher quantity of plant extractable P in soil.

DISCUSSION

Percent P derived from fertilizer (% Pdff)

In low P soils a decreasing trend in % Pdff with increase in the rate of inorganic P was due to the immediate adsorption of applied P by the Al and Fe hydrous oxides present in the soils. These hydroxides have the ability to absorb P on their surfaces and thus much of the added P is 'fixed' instead of being made available for crop use (Akinrinde, 2006). It was also noted that application of compost at higher levels resulted in a decrease of % Pdff at all levels of inorganic P. This would mean that higher levels of compost application resulted in more P absorption from soil pool rather than from fertilizer pool. It is assumed that rather than working directly by plants as a nutrient source, compost improves the soil properties and makes more P available from the native soil pool. The above results established the fact that teak plants showed a preference for native P over the fertilizer P especially with compost application. Comparatively low values of % Pdff observed in teak plants might be due to its perennial nature. Karanja et al. (1999) also reported low % Pdff (3 to 6%) in *Grevelia robusta* at three month after transplanting.

A value

Increased A value with every successive rate of inorganic P applied was due to the increase in the available P caused by the direct application of inorganic (Sharpley et al., 1987) also found that continued fertilizer P applications caused decreasing P-sorption thereby increasing the available P levels. Results also revealed that combined application of compost along with inorganic P increased A value. This increase might be due to the

release of organic acids during the decomposition of compost, which delay the crystallization and formation of Ca-P complexes and Ca-P minerals. At the same time, it may form complexes with iron (Fe) and aluminium (Al) and thus reduce the number of sites for P sorption. Illmer and Schinner (1995) also emphasized on the role of organic manures in the release of P as well as mobilization of native soil P into the soil matrix.

In general, it was seen that A value was high in medium P soil compared to low P soil. Medium P soil contains more soil organic carbon and this being an energy source for microbes, and their activity may be partly responsible in part for increased levels of labile P (Lee et al., 1990) in addition to its relatively higher level of inherent soil P.

Phosphorus use efficiency

Phosphorus use efficiency was found to be high in compost applied treatments. El-Ghamry et al. (2009) also reported higher P use efficiency due to application of increased rate of humic acid in faba bean. Results also indicated significant decrease of P use efficiency with increasing rate of inorganic P. This is due to at low levels of P, roots will compete for more P and this in turn leads to efficient use of P from applied P source in this soil. Similar observations were also made by Shrivastava et al. (2007). In general, low P use efficiency was seen in medium P soil compared to low P soil and this is attributed to relatively higher content of native P in this soil.

Shoot biomass

Fagbenro and Aluko (1987) also found positive correlations between rates of inorganic and organic fertilizer application and growth of leucaena and gliricidia in acid soils of Nigeria. Russo and Berlyn (1990) and Ulukan (2008) also reported that humates (granular and liquid forms) could reduce plant stress and enhance plant nutrient uptake.

Uptake of P

Results in general revealed the importance of inorganic P as well as compost application in both soil series. The low response of teak seedlings to compost application in low P soil might be due to the inadequate quantity of P coupled with slow decomposition of applied compost to elevate the inherent low level of available P pool to an optimum needed for easy absorption by plants. But in medium P soils, native soil P as well as applied compost might have been sufficient to get the desired level of available P pool for enhanced absorption by the plants. Integration of organic amendment with P fertilizers is

reported to increase P in labile pools and may have potential to enhance the availability in soil (Sanchez et al., 1997). Similar findings on enhanced dry matter yield and P uptake by rock phosphate enriched compost inoculated with fungus have been reported by Biswas and Narayanasamy (2006). Hence, the results in general indicated that application of inorganic P alone and in combination with compost resulted in enhanced P uptake. This can be attributed to increased P availability (Yasin et al., 2012) and a decrease in P sorption due to presence of the decomposition products of organic matter (Iyamuremye and Dick, 1996). This is in line with the finding of Rajan et al. (1991) that higher pH and low Al content in soil increased yield and P uptake in rye grass.

Conclusion

Results from the radiotracer investigation using ^{32}P revealed that in both soils with varying levels of P, application of inorganic P at 4 mg kg^{-1} increased % Pdff and P use efficiency compared to higher levels. Combined application of different levels of compost with inorganic P at 4 mg kg^{-1} resulted in significant improvement in % Pdff and P use efficiency. But shoot biomass, total P uptake and A value increased with increasing levels of inorganic P. Combined application of different levels of compost with inorganic P at 16 mg kg^{-1} also significantly improved shoot biomass, total P uptake and A value. From the above study it is concluded that combined application of compost with inorganic P fertilizer was more effective than application of inorganic fertilizer alone for enhanced absorption and use efficiency of P in teak seedlings. The findings of the study will help teak growers to make informed decision about integrated use of fertilizer and organic manure to improve productivity.

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Viability of maize pollen grains *in vitro* collected at different times of the day

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The viability of pollen grains is the essential precondition for obtaining enhanced or hybrid vigor genotypes and a good fixation of the fruit. It is a matter of great importance, especially for genetic improvement programs, which are used in various types of controlled pollination. This study aimed to evaluate the viability of the maize pollen grain through in vitro germination and stainability tests, collected at different times. The experimental design was a randomized block with factorial 2x5, two days of pollination at five times (9:00 a.m., 11:00 a.m., 1:00 p.m., 3:00 p.m. and 5:00 p.m.) with four replications. Each treatment consisted of 12 plants, which were taken randomly within each plot. The parameters evaluated were: germination percentage, the percentage of pollen grain stainability, the stigma receptivity and the best time for pollination. Through the analysis of variance, it was noted and interaction between the days and times of collection and highly significant differences for the following parameters: temperature percentage, humidity, germination and viability of the pollen grain, indicating that the days and times influenced the viability of pollen grains. We could observe that the best results of viable pollen grains were obtained at 09.00 a.m. regardless of the day. It was also noted that the ambient temperature and relative humidity were the main influencing factors on pollen viability, and not the collection times.

Key words: Genetic improvement, pollination, *Zea mays* L.

INTRODUCTION

The viability of pollen grains is essential precondition for obtaining enhanced or hybrid vigor genotypes and a good fixation of the fruit. It is a matter of great importance, especially for genetic improvement programs, which are used in various types of controlled pollination (Borém and Miranda, 2007).

The release of pollen grains can start from sunrise until noon, depending on the temperature, humidity and

genetic constitution of the plant. Under natural conditions, the maize pollen grain does not have a large strength and can lose viability within a range of one to four hours after being released into the atmosphere (Ferreira et al., 2007).

Various techniques are used to assess the viability of pollen grains, the most common being: germination *in vivo* and *in vitro*, besides the chemical dyes test, which is based on cytological criteria such as coloration (Almeida et al., 2011).

The germination *in vitro*, in culture medium, is a technique that emulates the style-stigma conditions, inducing germination of the pollen tube. Each species requires a specific formulation of culture medium to obtain good germination of the pollen grain. Among the elements that compose the culture medium, sucrose is considered essential, while the boron as boric acid, and calcium, as calcium dihydrate can maximize the medium efficiency. The agar is used to give consistency to the medium and avoid damage to the pollen tube during the evaluation (Ferreira et al., 2007).

The stainability is a quite simple procedure, inexpensive and provides results quickly, making it very attractive for works involving pollen grains. Considering that there is a correlation between viability and stainability, the estimation is given by counting the aborted and not aborted pollens showing stained and unstained, respectively (Alvim, 2008). Various dyes may be employed for this purpose, highlighting: acetic carmine, triphenyltetrazolium chloride, aniline blue and malachite green with acid fuchsin.

The viability of the pollen grain is not only influenced by intrinsic factors such as its state of physiological maturation, origin, genetic characteristics, the plant nutrition and by extrinsic factors such as the composition of the culture medium, pollen density in the medium, temperature and incubation time, collection period, but also by environmental conditions such as temperature, humidity, etc. (Stanley and Linskens, 1974).

Almeida et al. (2002) reported that *in vitro* germination of pollen grain is influenced by environmental conditions such as temperature and relative humidity during the collection and the maturing phase of the tassel, as newly formed gametes are more viable than pollen grains matured. The same authors also mention that the *in vitro* germination of pollen is highly correlated with fertilization in the field, in other words, *in vivo*. However, fertilization tends to be smaller than *in vitro* germination due to the influence of factors such as stigma receptivity, genetic barriers, temperature and relative humidity.

Considering these facts, it is necessary to raise and provide data about the feasibility of maize pollen grains, because it is a preliminary and essential condition for the success of the hybridization in the genetic improvement programs. The obtained results can contribute to future works, which aim at the storage of the pollen grain, besides enabling the crossed pollination between genotypes without reproductive synchrony, facilitating the search for superior individuals with greater genetic purity (Ferreira et al., 2007).

Few are the papers relating the influence of different times of collection in the feasibility of maize pollen grains, enhancing the importance of papers like these in improvement programs, aiming at better results as for pollination. In the light of the foregoing, this study aimed to evaluate the viability of the maize pollen grain through *in vitro* germination tests and the stainability, collected at different times.

MATERIALS AND METHODS

The experiment was conducted in the harvest of 2013/2014, in the experimental farm and Biotechnology Laboratory of Pontifícia Universidade Católica do Paraná, Campus Toledo, located in the city of Toledo, western Paraná 24°43'48 "S, 53°44' 24 "W, in an altitude of 560 m. Based on Köeppen classification, the climate is Cfa, mesothermal humid subtropical, with hot summers with a tendency of rainfall concentration, with an average temperature above 22°C. Winters with little frequent frosts with temperatures below 18°C, without a defined dry season. The average rainfall in the region is 1800 mm per year (Rubel and Kottek, 2010). The precipitation and air temperature, which occurred during the crop cycle can be observed in Figure 1. The soil used in this study was classified as typical Eutroferric Red Latosol, with smooth and wavy terrain and clayey texture (Embrapa, 2012).

The experimental design was a randomized block with factorial 2x5, two days of pollination at five times (9:00 a.m., 11:00 a.m., 1:00 p.m., 3:00 p.m. and 5:00 p.m.) with four replications. The size of each plot was 3 m long and 3.6 m wide, totaling 10.8 m², with 521.64 m² of total plot area. We used a population of four plants per meter, totaling 42 plants per plot. Each plot consisted of four rows of maize, in which the sample was done with 12 plants from the central area (useful area), discarding 1 m on each side of the plot. During the work, it was necessary to perform some crop practices such as soil analysis, covering fertilization, weed and pests control and protection of male and female inflorescences. We conducted a chemical analysis of soil and subsequent correction with 240 kg ha^{-1} of 8-20-20 NPK formulation. The seeds of CD384Hx maize hybrids were treated with imidacloprid at 0.25 L dose ha^{-1} and seeded on October 11, 2013. Fertilization in coverage was performed with 80 kg ha^{-1} of urea in the V6 stage of maize. The weed control was done by hand weeding at 14, 30 and 70 DAS (days after sowing). The application of physiological insecticide was made with Teflubenzuron 0.1 L ha^{-1} at 17; 26 and 40 DAS and contact insecticide Methomyl 0.4 L ha^{-1} at 40 DAS.

Once observed the emission of female inflorescence, they were covered with plastic bags to prevent contamination. Later, about 70 DAS, when the tassels had viable pollen, these were covered with paper bags so that it could be preceded with the pollen collection, and all the bags were informed with date. After seven days, when it was obtained a representative number of the sampling, we selected the plants with recently packaged pollen, and the tassels were beaten separately within each paper bag and made a bulk of pollen of the cultivar, which was conducted to the Biotechnology Laboratory of PUC-PR to carry out the *in vitro* tests.

Germination test

The pollen grains were incubated in culture medium, which was
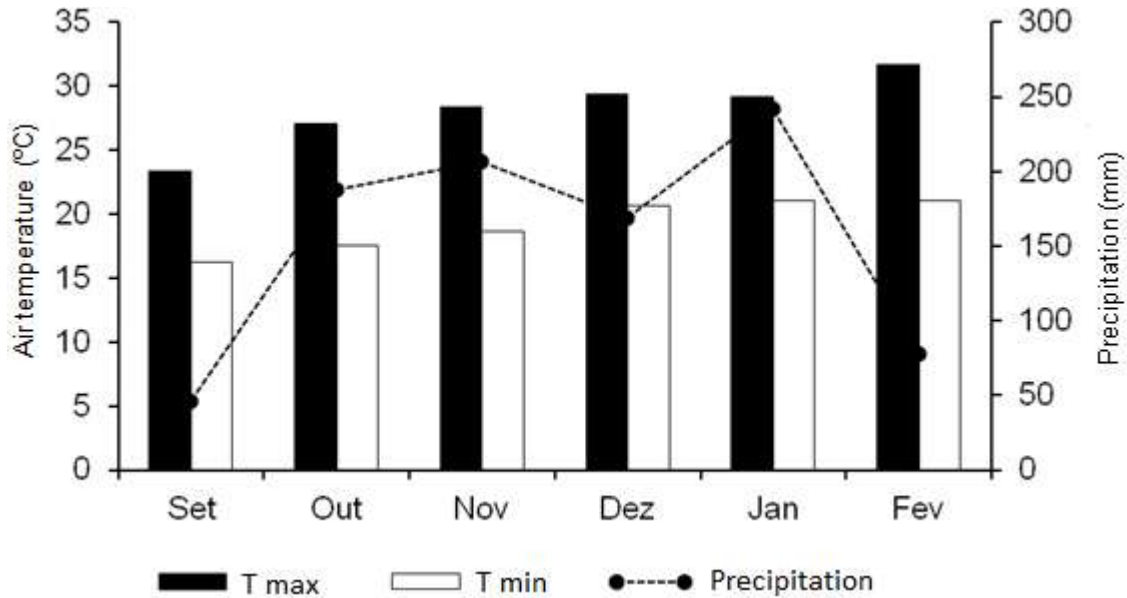
Figure 1. Representative graphic of maximum temperature, minimum and precipitation during the crop cycle by the Climatological station at Universidade Católica do Paraná - Campus Toledo. Toledo – PR, 2015.

determined prior to conducting the research (values are not shown). The culture medium used to determine the germination percentage was composed of 15% Sucrose; Boric Acid 0.01%; Calcium Nitrate 0.025%; 0.6% Agar, with pH 6.0. The incubation temperature was 25°C in a Biochemical Oxygen Demand (BOD). After two hours of incubation, the number of germinated grains was counted. The germinated pollen grains count was done with the aid of optical microscope with a 10x objective increase, evaluating four fields of view, corresponding to four repetitions. In each field of view there were on average 40 pollen grains. They were considered germinated, grains that had pollen tubes which exceeded the length of the diameter of the pollen grain itself (Pio, 2004).

Stainability test

For the determination of this parameter, we used the dye 2,3,5 triphenyltetrazolium (TTC) at 1%. A pollen sample was spread over a glass slide and then added a dye drop, closing the set with a cover slip. The observations of the number of viable and non-viable pollen were performed two hours after the preparation of the slides. The counting of viable and non-viable grains was made following the same germination procedure, in which it was considered viable pollen grains (red color) and non-viable (brown color) (Dafni, 1992).

Stigma receptivity

It was determined with the aid of a magnifying glass through observation of air bubbles formation when depositing hydrogen peroxide (H_2O_2) at 3% on the stigma surface, according to Dafni (1992). While performing the collection of pollen grains, temperature and humidity readings of air through the digital device Portable Digital Thermo-Hegrometro were carried out. The parameters used in the experiment were: percentage of germination, the percentage of stainability of the pollen grain and the stigma receptivity. The data were submitted to analysis of variance (ANOVA) and when detected significant effects between treatments, the regression test

was performed at a level of 5% probability, with the help of statistical program Costat 6.4 (COHORT SOFTWARE, 2003).

RESULTS AND DISCUSSION

Through the analysis of variance, it was found the existence of highly significant differences between the days and times of collection and the interaction between them, for the following parameters: temperature percentage, humidity, germination and stainability of the pollen grain, indicating that collection times were differently affected by days. Regarding the blocks there was no significant difference (Table 1).

Maize is a plant which is relatively tolerant to water stress during the growing season, but shows extreme sensitivity, with a decrease in grain yield, if there is no water in the stages of flowering and grain filling (Bergamashi et al., 2004). In this context, an important variable in estimating the water consumption of a culture is its evapotranspiration (ETc), which according to Doorenbos and Kassam (1994), is dependent on the knowledge of the reference evapotranspiration (ETo), with regard to weather conditions the site of interest, along with the physiological and morphological characteristics of the culture, represented by its culture coefficient (Kc), which incorporates plant characteristics (such as leaf area index) and effects evaporation of soil, varying over the cycle depending on the growth rate and, hence, the variation of the ground cover (Allen et al., 1998).

A large number of methods, with greater or lesser degree of empiricism, are being developed over the past

Table 1. Analysis of variance for the parameters temperature (°C); humidity (%); germination (%); viability (%) of maize pollen grain with *in vitro* cultivar CD 384Hx, Toledo, PR, 2015.

Fatores	Temperature (°C)	Humidity (%)	Germination (%)	Viability (%)
Day	0.001*	0.001*	0.001*	0.001*
Hours	0.001*	0.001*	0.001*	0.001*
Day x Hours	0.001*	0.001*	0.001*	0.001*
Block	0.853^{ns}	0.253^{ns}	0.191^{ns}	0.982^{ns}
Average	31.8	55.9	14.6	30.6
C.V. (%)	1.0	2.1	24.4	17.8

ns, *: Non- significant, significant at 5% probability, respectively, by Regression test.

$$y (\bullet) = 0.097x^3 - 4.16x^2 + 57.76x - 226.9$$
$$R^2 = 0.975$$

$$y (X) = 0.037x^3 - 1.618x^2 + 23.46x - 82.49$$
$$R^2 = 0.982$$
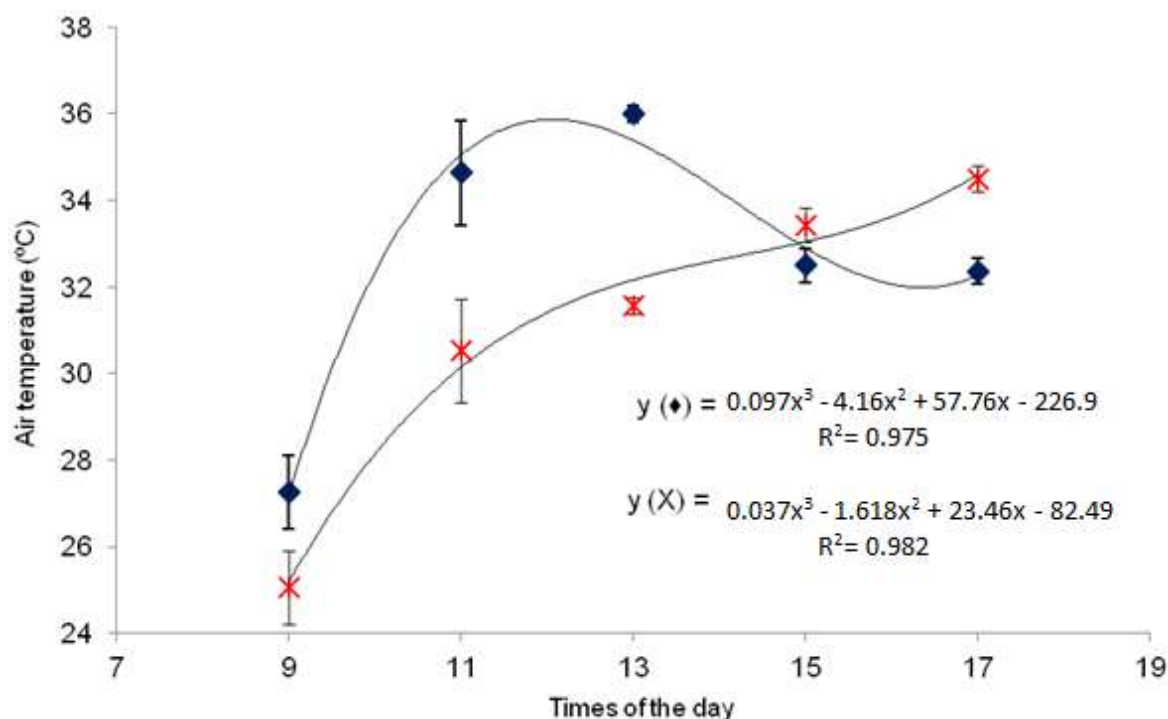
Figure 2. Air temperature (° C) of the two days of maize pollination in the field, day 1 (♦) and day 2 (X), and their respective time differences between each evaluation day. Toledo - PR, 2015.

decades in an attempt to estimate the evapotranspiration from different climatic variables (Valipour, 2014 a, b, d, e, f, g; 2015). The proposed correlations are often successfully tested in local calibrations, however, they are not universally applicable. To solve this problem, FAO has promoted studies to evaluate the available methods in order to obtain a standard method for calculating the ETo. After extensive testing, the Penman-Monteith method with the parameter proposed by the FAO came to be recommended as the standard method used in the daily scale. This is the method with the highest probability of correct answers in a wide variety of locations and climates (Allen et al., 1998). In this way, the most important weather parameters are temperature,

relative humidity, and wind speed for evapotranspiration models.

By analyzing the air temperature, it was found that there was a significant difference between the two days at all times of collection evaluated (Figure 2). On day 1, the lowest temperature was at 9:00 a.m. (27.2°C), with its peak at 1: p.m. (36.2°C), decreasing gradually in the following times. Regarding the second day, the lowest temperature was also in the first collection time at 9:00 a.m. (25.3°C). The temperature rose gradually in the following times, peaking at 5:00 p.m. (34.5°C). This difference in temperature between the days is due to the fact that the first day of collection was preceded by about seven days without rain, which contributed to the rise in
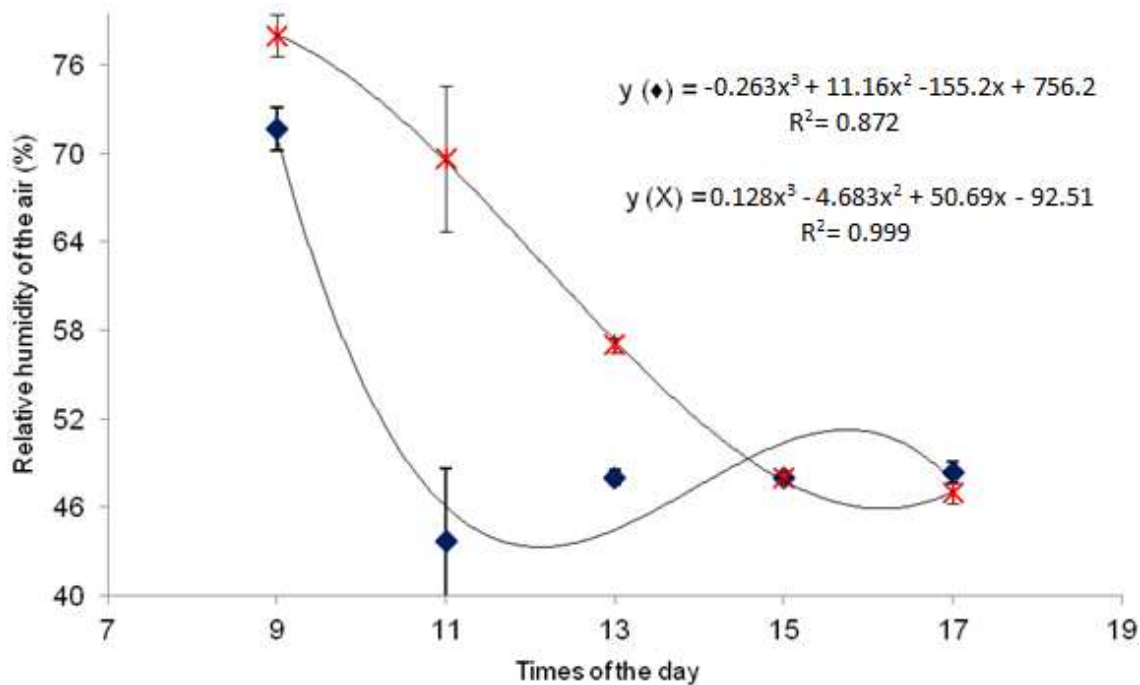
Figure 3. Relative humidity of the air (%) of the two maize pollination days in the field, day 1 (♦) and day 2 (X), and their respective differences between each evaluation time of the day. Toledo - PR, 2015.

temperature. It should also be borne in mind that one day before the collection on day 2, there was a shower of rainfall, increasing the relative humidity and consequently reducing the temperature.

Sorghum, being a C4 plant as maize, has its optimum range of air temperature during the vegetative period between 26 to 34°C (Hammer et al., 1993); and during the reproductive period between 25 to 28°C (Prasad et al., 2006, 2008). Decreases in pollen viability and consequently fewer pollen grains are a result of high temperature stress during pre-anthesis (sporogenesis), resulting in decreased seed set in sorghum grain (Prasad et al., 2008). High temperature stress during floret development alters pollen morphology and results in an abnormal exine wall, degeneration of tapetum cells, and membrane damage, leading to pollen sterility in grain sorghum (Djanaguiraman et al., 2014), wheat (Prasad and Djanaguiraman, 2014), and soybean (Djanaguiraman et al., 2013a,b).

The effect of thermal stress during reproductive development has been further investigated in tomato. Sato et al. (2002) reported that temperatures between 20 to 25°C are ideal for tomato. Surprisingly, increasing temperature to 29°C dramatically decreased the number of fruits and seeds formed. When evaluating the influence of the pollination temperature in the acquisition of haploid embryos in intergeneric cross wheat x maize, Silva et al. (2002) reported that 20 to 30°C temperature are optimal for obtaining embryos.

The relative air humidity differed significantly in two days and in all collection times, except for the fourth time (Figure 3). On day 1, the highest percentage of humidity was obtained at 9:00 a.m., with 72% RH, while the lowest percentage (44% RH) was observed at 11:00 a.m., with a small increase to 1:00 p.m. (48% RH), maintaining the same humidity in the following times. On day 2, the higher humidity was also at 9:00 a.m. (78% RH), reducing gradually the following times up to 5 h to 47% RH, which had lower results. This is due to the fact that humidity is correlated to the temperature, being inversely proportional behavior, and thus, these results may be explained in the same manner as above.

The effect of ambient relative humidity on pollen viability is evident in many investigated species. The response of high or low humidity, however, may differ among species and is generally associated with the intrinsic hydration state of the pollen in dehiscence (Nepi et al., 2001).

According to Aylor (2004), the maize pollen grain when exposed to dehydration by ambient conditions, can lose substantially all viability within three hours, as measured by *in vitro* germination of pollen grains. The author further states that the exact time of viability depended on the humidity during the experiment, that is, 20% RH, for example, the entire viability was lost in 50 min, whereas 75% RH pollen still existed after four hours.

Fonseca and Westgate (2005) found that after one hour in an environment of approximately 32°C and 50% RH, maize pollen grain loses approximately half of its water content (from 60 to 28.9%). For the same period of time, but 28.5°C temperature and 85% RH, the pollen

$$y\ (\blacklozenge) = -0.431x^3 + 17.57x^2 - 235.0x + 1038$$
$$R^2 = 0.858$$
$$y\ (X) = 0.240x^2 + 3.928x + 10.77$$
$$R^2 = 0.844$$
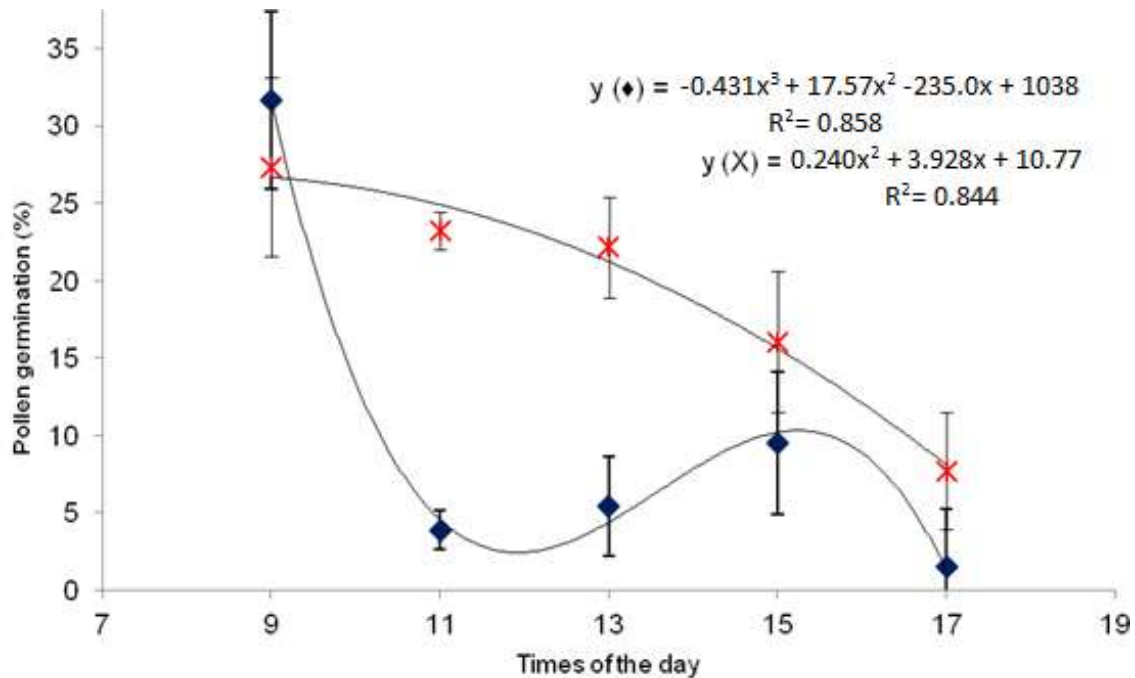
Figure 4. Maize pollen grains germination with *in vitro* using medium of germination (%) at different times and days of pollination in the field, day 1 (♦) and day 2 (X), and their respective differences between each evaluation time of the day. Toledo - PR, 2015.

grain water content remained around 54%. These values are relevant since the functional pollen life has a close relationship with its water content, and this turns out to be influenced by temperature and relative humidity.

For the germination of pollen grains we observed a significant difference between the two days and collection times (Figure 4). On day 1, the highest percentage of germination was observed at 9.00 (32%), and this time the relative humidity was 72% and the ambient temperature of 27.2°C. At the following times there was a great swing and decreased to 11.00 (4.8%) and rising to 13 and 15:00 and again, declining 17.00 (2%), obtaining lower germination. On day 2, the highest percentage of germination was also at 9.00 (27%), but unlike the first day, gradually declined to the 17.00 (9%), with the lowest percentage of germination. This shows that the percentage of pollen grain germination is directly related to the relative humidity, and conversely, to the ambient temperature.

For the germination percentage of pollen grains we observed a significant difference between the two days and collection times (Figure 4). On day 1, the highest percentage of germination was observed at 9:00 a.m. (32%), and at this time the relative humidity was 72% and the ambient temperature of 27.2°C. At the following times there was a great oscillation, having a decrease at 11:00 a.m. (4.8%) and rising at 1 and 3:00 p.m. and again, declining at 5:00 p.m. (2%), obtaining lower germination. On day 2, the highest percentage of germination was

also at 9:00 a.m. (27%), but unlike the first day, it gradually declined to 5:00 p.m. (9%), with the lowest percentage of germination. This shows that the percentage of pollen grain germination is directly related to the relative humidity, and conversely, to the ambient temperature.

Corroborating the results obtained, Almeida et al. (2002) reported that *in vitro* germination of pollen grains is influenced by environmental conditions such as temperature and relative humidity during the collection and the maturing of the tassel, as newly formed gametes are more viable than matured pollen grains.

Scorza and Sherman (1995) consider that a good pollen must present 50 to 80% of sprouted grains with well-developed tubes, and with the aging of pollen grains, the percentage of germination and the length of pollen tubes decrease. This explains the germination percentages not reaching higher rates because the protections of the banners were held for five days in a row until they reach 12 plants with viable banners.

Results of this work are evidenced by Alvim (2008), studying the feasibility and conservation of maize pollen grains. In his study, evaluating the best time to collect maize pollen grains (9:00 am, 2:00 and 4:00 p.m.) achieved the best results at 9:00 a.m., with 60% germination. At this time, the relative humidity was 80% and the ambient temperature of 19.5°C.

Costa et al. (2012) when studying the effect of collection time on the viability of maize pollen, reported

$$y = -0.569x^3 + 23.684x^2 - 323.33x + 1457.6$$
$$R^2 = 0.9964$$

$$y = -1.551x + 62.752$$
$$R^2 = 0.7278$$
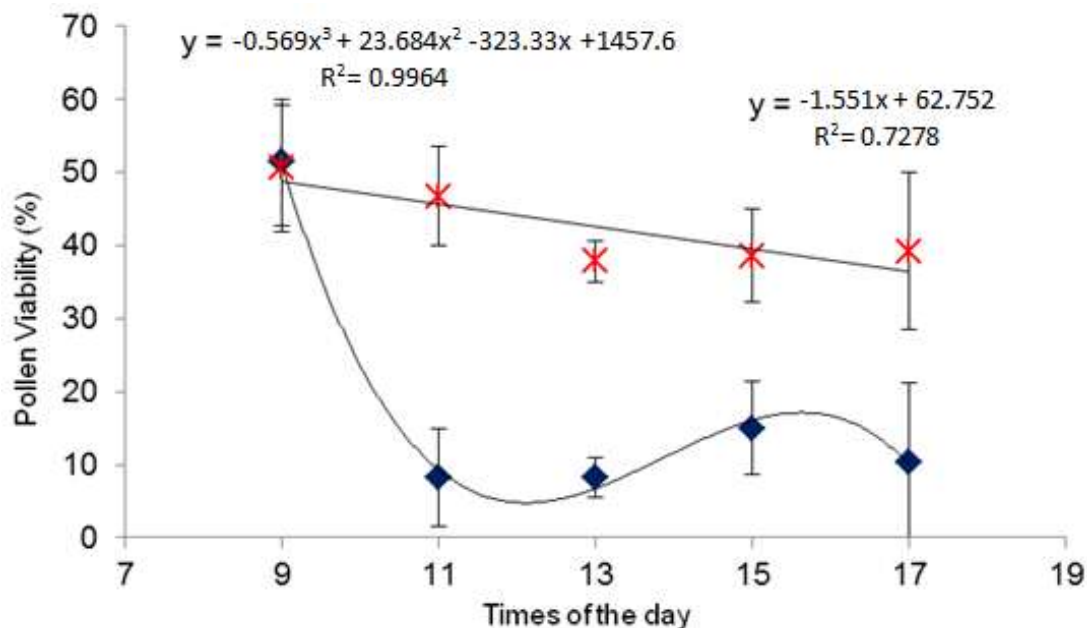
Figure 5. Viability of maize pollen grains with *in vitro* using Stainability Test (%) at different times of the day and pollination in field, day 1 (♦) and day 2 (X), and their respective differences between each evaluation time of the day. Toledo - PR, 2015.

that regardless of the cultivar, the first hours of release of maize pollen grains occur in the morning (8:00 to 12:00 a.m.), being more suitable for collection by providing higher germination values. Likewise, Maeda et al. (2012) in his work on *in vitro* assessment of the viability of maize pollen, found the best results at 10:00 a.m. for most crops during germination and in the viability test.

Figure 5 shows the analysis of the viability of maize pollen at different times of collection and days, and it can be seen that the cultivar is influenced by the collection times and the days of anthesis. The first collection time (9:00 a.m.) showed the best results 50.5 and 50% on days 1 and 2, respectively. The lowest viability results there were 12 and 35%, at 5:00 to 1:00 p.m. for days 1 and 2, respectively. The viability follows the germination curve, but always with higher values, showing the relationship between them.

The use of dyes is a very attractive technique, especially by the ease and speed in obtaining results (Alvim, 2008). However, the validity of this method has been questioned by the difficulty of distinguishing the color of viable pollen grains or immature and aborted pollen grains. For the realization of this method, trained and keen eye people are needed to realize these differences. Under favorable conditions, the pollen grain can remain viable for up to 24 h. Its longevity, however, can be reduced when subjected to low humidity and high temperatures (Aylor, 2002). In maize, it was found that the pollen grains do not support a humidity reduction higher than 50% without loss of its normal functions, with

the proper humidity content of around 20% (Ferreira et al., 2007).

According to Ferreira et al. (2007) when studying conservation and determining the viability of maize pollen grains, noted that the study of three different collection times (9: 00 a.m., 2: 00 and 4:00 p.m.) showed that the highest percentage of germination in vitro, consequently the viability, was at 9:00 a.m., with about 60%.

Regarding the style-stigma receptivity, there was 100% receptivity, showing that all plants were able to receive pollen grain in all the times. According to Ritchie and Hanway (1989), the establishment of direct contact between pollen grain and viscous pile of the stigma stimulates the germination of the first, originating a structure called pollen tube, which is responsible for the ovule fecundation inserted in the ear. Fertilization occurs in 12 to 36 h after pollination, a period which is variable depending on some factors involved in the process such as water content, temperature, contact point and style-stigma length.

Fanceli and Dourado (2000) also cite that the environmental stress in this stage, specially the hydric one, causes lower pollination and lower grain formation of the ear, once under drought, both the "hair" and the pollen grains tends to desiccation. Therefore, the number of fertilized ovule is closely related to the nutritional status of the plant, with the temperature as well as humidity condition of the soil and air. In this way, knowing all the factors that influence the viability of maize pollen grain is extremely important, as this is the main genetic material

used in plant breeding programs.

Conclusion

It was observed that the best results of viable pollen grains were obtained at 09:00 a.m. regardless of the day. It can be noted that the ambient temperature and relative humidity are the main factors of influence on pollen viability, not the times of the day.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of seasons on chemical composition and fungitoxicity of *Cymbopogon citratus* (DC) Staf essential oil

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The study aimed to evaluate the chemical composition and the fungitoxicity of *Cymbopogon citratus* (DC) Staf essential oil, obtained in different seasons of the year. Therefore, *C. citratus* leaves were harvest at four seasons (summer, fall, winter and spring) and essential oil extracted by hydrodistillation of freshly harvested and dried leaves, with a total of eight samples. Yield was expressed in percentage and the fungitoxicity was evaluated *in vitro* on *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc the causal agent of anthracnose at different concentrations (0, 0.25, 0.5, 0.75 and 1 µL mL^{-1}, respectively). The qualitative analysis of essential oil samples was made using gas chromatography coupled to mass spectrometry (GC-MS). The essential oil of freshly harvested leaves had a higher yield (0.25%) in the summer, whereas the essential oil of dried leaves had a better yield (0.48%) in the fall. In *in vitro* tests, concentrations of 0.75 and 1 µL mL^{-1} significantly inhibited mycelial growth and sporulation, in all samples. In the quantitative analysis, there was a variation in the levels of oil compounds observed in the different seasons. The most abundant compound in essential oil was citral (neral and geranial mixture) and myrcene. However, the variation in the compounds' levels had no influence on the fungitoxicity of *C. gloeosporiodes* (Penz.) Penz. & Sacc.

Key words: Lemongrass, citral, anthracnose, seasons, yield.

INTRODUCTION

Cymbopogon citratus (DC) Staf is an important species of Poaceae Family, considered as a medicinal plant commonly known in Brazil as capim-limão, capim-cidreira, capim-de-cheiro and capim-santo (lemon grass, lemon balm, smelling grass and holy grass). *C. citrates* has an essential oil rich in terpenoids such as neral,

geranial and myrcene (Correa et al., 1998; Souza Filho and Alves, 2002; Souza and Lorenzi, 2005; Tyagi and Malik, 2012, Tyagi et al., 2014) and it is widely used in cosmetic, perfumes and pharmaceutical industries (Verma et al., 2013). The analysis of *C. citratus* essential oil from plants of multiple regions determines citral (neral

Table 1. The experiment results of soil chemical analysis: pH, aluminum concentration, hydrogen, macronutrients and micronutrients.

pH	cmol dm^{-3}				mg dm^{-3}						
CaCl$_2$	H$^+$ + Al^{+++}	Ca^{++}	Mg++	K$^+$	P	S	Cu	Zn	Fe	Mn	B
6.50	2.64	5.76	1.83	0.51	3.20	6.85	11.30	2.20	19.00	56.00	0.25

From rural laboratory of Maringá– Maringá, Paraná (2011).

and geranial mixture) as the main constituent (Andrade et al., 2009). This terpenes act in the plant defense and can inhibit phytopathogenic fungi growth (Martins et al., 2004; Castro et al., 2007; Adamczyk et al. 2013) presenting no toxicity to humans (Erler et al., 2006).

However, the quality and quantity of essential oil compounds are often affected by environmental conditions (Martins et al., 2006; Blank et al., 2007; Nogueira et al., 2007). Gobbo-Neto and Lopes (2007), confirming that collection time of medicinal plant is one of the most important factors, since the amount and even the nature of active constituents is not constant throughout the year.

According to Costa et al. (2005), the steps of harvest and postharvest affect the chemical compounds' variation in medicinal plants, as well as, assist in obtaining a raw material with quality, resulting in the good quality of the final product. Also, according to Fennell et al. (2004), the chemical changes during postharvest periods of medicinal plants may affect essential oils or crude aqueous extracts' antimicrobial property.

The fungitoxicity of essential oils has been proven in several studies (Carnelossi et al., 2009; Benini et al., 2010; Moura et al., 2012; Sarmento-Brum et al., 2013), and according to Derbalah et al. (2012) the use of essential oils as antimicrobials is considered at low risk, because it is believed that it is difficult for a pathogen to develop resistance to the complex mixture of active components which comprise up the essential oils.

Thus, the current study aimed to assess the effect of harvest on the chemical composition and the fungitoxicity of *C. citratus*' essential oil harvest at different seasons of the year on *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc.

MATERIALS AND METHODS

The study was conducted in an experimental field at the State University of Maringá (UEM), Maringá-Paraná, Brazil, with geographic coordinates 23°23'18"S, 51°54'59"O and 526 m of altitude. The regional climate is classified as Cfa, subtropical, with hot summers, uncommonly frosts and a tendency of rainfall concentration during the summer, yet no dry season defined, according to the Köppen's classification (Neto, 2010). Soil chemical analyzes were performed before the experiment. Samples of soil were collected at a depth of 20 to 40 cm at different points, resulting in a composed sample. The pH values and concentrations of chemical elements are found in the soil results in Table 1. The soil

is characterized as eutrophic Red Oxisol (Souza and Gasparetto, 2010).

Cultivation of *C. citratus* seedlings

The seedlings were propagated by stem cuttings using polythene bags (10 × 20 cm), with 500 ml capacity of substrate (Red Oxisoleutroferric + sand + commercial substrate humus-based (2: 1: 1)). They remained in a greenhouse for one week before planting. Then the seedlings were removed from the greenhouse and relocated to an acclimatization area. The seedlings' transplant was conducted at the beginning of June, 2011, in single beds of 50 m^2. The plants leaves were harvested randomly during the different seasons of the year.

Harvesting of *C. citratus* leaves

The harvesting of *C. citratus* leaves were carried out in late summer (March, 2012) nine months after transplanting the seedlings, in late fall (plants with twelve months of age), late winter (plants with fifteen months) and in late spring (plants eighteen months), with a total of four harvesting. The essential oil was extracted from both freshly harvested and dried leaves, in a total of eight extractions. For each sample was weighed 4 kg of leaves and 50% were placed for natural drying in the shade at room temperature for four days to obtain the dried leaves, during this period the leaves lost an average of 57% of its initial content. The other 50% of freshly harvested leaves remained were used for essential oil extraction. All samples were harvested in the late afternoon, between 4:30 and 5 p.m. Climate data (average temperature, relative humidity and rainfall recorded in the previous thirty days of collection) at the moment of harvest and the leaves' drying are described in Table 2. These data were obtained from the Experimental Farm of Iguatemi (FEI / UEM) meteorological station and Seeds analysis and Medicinal plants Laboratory.

Essential oil extraction

The essential oil was obtained using the extraction process by steam distillation, which consists of placing the plant material in the distiller, then by passing the plant material, the steam drags the essential oil (volatile) out of the plant; it passes the condenser where it is collected by a separatory funnel, then the immiscible liquids of different densities separated naturally; removal from the container is made through a stopcock. The extraction process had duration of four hours from the beginning of the first condensate drop (Worwood, 1995). The essential oil was stored in amber glass container in the refrigerator at a temperature of 4°C (+/- 2°C). The essential oil harvested was weighed on an analytical scale for later yield calculation, which was expressed in percentage by the ratio between the amount of used distillable leaves and the amount of essential oil produced(g/g) (Furlan et al., 2010).

Table 2. Climate data (average temperature, rain fall and relative humidity) in the harvest seasons. FEI / UEM laboratories –Maringá, 2012.

Harvest seasons	Temperature (°C)	Rain fall (mm)	Relative humidity (%)
Field			
Summer	26 (+/-1)	90	58 (+/-5)
Fall	15 (+/-1)	344	67 (+/-5)
Winter	27 (+/-1)	4.8	55 (+/-5)
Spring	28 (+/-1)	113	68 (+/-5)
Laboratory			
Summer	27 (+/-2)	-	65 (+/-5)
Fall	18 (+/-2)	-	69 (+/-5)
Winter	30 (+/-2)	-	47 (+/-5)
Spring	30 (+/-2)	-	66 (+/-5)

*Rainfall recorded in the previous 30 days to harvest.

Essential oil qualitative analysis

Qualitative analysis was determined by gas chromatography coupled to mass spectrometry (GC-MS) and determination of their Kovats retention indices.

Gas chromatography coupled with mass spectrometry

The analyses of eight essential oils resulting from the extractions were performed using a GC-MS system in the Department of Analytical Chemistry of the State University of Maringá (UEM), Paraná, Brazil. The chromatograph used was FOCUSGC model ("Thermo Electron"), equipped with a fused silica capillary columnDB-5ms (30 mm x 0.25 mm, 0.25 µm of stationary phase) and mass spectrometer model DSQII ("Thermo Electron"). The operating conditions were: injector temperature 200°C; drag gas flow (helium) 1.0 mL min^{-1}; injection volume of 0.5 µL (dichloromethane solution) withdivision of flux ("split") at a ratio of1:40. The programming of oven temperature was 50°C for 1 min, elevated to 180°C at a rate of 3°C min^{-1} and180°C to 240°C at a rate of 10°Cmin^{-1}, remaining at 240°C for 5 min. Mass spectrum were obtained by ionization of electron impact 70eV, in the range of 50 to 550 mz^{-1}; with an inter face temperature of 250°C and ion source of 200°C (Maia et al., 2013).

Kovats retention index

The calculation of Kovats retention index (KI) was based regarding the homologous series of n-alkanes, analyzed under the same experimental conditions. The retention indices of the compounds were determined according to the equation proposed by Van den Dool and Kratz (1963). The identification of main compounds was performed based on a comparison of its mass spectrum with those calculated from the mass spectrum of the spectral library" NIST MS Search2.0" contained in the soft ware "Xcalibur" accompanying the appliance, comparing their Kovats retention indices obtained experimentally with Kovats retention index data obtained from the literature (Boulanger et al., 1999; Beaulieu and Grimm, 2001; Siegmung et al., 2001; Jordán et al., 2003) for the same compounds analyzed using the same column. Quantitative data of compounds were obtained from percentages of the chromatogram are as through normalization.

Essential oil fungi toxicity analysis

The phytopathogen *C. gloeosporioides* (Penz.) Penz. & Sacc was isolated from mango fruit presenting typical anthracnose symptoms. Fragments from fruit were transferred into petri plates containing PDA growth medium (potato, dextrose, agar) which was maintained at 25°C in biochemical oxygen demand incubator (BOD) for five days. Later the plates with mycelia were transferred to a room with continuous light to stimulate sporulation.

After the formation of their productive structures (spores mass with rose color) *C. gloeosporioides* were transferred to new plates and incubated at 25°C in BOD. After seven days of incubation, *C. gloeosporioides* isolates were used in separate trials. The essential oil was tested at concentrations of 0 µL mL^{-1}, 0.025 µL mL^{-1}, 0.05 µL mL^{-1}, 0.075 µL mL^{-1} e 0.1 µL mL^{-1}, increased by 0.25µL mL^{-1}Tween 20 (emulsifying agent), to inhibit the growth and sporulation of *C. gloeosporioides*. The essential oils at the different concentrations were added to PDA growth medium after sterilized and still liquefied, poured into petri plates. After PDA solidification, a mycelialdisc (7 mm diameter) of the isolated fungus was placed in the plates center, sealed with plastic wrap and kept in a growth chamber at 25°C and photoperiod of 12 h.

From the second day after replication, evaluations were performed every 24 h until one of the isolates reached the plates edge (144 h). The mycelial growth was measured by the colonies' radius in two perpendicular directions, using a digital paquimeter. With the obtained data it was determined the mycelia growth area of the isolated fungus, from this we calculated the percentage of my celial growth inhibition (MGI), using the formula of Bastos (1997).

For each treatment, five plates were replicated each plate represented a repetition in a completely randomized design. Data were subjected to analysis of variance (ANOVA) and when significant regression equations were applied, adjusted with a coefficient of 5% probability and the coefficient of determination (R^2). Analyses were performed using SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

Essential oil qualitative and quantitative analysis

The highest yield was obtained in the extraction of freshly

Table 3. *Cymbopogon citratus* essential oil yield of freshly harvested and dried leaves harvested in different seasons (Maringá, 2012).

Harvest season	Yield (%)	
	Fresh mass	Dried mass
Summer	0.25	0.43
Fall	0.16	0.48
Winter	0.20	0.44
Spring	0.21	0.29

harvested leaves essential oil in summer harvest (0.25%), followed by yields of essential oils collected in the spring, winter and autumn seasons with income 0.21, 0.20 and 0.16%, respectively (Table 3). According to Nascimento et al. (2003), the essential oil yield of *C. citrates* is 0.28 to 0.50% of freshly harvested weight, higher than those found in this study. They observed that collection during the morning period (between 9 and 11 a.m.) showed higher oil yield compared to other periods. Similar results were observed by Marco et al. (2002). This explains (or justify) the lower values found in this study.

The yield variation between same species plants is attributed mainly to differences in harvest season, soil type, region climate and relative humidity (Burt, 2004). The seasonal variation of secondary metabolites can be caused by physiological demands as growth, defense and plant reproduction, as well as differences in the environment as water stress, light, nutrient deficiency and extreme temperatures. The study of this variation is very important as it allows knowing the exact time, approximately, in which some constituents are in greater proportion (Kumar et al., 2011).

According to Silva and Casali (2000), decreasing the amount of water in the plant, the amount of active ingredients in terms of dry mass increases. Justifying the findings of this study, where the highest yield values were observed in essential oil resulting from dried leaves extracts comparing to freshly harvested leaf extracts (Table 3). Yield was expressed by the ratio between the mass of distillable leaves used and the amount of essential oil produced (g/g), thus decrease the dried leaves mass (57%) has optimized performance values. The results also agree with Martins et al. (2002), who observed an increase in oil yield of 21% when compared to essential oil amount extracted from the dried leaves to that obtained from freshly harvested product of *C. citratus*.

For the variation observed in the essential oil yield of freshly harvested mass, from summer to fall harvest, it might have been caused by the change in micro climate parameters of temperature and rain fall recorded in the thirty days prior to harvest. The excess of rain during the thirty days prior to fall harvest (344 mm) provided a decrease in secondary metabolites accumulation in plants. According to the results of Koshima et al. (2006), when they analyzed the essential oil yield extracted from freshly harvested leaves of *C. citratus* grown with mulch and harvested in all seasons of the year, found a higher yield of essential oil in the period with lower rainfall.

In relation to dry mass to yields, fall presented extraction yield of 0.48% and spring presented the lowest yield of 0.29%. In summer and winter extraction yield was 0.43 and 0.44% respectively (Table 3). The lower essential oil yield extracted from the dried leaves during the spring compared to fall, is possible due to temperature differences. During the drying process in fall, it had mild temperature of 18°C, whereas during the spring it had a higher temperature (30°C) (Table 2). Blanco et al. (2002), studied the drying temperature effect on the essential oil yield of *Mentha piperita* and observed that drying temperature elevation caused a reduction in essential oil content. As explained by Calixto (2000), some plants constituents are labile when exposed to heat, thus plants containing them must be dried at low temperatures. In the drying process, water loss occurs naturally, but can also result in volatiles loss depending on the drying temperature. Also, Ortiz et al. (2002) related the essential oil yields with variations in temperature and found that the increase or decrease on it has a direct influence at essential oil yield. In chromatographic, analysis was possible to identify six mainly compounds in the essential oil of *C. citratus*: myrcene; linalool; verbenol; neral; geranial and 2-undecanone (Table 4).

The most abundant compound in the essential oil of *C. citratus*, obtained in different seasons, was the citral composed by a mixture of neral and geranial isomers, this had a higher area in the essential oil from freshly harvested leaves in spring (65.6%), followed myrcene presented an area of 21.48%. For its part, the essential oil extracted from dried leaves in winter, myrcene compound showed greater area of 41.28% and citral with an area of 44.94% (Table 3). These results agree with other authors, who claim the presence of citral as a major component of *C. citratus* essential oil (Martins et al., 2002; Leal et al., 2003; Costa et al., 2005; Furlan et al., 2010; Kumar et al., 2013; Pinto et al., 2015).

Lima et al. (2008), found that essential oil of *C. citratus* presented geranial (43.8%), neral (34.5%) and myrcene (14.6%) as major compounds. Barbosa et al. (2008), reported a variation in citral levels of the essential oil marketed in Brazil from 40.7 to 75.4% and myrcene from 0.24 to 7.29%, being the myrcene levels below what is found in the essential oils studied. Early investigation of the leaf essential oil of *C. citrates* from Rio de Janeiro were also identified and quantified geranial with 53.2% and neral with 36.37% as the major components but the monoterpene myrcene was not observed (Pinto et al., 2015).

Still analyzing the compounds content of the essential oil from freshly harvested leaves it can be observed that, citral presented a larger area (65.56 and 65.49%) in the

Table 4. The main components contents of *C. citratus* essential oil coming from the freshly harvested and dried leaves extractions, harvested at four seasons, identified by GC-MS and expressed as normalization area (%).

Extractions seasons		Compounds (KI cal / Yield (%))					
		Myrcene	Linalool	Verbenol	Neral	Geranial	2-Undecanone
Summer	FL	992 / 20.15	1101 / 1.15	1184 / 2.34	12411 / 28.94	1271 / 36.55	1294 / 0.19
	DL	991 / 25.70	1101 / 1.20	1184 / 2.21	1241 / 27.02	1271 / 34.90	1294 / 0.27
Fall	FL	991 / 28.90	1100 / 1.28	1183 / 1.67	1241 / 25.57	1271 / 32.58	1294 / 0.80
	DL	991 / 28.23	1101 / 1.25	1184 / 2.02	1241 / 26.31	1271 / 33,87	1294 / 0.74
Winter	FL	991 / 25.35	1100 / 1.41	1183 / 2.28	1241 / 26.30	1271 / 34.71	1294 / 0.60
	DL	992 / 41.28	1101 / 2.21	1184 / 1.70	1241 / 19.29	1271 / 25.65	1294 / 0.89
Spring	FL	991 / 21.48	1100 / 1.12	1183 / 2.25	1241 / 28.40	1271 / 37.16	1294 / 0.27
	DL	992 / 32.25	1102 / 1.24	1184 / 1.33	1242 / 16.75	1272 / 22.28	1295 / 0.25
KItab	-	991	1101	1188	1240	1270	1294

KIcal: Kovats retention index obtained experimentally; KI tab: Kovats retention index data obtained from the literature. FL: freshly harvested leaves; DL: dried leaves.

spring and summer seasons, respectively, when compared to fall and winter (58.15 and 61%). However, there was not just the increase of citral content in these conditions, but also the decrease of myrcene content (21.48 and 20.15%), while in fall and winter presented area of 28.9 and 25.35%, respectively (Table 4).

This change may be caused by micro climate factors (Gobbo and Lopes,2007) and mostly rainfall, since in the climatologic seasons rainfall records presented median (113 and 90 mm) in the thirty days prior to harvest, while in the fall and winter the indexes present significant variation (344 and 4.8mm) respectively (Table 2).

**Essential oil fungitoxicity**

The essential oil extracted from freshly harvested and shaded dried leaves harvested in all the four seasons, presented significant inhibition on the mycelial growth of *C. gloeosporioides* with a 29% coefficient of variation (Figures 1, 2, 3 and 4). According to Pereira et al. (2011), essential oils can inhibit or reduce phytopathogens growth due to the action of substances present in its composition.

Guerra et al. (2000), attribute the antimicrobial and antifungal activities of *C. citrates* essential oil to citral monoterpene. Guimarães et al. (2011), observed that myrcene compound has no fungi toxic activity, since its celial inhibition was equal to zero for all phytopathogens. The essential oils obtained from the eight extractions, it was observed that the variation in the chemicals levels, mainly citral (neral and geranial mixture) to which the anti fungal action is assigned, had no effect at the fungitoxicity of the essential oil on *C. gloeosporioides*. It

was demonstrated that even the lowest citral content (39.03%), found in the essential oil from the dried leaves in spring, showed high fungi toxicity (99%) (Figure 4B). In relation to citral, Guimarães et al. (2011) also demonstrate the citral fitotoxicity and its importance activity in the *C. citrates* essential oil. Lee et al. (2008), observed that compounds as citronellal, neral, geraniol and geranial at a concentration of 28×10^{-3} mg mL^{-1} of air are able to inhibit 100% mycelial growth of *Phytophthora cactorum.*

It was observed that the essential oil obtained in all extraction seasons demonstrated to be efficient in the control of the phytopathogen mycelial growth. The effect expressed as quadratic for the majority of samples with mycelial growth inhibition (MGI) of 102% at a 0.8 µL mL^{-1} concentration (Figure 1A), of 103% ata 1 µL mL^{-1} concentration (Figure 1B), of 113% at a 0.7µL mL^{-1} concentration (Figure 3A) and 99% at a 0.9 µL mL^{-1} concentration. The essential oil of freshly harvested leaves harvested in fall, although it had a cubic effect, also obtained MGI maximum point of 97% at a 0.9 µL mL^{-1} concentration and with minimum inhibition (1%) at a 0.078 µL mL^{-1} concentration 1 (Figure 2A). The mycelia growth rate of *C. gloeosporioides* in the presence of *C. citrates* essential oil from dried leaves, harvested in the fall and winter, and freshly harvested leaves, harvested in the spring, has expressed positively linear form depending on the essential oil doses, in other words, it presented dose-dependent effect (Figure 2B, 3B and 4A).

These results confirmed the *C. citratus* essential oil potential in control of the phytopathogen that causes anthracnose in mango fruits. Santos et al. (2013), also verified that the *C. citrates* essential oil was effective in reducing the mycelia growth of *Helminthosporium* sp.
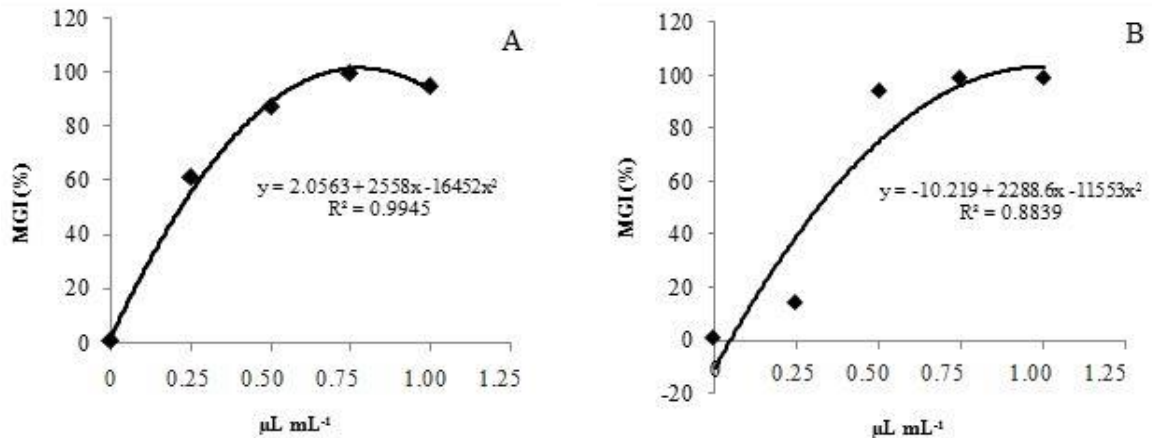
Figure 1. *C. gloeosporioides* mycelial growth inhibition (MGI %) in presence of *C. citratus* essential oil obtained from freshly harvested (A) or dried (B) leaves harvested in summer.
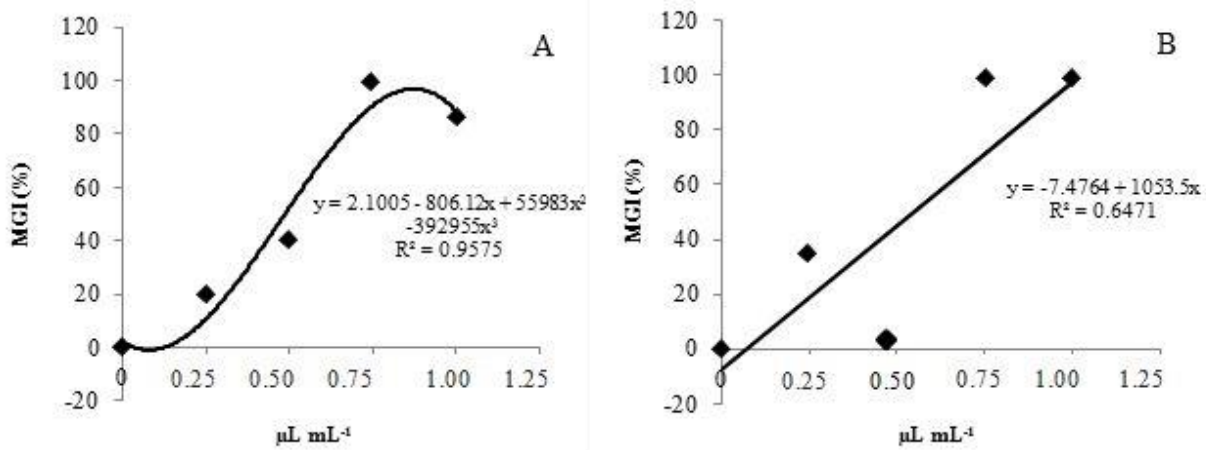
Figure 2*. C. gloeosporioides* mycelial growth inhibition (MGI %) in presence of *C. citratus* essential oil obtained from freshly harvested (A) or dried (B) leaves harvested in fall.
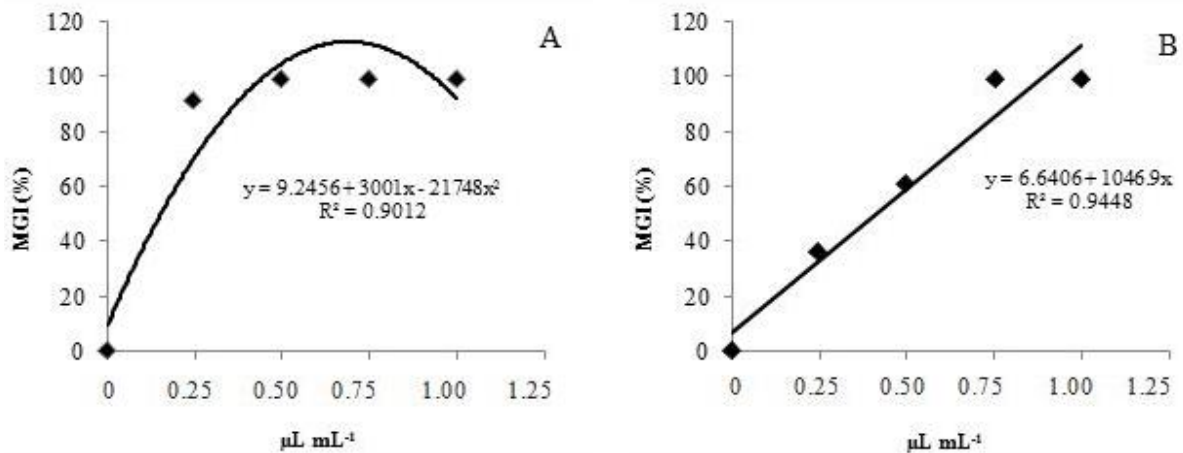
Figure 3. *C. gloeosporioides* mycelial growth inhibition (MGI %) in presence of *C. citratus* essential oil obtained from freshly harvested (A) or dried (B) leaves harvested in winter.
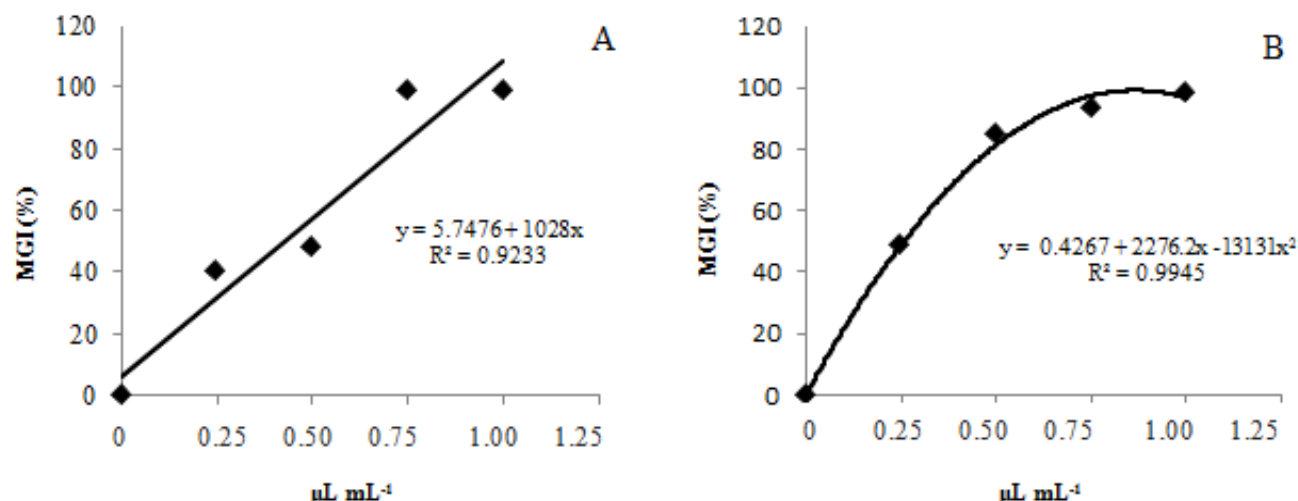
Figure 4. *C. gloeosporioides* mycelial growth inhibition (MGI %) in presence of *C. citratus* essential oil obtained from freshly harvested (A) or dried (B) leaves harvested in spring.

5 and 7 μL mL^{-1}).

Conclusion

The results presented in the current study, corroborated with the cited literature demonstrate that the *C. citratus* essential oil has effective control of *C. gloeosporioides,* due to the activity of chemical compounds present in this oil.

Conflicts of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Striga hermonthica reduction using *Fusarium oxysporum* in Kenya

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The biological control agent *Fusarium oxysporum* f.sp. *strigae* isolate Foxy 2 had shown potential as a biological control in West Africa, however it failed to achieve the same results when used on Kenyan *Striga hermonthica*. A *F. oxysporum* isolate FK3 was obtained from infected *S. hermonthica* in a maize crop in Kenya and tested as a potential control agent of *S. hermonthica* in Kenya. Two pot trials were conducted where the *S. hermonthica* seed and varying rates of isolate FK3 (7.5 to 60 × 10^7 CFUs per pot) were added prior to planting of maize. Numbers of *S. hermonthica* plants were reduced significantly at the lowest rate of FK3 application when compared to the control from 10.7 to 5.5 per pot in the first trial and 21.3 to 7.9 plants per pot in the second trial. Where a more susceptible variety of maize was used in the second trial evidence of improved maize growth (fresh root mass and stover weight) and yield (weight of grains and whole cob weight) was associated with the reduction in *S. hemonthica* numbers. More extensive field trials are recommended to fully assess the impact of FK3 on maize yield. The findings support the idea that regional genetic variation in both *S. hermonthica* and the pathogen *F. oxysporum* may explain the regional specificity of *F. oxysporum* isolates as a potential biological control agents.

Key words: Biological control, maize, mycoherbicide, parasitic plants, striga, *Fusarium*.

INTRODUCTION

Maize is grown throughout the world but there are large differences in yield and consumption. African countries are relatively small producers in total tonnage, for instance the USA and China are the largest producers with 274 and 208 million MT/year while South Africa, with 12 million MT/year is the largest African producer. However consumption as measured in g/person/day is highest in Africa with six out of the top ten consuming countries being in Africa (Ranum et al, 2014). Maize is by far the most important food crop in Kenya, playing an integral role in national food security. Maize is the primary staple food for most Kenyans, accounting for 36% of all calories consumed and 65% of staple food calories consumed. Kenya produces around 3 million tonnes of maize per year on about 2 million ha of land annually (Short et al., 2012). *Striga hermonthica* (Del) Benth (Witch weed) is a wide spread and endemic parasitic weed of a wide range of gramineous species, including

maize, sorghum, millet and rice. The weed is found across sub-Saharian Africa (Beed et al., 2007). In East Africa, *S. hermonthica* is found around the Lake Victoria basin in Kenya, Tanzania and Uganda. In Kenya, *Striga* infestation is most severe in Nyanza and Western provinces. The parasitic weed is found in about 75,000 hectares of farmland and results in crop losses estimated at about US$ 10 to 38 million per annum (Manyong et al., 2008). *S. hermonthica* is a major cause of yield loss in Western Kenya in maize where up to 70% losses have been reported (Khan et al., 2008). Despite extensive research and extension efforts the problem of this parasitic weed has not receded and with poor farming practices and potential climatic change the problem may further increase (Jamil et al., 2012).

There are numerous control strategies that have been developed for the management of *S. hermonthica* including push pull technology which involves establishing *Desmodium uncinatum* plants in the understory of the maize which are allelopathic to *S. hermonthica* (Khan et al., 2008), herbicide coated resistant maize (IR maize) (Kanampiu et al., 2002) and breeding of tolerant and resistant varieties of maize (Kamara et al., 2012). These technologies have shown to be effective under on-farm trial conditions; however their widespread adoption and uptake has been limited. Reasons for slow uptake are a complex mix of social and economic factors that influence the decisions of risk averse small-scale subsistent farmers.

In the 1990s interest in finding a disease of *S. hermonthica* for use as a potential biological control was investigated. In North Ghana *Fusarium oxysporum* isolate Foxy 2 was isolated from a diseased *S. hermonthica* plant (Abbasher et al., 1995) and a new forma specialis named *F. oxysporum* f. sp. *strigae*, which causes *Fusarium* wilt of *Striga* species was identified (Elzein et al., 2008). In 2009, the same isolate was imported into Kenya and has been subsequently tested against *S. hermonthica*. Foxy 2 was shown not to control *S. hermonthica* in Western Kenya (Avedi et al., 2014). As part of the efforts of developing a possible biological control in Kenya, diseased *S. hermonthica* plants were selected from a maize field and the pathogens were isolated and subsequently cultured which included the isolate FK3. In order to confirm whether the selected Kenya *F. oxypsorum* isolate FK3 had potential as a biological control agent of *S. hermonthica* in Kenya, our hypothesis is that pathogen, isolate FK3 taken from infected *S. hermonthica* plants in Kenya, reduces the presence of *S hermonthica* when applied to the planting hole and secondly, the use of FK3 improves the growth and yield of maize in *S. hermonthica* infested soil.

MATERIALS AND METHODS

Fungal isolate FK3

Diseased *S. hermonthica* plants were selected in a maize

field in Western Kenya. Based on the speed of growth on potato dextrose agar (PDA) plates an isolate FK3 was selected as a fast growing isolate. Subsequently, this isolate was confirmed as a *F. oxysporum* (Wainwright and Viljoen, 2014). The production of FK3 was done by inoculating sterilised rice grains in plastic bags and allowing to grow for 7 days at 22°C, the rice was then removed from the bags and dried to a moisture content of approximately 5%. The dried rice was then ground to a coarse powder prior to use. Each gram contained 1.5×10^7 colony forming units (CFUs).

Pot trial protocols

Two pot experiments were undertaken during 2013 and 2014 at KARI/CIMMYT collaborative site in Kibos, Kisumu, Kenya. Plants were grown in a shade house. Each experiment consisted of a factorial design with two maize varieties and four rates of *F. oxysporum* isolate FK3. Five litres pots containing un-sterilized field soil were used with 20 pots per treatment. To each pot, 5 g of Di ammonium phosphate fertiliser (18-0-46) and a teaspoon of *S. hermonthica* seed and sand mix (approximately 1000 seeds) were mixed to the upper 5 cm layer of pot soil. Three maize seeds were sown per pot and thinned to a single seedling after germination. In both experiments, the rates of FK3 were the same; these being 7.5×10^7 (low rate), 1.5×10^8 (medium rate) and 6×10^8 (high rate) of CFUs per pot. Top dressing with CAN fertiliser was done at 6 weeks after sowing at a rate of 10 g per pot (27:0:0) (DEFRA, 2010).

Maize varieties

In the first pot trial the two varieties of maize sown were WH507, a susceptible hybrid variety from Western Seed Company Ltd, and a local farmer saved white seeded maize variety locally known as Rachar. Both varieties are short season varieties (3 month). In the second pot trail, the two varieties of hybrid maize sown were WH507 and WH403, both considered susceptible varieties from Western Seed Company Ltd.

Data collection and analysis

Parameters assessed to validate the effect of *F. oxysporum* were *S. hermonthica* plant numbers per pot at week 14 and maize plant height and leaf number. In the second pot trial, additional parameters assessed were total weight of harvested cobs in each treatment, stover weight, cob length, cob diameter, 100 seeds grain weight and fresh root weight. Data obtained was subjected to analysis of variance and significant differences determined between the means by Fishers protected LSD at p<0.05, using GenStat software. The interaction of two-way analysis wase not presented as none were significant.

RESULTS

The first pot trial showed that there was a significant difference in *S. hermonthica* plant numbers between the untreated pots and those treated at any rate with FK3 isolate of *F. oxysporum*, however there were no differences in *S. hermonthica* number with different rates of FK3. There were no differences in plant height or leaf number with different rates of FK3. There were no differences in *S. hermonthica* numbers for each variety; however variety WH507 gave short plants and more

Table 1. Mean number of Striga plants per pot and growth of maize (height and leaf number) in the first season pot trial.

Treatments	Striga plant number per pot (Week14)	Maize plant height (Week 11) (cm)	Maize leaf number (Week 11)
Rate of FK3			
Untreated	10.73[a]	159.2[a]	17.05[a]
FK3 lower rate	5.55[b]	162.7[a]	17.01[a]
FK3 mid-rate	5.03[b]	164.9[a]	17.18[a]
FK3 high rate	3.30[b]	163.7[a]	17.12[a]
P value	<.001	0.732	0.075
S.E	1.526	5.26	0.2252
Variety			
WH507	6.36[a]	144.5[a]	18.20[b]
Rachar	5.94[a]	180.8[b]	15.94[a]
P value	0.694	<.001	<.001
S.E	1.079	3.72	0.2372

Means in the same column and the same variable (FK3 rate or variety) followed by the same letter are not statistically different from each other.

Figure 1. The effect of applying four rates of *F. oxysporum* f. sp. *strigae* isolate FK3 at the time of maize planting (variety WH507) on the emergence of Striga plants. From left to right, Control (untreated); Low rate; Medium rate and High rate.

leaves than Rachar irrespective of treatment (Table 1).

The second pot trial showed that there was a significant difference in *S. hermonthica* plant numbers between the untreated pot soil and those treated at any rate with FK3 isolate of *F. oxysporum*, and also that the lower rate FK3 treated had higher number of *S. hermonthica* plants than the middle and higher rates of FK3. Single pot examples of the different treatments of variety WH507 showing the different Striga numbers are shown in Figure 1. There

were no differences in plant height or leaf number with different rates of FK3. There was significant reduction in internode length in the untreated when compared to the medium rate FK3. Fresh root mass gave significant differences with the untreated giving the least root mass and the highest rate of FK3 gave the highest root mass. Stover weight showed that there was a significant difference between the untreated pot soil and those treated at any rate with FK3. For varieties, WH403 had

Table 2. Mean number of Striga plants per pot and growth of maize (height, leaf number, internode length, fresh toot mass and stover weight) in the second season pot trial.

Parameter	Striga plant number per pot (Week14)	Maize plant height (Week 11) (cm)	Maize leaf number (Week 11)	Maize internode length (Week 11) (cm)	Maize Fresh root mass (g)	Stover weight (kg)
Rate of FK3						
Control	21.274[c]	195.3[a]	16.58[a]	13.27[a]	182.5[a]	1.695[a]
FK3 lower rate	7.881[b]	205.4[a]	16.63[a]	14.34[ab]	209.4[b]	2.074[b]
FK3 medium rate	4.274[a]	206.1[a]	16.70[a]	14.80[b]	188.1[ab]	2.083[b]
Fk3 higher rate	3.996[a]	204.8[a]	16.46[a]	14.32[ab]	261.2[c]	2.196[b]
P value	< 0.001	0.119	0.811	0.043	0.002	0.047
S.E	2.032	5.843	0.2369	0.5741	22.11	0.2094
Variety						
WH 403	11.480[b]	197.6[a]	16.27[a]	13.84[a]	152.5[a]	1.852[a]
WH 507	7.507[a]	207.8[b]	16.89[b]	14.50[a]	268.1[b]	2.171[a]
P value	0.006	0.009	< 0.001	0.125	<.001	0.061
S.E	1.412	4.071	0.1634	0.3990	15.64	0.1223

Means in the same column and the same variable (FK3 rate or variety) followed by the same letter are not statistically different from each other.

significantly more *S. hermonthica* plants than WH507, but WH403 had significantly shorter plants, fewer leaf number and less root mass than WH507 (Table 2).

The maize yield parameters of the second pot trial gave significantly higher maize seed weight (100 grains) and total cob weight using the higher rate of FK3 when compared to the untreated control. However there were no significant differences in the cob diameter, length or moisture content with any rate of FK3. The variety WH403 gave significantly lower grain weight and higher total cob weight than WH507 but no other differences between cob diameter, length or moisture content (Table 3).

DISCUSSION

The two trials consistently show that the *F.* *oxysporum* isolate FK3 reduced the number of *S. hermonthica* plants when maize of different varieties was grown in pots. The reduction of *S. hermonthica* was greatest with the two highest rates of FK3 in trial 2. The use of the isolate Foxy 2 in Benin and Burkina Faso showed that *S. hermonthica* numbers were reduced in maize and sorghum crops (Venne et al., 2009), however when used in Kenya Foxy 2 had no effect on S. hermonthica reduction (Avedi et al., 2014). A range of *S. hermonthica* pathogens with visible characteristics of *F. oxysporum* was sampled from maize fields in Western Kenya and West Africa in 2012. These pathogens were isolated and comparative analysis undertaken showed that the populations from the two regions, Kenya and West Africa, were genetically different from each other (Wainwright and Viljoen, 2014). *S. hermonthica* is present in both East and West Africa; however a recent genetic study with

microsatellite markers showed that a small portion (8%) of the variation occurred between regions of origin of the populations (Bozkurt et al., 2015). The genetic variation in both *S. hermonthica* and *F. oxysporum* pathogens may explain the regional specificity of *F. oxysporum* isolates as a potential biological control agents.

The preparation of the FK3 isolate for soil Inoculation was based on rice media that was dried and coarsely ground prior to application to the planting hole. This method of preparation showed considerable promise as a simple low cost method of manufacturing the inoculum and then treating the soil. This is similar to the concept of the Pesta granule as a means of inoculating the soil (Elzein and Kroschel, 2006), however the preparation based on rice grains is simpler than the Pesta granule that contained semolina, kaolin, and sucrose as well as the fungal inoculants. The efficacy of soil applied biological control agents

Table 3. Mean maize yield parameter of maize (grain weight, cob diameter, cob length, seed moisture content and total cob weight per plant) in the second season pot trial.

Treatments	Grain weight (100 seeds) (g)	Cob diameter (cm)	Cob length (cm)	Seed moisture content (%)	Total cob weight (kg)
Rate of FK3					
Control	28.50[a]	3.00[a]	10.65[a]	26.80[a]	1.689[a]
FK3 lower rate	29.00[ab]	3.10[a]	11.20[a]	29.80[a]	1.797[ab]
FK3 medium rate	28.70[a]	3.10[a]	10.90[a]	27.45[a]	1.776[ab]
FK3 high rate	30.00[b]	3.15[a]	10.95[a]	30.70[a]	1.995[b]
P value	< 0.001	0.816	0.056	0.624	0. 031
S.E	1.637	0.1229	0.3432	1.112	1.562
Variety					
WH 403	24.75[a]	3.08[a]	11.45[a]	28.33[a]	2.072[a]
WH 507	33.25[b]	3.10[a]	10.40[a]	29.05[a]	1.556[b]
P<0.05	0.026	0.57	0.063	0.42	0.038
S.E	1.223	0.0869	0.2760	1.047	1.353

Means in the same column and the same variable (FK3 rate or variety) followed by the same letter are not statistically different from each other.

such as FK3 appears to rely on dose, as increasing the rate showed a decrease in *S. hermonthica* plant numbers. However there was no interaction between dose rate and variety on *Striga* numbers. There are practical and economic limitations on how much inoculum will be able to be applied to the field situation and further evaluation of this aspect will need to be assessed before such an application method may be applied in the field.

The performance of maize showed there were indications that when exposed to *S. hermonthica*, growth parameters were improved with the use of FK3 such as internode length, fresh root mass and stover weight. Similarly harvested yield of maize showed increases in grain and cob weight. However pot trials are not a reliable enough method to evaluate growth and yield studies based on twenty plants per treatment. However the pot trials results reported here strongly suggest the need to move to open field trials to assess the impact of FK3 on maize yield when stressed by S. hermonthica infestations. The maize variety WH403 produced significantly more *S. hermonthica* plants than WH507, whilst there were no differences between WH507 and the local variety Rachar. Therefore, variety susceptibility with *S. hermonthica* is important, but the response to FK3 in *Striga* number reduction was evident in all varieties which indicates the wider applicability of this technique to a range of germplasm. The level of *S. hermonthica* in the first trial for WH507 (6.36 plants per pot) were very similar to those in the second trial (7.5 plants per pot) for the same variety which demonstrates the consistency of the methodology used.

The use of biological control treatment for the reduction of *S hermonthica* has numerous benefits for the control or reduction *S. hermonthica.* The inoculum can be used on

both hybrid and farmer saved seed, the latter being of continuing importance as a farming practice in many sub-Saharan African regions. The biocontrol has the potential to be used on a range of species that are affected by *S. hermonthica* such as sorghum, rice and millet as well as maize though this needs verification. However this work in conjunction with other findings suggests that *S. hermonthica* pathogens are region specific.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Optimization of culture media for *Desmodium incanum* micropropagation

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Desmodium incanum DC., popularly known as pega-pega, is a wide spread leguminous plant in fields in the state of Rio Grande do Sul in Southern Brazil and is well accepted by the cattle as forage. However, its forage potential is currently threatened, due to the replacement of natural grasslands by agricultural crop and grazing lands and because of the poor pasture management associated with high stocking rate, making it necessary to search for alternatives for the preservation of this genetic resource. The aim of this study was to test variations in the composition of culture media in order to optimize the development of *in vitro* plantlets of *D. incanum.* The study was divided into three stages: the first test, evaluated different compositions of MS medium, varying concentrations of nutrients, especially macronutrients (MS 50%; MS 50% and macronutrients at 25%; MS 100% and macronutrients at 50%; MS 100% and macronutrients 25%); the 2nd test evaluated different concentrations of MS medium nutrients, especially micronutrients (MS 50%; MS 100% and micronutrient at 50%; MS 50% and micronutrients at 25%); and the 3rd test, in which varying concentrations of IBA (0, 0.5, 1.0 and 1.5 mgL^{-1}) were evaluated. In Test 1, the culture media with components diluted to 50% provided better development for *D. incanum* in relation to the media with 100% of the components, and MS 50% was the best treatment. In Test 2, the MS medium with 25% of micronutrients and 50% of other components provided the best growth. In Test 3, the species *D. incanum* responded positively to the addition of IBA with an increase in root development.

Key words: *In vitro,* forage, pampa biome, leguminous plant, mineral nutrition.

INTRODUCTION

Desmodium incanum, popularly known as pega-pega, is a perennial, summer crop and native leguminous plant which presents a prostrated and growing growth habit

(Oliveira, 1983). According to Burkart (1939), the species is widely spread throughout fields in the state of Rio Grande do Sul (RS) fields in Southern Brazil and Damé

(1999) found that *D. incanum* was predominant in the Fabaceae family, with a frequency above 50%, except during the winter period, when its population was considerably diminished. It is an important forage due to its favorable qualitative characteristics and its good acceptance by the animals (Boldrini, 1993). The effective dispersion of *D. incanum* is associated to the trichomes present on the fruit epicarp, which facilitate t h e spread of seeds through the epizoocoria (Souza et al., 2006).

However the information available about the biodiversity of native grasslands is still lacking (Overbeck et al., 2007), and their forage potential is still neglected by most technicians and producers, due to improper management of native pastures, combined with intense pressure from grazing due to high stock densities, leading to the extinction of many field species.

Between 1970 and 2005, Pillar et al. (2009) estimated that 4.7 million hectares of native grasslands in the state had been converted to other agricultural uses, such as farming and afforestation areas. In this context, there is a need for preservation of forage and *D. incanum* appears as a promising alternative because of its potential for recovery of native grassland areas affected by crops (Favreto, 2004).

One efficient strategy for the preservation of germplasm is *in vitro* growth, where whole plants can be obtained from the growth of cells, tissues or plant organs (Pence, 2011). *In vitro* growth techniques allow for the control of climate variables such as light intensity, temperature and humidity, and also the availability of nutrients and vitamins that the plant can acquire from the culture medium. Nutritional requirements vary among different species, making it necessary to perform initial tests and adjustments for the *in vitro* establishment of any species.

In order to induce the processes responsible for generating tissues and organs, growth regulators are often used to stimulate the formation of shoots and roots (Kielse et al., 2009). Auxins act on cell expansion, elongation and division, their main function being root induction (Blakesley et al., 1991; Werner and Pla, 2012).

Successful *in vitro* development necessitates knowledge of a species' nutritional requirements, thus it is necessary to verify the best growth medium composition for each species. As *D. incanum* is native to acid soils with low fertility (Miotto, 2011), it can be assumed that it does not have a high nutritional requirement, but this information needs to be tested. In addition, little is known about the tolerance of this species to salinity (Marques, 1991). Thus, the aim of this study was to test variations in the nutritional composition of growth media in order to optimize the *in vitro* development of seedlings of *D. incanum*.

MATERIALS AND METHODS

In all tests, the growth media were based on variations of MS growth medium (Murashige and Skoog, 1962) (Table 1). Besides the macro and micronutrients and vitamins, 30 gL^{-1} of sucrose and 100 mgL^{-1} of myo-inositol were added to the MS media. After dilution, the media were completed to a volume of 1 L. The pH of the culture media was adjusted to 5.8 and 7 gL^{-1} of agar were added before autoclaving. The seeds of *D. incanum* were collected at the Iwar Beckman Research Center - Fepagro Campanha, lat. 31.48'S and long. 53.88'W, mechanically scarified on sandpaper number 120 (sand grains cm^{-2}), and then disinfected according to the methodology proposed by Maldaner et al. (2014). After inoculation, the material was kept in a growth room at a temperature of 25 ± 2°C, photoperiod of 16 h of light and photon fluency rate of 35 µmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps.

All tests were conducted in a completely randomized design with 20 replications, and the experimental unit consists of a glass flask (100 ml) with approximately 20 ml of culture media containing three seeds of *D. incanum*. Subsequently, the tallest seedling of each flask was used for evaluations.

Test 1: Nutrient concentration - Effect of macronutrient in *D. incanum* development

Four different growth medium compositions were evaluated as shown in Table 2. At the thirtieth day of growth, the following aspects were evaluated: height, number of nodes and number of leaves of *D. incanum* seedlings. The resulting data were subjected to analysis of variance and means of each treatment were compared by Tukey test at 5% probability (p ≤ 0.05). Statistical analyses were performed using the software ASSISTAT 7.7 (Silva and Azevedo, 2002).

Test 2: Nutrient concentration - Effect of micronutrients in *D. incanum* development

The different growth medium compositions are presented in the Table 3. After thirty days of growth, the following morphological data were recorded: height, number of roots, number and color of leaves, number of shoots, root dry weight and shoot dry weight. For the color of leaves notes were given from 1 to 4, with 1 for lightest and 4 for darkest green. The resulting data were subjected to analysis of variance and means of each treatment were compared by Tukey test at 5% probability (p ≤ 0.05). Statistical analyses were performed using the software ASSISTAT 7.7 (Silva and Azevedo, 2002).

Test 3: Effect of different concentrations of indolebutyric acid – IBA in *D. incanum* development

After being sterilized and scarified, the seeds were germinated in a growth medium with half of the concentration of all mineral nutrients, vitamins and FeEDTA of the MS culture medium (MS to 50%), and subjected to different concentrations of indolebutyric acid (IBA) concentrations (0, 0.5, 1.0 and 1.5 mgL^{-1}). At the end of thirty days, height, number of shoots, number of leaves, number of roots, root dry weight and shoot dry weight were evaluated. The resulting data were subjected to analysis of variance and when there was a significant difference, with α = 0.05, the means of each treatment were subjected to regression analysis. Statistical analyses were performed using the software ASSISTAT 7.7 (Silva and Azevedo, 2002).

RESULTS

The treatments in Tests 1 and 2 were chosen based on

Table 1. Composition of MS culture midium (Murashige and Skoog, 1962).

Component	Formula	Concentration (mg L-[1])
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride	$CaCL_2.2H_2O$	441
Magnesium sulphate	$MgSO_4.7H_2O$	370
Potassium phosphate	KH_2PO_4	170
EDTA disodium	Na_2EDTA	37,25
Potassium iodide	KI	0,83
Micronutrients		
Iron sulfate	$FeSO_4.7H_2O$	27.85
Manganese Sulfate	$MnSO_4.H_2O$	16.9
Zinc sulfate	$ZnSO_4.7H_2O$	8.6
Boric acid	H_3BO_3	6.2
Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.25
Cobalt Chloride	$CoCL_2.6H_2O$	0.025
Copper sulphate	$CuSO_4.5H_2O$	0.025
Vitamins		
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine hydrochloride	$C_6H_{12}ClNO_2$	0.5
Thiamine hydrochloride	$C_{12}H_{18}CL_2N_4OS$	0.5
Glycine	$C_2H_5NO_2$	2.0
Myo-inositol	$C_6H_{12}O_6$	100.0
Agar		7.000
Sucrose	$C_{12}H_{22}O_{11}$	30.000

Source: Adapted from Oliveira et al. (2005).

Table 2. Treatments (T1, T2, T3 and T4) that have been derived from the standard composition of MS medium (table 1) and their respective percentage of each constituent.

Test 1	Vitamins (%)	Macronutrients (%)	Micronutrients (%)	FeEDTA (%)
T1	50	25	50	50
T2 – standard*	50	50	50	50
T3	100	50	100	100
T4	100	25	100	100

T2 was choice as standard treatment due results from previous experiments.

Table 3. Treatments (T1, T2 and T3) that have been derived from the standard composition of MS medium (table 1) and their respective percentage of each constituent.

Test 2	Vitamins (%)	Micronutrients (%)	Macronutrients (%)	FeEDTA (%)
T1 –standard *	50	50	50	50
T2	100	50	100	100
T3	50	25	50	50

*T1 was choice as standard treatment due results from previous experiments.

results of previous experiences (Schwalbert et al., 2014), in which reducing the medium components to half (MS medium to 50%) showed better results than the full culture medium for *D. incanum*. Therefore the medium
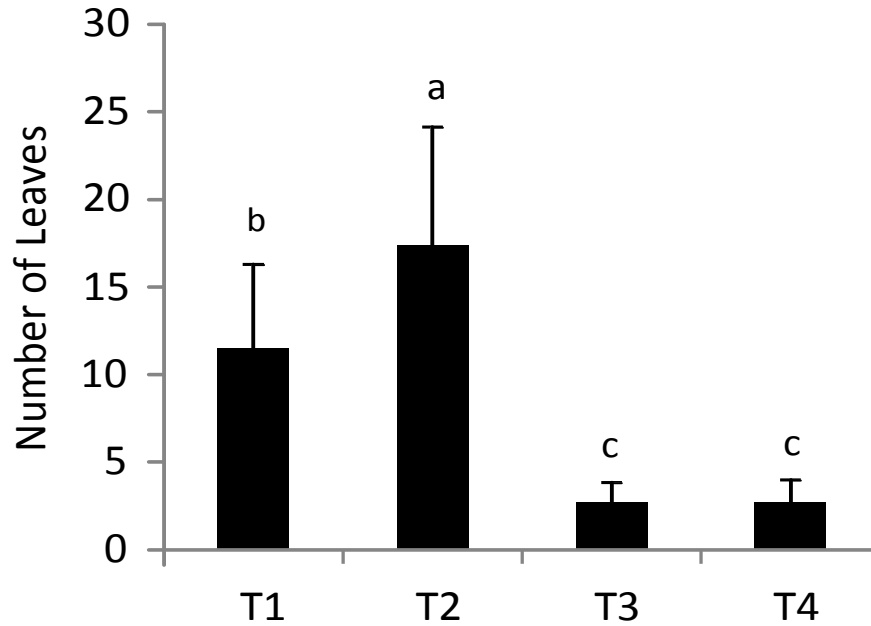
Figure 1. Effect of different compositions of MS culture media in seedling height (A), number of nodes (B) and the number of leaves (C) of *D. incanum* cultivated *in vitro*. Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

with half of all the components was adopted as the standard in both tests. The other treatments were an attempt to find out which medium component, when reduced in relation to the complete medium, favored the growth of the species. The best treatment found in Tests 1 and 2 was used for Test 3.

Test 1: Nutrient concentration - Effect of macronutrient in *D. incanum* development

Variation of macronutrient concentrations significantly affected the height of shoots, as well as the number of nodes and the number of leaves of *D. incanum* grown *in vitro*. Half of the concentration of the basic nutrient composition of MS culture medium (T2) promoted growth in terms of height and production of nodes and leaves (Figure 1) when compared to the other treatments.

Test 2: Nutrient concentration - Effect of micronutrients in *D. incanum* development

The darker color of leaves, which visually indicates a healthy pattern was obtained with 50% of the concentration of micronutrients, keeping the other components of MS medium at the standard concentration (T2). However, when all nutrients were reduced by 50% (T1), the color of green was less intense, while the reduction to one fourth part of micronutrients and 50% of macronutrients, vitamins and FeEDTA (T3) produced clearer leaves, which were visibly affected and chlorotic (Figure 2).

On the other hand, reducing the concentration of micronutrients to one fourth part and macronutrients, vitamins and FeEDTA to one half (T3), promoted the development of shoots, leading to a higher number of roots and increasing dry weight of roots and shoots (Figure 3). The number of *D. incanum* roots was 1.2 times higher in the more diluted micronutrient treatment (T3) when compared to T2, which was the second best treatment (Figure 3C), while the root dry weight in T3 exceeded T2 by 4.2 times (Figure 3E), demonstrating that the plants not only produced new roots, but showed increased weight of the root system. According to Silveira and Monteiro (2011), under a low supply of nitrogen, the plant produces longer roots, while an increased supply of this nutrient reduces the length of the roots.

The number of leaves and the number of shoots per plant was not significantly affected by the treatments (Figure 3D, B). However, the plant morphology changed, despite these variables not being statistically different, as plant height and shoot dry mass were increased in treatment 3 Figure 3A, F), with lengthened internodes and larger leaves.

Test 3: Effect of different concentrations of indolebutyric acid – IBA in *D. incanum* development

Regression analysis resulted in a linear fit for root dry
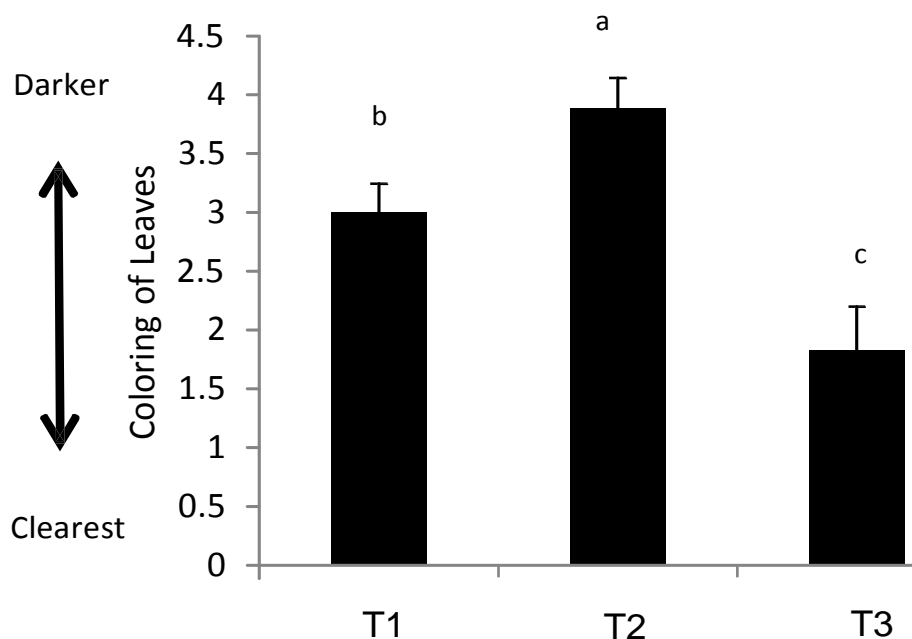
Figure 2. Effect of different compositions of MS culture media in the coloration of *Desmodium incanum* leaves cultivated *in vitro.* Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

weight and IBA concentrations (Figure 4). Root dry weight was increased linearly by IBA concentrations (Figure 4). On the other hand, IBA concentrations were not sufficient to promote significant differentiation in the components of shoot morphology. However, a tendency to increase was observed for height of shoots and shoot dry weight (Figure 5).

DISCUSSION

Test 1 results indicate a likely intolerance of the species to high concentrations of nutrients, what may be attributed to the fact that the species *D. incanum* is native to acid soils with low fertility and adapted to these conditions (Miotto, 2011). In addition, the results suggest some toxicity especially from micronutrients, because the reduction only in the concentration of macronutrients (treatments 3 and 4) led to a decrease in all parameters (Figure 1).

The toxicity of micronutrients has long been studied. Increasing the availability of manganese in the soil, for example, leads to an increase in its content in the aerial part of the plant, reducing the production of chlorophyll, and therefore the photosynthetic capacity, hindering the growth of roots and total dry weight (Smith et al., 1983). Copper toxicity appears primarily in the roots, damaging the permeability of membranes (Seliga, 1993). Corn plants fertilized with high doses of boron showed toxicity symptoms as chlorosis in older leaves evolving to necrosis in a "V" (Leite et al., 2003).

Antagonistic effects on the absorption of some elements may also occur due to an increase in the concentration of others. According to Malavolta (1994), the presence of zinc can prevent the absorption of other ions by competition and thus can cause chlorosis of the leaves. Moreover, Disarz and Corder (2009) observed that a 25% reduction of the nutrient concentration in the MS medium was beneficial for the formation of buds and leaves of *Acacia mearnsii.* Other authors also found satisfactory results by reducing the macronutrient content in MS culture medium for several species (Mercier and Kerbauy, 1992).

Tamaki and Mercier (2007) noted that *Ananas comosus* plants grown in MS culture medium with a fifth of the nutrient concentration had absorbed and assimilated sufficient nitrogen quantity for normal development of plants, as that obtained in basic MS medium.

In Test 2, the nitrogen content probably interfered directly in the staining of leaves, since according to Booij et al. (2000), the amount of chlorophyll is directly correlated with the N concentration in the plant. Similarly, other micronutrients were also correlated to chlorophyll content in tissues (Taiz and Zeiger, 2004). For this variable, the reduction of the concentration of micronutrients to 25% (T3) appears to be too high, resulting in chlorotic leaves, which may indicate some micronutrient deficiencies. Many authors have reported the symptoms of chlorosis with a deficiency of micronutrients such as boron, zinc, iron and manganese (Malavolta et al., 1997). The other results from Test 2 (Figure 3), agree with those presented in Test 1,
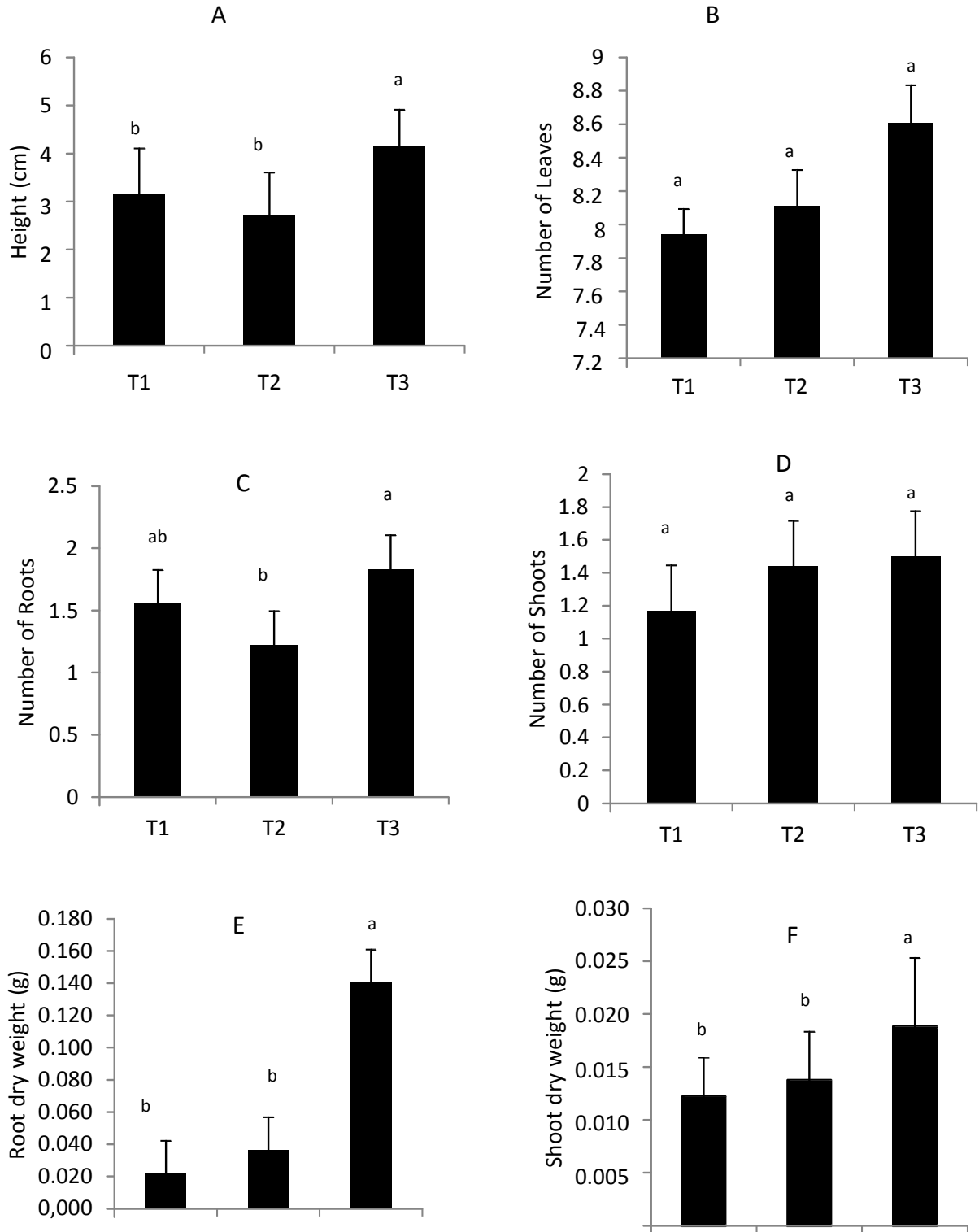
Figure 3. Effect different compositions of MS culture media in height (A), number of leaves (B), number of roots (C), number of shoots (D), roots dry weight (E) shoot dry weight (F) of *Desmodium incanum* cultivated *in vitro.* Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

indicating that the reduction in the concentration of nutrients, particularly micronutrients is favorable for the

growth in height of *D. incanum.* Comparing the results for height of shoots (Figure 3A) and number of roots
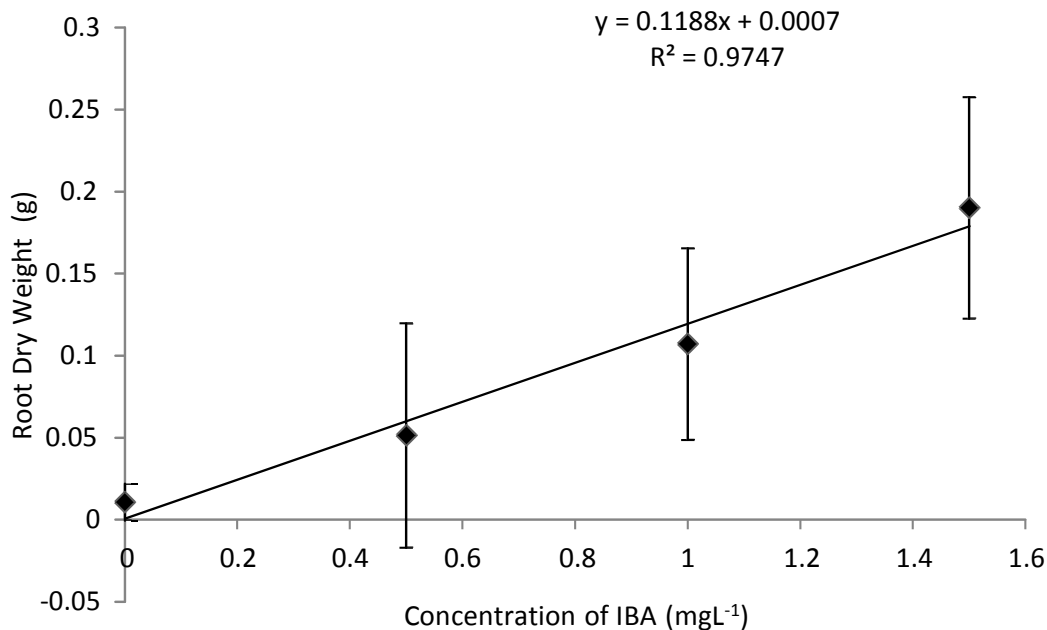
Figure 4. Effect of different concentrations of IBA (mgL^{-1}) in root dry weight of *Desmodium incanum* cultivated *in vitro* $p < 0.01*$.

per plant (Figure 3C) it is also possible to link the increased height in treatment 3 with the highest number of roots also verified in this treatment. Plants grown in saline media often have restricted vegetal growth, when the concentration of mineral ions reaches levels that limit water availability or exceed the appropriate amount of a particular nutrient. The mechanisms by which plants tolerate salinity are complex and involve molecular synthesis, enzyme induction and membrane transport (Taiz and Zeiger, 2009).

In some cases, a restriction of nutrients leads to increased mass allocation to the roots when compared to the shoot, in unrestricted light conditions (Chapin, 1980). In most cases, the explants do not start the rooting process in culture media with high concentrations of salts, even in the presence of auxin. Reducing the salt concentrations in the culture media to 1/2, 1/3 or 1/4, enables enhanced rooting (Hu and Wang, 1983).

The increase in plant height and number of roots and the healthy coloration of the plants when the concentration of micronutrients was reduced to 50% may also indicate the toxicity of some micronutrient(s) as already discussed with regard to the results obtained in Test 1. For shoot dry weight (Figure 3F), similar results were found by Kanashiro et al. (2007) in *Aechmea blanchetiana* seedlings, in which this variable decreased linearly with increasing nitrogen concentration in modified MS medium. Moreover, the number of leaves was increased, according to the quadratic regression. According to Illenseer and Paulilo (2002), with a change in nutritional regimen, species can show morphological

and physiological changes to maximize the dry weight gain in the new conditions, or vary the distribution of biomass between root and shoot (Osunkoya et al., 1994). In addition, plants subjected to low levels of nitrogen showed higher use efficiency of this nutrient (Illenseer and Paulilo, 2002).

For Test 3, the results presented in Figure 4 can be explained by the change in the standard balance of auxin/cytokinin. For differentiation and tissue formation, as well as growth *in vitro*, a suitable balance between auxins and cytokinins is necessary, maintaining the auxin/cytokinin ratio less than one (Pierik, 1990). When the auxin level is high in relation to cytokinin, root formation occurs, while the opposite leads to shoot formation and even when the proportions are approximately the same, a mass of callus is produced (Krikorian, 1995). Similarly, Nascimento et al. (2008) found the highest percentage of root formation (60%) of *Eugenia pyriformis* in a treatment with 1.0 mgL^{-1} of IBA. Machado et al. (2011) observed an increase in lavender rooting percentage with increasing concentrations of IBA, up to 5.0 mM.

Although it is possible to observe an upward trend in height and shoot dry weight in response to IBA concentration (Figure 5D), available data did not show statistical differences. A similar trend was observed by Silva et al. (2011). Moreover, Machado et al. (2011) observed that increasing concentrations of IBA reduced plant height and root length of lavender plants. The number of leaves was constant for all IBA concentrations tested, with no significant difference between means
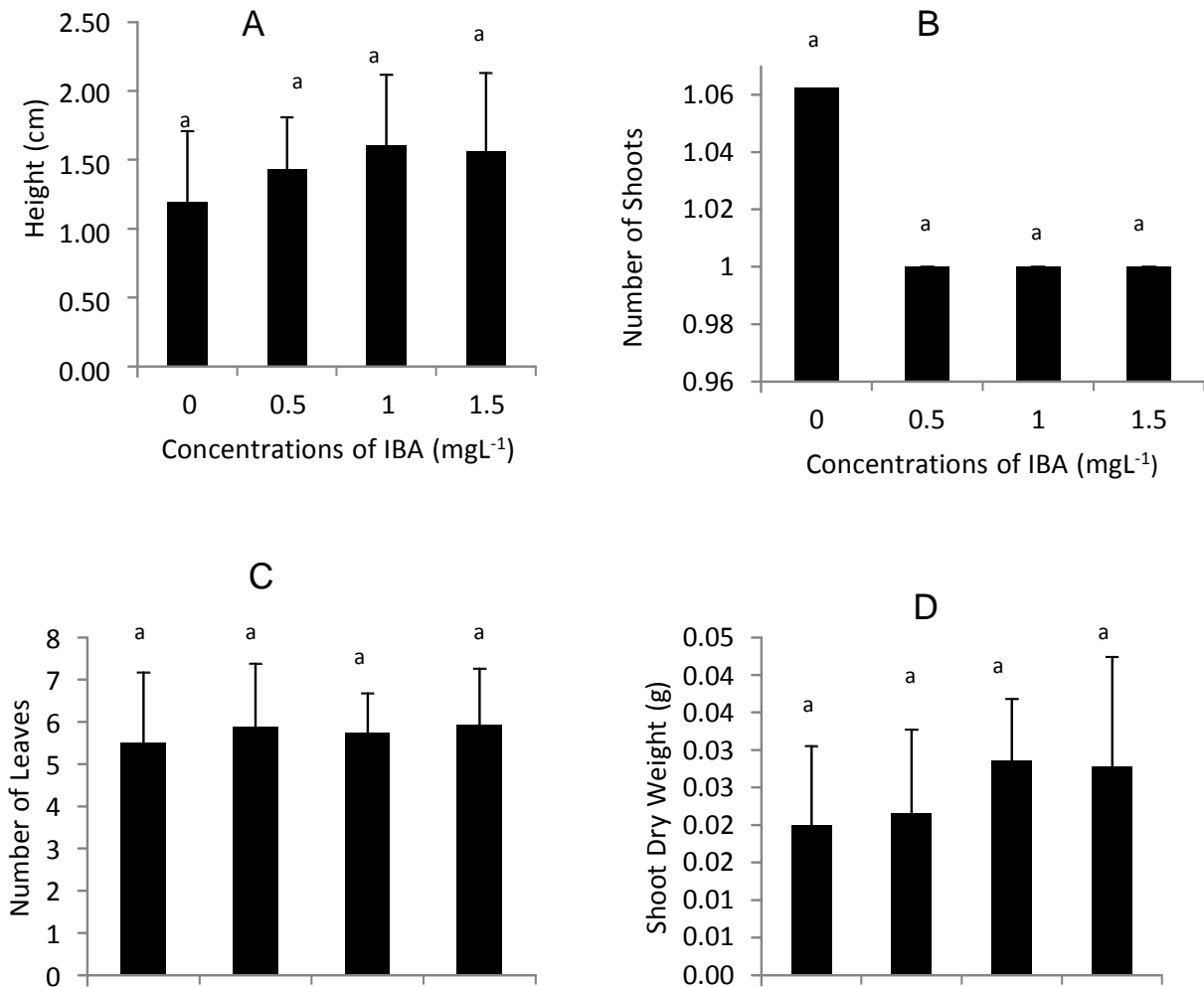
Figure 5. Effect of different IBA concentrations (mg L^{-1}) on height (A), number of shoots (B), number of leaves (C) and shoot dry weight (D) of *Desmodium incanum* cultivated *in vitro*. Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

(Figure 5C). Similar results were found by Horbach et al. (2011). The number of shoots tended to increase in the absence of IBA, although without statistical difference (Figure 5B). Similar results were observed by Maia and Botelho (2008), with a constant number of one shoot, regardless of IBA dose.

Conclusion

Reducing the concentration of macronutrients, micronutrients, vitamins and FeEDTA to 50% provided the greatest growth and development of *D. incanum* grown *in vitro*. Moreover, a greater reduction (to 25%) of micronutrients appears to have further promoted the growth of this species. In addition, *D. incanum* responded positively to the addition of IBA in terms of root growth.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Efficiency of optimal pheromone trap density in management of red palm weevil, *Rhynchophorus ferrugineus* Olivier

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The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier is an invasive, hidden pest of date palms causing significant economic losses in Middle East. Mass trapping of weevils with synthetic male aggregation pheromone and food baited traps has been an important component of Integrated Pest Management (IPM) program against RPW. As the viability of pheromone trapping system depends on the optimum trapping density, field experiments were conducted in two locations of Eastern Province of Saudi Arabia. The efficiency of four pheromone trap densities viz., 1, 2, 4 and 8 traps/4 ha, were evaluated using the Standard Saudi Trap. In Al Hassa experiment, the treatment with 1 trap / 4ha captured an average 10.0 weevils as compared to 61.5 weevils in 8 traps / 4 ha, while at Al Qatif experiment an average of 5.0 and 49.8 weevils were captured in 1 trap and 8 traps / 4 ha respectively in 10 weeks indicating the superiority of high density trapping. Although RPW adults are strong fliers, these experiments showed that more traps per unit are necessary to capture the resident adults in a shorter period of time. Hence, our studies provide information for optimizing trap density for mass trapping program.

Key words: Red palm weevil, *Rhynchophorus ferrugineus,* pheromone, trap density, date palm.

INTRODUCTION

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier, is the most dangerous pest of date palm (*Phoenix dactylifera*) in Middle East region responsible for the death of a large number of palms and consequent yield losses (Abozuhairah et al., 1996; Abraham et al., 1998). It was reported as a serious invasive pest of Canary Island date palm in Europe from the year 1996 and continues to remain a serious threat even today

(Barranco et al., 1996; Esteban-Durán et al., 1998; Ferry and Gómez, 2002; Haddad, 2009). More recently *R. ferrugineus* has been reported for the first time in the year 2010 from a location in Orange County, California, USA (CDFA, 2011). The larvae of RPW bore in to the stem tissues of a palm to complete the life cycle. Upon emergence the adults fly out and infest new palms or remain in the same palm and cause re-infestations at a new site on the same palm (Nirula, 1956; Esteban-Durán et al., 1998). Since the destructive stage is hidden inside the palm stem tissue, it is difficult to administer an appropriate control method (Abraham et al., 1998; Murphy and Briscoe, 1999). Among recent works an improved instrumentation and signal analysis software were described for early detection of RPW larval stages in groves and greenhouses (Mankin, 2011).

For the most effective management of this pest, integrated pest management (IPM) developed earlier was applied in most of the Gulf countries. As an important module of IPM program, the Agriculture Ministry of Saudi Arabia has been organizing the mass trapping of RPW for over 15 years (Abraham et al., 1998; Faleiro, 2006). Recent researches have also been focusing on finding environment friendly natural products as alternatives for the management of *R. ferrugineus*.

The male aggregation pheromone of *R. ferrugineus* was identified as 4-methyl-5-nonanol and 4-methyl-5-nonanone by Hallett et al. (1993) and soon commercial formulations were available for monitoring or mass trapping. The pheromone components 4-methyl-5-nonanol and 4-methyl-5-nonanone in the ratio of 9:1 was found to be effective for attracting both male and female adults of RPW (Hallet et al., 1999; Abozuhairah et al., 1996). Pheromone trapping of adult palm weevils was used to capture and kill the insect to reduce the insect populations in the field (Oehlschlager et al., 1993; Abraham et al., 1998; Vidyasagar et al., 2000). The aggregation pheromones have high potential in the management of palm weevils especially the American palm weevil, *Rhynchophorus palmarum* and *R. ferrugineus* as it attracts both males and females (Rochat et al., 1991; Oehlschlager et al., 1993; Giblin-Davis et al., 1996; Hallett et al., 1999). The trapping methods are also advantageous as they are efficient in attracting a much higher percentage of females in comparison to males and this kind of pheromone based system helps in further reducing progenies (Vidyasagar et al., 2000; Faleiro et al., 2002; Soroker et al., 2005). However, the trap requires addition of food bait and pesticide to kill the attracted weevils. Most studies have not been transferred to the field. Moreover, for the success of IPM it is necessary to know the optimum number of traps for a specific unit area.

In the present investigations, large scale field studies to find solutions for optimization of trap density have been conducted. This study will help in revising the present trapping methods for area-wide trapping of red palm weevil not only in Saudi Arabia but also in the entire Middle East region. It attempts to find a more efficient way of augmenting weevil capture in a unit area within a shorter period of time by testing variable number of traps. For this purpose, a field experiment with variable number of trap densities was designed and conducted it in two locations. These studies are planned to provide answers regarding the optimum density of traps for mass pheromone trapping program of *R. ferrugineus*.

MATERIALS AND METHODS

The field experiments were conducted in Al Hassa (25° 23' 24" N latitude, 49° 35' 5" E longitude) and Al Qatif (26° 34' 36"N latitude, 49° 59' 9"E longitude) regions of Eastern Province of Saudi Arabia in the year 2010 during early summer months. In each location several date farms were selected for setting up traps based on the experimental design. A careful choice was made to ensure the weevil capture rates in all these farms were more or less uniform prior to the start of field trials. Before the start of each experiment, regular trapping was discontinued for about one week to avoid any population changes due to emerging adults. The trap density experiment was organized for 10 weeks.

Al Hassa

The first experiment was organized in Al Hassa in Eastern Province of Saudi Arabia. This region has more than three million date palms and produces the finest date fruits in Saudi Arabia. The experiment was conducted with a randomized block design (RBD) having 4 treatments and 10 replications (Figure 1). For each treatment a block of 4 ha of date farm was selected and all treatments in each replication were in one continuous area. The distance between replications was kept at more than 200 m to avoid interaction. Standard Saudi Trap described in detail below was used in this trial. At this location date fruits were used as food bait in traps. The four treatments imposed were one trap per 4 ha; two traps per 4 ha; four traps per 4 ha; and eight traps per 4 ha. Total area under this field experiment was about 160 ha with about 16,000 palm trees.

Al Qatif

The second trial was conducted in Al Qatif in the Eastern Province of Saudi Arabia. This region has about one million date palms and the infestations by RPW were first reported from this region. We followed RBD with four treatments and 8 replications as in Figure 1. The pheromone trap used was also of the same design and lure except that the food bait used at this center was freshly cut date stem pieces in place of date fruits. According to the spacing of palms and replications, the total area under the field experiment was about 120 ha accommodating approximately a palm population of 12,000.

Standard Saudi Trap

The Standard Saudi Trap was prepared by making four square holes in the top half of the 6 L plastic buckets. The buckets were wrapped with jute cloth keeping 4 holes open. The pheromone lures used in this study were the Ferrolure+ (with composition of 4-methyl-5-nonanol and 4-methyl-5-nonanone in 9:1 ratio and purity of 98%) manufactured by ChemTica International, Costa Rica. Each lure contained approximately 600 mg of the lure in a bubble
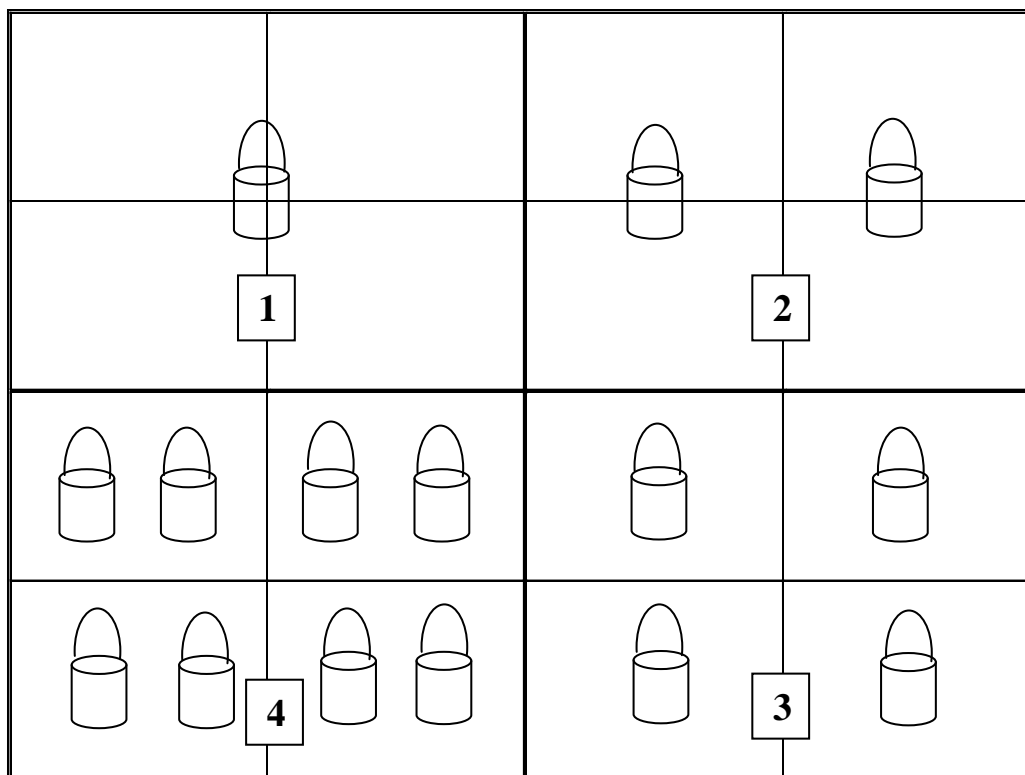
Figure 1. Diagram showing the schematic distribution of Standard Saudi Traps under trap density experiment. Each block of four squares represents four hectares. Treatments are 1. 1 trap/4 ha, 2.2 traps/4 ha, 3. 4 traps/4 ha and 4. 8 traps/4 ha.

formulation. One lure was attached to the lid of bucket from inside and bottom of the bucket was filled with 200 g of ripe date fruits and about 2 L of water. The food baited pheromone traps were mixed with pesticide carbaryl 10 WP 2g per trap to kill the attracted insects.

Weekly servicing and data collection

All the traps were examined every week and RPW adults captured in each trap were recorded in both the experiments. After removing dead insects all traps were replenished with fresh date fruits/stem pieces, water and pesticide. The Ferrolure+ sachets were replaced with new ones in all traps after six weeks to eliminate any differences in available pheromone.

Statistical analysis

The data collected every week at weekly interval for 10 weeks from two locations were subjected to statistical analysis of (one factor) One-Way ANOVA. Both experiments were statistically analyzed with Opstat software available online at CCS, Haryana Agricultural University, Hissar 125 004, India (website: www.hau.ernet.in). The values are presented in the form of table / figures and interpreted according to significance under results for each of the locations.

RESULTS

Generally the efficiency of pheromone trapping was

correlated to the number of weevils captured in each trap for a specific period of time. However, in the case of trap density, it was very important to take in to account the total number of weevils trapped in a unit area with variable number of traps. The data were collected at weekly intervals for 10 weeks and statistically analyzed.

Figure 2A and B illustrates the results on the adult weevils captured in the trapping density experiment in Al Hassa region. Results indicated that the treatment comprising 8 traps / 4 ha captured higher number of adults than all other treatments. The treatments with 4 and 8 traps / 4 ha at weeks 1, 2, 3, 6, 8 and 9 were not significantly different in captured number of adults. Moreover, treatments with 1, 2 and 4 traps / 4 ha were not significantly different in adult catches through the study period.

Figure 3A and B show the results on the adult weevils caught in the trapping density field experiment at Al Qatif location. It showed that the treatment comprising of 8 traps / 4 ha trapped significantly higher number of adults in weeks 1, 3, 7 and 10 than all other treatments but were not significantly different than the treatment 4 traps / 4 ha in other weeks. Also the treatments with 1, 2, and 4 traps/ha were not significantly different in captured number of weevils in the weeks 1, 3, 7 and 10.

Table 1 indicates the mean number of captured weevils / trap / week for 10 weeks in different trap densities at
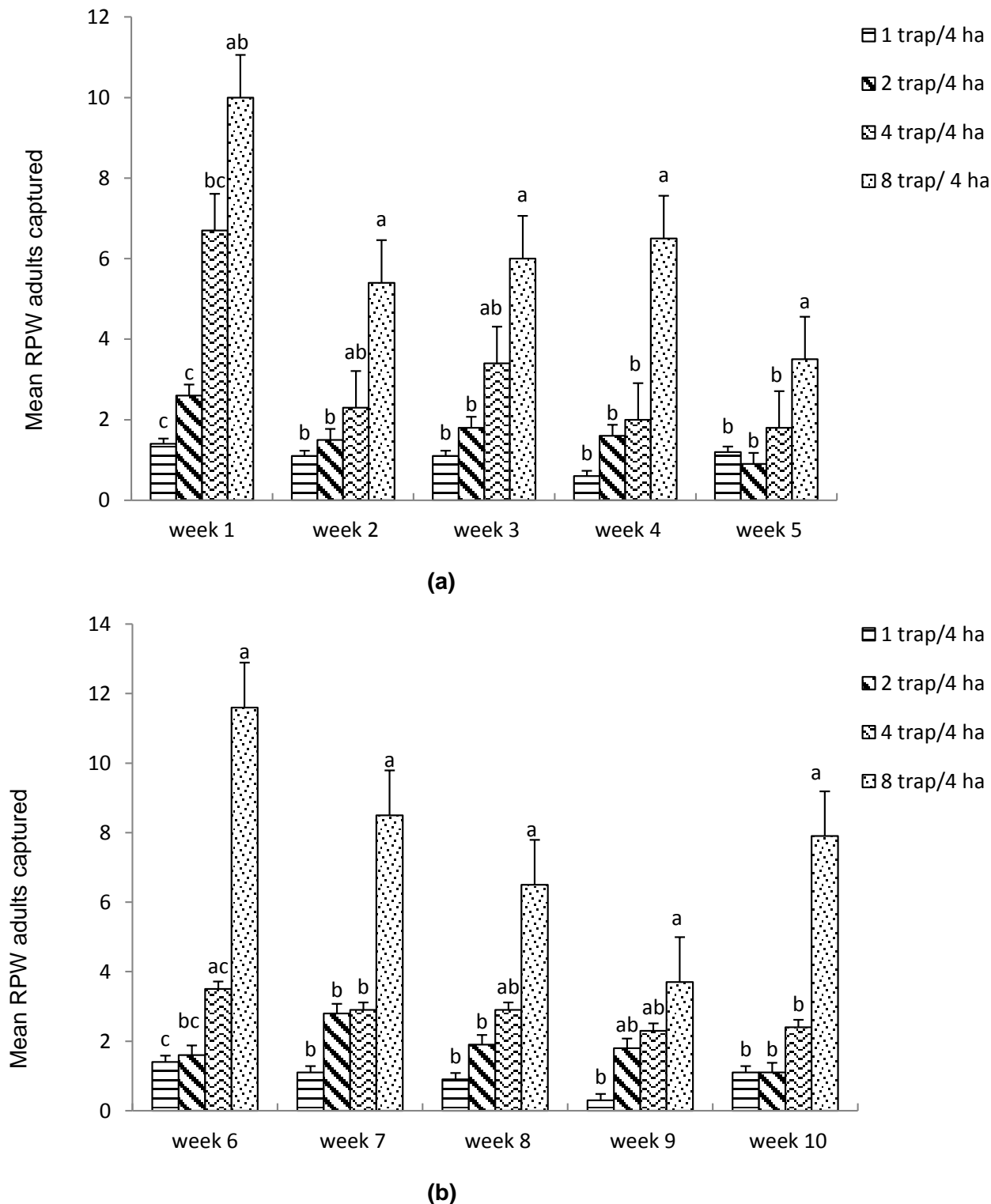
Figure 2. Mean numbers of adult *R. ferrugineus* (RPW)captured in one unit area of 4 ha with different trap densities from **(a)** 1-5 weeks in Al Hassa. One-way ANOVA. (i) Week 1. , P = 0.000076; CD 3.490; SE(m) 1.196;(ii)week 2 P = 0.028782; CD 3.036; SE(m) 1.041; (iii) week 3 P = 0.000499; CD 2.220; SE(m) 0.761; (iv)week 4 P= 0.009723; CD 3.478; SE(m) 1.192; week 5 (v)P = 0.001772; CD 1.323; SE(m) 0.453, and **(b)** 6-10 weeks in Al Hassa. One-way ANOVA. (vi) week 6 P= 0.00287; CD 2.255; SE(m) 0.773; (vii) week 7P= 0.002693; CD 3.818; SE(m) 1.309; (viii)week 8 P = 0.001016; CD 2.644; SE(m) 0.906; (ix)week 9 P= 0.048798; CD 2.370; SE(m) 0.812; (x) week 10 P= 0.00957; CD 4.387; SE(m) 1.504. Higher trap numbers caught significantly more weevils. Bars labelled with same letters are not significantly different (P < 0.05). Standard errors are indicated.
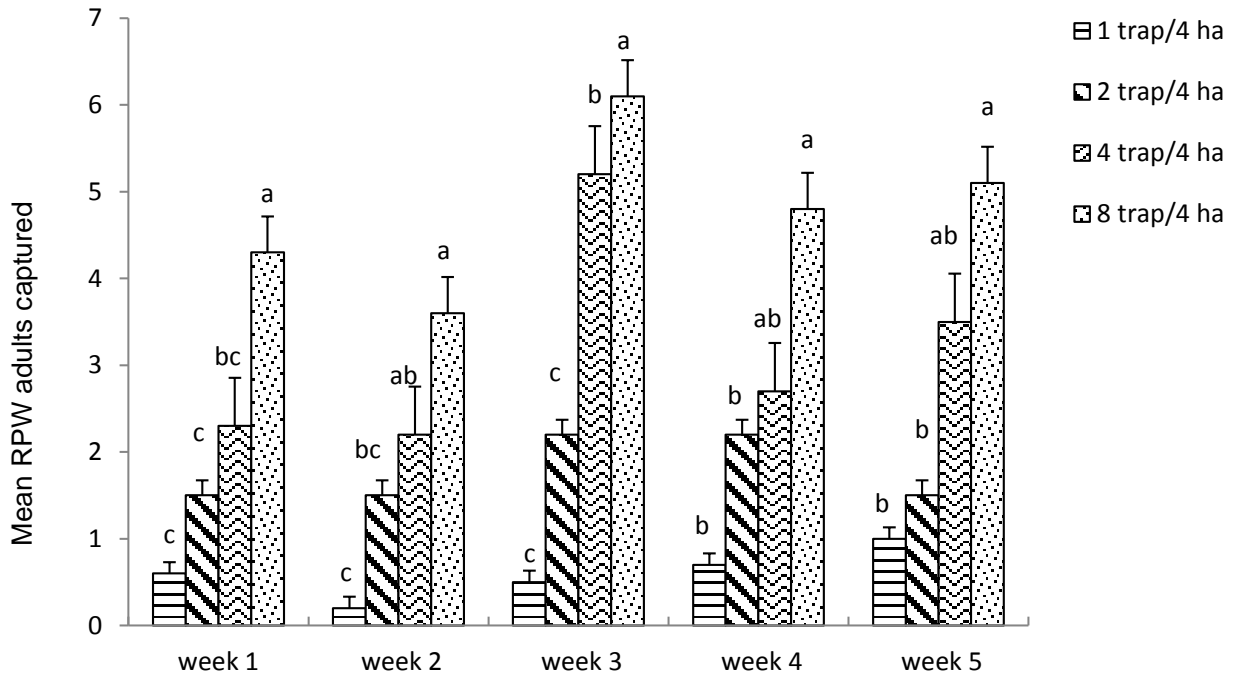
(a)
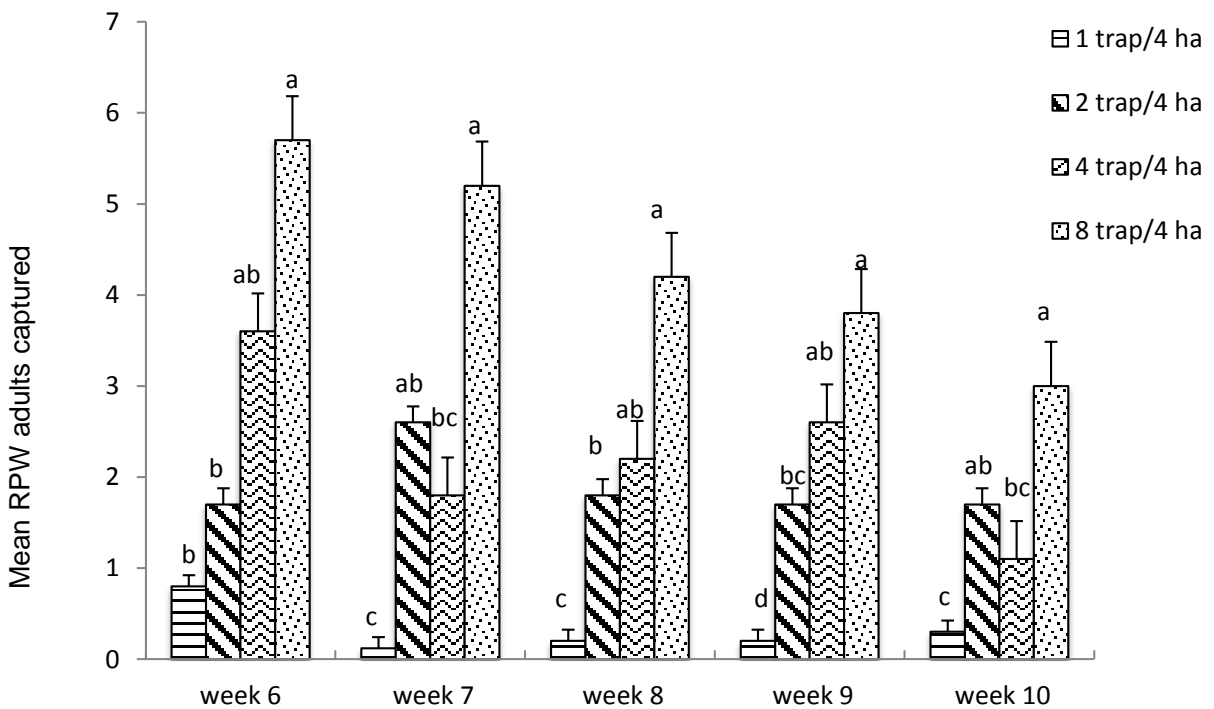
(b)

Figure 3. Mean numbers of adult *R. ferrugineus* (RPW)captured in one unit area of 4ha with different trap densities in **(a)** 1-5 weeks in Al Qatif One-way ANOVA (i) Week 1 *P*= 0.000022; CD 2.187; SE(m) 0.739. (ii)Week 2 *P*= 0.006449; CD 1.797; SE(m) 0.607; (iii) Week 3*P*= 0.000022; CD 2.187; SE(m) 0.739; (iv) Week 4 *P*= 0.003595; CD 2.036; SE(m) 0.688; (v) Week 5 P= 0.015488; CD 2.619; SE(m) 0.884 and **(b)** 6-10 weeks in Al Qatif One-way ANOVA (vi) Week 6 *P*= 0.037036; CD 3.478; SE(m) 1.175; (vii) Week 7*P* = 0.00076; CD 2.444; SE(m) 0.838; (viii) Week 8 *P* = 0.016226; CD 2.345; SE(m) 0.792; (ix) Week 9 *P*= 0.000203; CD 1.394;SE(m) 0.471; (x) Week 10 *P*= 0.005438; CD 1.387; SE(m) 0.469. Bars labelled with same letters are not significantly different (*P* < 0.05). Standard errors are indicated.

Table 1. Mean of captured weevils / trap / week for 10 weeks in different trap densities at Al Hassa and Al Qatif locations.

Location	Treatments	Weevils captured / Trap / Week									
		1	2	3	4	5	6	7	8	9	10
Al Hassa	1 Trap/4 ha	1.4	1.1	1.1	0.6	1.2	1.2	1.1	0.9	0.3	1.1
	2 Traps/4 ha	1.3	0.8	0.9	0.8	0.5	0.5	1.4	1.0	0.9	0.6
	4 Traps/4 ha	1.7	0.6	0.9	0.5	0.5	0.5	0.7	0.7	0.6	0.6
	8 Traps/4 ha	1.3	0.7	0.8	0.8	0.4	0.4	1.0	0.8	0.5	1.0
Al Qatif	1 Trap/4 ha	0.5	0.2	0.5	0.7	1	0.8	0.1	0.2	0.2	0.3
	2 Traps/4 ha	1.1	0.8	1.1	1.1	0.8	0.9	1.3	0.9	0.9	0.9
	4 Traps/4 ha	1.3	0.6	1.3	0.8	0.9	0.9	0.5	0.6	0.7	0.3
	8 Traps/4 ha	0.8	0.5	0.8	0.6	0.6	0.7	0.7	0.5	0.5	0.4

Al Hassa and Al Qatif locations. The mean number of captured weevils in Al Hassa with 8 traps / ha in 10 weeks was 61.6 weevils as compared to 10, 17.4 and 30.8 weevils in treatments 1, 2 and 4 traps / 4 ha respectively. Whereas the mean number of weevils caught in Al Qatif location with 8 traps / 4 ha was 48.8 weevils when compared with 4.5, 19.6 and 31.6 weevils in treatments with 1, 2 and 4 traps / 4 ha respectively.

In 10 weeks duration the 4 ha area with 8 traps on average captured 61.5 weevils whereas, treatments with 1, 2, and 4 traps / 4 ha trapped only 10, 16.9 and 28.5 weevils respectively. Overall best per unit area capture rates were recorded in high density trapping with 8 traps / 4 ha in all weeks and trap density of 4 traps / 4 ha was superior only to treatments with lower trap densities. The statistical values of each week are given in the legends of the corresponding graphs.

DISCUSSION

The studies carried out in Saudi Arabia during mid-nineties with mass pheromone trapping gave positive results in reducing the infestation levels in date farms (Abozuhairah et al., 1996). Abraham et al. (1998) described various methods under IPM for the management of this pest including surveillance and trapping of weevils with pheromone lures. Though mass trapping of RPW was an important component of IPM in Saudi Arabia, the trapping density was not followed systematically and was based on the ease of servicing regularly (Vidyasagar et al., 2000).

Our studies on trap density revealed that 8 traps / 4 ha of area were necessary to capture the maximum number of adults. For example in Al Hassa region the highest density of 8 traps attracted 61.5 weevils in 4 ha area in 10 weeks duration as compared to just 10.0 weevils captured in lowest density of 1 trap treatment during the same period. Similar results were obtained in Al Qatif region as well with 4 traps and 8 traps / 4 ha capturing an average of 28.75 and 49.88 weevils respectively in 10

weeks, suggesting the efficacy of higher density of traps. It was interesting to note that when comparison was made on the weevil capture rate per trap, lower density traps caught numerically more number of weevils than in higher density. But the overall performance of the higher density traps of 4 and 8 traps per 4 ha was much better than other treatments.

By using pheromone trapping in three date palm farms of UAE during 2000 and 2001, the populations of RPW was reduced by 29.7-51.7% (Abbas et al., 2002). Their studies also reported that insect populations peaked in March, April or May months and the marked weevils migrated 1-7 km from the release site and were recaptured within 3-5 days of release. A few trapping studies on RPW in date farms of India were conducted with ferrugineol as pheromone lure and different food baits for their preference (Muralidharan et al., 1999). For monitoring the presence and distribution of the pest the pheromone traps were used in the same study (Martorana, 2008).

The results from *R. palmarum* trapping experiments showed that peripheral traps were not effective in reducing adult weevil populations in oil palm plantations of Costa Rica (Oehlschlager et al., 1993). But, Jayanth et al. (2007) used indigenously synthesized pheromone for mass trapping studies in coconut plantations in South India for 10 months and reported that peripheral traps caught more adults especially in the south and south-eastern directions.

The studies conducted in Israel tested the density parameters up to 10 traps per hectare which was fairly high and required efficient management (Soroker et al., 2005). Their field trials were organized for monitoring with 1 trap per 3 ha in 2200 ha area and mass trapping with 5000 traps @ of 10 traps per ha in an area of 450 ha at 10 regions in Israel. A total of 600 weevils only were caught in five years and ~20% traps only captured weevils suggesting very low weevil populations. These studies had not thrown much light on the trap density for improved trapping of weevils but suggested the usefulness of mass trapping. Hence, one has to consider

the practical application of such large number of traps per unit area in relation to the number of palms in a country and the area covered.

Studies on mass trapping of *R. palmarum* conducted in mid-nineties in Costa Rica gave good results with one trap per 5 ha in oil palm plantations (Oehlschlager et al., 1995). But the same density of trapping when followed in the Middle East region appears to have not given the same level of RPW population reduction. In a field study out of two trap densities of 1 and 2 pherolure traps per 5 acres tested, the pherolure traps @ 1 trap/5 acres gave better results (Jose et al., 2008).

A total of 2,252 pheromone traps were used in the mass trapping system in an area of more than 10,000 ha Al Qatif region in Eastern Province of Saudi Arabia (Vidyasagar et al., 2000). They reported that from an initial level of 4.12 weevils per trap per week in 1994, the adult population was reduced to 2.02 weevils per trap per week at the end of 1997.

In an endemic area of RPW infestation, the higher trap density is needed to reduce insect population in a much shorter time frame. It may be suggested to increase the trap numbers sufficiently to remove higher numbers of weevils rapidly to reduce the risk of new and re-infestations. Consideration should also be given about the pheromone dispersal, infestation levels, available resources and logistics before embarking on a plan to increase the number of traps in a given region. Therefore, in our studies presented in this paper we have tested up to 8 traps as the maximum per 4 ha area, because of the above mentioned reasons.

Faleiro et al. (2011) suggested the use of 4-7 traps / ha, where the infestation levels were >1%, and 1 trap / ha in farms with <1% infestation. However, their studies were based on data from one region and the experimental fields were also smaller. But our studies were conducted in very large experimental plots, representing two important regions namely Al Hassa and Al Qatif with high and low weevil populations respectively. Moreover, these two areas where we organized the study were the regions with earliest report of RPW infestations in Saudi Arabia, and despite regular area-wide trapping with Standard Saudi Trap for more than a decade, the insect populations still remain high. Hence, this investigation provides information for mass pheromone trapping of RPW in Saudi Arabia.

From our studies it was clear that by raising the number of pheromone traps per unit area, the number of trapped weevils could be drastically increased as compared to a sparsely distributed trapping system. The current practice of mass pheromone trapping appears to be inadequate in reducing the resident population of adult RPW in date palm farms.

Conclusion

This study revealed that trap density of 8 traps / 4 ha, or

in other words 2 traps / ha is necessary to optimize the capture rate and drastically reduce weevil population and new infestations. There is a need to break the equilibrium between the emerging adult weevils that add to the resident population and the capture rates, so as to achieve satisfactory results in IPM program. The results from the study will help in revision of protocols for area-wide mass trapping and their integration in the management of RPW in Middle East.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Profitability of yellow passion fruit as a function of irrigation depths under semiarid conditions

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In regions where rainfall is limited and irregular, irrigation technique is important for optimizing fruit plants production and profitability, such as yellow passion fruit. Therefore, this study investigates the economic viability of irrigations depth applied on passion fruit cultivation in the semiarid region of Paraíba State. The treatments were arranged on a factorial scheme that comprises four levels of ETo replacement (33, 66, 100 and 133%) and two yellow passion fruit hybrids (BRS Sol do Cerrado and BRS Gigante Amarelo), in a randomized block design, with five replications. Effective operational cost, administrative cost, total water cost, total production cost, gross income, net income, benefit/cost relation, balance of price and profitability index were evaluated. The best economic results were achieved with 'BRS Sol do Cerrado' hybrid under irrigation with 133% of ETo.

Key words: *Passiflora edulis,* hybrids, economic viability, drip irrigation.

INTRODUCTION

Yellow passion fruit (*Passiflora edulis* SIMS f. *Flavicarpa* DEG) is one of the most important fruit exploitation in Brazil, due to its use in nature as well as industrial beverages, besides excellent perspectives for exporting concentrated juice to Europe and USA (Ataíde et al., 2012).

Brazil is the biggest producer and consumer of this fruit with 61,842 ha of growing area and estimated fruit production of 923,035 ton per year. The northeast of Brazil is the main producing area with 46,159 ha and 671,421 ton of fruit harvested, which correspond to 63% of national production (IBGE, 2014). According to Silva et al. (2015), the passion fruit has significant social and economic importance in Brazil and it is a versatile fruit

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that can be marketed in different forms, such as raw fruit, frozen pulps, juice, jams, yogurts, milk drinks and ice cream.

In Brazilian semiarid, specifically in the Paraíba State, the amount and quality of water are the factors that limit agricultural production. At this region the limited and irregular rainfall difficult the development of a non-irrigated fruit plant cultivation.

Nevertheless, in other Brazilian regions, where rainfall is regular year-round, passion fruit plants can be cultivated without irrigation techniques or with water supplementation when rainfall is below of the plant necessities (Arêdes et al., 2009). In this context, there is a history of research on the technical feasibility of irrigation methods according to Valipour (2012a) in determining optimal values for these systems. Thereafter, many attempts have been made to access appropriate design of sprinkle and trickle irrigation system (Valipour, 2012b).

By the benefits of localized irrigation system into the other techniques especially surface irrigation, more accurate design of this systems for saving in water resources, increasing irrigation efficiency, and finally encourage farmers to use of this system in order to save water resources (Valipour, 2012a).

Research with yellow passion fruit, in several regions of Brazil, have shown that this specie, in spite of having a high production cost and also a negative price fluctuations in the consumer market, has economic potential to increase employment and as well financial return; which has been matched and has often exceeded the rates achieved by the main crops in the country such as other species of fruit trees, cereals and vegetables crops (Kits et al., 1996; Araújo Neto et al., 2008; Arêdes et al., 2009; Hafle et al., 2011).

Economic analyses of the production chain is a significant index to show the implementation of a new technology and it has been studied for some researchers as Araújo Neto et al. (2008), Arêdes et al. (2009), Hafle et al. (2011) and Silva et al. (2015). The viability of a production chain depends directly on correct agricultural management, however, one should take into account all factors involved in this process (Hafle et al., 2012). Although there are researches with irrigated passion fruit cultivation (Araújo et al., 2012; Dias et al., 2012; Freire et al., 2014), there is still the lack of information about using this technology and the profitability of BRS Sol do Cerrado and BRS Gigante Amarelo hybrids under Paraíba State semi-arid conditions. These information are necessary to the farmers because they will help them to make the right decision of the resources used in its production, as also improving the performance of this fruit plant by enabling the expansion of areas under cultivation and guide the proper choice of the variety most adapted to the soil and climate of the semi-arid region.

Thus, this research was carried out to evaluate the economic viability of the application of different irrigation depths in yellow passion fruit crop in the semi-arid region of Paraíba State.

MATERIALS AND METHODS

Plant material

The 'BRS Gigante Amarelo' and 'BRS Sol do Cerrado' are genotypes obtained by breeding program of Embrapa and has been indicated for all Brazilian regions, especially due its productivity and fruit quality when subjected to the adoption of technologies such as irrigation. The great interest according to the Embrapa researchers is due to the adaptation of these cultivars to all regions of the Brazil, which were proven after extensive evaluations in different regions, including in states in the Northeast.

According to Embrapa researchers, 'BRS Gigante Amarelo' is a highly productive hybrid under irrigation, reaching around 60 tons/ha in the first production year. Its fruit is yellow, has an oblong shape, and weighs 120 to 350 g. Already 'BRS Sol do Cerrado' is a hybrid of passion fruit that can be grown throughout the year under irrigation in different soil types, except in areas subjected to frost. Flowers occur year-round, with the highest concentration in the dry season, which improves its cultivation in Alto Piranhas region in the Paraiba State, being more tolerant to leaves diseases such as bacterial blight, virus and anthracnose (Andrade Neto et al., 2015).

Study site and experimental conditions

The experiment was conducted at Alto Piranhas region located in Paraíba State. This region has 6º 21' S of latitude and 37º 48'W of longitude and height of 250 m from sea level.

The climate type in the region, according to Köppen classification, is BSw'h', belonging to the group of semiarid climate, with average annual rainfall of 870.0 mm, average annual temperature of 27°C, relative air humidity of 75% and monthly average evaporative demand of 120 mm, with average annual rainfall concentrated in the months from February to April.

The soil from experimental area was classified as Eutrophic fluvial neosol with sandy loam textural classification. The physical and chemical characterization of the soil layer from 0.00 m to 0.30 m were: pH (H_2O) = 7.1; P (Mehlich^{-1}) = 36 mg dm^{-3}; K = 0.83 cmolc dm^{-3}; Ca = 2.8 cmolc dm^{-3}; Mg = 0.7 cmolc dm^{-3}; Na = 0.16 cmolc dm^{-3}; Al = 0.0 cmolc dm^{-3}; H+Al = 0.49 cmolc dm^{-3}; organic matter = 9.54 g kg^{-1}; bulk density = 1.51 g cm^{-3}; total porosity = 0.47 m^3 m^{-3}.

Treatments and experimental design

The experimental design was a randomized block with 5 replications, on a factorial scheme 4 x 2 composed of four irrigation depths (33, 66, 100 and 133% from ETo, corresponding to 323, 646, 978 and 1300 mm cycle^{-1}, respectively) and two yellow passion fruit hybrids (BRS Sol do Cerrado e BRS Gigante Amarelo). The experimental unit was composed of 3 plants. Seedlings were grown in greenhouse and they were transplanted to the field in the spacing 4 m x 3 m and were conducted in vertical espalier with one wire (Figure 1A).

Irrigation management

For irrigation management, the reference evapotranspiration was calculated by Penman-Monteith (Equation 1). The data used to estimate the ETo were obtained daily from an automated weather station installed near the experimental area. According to Valipour

Figure 1. Yellow passion fruit plants cultivated in vertical espalier with one wire (A) and irrigation management (B).

(2014), the Penman-Monteith method is suitable for estimating the water requirement of crops, despite showing variation around 10% compared with the lysimetric measures. Although this method has been applied in various regions of the world, it needs too many parameters to estimate reference crop evapotranspiration (Valipour 2015), hence it is important to assess its use in this research.

$$ETo = \frac{0.408\Delta(R_n - G) + \gamma\left(\frac{900U_2}{T+273}\right)(e_s - e_a)}{\Delta + \gamma(1 + 0.34U_2)} \qquad (1)$$

In which: ETo = reference transpiration (mm day^{-1}); Rn = net radiation on culture surface (MJ m^{-2} day^{-1}); heat flow in the soil (MJ m^{-2} day^{-1}); Δ = slope of the vapor pressure curve versus air temperature (kPa °C^{-1}); U_2 = wind speed measured at two meters high (m s^{-1}); T = average air temperature (°C); es = water vapor saturation pressure (kPa); ea = actual pressure of water vapor (kPa); γ = psychrometric factor (MJ kg^{-1}).

The crude depth, water application rate and the time of irrigation were determined by Equations 2, 3 and 4, respectively (Mantovani et al., 2006).

$$LB = \frac{ETo\ Kc\ KL - Pe}{Ef}; \quad LB \leq 0; \quad LB = 0 \qquad (2)$$

In which: LB = crude depth (mm); ETo = reference evapotranspiration according to Penman-Monteith (mm); Kc = crop coefficient, considered 1 for application of ETo; KL = percentage of wetted area by the water emitter; Pe = rainfall during the experimental period (mm); Ef = irrigation method efficiency (decimal).

$$Ia = \frac{n \times v}{ec} \qquad (3)$$

In which: Ia = water application rate (mm h^{-1}); n = number of emmiter per plant; v = emitter flow (L h^{-1}); ec = area occupied by the plant (m^2).

$$Ti = \frac{LB}{Ia} \qquad (4)$$

In which: Ti = irrigation time (h); LB = crude depth (mm day^{-1}); Ia = water application rate (mm h^{-1}).

Management of the application of different water depths was carried out by varying the number of drippers per plant, using 1, 2, 3 and 4 pressure compensating drippers, with a nominal flow rate of 4 L h^{-1}, corresponding to depth of 33, 66, 100 and 133% of ETo respectively (Figure 1B).

The fertilizers were applied through fertigation, every 15 days, in such a way to distribute nutrients throughout the crop cycle. Thus, a Ventury injector was used with a flow rate of 70 L h^{-1}.

Profitability and economic analysis

Fruit yield was estimated taking into account weight and number of fruits per plant (Mg ha^{-1}), and the estimated net income (RL) (Equation 5) was obtained through the response function (Y(W) = $\beta_0 + \beta_1 W_1 + \beta_2 W_2 + e$), cost of water (CW = $\frac{CEE}{LL} \times Pee$ (Andrade Júnior et al., 2001) and the cost of production (CP) (Pimentel et al., 2009).

$$RL = \frac{(Yw \times P - (CP + Cw \times W)}{10 \times W} \qquad (5)$$

In which:
$Y(W)$ - Fruit yield as a function of applied water depth (Mg ha^{-1});
β_0, β_1 e β_2 – Regression parameters;
W_1 e W_2 – Irrigation depths;
e – Random error;
CW = Cost of irrigation water (US$ mm^{-1});
CEE = Electricity consumption during the crop cycle (kwh ha^{-1});
LL = Total water depth applied during the crop cycle (mm);
Pee = Price of kwh (US$ kwh^{-1}), was obtained from Paraíba electrical power company;
P = Price of passion fruit on Catolé do Rocha county market (Paraíba State) was calculated on the basis on a weekly survey in street markets. The price paid to the farmer, that is, 50% of the

Table 1. Description of the absolute values and percentages of the cost of production on the first year of cultivation of passion fruit hybrids (*P. edulis* f. *Flavicarpa*) BRS Sol do Cerrado and BRS Amarelo Gigante irrigated with different replacement rates of ETo.

Description of costs	Irrigation depths							
	33% of ETo		66% of ETo		100% of ETo		133% of ET	
	Value	%	Value	%	Value	%	Value	%
COE (US$ ha^{-1})								
Inputs	2,281.09	39.00	2,281.09	38.40	2,281.09	37.50	2,281.09	36.90
Soil tillage and planting	400.00	6.80	400.00	6.70	400.00	6.50	400.00	6.40
Phytosanitary treatment	417.39	7.10	417.39	7.00	417.39	6.80	417.39	6.70
Harvest	434.78	7.40	434.78	7.30	434.78	7.10	434.78	7.00
Purchase of equipment	3,500.00	26.00	3,500.00	25.60	3,500.00	25.00	3,500.00	24.6
Subtotal (US$ ha^{-1})	5,055.00	86.40	5,055.00	85.10	5,055.00	83.20	5,055.00	81.80
CEA (US$ ha^{-1})								
Financial charges	685.50	11.70	685.50	11.55	685.50	11.20	685.50	11.10
CTA (US$ ha^{-1})	108.26	1.80	195.31	3.29	328.60	5.41	436.52	7.00
CTP (US$ ha^{-1})	5,848.85	100	5,935.81	100	6,069.10	100	6,177.02	100.0

average price paid at these locations during the years 2011 and 2012 was also taken into account.

To analyze passion fruit production costs (CP), the expenditures and charges were grouped into the related categories:

(a) Effective operational cost (EOC): this comprises direct costs with financial disbursement to the activities from soil tillage and fruit harvest;

(b) Costs and administrative expending: it reflects fixed costs or indirect expenses for interest, social charges, administration fee and equipment depreciation:

(b.1) Remuneration of the farmer's capital calculated on the basis of 0.5% per month on the COE's half value: it aims to remunerate the alternative use of farmer's capital if he or she chooses for financial savings application;
(b.2) Land remuneration that correspond to the real value of 1.0 ha rentals in the region;
b.3) Depreciation of machinery and equipment: this includes financial resources to acquire spare parts which should correspond to 10% of the irrigation equipment;
b.4) Management fee calculated on the basis of 6% of the COE; and

(c) Total operating costs (COT), corresponding to the sum of the overall expenses of (a) + (b).

In addition to net income, other profitability indicators such as benefit/cost relation, equilibrium price and index of profitability were calculated by the following equations:

$$B/C = RB/CTP \tag{6}$$

$$PE = CTP/Y \tag{7}$$

$$IL = RL/RB \times 100 \tag{8}$$

In which:
B/C = benefit/cost relation (non-dimensional);
RB = gross income (US$ ha^{-1} year^{-1});
CTP = total production costs (US$ ha^{-1} year^{-1});
PE = equilibrium price (US$ Mg^{-1});
Y w= estimated productivity (Mg ha^{-1});

IL = profitability index (%).

RESULTS

Cost of production

On Table 1 are shown the production costs for both 'BRS Sol do Cerrado' and 'BRS Gigante Amarelo' passion fruits hybrids. The cost generated by this factor is related to the price of seed, being equal to both genotypes. It should be observed it was included at disbursement stage of seedlings production, as a component of costs to calculate the effective operational cost (COE); it had the absolute value of US$ 5,055.00 ha^{-1} year^{-1}, which corresponded to 86.43, 85.16, 83.19 and 81.84% of the total production cost (CTP), in relation to water replacement depths 33, 66, 100 and 133% of ETo, respectively (Table 1). When the COE was fractionated, it was observed that the inputs accounted for 39 and 36.93% of the total cost of production process for the irrigation depths of 33 and 133%, respectively. These values corresponded to 1.95, 3.29, 5.41 and 7.07% from total cost of fruit production, respectively.

Technical indexes used to calculate costs of the research were obtained by monitoring the current field experiment. Labor, tractor rental and inputs considered were those used in Catolé do Rocha County-PB from 2009 to 2012. COE (effective operational cost), CEA (costs of administrative burden), CTA (Water total cost) and CTP (total cost of production).

Regarding costs of administrative burden (CEA), this value was US$ 685.50 ha^{-1}. To the costs of irrigation water, these values were US$ 108.26 (33% of ETo), US$ 195.31 (66% of ETo), US$ 328.60 (100% of ETo) and US$ 436.52 per hectare (133% of ETo), which corresponded to 1.95, 3.29, 5.41 and 7.07% of the total

Table 2. Economic indicators on the first year of cultivation of passion fruit hybrids (*P. edulis* f. *Flavicarpa*) BRS Sol do Cerrado and BRS Gigante Amarelo irrigated with different replacement rates of ETo.

Economic indicators	Irrigation depths							
	33% of ETo		66% of ETo		100% of ETo		133% of ETo	
	SC	GA	SC	GA	SC	GA	SC	GA
Productivity (Mg ha^{-1}) ha^{-1})	10.80	16.30	19.10	20.60	25.00	22.80	28.00	22.60
Price (US$ Mg^{-1})	569.56	569.56	569.56	569.56	569.56	569.56	569.56	569.56
Gross income (US$ ha^{-1})	6,151.30	9,328.90	10,926.20	11,772.34	14,265.87	12,992.40	16,008.8	12,917.10
Net income (US$) ha^{-1})	302.4	3,480.06	4,990.36	5,836.53	8,196.78	6,923.25	9,831.8	6,740.11
Benefit/Cost (B/C)	1.05	1.59	1.84	1.98	2.35	2,14	2.59	2.09
Equilibrium price (US$ Mg^{-1})	541.55	358.82	310.77	288.143	242.76	266.18	220.61	273.32
Profitability index (%)	4.92	37.30	45.60	49.50	57.40	53.20	61.40	52.10

SC (BRS Sol do Cerrado) and GA (BRS Gigante Amarelo).

production cost for replacement depths 33, 66, 100 and 133% of ETo, respectively (Table 1).

Passion fruit hybrids cultivation had a total production cost of 5,848.85 US$ ha^{-1} (33% of ETo), 5,935.81 US$ ha^{-1} (66% of ETo), 6,069.10 US$ ha^{-1} (100% of ETo) and 6,177.02 US$ ha^{-1} (133% of ETo) (Table 1).

Yield and economic indicators

Regarding economic indicators (Table 2), there was no economic deficit for any of the hydric replacement depths studied. The lower net income (302.45 US$ ha^{-1} year^{-1}) was attained by 'BRS Sol do Cerrado' irrigated with 33% of ETo. The bigger net income (9,831.0 US$) was also attained by this hybrid irrigated with 133% of ETo. It was observed that the best benefit/cost relation with 2.59 value for that same water replacement, which shows for each US$ 1.00 invested generated a net income of US$ 1.59.

The higher profitability index (61.41%) was attained by 'BRS Sol do Cerrado' under 133% ETo of water replacement, however, under 33% ETo, the highest profitability index (37.30) was obtained by 'BRS Gigante Amarelo', higher than 'BRS Sol do Cerrado' under lower water availability. For equilibrium price, it was observed that when 'BRS Sol do Cerrado' was irrigated with 133% of ETo, the price paid by the ton of fruit decreased the price from US$ 541.56 to US$ 220.60, even so, this cultivation system ensures the production costs.

When the productivity was reduced due to water stress by low water availability to plants (from 28.00 and 22.6 Mg ha^{-1} at 133% of ETo to 10.80 and 16.30 Mg ha^{-1} at 33% of ETo by 'BRS Sol do Cerrado' and 'BRS Gigante Amarelo', respectively), the price paid for the product was the determining factor for which there was no prejudice to the cultivation system (Table 2). However, under water scarcity the 'BRS Gigante Amarelo' shows higher profitability index per unit of water depth applied (Figure 2).

DISCUSSION

Differences regarding the total production cost occurred due to the amount of water applied to replace the hydric status of the plants, taking into account that its cost is based on the amount of electricity used during the water pumping process from water source to the plants.

Some researchers such as Kits et al. (1996) have evaluated agricultural and economic aspects of yellow passion fruit plants, whom, in a study on planting densities, observed that expenses on inputs and labor accounted for 98 and 97% of the total cost of production, when the fruit plants were grown at a spacing of 2 m x 1.25 m and 2 m × 3.75 m, respectively. Araújo Neto et al. (2008) reported that inputs along with labor were 57.83% compared to minimal cultivation and 60.46% compared to conventional tillage. Hafle et al. (2011) reported that passion fruit plants training pruning account for 73.9% from inputs along with labor.

In this context, labor costs studied in this research (around 80%) are less than those achieved by previous authors. This current result may be related to the growing technology in use, that is, one in which weeds are controlled by herbicide applied in the planting lines and mechanical mowing between planting lines which requires few labor. These agricultural practices comprise labor price and machinery working time. Moreover, the use of genetic material more resistant to pests and diseases decreased spraying during passion fruit growing and, therefore, the use of labor.

The economic profitability is dependent on the quantity produced and the price paid for the product. In relation to the passion fruit growing system in the semiarid region of Paraíba State, good productivity was observed considering mainly the potential of the hybrids evaluated in this study under irrigation.

By analyzing yellow passion fruit profitability, Kits et al. (1996) recorded net income corresponding to US$ 3,560.68 ha^{-1}. Araújo Neto et al. (2008) cited a higher value of net income around US$ 5.524,77 ha^{-1}. In another

Figure 2. Yield of yellow passion fruit 'BRS Gigante Amarelo' irrigated in the Brazilian Semiarid.

study, Arêdes et al. (2009) found a net income of US$ 5,749.29 ha^{-1} and the benefit/cost relation of 1.24. The results found in this study (US$ 8,196.7 ha^{-1} and US$ 9,831.8 ha^{-1}) mainly with 'BRS Sol do Cerrado' were higher than those presented by Kits et al. (1996) Araújo Neto et al. (2008) and Arêdes et al. (2009).

This may be related to the high productivity of the genotypes in this research and the high price paid for the fruits in the local market. These results show that irrigated fruit activity enables good economic returns, generates currency from outside and jobs mainly in areas where irrigation technology can be used, promoting greater water savings and economic returns.

Moreover, the productivity of 'BRS Gigante Amarelo' was higher than the 'BRS Sol Cerrado' under lower water availability. In this case, 'BRS Sol Cerrado' is more productively irrigated with larger volume of water, but water use efficiency is reduced. On the other hand, increase in productivity may not compensate for increase in water availability for this genotype as well as reduce its efficient use (Sousa et al., 2005).

According to Sousa et al. (2005), the water use efficiency relates accumulation of biomass or commercial production to the amount of water applied or evapotranspired by the crop. Thereafter, proper irrigation management stands out in irrigated agriculture for improved efficiency in water use. Among the means and techniques used to increase the water use efficiency in irrigated agriculture, is the use of drip irrigation and water supply along with high frequency and low amount.

Conclusions

Irrigated passion fruit cultivation provides economic returns in the semiarid region of Paraíba State. The best economic results were obtained with 'BRS Sol do Cerrado' under irrigation depth of 133% of ETo. Under water scarcity the 'BRS Gigante Amarelo' shows higher profitability index per unit of water depth applied.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Production of yellow passion fruit seedlings on substrates with different organic compounds

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The production of passion fruit seedlings constitutes one of the important stages of the production system and the importance of substrate for the seedlings growth and plant performance in the field. Therefore, the objective of this study was to evaluate the effects of different composting organic wastes in the substrate composition for production of yellow passion fruit seedlings. The experiment had been conducted in the Centre of Human, Social and Agricultural Sciences at The Federal University of Paraíba. The experimental design was randomized block design, with six treatments, goat composite + soil (CCP+S), poultry composite + soil (AC+S), bovine composite + soil (CB+S), rabbit composite + soil (CCO+S), earthworm humus + soil (MH + S), and soil (S) and S in the ratio of 2:1 (v/v) and five repetitions. Emergency speed index, emergency percentage, stem diameter, seedling height, leaf number, root length, fresh root mass, shoots fresh mass, total of fresh mass, dry mass of root, dry mass of branches, total dry mass, chlorophyll *a, b* and total, and Dickson quality index were assessed. The compounds in the constitution of substrates exerted significant effect on the characteristics evaluated in the passion fruit, which may be related to the availability of nutrients to the substrate. The substrates containing CCP+S and CCO+S provided better growth, chlorophyll contents and quality of "Serra" yellow passion fruit seedlings.

Key words: Initial growth, *Passiflora edulis* Sims f. *flavicarpa* Deg., waste reuse, chlorophyll indexes.

INTRODUCTION

The yellow passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Deg.) is native from tropical America, with more than 150 native species of Brazil, intensively cultivated in tropical and subtropical countries (Faleiro et al., 2008). This culture has a significant importance in the Brazilian agricultural sector, mainly due to the physicochemical qualities and pharmacy-therapeutic fruits, besides the high acceptance by the consumer

market (Natale et al., 2006).

It is a fruit with wide adaptation in Brazil and considered a cultural entity that employs large amount of workers, characterizing it as a family agricultural activity (Silva et al., 2010). Brazil is the world's largest producer of passion fruit and domestic production currently accounts for about 1.5% of the fruits produced in the country, totaling in 2005 more than 479,000 tons of fruit (IBGE, 2012).

For Cavalcante et al. (2005), the Brazilian Northeast is recognized as a region that offers edaphic aptitude and climate for fruticulture and among the fruit the yellow passion fruit stands out, with favor conditions for their growth, production and fruit quality (Pires et al., 2008).

This species may be sexually and asexually propagated but cultivation through seed is the most used way (Ferreira et al., 2001). The seedling production constitutes one of the most important stages of the production system in the horticulture, since it determines the final performance of the plant in the field (Echer et al., 2007). In this sense, the substrate has a fundamental role in the plant growth requiring secure the shoot growth and development of root system (Silva et al., 2010).

According to Silva et al. (2001) for good seedlings quality, it is necessary to use substrates, which must provide appropriate physical and chemical properties and provide nutrients necessary for the plant development. Allied to this, the quality of the substrate depends primarily on the proportions and materials that make up the mixture.

Composting is the controlled decomposition of plant debris and manure. This decomposition occurs by the action of microorganisms and the soil fauna, the compound being a source of slow release of macro and organic micronutrients, serving as a soil builder and allows the increase in organic matter, favoring increased retention capacity water, increasing the CTC, nutrient retention in the soil and reducing the acidity of the soil over time, to form organic complex and retain bases providing best condition for resistance to pests and diseases (Penteado, 2007). The organic inputs provide improved microbiological substrate and increasing population and diversity of soil fauna of the soil (Sall et al., 2015).

In this context, the objective was to evaluate the effects of different composting organic waste in the substrate composition for production of yellow passion fruit seedlings (*P. edulis* Sims f. f*lavicarpa* Deg.).

MATERIALS AND METHODS

The experiment was conducted in the seedling production nursery in Sector of Agriculture in the Centre of Human, Social and Agricultural Sciences at The Federal University of Paraíba., located in the Paraíba's swamp region, Bananeiras-PB county. The climate according to Koppen is As' type and corresponds to sub-Mediterranean climate (BRASIL, 1972). The values for rainfall and temperature during the months of experiment conduction (June to
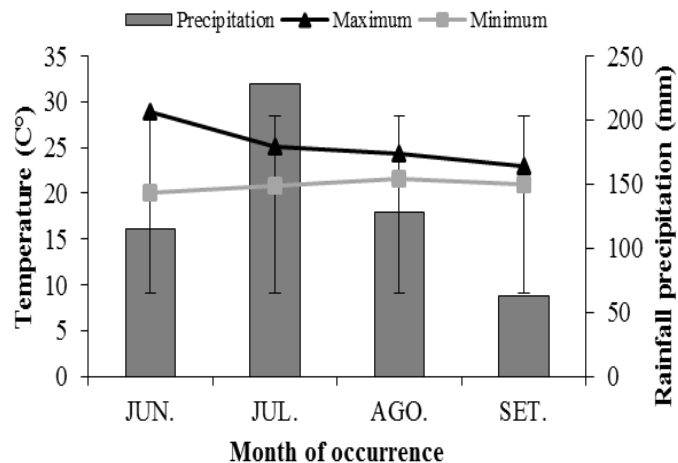
Figure 1. Rainfall precipitation and temperature in the Bananeiras-PB County, 2013.

September) are as shown in Figure 1.

Initially, four composting cells were prepared, using different manure (cattle, goats, poultry and rabbits), crop residues (bean, corn, citronella, jack fruit leaves and jambolan) and grasses, attempting to use the material available on site. After the material had been collected, all plant materials were ground for better uniformity and decrease the decomposition time.

The pile built with dimensions of 1.5 m wide × 1.20 m high. To better preparation was placed around each pile, a nylon screen in order to prevent loss of the used material. The pile temperature and humidity were monitored and 90 days after the compost piles, it was observed that they were ready to start the sieving process and use. The experiment was carried out using the passion fruit Serra cultivar, obtained by mass selection by producers in the Nova Floresta County donated by Canteiro Cheiro Verde in the Picuí-PB. Sowing was done using polyethylene bags with dimensions of 18 × 30 cm, placing three seeds per bag. After the emergency, the thinning was carried out, leaving only one plant/bag and the experimental plot consisted of five seedlings.

The experimental design was a randomized block with six composite treatments goat composite + soil (CCP+S), poultry composite + soil (AC+S), bovine composite + soil (CB+S), rabbit composite + soil (CCO+S), earthworm humus + soil (MH + S) and soil (S), with the ratio of 2: 1 v/v and five replications. The results of chemical analysis are shown in Table 1.

Plants were harvested at 60 days after emergence, when they reached the point of being taken to the field. Emergency speed index (ESI), emergency percentage (% EER), stem diameter (SD), height plant (HP), number of leaves (NL), root length (RL), fresh root mass (FRM), fresh weight of aerial part (FWA), total fresh weight (TFW), root dry mass (RDM), dry mass of shoots (DMS), total dry matter (TDM), chlorophyll *a* indexes (Cl *a*), chlorophyll *b* indexes (Cl b), total chlorophyll indexes (Cl *t*) and Dickson's quality index (IQD) were evaluated.

The determination of plant height and root length was performed with a ruler graduated in centimeters, for determining the stem diameter, a digital caliper was used with values expressed in millimeter. Chlorophyll levels were quantified (a, b total = a + b) by reading made in chlorophyll. The ClorofiLOG® model was used and the readings were taken from three sheets exposed to solar radiation (from top to bottom) starting from the third changes of the sheet. To determine the fresh weight of roots and shoots, the plants were weighed after harvest, dry mass of roots and shoots was determined with the dry material in an oven with forced air at 65°C

Table 1. Chemical characterization of substrates consisting of compounds obtained from different animal manures and crop residues

Source	$**pH$ H_2O	P	K^+	Na^+	H^+Al^{+3}	Al^{+3}	Ca^+	Mg^{+2}	SB	CTC	V	m	M.O.
		mg/dm³					cmol$_c$/dm³				%		g kg⁻¹
*CB + S	7.67	136.0	12.31	1.51	0.91	0.00	6.40	5.40	25.59	26.50	96.57	0.00	179.6
CCO + S	6.84	233.5	9.53	2.67	4.54	0.00	6.30	6.70	25.18	29.72	84.72	0.00	164.0
CA + S	6.71	477.3	9.98	3.53	2.56	0.00	8.40	7.95	29.84	32.40	92.10	0.00	29.66
CCP + S	6.83	136.8	9.53	1.22	4.62	0.00	8.50	5.45	24.68	29.30	84.23	0.0	141.1
HM + S	6.67	397.8	9.68	1.8	5.69	0.00	7.40	12.00	30.86	36.55	84.43	0.00	142.0
S	4.57	16.17	0.26	0.09	12.46	0.55	2.40	1.45	4.21	16.67	25.25	11.55	9.48

**pH = active acidity, P = available phosphorus, K^+ = available potassium, Na^+ = exchangeable sodium, $H^+ Al^{+3}$ = potential acidity, Al^{+3} = exchangeable acidity, Ca^+ = calcium exchangeable Mg^{+2} = magnesium exchangeable, SB = sum of bases, CTC = effective cation exchange capacity, V = base saturation, m = Al^{+3} saturation, MO = Organic matter.
*Goat composite + soil (CCP+S), poultry composite + soil (AC+S), bovine composite + soil (CB+S), rabbit composite + soil (CCO+S), earthworm humus + soil (MH + S) and soil (S), with the ratio of (2: 1, v / v).

until they reach constant weight.

The IQD proposed in the present research is a balanced formula that includes the relations of morphological characteristics, such as total dry weight, shoot dry weight, dry weight of the root system, shoot height and stem diameter (Dickson et al., 1960) by the formula:

$$IQD = \frac{MST}{\left(\frac{ALP}{DIC}\right)+\left(\frac{MSP}{MSR}\right)} \tag{1}$$

Where IQD = Dickson's quality index, MST = total dry matter, ALP = plant height, DIC = stem diameter, DMAP = shoot dry mass, MSR = root dry mass.

Data were subjected to analysis of variance and means were compared by Tukey test at 5% probability, using the statistical software ASSISTAT version 7.7 beta (Silva and Azevedo, 2002).

RESULTS AND DISCUSSION

Treatments with CCP+S and CCO+S provided better speed of emergence index and emergency percentage (Table 2), possibly these substrates have adapted better moisture retention conditions sufficient to allow for better germination, combined with good aeration of the substrate (Smiderle and Minami, 2002; Penteado, 2007). In work carried out by Silva et al. (2010), the best results for the IVE, the possible maintenance of humidity Plantmax® substrate.

The treatment used consisting of CCP + S provided increment in the height of passion fruit seedlings "Serra" (Table 2). The lower growth was observed in the control, data that can be explained by the chemical conditions and soil fertility. The pH was considered with high acidity and all other attributes in conditions that do not favor plant growth, results corroborate that by Fey et al. (2010) where they evaluated the initial growth of yellow passion fruit seedlings due to increasing doses of superphosphate.

The substrates containing CCP+S+S and CA provided increases in stem diameter of passion fruit seedlings (Table 2). Cavalcante et al. (2009) when working with different textures on substrates significant effects on stem diameter of passion fruit seedlings. Palácio et al. (2011) when using different substrates formulated with dung: sand: soil and manure: Land noted that the yellow passion fruit seedlings had increase in stem diameter when cattle manure added to the substrate.

The best responses to the variable number of leaves were observed for the treatments PCC+S and S+CCO (Table 3). Negreiros et al. (2005), studying different substrates in the formation of the papaya seedlings from "Solo" group, it was observed that a greater number of leaves per plant in the substrate consisting of cattle manure, soil, sand and vermiculite in the proportion of 2: 1: 1: 1 v/v.

Dantas et al. (2012) using different percentages of bovine manure on substrates, observed that the use of cattle manure in the substrate promoted positive effects on the number of leaves and in the initial development of yellow passion fruit.

There was no significant effect on root length of seedlings of yellow passion fruit (Table 3). Costa et al. (2011) when using different volumes of commercial substrates, soil and organic compound observed no significant effect of treatment on the formation of yellow passion fruit

Table 2. Emergence speed index (ESI), emergency percentage (EP), plant height (PH) and stem diameter (SD), passion fruit "Serra" grown in different compositions of substrates.

Treatment	ESI	EP (%)	PH (cm)	SD (mm)
CB + S	1.14[b]	78.40[b]	26.83[b]	4.73[ab]
CCO + S	1.47[a]	95.80[a]	30.24[ab]	5.12[a]
CA + S	1.12[b]	75.60[b]	18.40[c]	4.17[b]
CCP + S	1.52[a]	97.20[a]	32.92[a]	5.30[a]
HM + S	1.11[b]	71.60[b]	17.28[c]	4.29[b]
S	1.00[b]	61.20[c]	6.90[d]	2.42[c]
Mean	1.23	79.96	22.09	4.33
CV (%)	7.28	6.26	12.78	7.91

Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 3. Number of leaves (NL), root length (RL), fresh weight of root (FWR) and fresh mass of the aerial part (FMAP) of passion fruit of the "Sierra" grown in different compositions of substrates.

Treatment	NL	RL (cm)	FWR (g)	FMAP (g)
CB + S	8.16[ab]	19.20[a]	9.56[b]	10.59[b]
CCO + S	9.00[a]	19.55[a]	13.74[a]	13.99[a]
CA + S	7.60[ab]	19.35[a]	8.20[b]	7.41[c]
CCP + S	9.80[a]	21.83[a]	14.26[a]	15.55[a]
HM + S	7.00[ab]	20.88[a]	10.69a[b]	7.98[bc]
S	5.24[b]	17.76[a]	1.96[c]	1.61[d]
Mean	7.80	19.76	9.73	9.52
CV (%)	19.03	12.20	19.40	14.00

Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

seedlings.

The fresh weight of roots and shoots were increased on substrates with CCP and CCO+S+S (Table 3). Treatment with soil was inferior to other substrates in the fresh matter accumulation, a result that may be related to the conditions of fertility, even as high acidity, low base saturation and organic matter content, conditions that do not favor the development of seedlings with quality.

Using different settings, containers and substrates, Costa et al. (2010) observed that in individual, cages environments with shading of 50% polyethylene bags containers and substrates using soil and compost had the best fresh mass accumulation of roots. Ramos et al. (2008) observed a significant effect on fresh weight of aerial part of yellow passion fruit trays and testing tubes with Plantmax® substrate, charcoal, earth and sand, 1:1:1:1 v/v.

For total fresh weight, dry weight of roots, dry weight of shoot and total dry matter (Table 4), it was observed that the substrates that showed superiority contained CCP and CCO+S+S.

Barros et al. (2013) found significance in the dry root mass when evaluating different substrates made with compounds of green manure and application of foliar

biofertilizers in seedlings of yellow passion fruit, however, the substrate with manure, provided dry mass increase and should be the best fertility conditions provided to the substrate.

Cruz et al. (2008) observed that the use of swine wastewater in irrigation of sour passion fruit seedlings provided increase in the amount of dry matter of seedlings, supplying the nutritional demand of seedlings without the supply of commercial fertilizers. Costa et al. (2011) observed that the containers with dimensions of 15.0 × 21.5 cm in environments with shading showed higher accumulation of total dry matter in the yellow passion fruit seedlings.

It is observed (Table 5) that the substrate containing goat compost expressed the best results for chlorophyll indices, b and total, which can be explained by the balanced availability of nutrients, aeration, and the possible increment of humic substances to the substrate. The plant chlorophyll is a factor that is directly linked with the photosynthetic efficiency. It is from the photosynthesis process the way that the plant gets energy to grow and develop (Cavalcante et al., 2013).

Silva et al. (2010) when using different substrates found that treating soil+cattle manure afforded increase in

Table 4. Total fresh weight (TFW), root dry weight (RDW), dry matter of the aerial part (DMAP), total dry matter (TDM) of yellow passion fruit plants "Sierra" grown in different compositions of substrates.

Treatment	TFW (g)	RDW (g)	DMAP (g)	TDM (g)
CB + S	20.16[b]	3.17[b]	2.43[b]	5.61[bc]
CCO + S	27.73[a]	5.06[a]	4.26[a]	9.32[a]
CA + S	15.61[b]	2.61[b]	1.57[c]	4.18[c]
CCP + S	29.81[a]	5.16[a]	4.89[a]	10.05[a]
HM + S	18.67[b]	3.48[b]	2.38[b]	5.83[b]
S	3.57[c]	0.43[c]	0.51[d]	0.9[4d]
Mean	19.25	3.31	2.67	5.98
CV (%)	13.13	17.65	12.24	12.40

Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

Table 5. Average of chlorophyll indices (CI), indexes chlorophyll b (CI b), chlorophyll index (CI t) and Dickson quality index (IQD) of yellow passion fruit plants "Sierra" grown in different substrate compositions.

Treatment	CI *a*	CI *b*	CI *t*	IQD
CB + S	27.34[ab]	6.92[ab]	34.50[ab]	1.80[cd]
CCO + S	28.02[ab]	8.10[a]	36.12[ab]	2.42[ab]
CA + S	24.14[bc]	6.98[ab]	31.12[bc]	1.58[d]
CCP + S	29.76[a]	8.90[a]	38.66[a]	2.60[a]
HM + S	25.98[ab]	6.92[ab]	32.90[ab]	2.13[bc]
S	20.36[c]	4.92[b]	25.28[c]	1.54[d]
Mean	25.93	7.12	33.09	2.01
CV (%)	10.16	20.15	8.94	10.15

Means followed by the same letter in the columns do not differ by Tukey test at 5% probability.

chlorophyll content in passion fruit leaves, probably not only by nutrient supply provided by the presence of organic matter, but also the improvement of microbiological content and increase in population and diversity of soil fauna in the substrate (Sall et al., 2015).

For the IQD, CCP + S substrates and CCO + S, provided better quality of passion fruit seedlings (Table 5). Almeida et al. (2011) also noted the significance of IQD when substrates containing soil + cattle manure in 1: 1 v/v were used for the production of passion fruit seedlings in trays. According to the authors, the results may be related to water retention, since this mixture has hardened earth and cattle manure, materials having high water retention and sand, which is highly porous, and facilitates aeration. IQD has been mentioned as a promising integrated morphological as Johnson and Cline (1991) and cited as good indicator of quality seedlings.

Conclusion

Substrates made up of goat composite +soil and rabbit composite + soil provide better growth, chlorophyll contents and quality of yellow passion fruit "Serra"

seedlings.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Comparative study on cross-compatibility between *Camellia sinensis* var. *sinensis* (China type) and C. *sinensis* var. *assamica* (Assam type) tea

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Tea has long been a well-known crop for its economic value and widening the genetic variability of tea family is often necessitated. Hybridization programs at intraspecific level have been greatly fascinated as potential and useful methods in tea plant breeding to widening the genetic diversity. This comparative study was intended to explore a new avenue to develop the tea plant breeding programs through evaluating remote intraspecific cross-compatibility between *Camellia sinensis* var. *sinensis* (L.) O. Kuntze and *C. sinensis* var. *assamica* (Masters). Remote intraspecific cross-compatibility was assessed by comparing and contrasting the *in-vivo* pollen germination and pollen tube growth using fluorescence microscopy and the subsequent fruit set following controlled self- and cross-pollinations. *In-vivo* pollen germination and pollen tube growth was examined at 1 day, 3 days, and 14 days after pollination treatments, but disparity was not observed in pollen germination and pollen tube growth between self- and cross-pollinations. Early fruit set was evaluated at 3 months and 6 months after pollination. Fruit set was observed in cross-pollination except self-pollination. A late-acting self-incompatibility system or post-zygotic barriers and close intraspecific cross-compatibility were confirmed within *C. sinensis* var. *sinensis* (L.) O. Kuntze. Potential remote intraspecific cross-compatibility was recorded from cultivars crossed between China type and Assam type tea. The present findings bestow the significant contribution to develop the future tea breeding programs.

Key words: Intraspecific cross, pollen germination, pollen quality, pollination, tea breeding.

INTRODUCTION

Tea has been devoted as the most versatile non-alcoholic beverage in the world. This perennial crop is commercially cultivated for its tender leaves and has been playing an important role in the world economy. Whilst the Yunnan province in China rewarded as the seedbed of tea plant, currently it has been grown mostly in Southeast

and South Asian countries (China, Japan, Korea, Sri Lanka, India, and Indonesia), African countries (Kenya, Uganda, and Malawi), and South America as well as up to some extent in North America, Australia, and Europe (Mondal, 2011; Chen et al., 2012; Wachira et al., 2013; Mondal, 2014). The commercially cultivated tea populations belonging to the family Theaceae and the genus *Camellia* have been categorized into three distinct taxa; *Camellia sinensis* var. *sinensis* (L.) O. Kuntze or China type, *Camellia sinensis* var. *assamica* (Masters) or Assam type, and *C. sinensis* var. *assamica* subssp. *lasiocalyx* (Panchon ex Watt.) or Cambod or Southern type (Wachira et al., 2013; Mondal, 2014). The classification of the genus *Camellia* was initially put forwarded by Sealy in 1958 following a revision in 1962 by Wight, principally based on leaf morphological characters of tea plant (Banerjee, 1992; Chen et al., 2012; Wachira et al., 2013; Mondal, 2014). In brief, China type has small size leaves, Assam type has large size leaves, and Cambod or Southern type leaf size is intermediate between of Assam and China types (Banerjee, 1992; Mondal et al., 2004; Chen et al., 2012; Mondal, 2014). Owing to the out-breeding nature of tea plant, the cultivated germplasm consists of extreme China types to extreme Assam types with the continuous variation between them (Banerjee, 1992; Mondal et al., 2004; Rajkumar et al., 2010; Ariyarathna et al., 2011; Chen et al., 2012; Wachira et al., 2013; Mondal, 2014).

Tea has long been a well-known crop for its economic value and widening the genetic variability of tea family is often necessitated. In the recent past, tea plant breeding has been intensified and expanded to widening the genetic variability through accelerating the production of new improved plant materials. Hybridization programs at intraspecific level have been greatly fascinated as potential and useful methods in tea plant breeding to widening the genetic diversity. The existing tea populations all over the world might be as a result of the intensive natural hybridization between three main taxa and other non-tea *Camellia* species (Bezbaruah and Gogoi, 1972; Banerjee, 1992; Mondal et al., 2004; Rajkumar et al., 2010; Ariyarathna et al., 2011). To the best of our knowledge attempts on remote intraspecific hybridizations between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* have not been reported even though close intraspecific hybridization within each China or Assam type has been undertaken extensively in *C. sinensis*. Novel tea cultivars with blended desirable traits such as biotic and abiotic stress resistance, new aroma of tea, and specific characters in chemical components might be accomplished fruitfully via remote intraspecific hybridization between China and Assam types.

Thus, this comparative study is intended to explore a new avenue to develop the tea plant breeding programs through evaluating remote intraspecific cross-compatibility between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* by comparing and contrasting the *in-vivo* pollen germination and pollen tube growth using fluorescence microscopy and fruit set following controlled self- and cross-pollinations.

MATERIALS AND METHODS

Plant

Present experiment used three cultivars of *C. sinensis* var. *sinensis* (L.) O. Kunze namely, 'Yabukita', 'Yutakamidori', 'Okuhikari' and one cultivar of *C. sinensis* var. *assamica* (Masters) namely, 'AI-37'. The tea plants were grown in plastic greenhouse located at Jeju National University, South Korea. *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' were used as pollen donors for artificial pollination. *C. sinensis* var. *sinensis* 'Yabukita' is a mid-plucked and leading tea cultivar in Japan, cultivated in about 75% of country's tea fields. It has been proved to be a good yielding, cold resistant, and good seed setting cultivar with intense green tea flavor (Ogino et al., 2009; Yagi et al., 2010; Chen et al., 2012). *C. sinensis* var. *sinensis* 'Yutakamidori' is an early-plucked and second most cultivated tea cultivar in Japan and has good yield as well as highly resistant to anthracnose (Yagi et al., 2010). *C. sinensis* var. *sinensis* 'Okuhikari' is late-plucked and resistant to anthracnose, blister blight, and gray blight but susceptible to bacterial shoot blight. This cultivar has yield potential similar to *C. sinensis* var. *sinensis* 'Yabukita' (Yagi et al., 2010). Assam type tea cultivars have been renowned as potential genetic resources in tea plant breeding due to their disease resistant traits and fine tea aroma (Takeda, 1990).

Pollen collection

Late balloon phenophase flower buds at pre-anthesis stage were collected for pollen collection. Anthers were collected using fine forceps onto tracing papers laid in petri dishes and air dried at room temperature (20 to 25°C) for 2 to 3 days until anthers were dehiscent and released pollen grains. Collected pollens were stored at -4°C in glass tubular vial bottles with air-tight caps until used for pollination.

Pollen quality tests

Collected and stored pollens were initially subjected to pollen quality tests viz. viability and *in-vitro* germinability using staining method and *in-vitro* germination method, respectively.

Pollen viability was determined with two different staining tests, that is, fluorescein diacetate-FDA test (Heslop-Harrison and Heslop-Harrison, 1970) and iodine potassium iodide-I_2KI test. The pollen grains were separately immersed in a trace of 1% I_2KI solution and FDA solution (200 µg·mL^{-1} FDA in 0.5 M sucrose) in eppendorf tubes following 5 min incubation at room temperature in dark condition for proper staining of pollen grains. A drop of stained pollen mixture was mounted on a glass slide and covered with a coverslip as the drop of pollen evenly distributed. Pollen viability counts were made under the light microscope and the fluorescence microscope for I_2KI and FDA tests, respectively. Six microscopic slides were used for each cultivar in each staining method. In relation to I_2KI test, pollen grains stained with dark brown in color were counted as viable while yellowish or unstained pollen were counted as non-viable (Figure 1A). In contrast, in FDA test the viable pollen grains fluoresced brightly and non-viable pollen emitted the ghost fluorescence (Figure 1B). The percentage of pollen viability was determined as ratio of the number of viable grains to the total number of grains per viewed area.
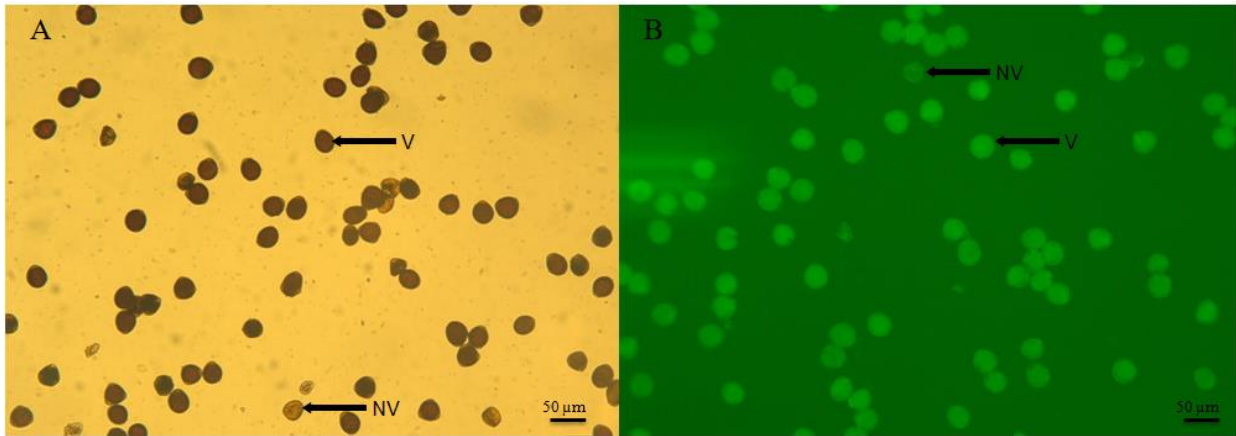
Figure 1. Viable and non-viable pollens in I_2KI (A) and FDA (B) staining tests. V: viable; NV: non-viable.
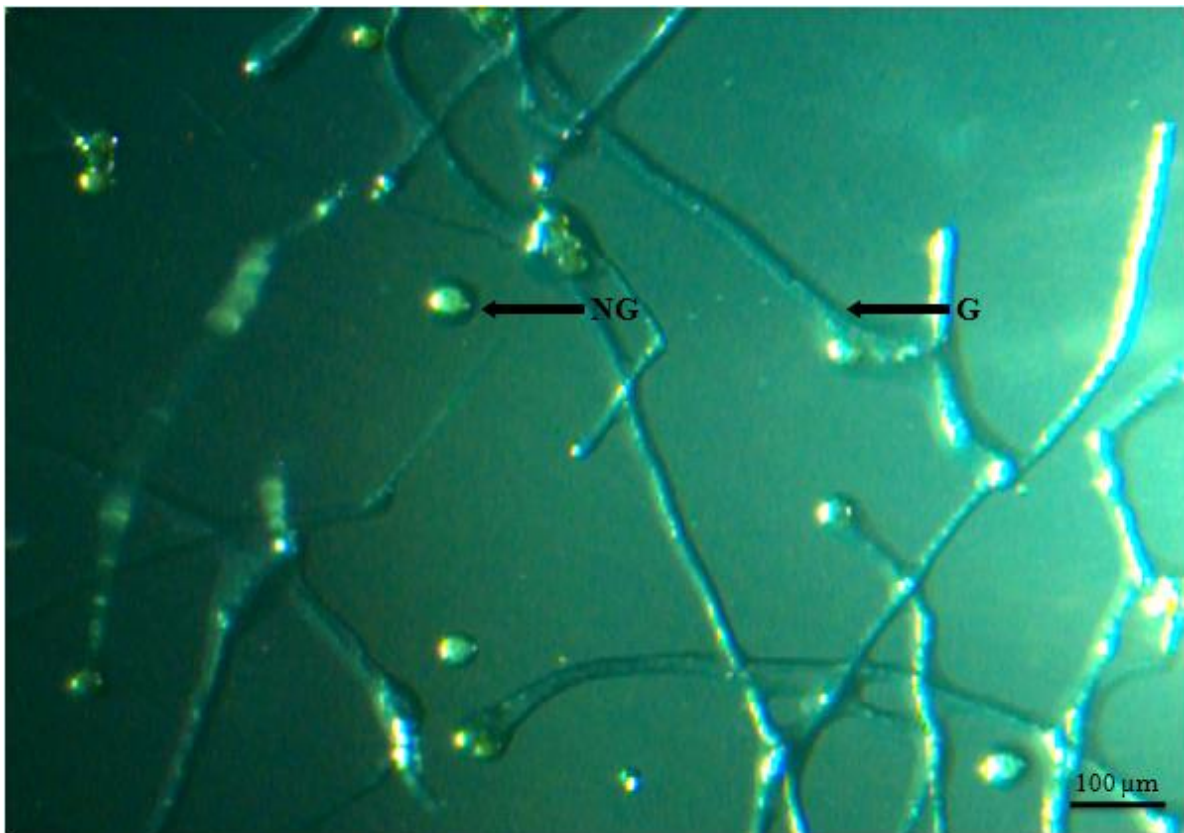
Figure 2. Germinated and non-germinated pollen grains in *in-vitro* germination medium. G: germinated; NG: non-germinated.

In-vitro pollen germination was assessed via "Hanging Drop" method described by Yang et al. (2008). Pollen were uniformly scattered on to the media consisted with 1% agar and 10% sucrose with pH 5.6 in petri plates. Pollen germination was observed under the light microscope after 4 h incubation period in dark. Pollen grains were considered as germinated when the pollen tube length was equal to or greater than the diameter of pollen grain (Figure 2). Five plates were made for each cultivar. The percentage of pollen germination was calculated as ratio of the number of germinated grains to the total number of grains per viewed area.

Pollination treatments

Artificial pollination was carried out in plastic green house in October, 2014 when the peak flowering occurred. Late balloon

Table 1. The percentages of pollen viability and *in-vitro* germination of *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37'.

Cultivar	Viability (%)		*In-vitro* germination (%)
	I_2KI	FDA	
'Yabukita'	88.5 ± 1.5^z	82.2 ± 1.0	69.43 ± 0.9
'AI-37'	87.5 ± 1.2	81.3 ± 1.1	81.05 ± 1.3

[z]Values indicate means ± S.E.

phenophase flower buds of ovule parents at pre-anthesis stage were emasculated by removing petals and stamens using fine forceps and hand pollinated with the aid of a small camel's hair brush and then bagged. Pollination treatments were as follows: 'Yabukita' x 'Yabukita' self pollination and 'Yutakamidori' x 'Yabukita' close intraspecific cross pollination within *C. sinensis* var. *sinensis*, and 'Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37' remote intraspecific cross pollinations between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. Fifty flower buds for each combination were used.

In-vivo pollen germination and pollen tube growth

In-vivo pollen germination test was done using aniline blue fluorescence microscopy assay following Yang et al. (2008). Self- and cross-pollinated flower pistils were collected at 1 day, 3 days, and 14 days after pollination and fixed in FAA (70% ethanol: formalin: acetic acid, 18: 1: 1, v/v/v). Five pistils from each treatment were collected. Fixed pistils were rinsed with distilled water for 4-5 times. Cleared pistils were hydrolyzed in 2 N NaOH at 60 ^{0}C for 1 h until the tissue became transparent. Hydrolyzed pistils were rinsed in distilled water for 4-5 times and stained with 0.1% aniline blue dissolved in 0.1 N K_3PO_4 for 24 h at room temperature in dark place. The stained pistil was placed on a microscopic slide and squashed under a glass coverslip to spread the material evenly and observed under the fluorescence microscope (Leica DMREB, Leica Co., CA, US).

Early fruit set

Some of pollinated flowers were left on plant to monitor the early fruit set for self- and cross-pollinations which were recorded at 3 months and 6 months after pollination. The percentages of fruit set and fruit diameters were recorded.

RESULTS

Pollen quality tests

The data on pollen quality tests viz. viability and *in-vitro* germinability were presented in Table 1. In I_2KI test, *C. sinensis* var. *sinensis* 'Yabukita' had the highest (88.5%) pollen viability followed by *C. sinensis* var. *assamica* 'AI-37' (87.5%). Pollen viability determined by FDA test was 82.2% for *C. sinensis* var. *sinensis* 'Yabukita' and 81.3% for *C. sinensis* var. *assamica* 'AI-37'. The highest *in-vitro* pollen germination was obtained for *C. sinensis* var. *assamica* 'AI-37' (81.05%) and it was significantly low in *C. sinensis* var. *sinensis* 'Yabukita' (69.43%).

In-vivo pollen germination and pollen tube growth

In the present study, the *in-vivo* pollen germination and pollen tube growth related to the controlled self- and cross-pollinations were examined at 1 day, 3 days, and 14 days after pollination using fluorescence microscopy. A specialized polysaccharide viz. "callose" found in pollen tube wall has a great affinity to aniline blue and produces a bright yellow-green fluorescence when illuminated by ultraviolet light. The growing pollen tubes are characterized by callose outlining and irregularly spaced callose plugs in pollen tubes (Kho and Baer, 1968; Unal et al., 2013). This phenomenon was used to detect pollen germination and pollen tube growth in pistils using fluorescence microscopy. Four broad sites viz. stigma, upper style, lower style, and ovary in pistil were examined for pollen germination and pollen tube growth. The style of tea flower pistils consists of three arms which are united for varying length into a column (Banerjee, 1992; Mondal et al., 2004; Ariyarathna et al., 2011). Therefore, most of pollen tubes were overlapped with each other subsequent to squashing of pistils. Consequently, the quantification of precise number of pollen tubes at each site of pistils was not feasible in our study.

Fluorescent microscopy revealed that the copious pollen grains had successfully germinated on stigma and grew rapidly through style in a dense cluster within 1day after pollination in both self- and cross-pollination (Figure 3). Within one day of pollination the elongated pollen tubes were found in upper and lower style and tails of pollen tubes were observed in ovary of both selfed and crossed flower pistils (Figure 3). The squashed selfed and crossed pistil samples were not clear enough to examine the pollen tubes in or near ovules. The magnitude of the pollen tubes germinated on stigma was higher than that of reaching to style base and to ovary in all tested crosses. The present study further revealed that at 3 days and 14 days after pollination there were less pollen grains and pollen tubes in all pistils than those found at 1 day after pollination. Moreover, as the time after pollination prolonged, the fluorescence of pollen tubes disappeared and recording the presence of pollen tubes based on callose deposition was not feasible with 3 days and 14 days pistil samples. Even so, the obvious morphological or structural dissimilarities in pollen grain germination and pollen tube growth were not found in self- and cross-
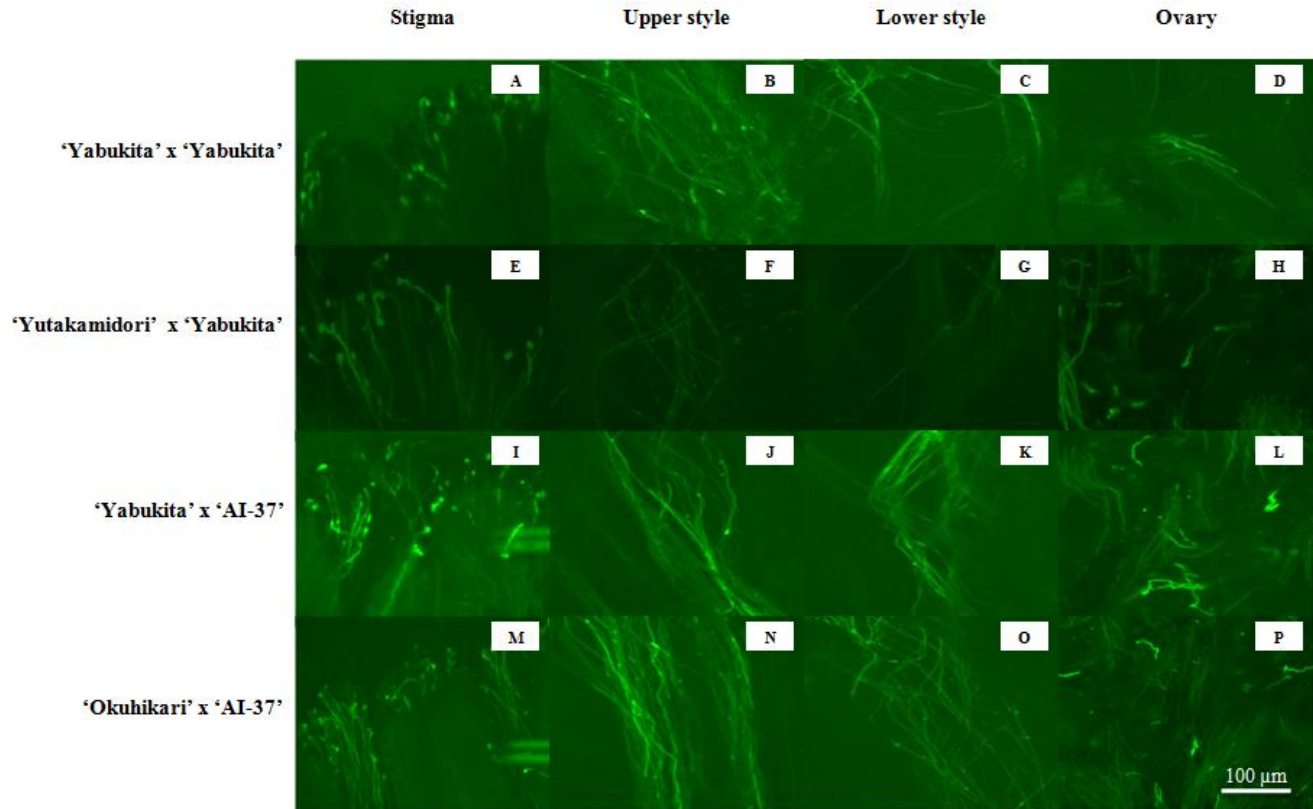
Figure 3. *In-vivo* pollen germination and pollen tube growth from stigma to ovary visualized using fluorescence microscopy at one day after different pollination treatments. (A–D) 'Yabukita' x 'Yabukita'; (E–H) 'Yutakamidori' x 'Yabukita'; (I–L) 'Yabukita' x 'AI-37' and (M–P) 'Okuhikari' x 'AI-37'.

combinations. Pollen tube growth was normal without any considerable inhibition of pollen germination and pollen tube growth in pistils and showed same pattern for all type of crosses in present investigation. Disregard to the normal growth pattern an unusual zigzag growth was discriminated at very low frequency in self-cross ('Yabukita' x 'Yabukita') and remote intraspecific crosses ('Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37') (Figure 4).

Early fruit set

Early fruit set percentages and fruit diameters were recorded at 3 months and 6 months after pollination (Table 2). The estimated fruit set percentages at 3 months after pollination were, 75%, 60%, and 80% for crosses 'Yutakamidori' x 'Yabukita', 'Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37', respectively. The fruit set percentages declined at 6 months after pollination as 70%, 35%, and 50% for above crosses, respectively. Fruit diameters ranged between 4.4 to 4.8 mm and 4.5 to 4.9 mm at 3 months and 6 months after pollination, respectively. In self-cross 'Yabukita' x 'Yabukita' most of pistils were withered and dropped within a few days after pollination.

DISCUSSION

Pollen quality tests

Microscopic pollen grain contains the male gamete to be used in fertilization. Assessing the pollen quality for a cultivar to be used as a pollinizer is essential in plant breeding to ensure the success of artificial pollination. Pollen quality of *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' was evaluated before using them in controlled pollination. Heslop-Harrison et al. (1984) perceptively reviewed three general approaches for evaluating pollen quality viz., histochemical, *in-vitro* and *in-vivo* pollen germination and pollen tube growth. These tests estimated the potential of pollen to germinate and grow on stigma in artificial pollination. Histochemical tests are based either on the ability to stain specific constituents of vegetative cell of pollen grain or on the activity of specific enzymes (Heslop-Harrison et al., 1984).

In the present study, I_2KI and FDA histochemical tests were used for pollen viability assessment. A significant difference was not found between *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' for each of viability tests. Pollen viability detected by I_2KI
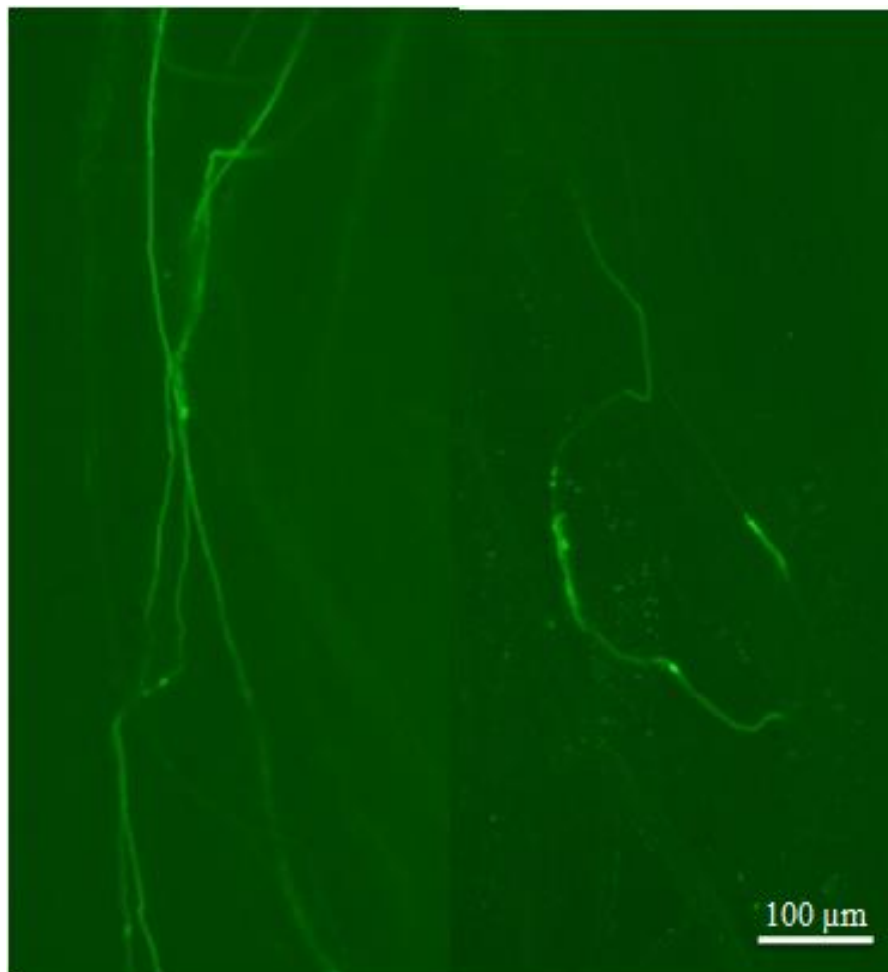
Figure 4. Abnormal 'zigzag' growth pattern observed at 3 days after pollination in lower styles at very low frequency in self cross 'Yabukita' x 'Yabukita' and remote intraspecific crosses 'Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37'.

Table 2. The percentages of fruit set and fruit diameters in cross-pollination.

Cross	Fruit set (%)		Fruit diameter (mm)	
	3 Months AP	6 Months AP	3 Months AP	6 Months AP
'Yutakamidori' x 'Yabukita'	75	70	4.4	4.5
'Yabukita' x 'AI-37'	60	35	4.7	5.0
'Okuhikari' x 'AI-37'	80	50	4.8	4.9

AP: After pollination.

test has given higher values for each cultivar than by FDA (Table 1). The I_2KI indicates the presence of starch while FDA implies the integrity of plasmalemma of vegetative cell of pollen grains (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984). Hence, FDA test was more effective in tea pollen viability assessing than I_2KI test.

In *in-vitro* pollen germination and pollen tube growth test involves germinating pollen on artificial media and determining the germinability and pollen tube growth (Heslop-Harrison et al., 1984). The percentage of *in-vitro* pollen germination of two pollen parents *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' was found to be significantly different (Table 1). The percentage of *in-vitro* pollen germination is low in both cultivars when compared to pollen viability percentages.

This clearly indicated that all the pollen estimated by staining methods to be viable was not germinated in *in-vitro* medium. Hence, compared to *in-vitro* germination test the pollen staining tests overestimated the viability of pollen. *In-vitro* pollen germination is generally believed to provide more reliable estimate of pollen viability (Muoki et al., 2007). Additionally, both viability and *in-vitro* germinability tests together provided important insight into understanding about the pollen quality. Notably, disparities in pollen quality are evidenced for potential male gametophyte competition and unequal reproductive success among *C. sinensis* genotypes (Muoki et al., 2007). The paternal traits, that is, phenology of male organ and amount of pollen produced, and pollen grain traits, that is, germination percentage, germination time, pollen tube growth rate, and selective fertilization are the factors that determine the fitness of pollinizers (Muoki et al., 2007).

In this study, paternal parents *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' possessed considerable good quality in both viability and *in-vitro* germinability tests which is a prerequisite for successful pollination and fertilization. Therefore, both of tea cultivars, *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' can be considered as good pollinators.

In-vivo pollen germination and pollen tube growth

Monitoring the pollen grain germination and pollen tube growth in pistils using aniline blue fluorescence microscopy assay subsequently with the fruit and seed set are included in cross-compatibility test in intraspecific hybridization (Heslop-Harrison et al., 1984; Muoki et al., 2007). Although fluorescence microscopy is a practicable approach for examining the pollen tube growth in pistil, this method is relatively time consuming, unfeasible for testing many samples. Further, seed set may depend not only on fertilization, but also on post-pollination development of ovary, pistil receptivity, and incompatibility reactions (Heslop-Harrison et al., 1984).

In our study, successful germination of pollen grains on stigma, rapid growth of pollen tubes through style, and tails of pollen tubes in ovary were examined within one day after pollination of both selfed and crossed flower pistils. This was supported by further evidenced from Wachira and Kamunya (2005) with an account of tea pollen germination on stigma and the succeeding pollen tube growth along style of self- well as cross-pollination within one day. In addition, Simura and Oosone (1956) monitored satisfactory pollen grain germination on stigma within about 1 h after both cross- and self-pollination of *C. sinensis*. The successful pollen germination on stigma in all cross combinations indicated the pistils' receptivity during the pollination. Stigmatic receptivity showed the ability of stigma to support the pollen germination. The selected late balloon phenophase flower buds of ovule

parents in this study proved to being well receptive at the time of pollination. This fact was further emphasized by Ariyarathna et al. (2011), by examining the adequate pollen adhesion and germination in manually pollinated floral buds at balloon stage. Tea flowers own a group III wet type stigma (Heslop-Harrison and Shivanna, 1977; Ariyarathna et al., 2011) and stigma surface is the first site of the cross compatibility and incompatibility responses that govern the success of the breeding system (Heslop-Harrison and Heslop-Harrison, 1985). Further, this implies the affinity of plant materials used in hybridization program. In view of the self-pollen grains germinated on stigma in our study it is convinced the gametophytic self-incompatibility of tea plant (Fuchinoue, 1979; Chen et al., 2012). The occurrence of pollen tubes in ovary might be applied as a reliable appraisal to persuade the ovule penetration in self-/cross-pollinated pistils. Our results are compatible with the findings of Chen et al. (2012) who observed the successful pollen tubes elongation through style to ovary at 24-48 h after self-/cross-pollination in *C. sinensis*. Supporting to our observations, Rogers (1975) also reported that by 24 h crossed/selfed pollen tubes had entered ovary and probably penetrated as far as the ovules. Analogous growth pattern of cross- as well as self-pollen tubes in tea plant flowers has been reported by Wachira and Kamunya (2005).

Higher magnitude of pollen tubes was also observed to have germinated on stigma than that of reaching to style base and to ovary in all tested crosses. Further, at 3 days and 14 days after pollination the fluorescence of pollen tubes disappeared and recording the presence of pollen tubes based on callose deposition was not feasible. These observations might have resulted from the degradation of growth substances in pollen grains and pistils. According to the information generated by Rosen (1971), the pollen germination and pollen tube growth on stigma rely on reserves within the pollen, hence, this phenomenon is known as autotrophic. The pollen tube growth in styles is heterotrophic, since, the growing pollen tube depends on stylar reserves. Thus, the growing pollen tubes might be competing for nutrients and space during their autotrophic and heterotrophic growth in pistil and number of pollen tubes gradually decreased from stigma to ovary.

Seeing as the pistil sampling was not done before one day after pollination and pollen tubes grew along style and reach to ovary within one day the speed of pollen tube elongation was not distinguishable between self- and cross-pollinations in current investigation. Nevertheless, the review of literature on this subject reported by many scholars helped to have a clear understanding of our observations comparatively. Chen et al. (2012) have determined the pollen tube elongation rate in *C. sinensis* with the ration of length of the longest pollen tube to that of the style and differences was found based on cultivars. It was found that the higher elongation rate

for cross-pollination and lower rates for self-pollination in some cultivars while there was not a substantial difference in pollen tube elongation rate between cross- and self-pollination in some other cultivars (Chen et al., 2012). Liao et al. (2014) also supportively depicted that the growth speed of crossed pollen tubes of *C. oleifera* was slightly faster than selfed pollen tubes as pollen tubes reached style base at 48 h after cross-pollination and 60 h after self-pollination. Comparable remarks were reported by Simura and Oosone (1956) where, in crossed flowers the pollen tubes grew rapidly and reached funiculus base in about 36 to 40 h after pollination, while in selfed flowers they grew uniformly and scarcely reached it by 72 h. Moreover, pollen tubes grew slower in styles of a different species and protruded ovules within 3 to 5 days after pollination (interspecific crosses) whereas that of within the same species (intraspecific crosses) occurs within 1-2 days after pollination (Hwang et al., 1992). Wachira and Kamunya (2005) found that the cross- and self-pollen tubes of tea plant flowers grow at different rates and compete to fertilize the ovule.

The pollen grain germination and pollen tube growth pattern was normal and similar in self- and cross-combinations in our study. Tanaka (1988) and Liao et al. (2014) reinforced our appraisal by particular studies on self-incompatibility in the genus *Camellia*. Aside from the normal growth of pollen tubes we observed an unusual zigzag growth at very low frequency in self and remote intraspecific crosses. Conspicuously, Hwang et al. (1992) has been reported a small frequency of abnormal pollen tubes with a zigzag or branching growth habit in inter-specific crosses between *C. japonica* and *C. chrysantha*. Apart from that the distorted pollen tubes containing reversal tubes, swelling tube tips with callose deposits, irregular tubes and furcal tubes have been noted in selfing pistils of *C. oleifera*, an another member of the genus *Camellia* (Liao et al., 2014). Another report by Rogers (1975) has been documented the presence of self-pollen tubes with swollen and distorted tips in some tea clones.

Early fruit set

So as to confirm remote intraspecific cross-compatibility of crosses attained in this experiment, *in-vivo* pollen tube growth is not a sufficient witness since, pollen tube growth patterns in selfed and crossed pollinated pistils were similar. In view of that, the cross- as well as self-pollen tubes had reached ovary and might be near the ovules at 24 h after pollination. Therefore, it was intended to go into an estimation of fruit set and retention subsequent to the self-and cross-pollination.

Early fruit set and retention of all cross combinations was estimated at 3 months and 6 months after pollination. In our observations the cross-pollinations bore fruits whereas self-pollination failed in fruiting. The fruit set percentages declined at 6 months after pollination and

obvious reductions were recored for the remote intraspecific cross pollinations, 'Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37' compared to the close intraspecific cross pollination, 'Yutakamidori' x 'Yabukita'. Aborted or under developed ovaries were found very often in tea bushes soon after anthesis and intensive abortion of fruitlets/seeds were recorded during the initial 15 to 20 days after pollination despite to the succeeded pollination (Ariyarathna et al., 2011). Hence, comparatively obvious decline in fruit set percentages in remote intraspecific cross pollinations might be caused by the high intensive abortion of fruitlets/seeds. In self-pollination most of pistils were withered and dropped within a few days after pollination. According to the depiction by Ariyarathna et al. (2011) in incompatible cross combinations more than 90% of pollinated flowers withered and fell in less than one week. Similar circumstance has been documented by Ozaki et al. (2003), that is, unfertilized fruitlets dropped before one month after pollination in the genus *Camellia*. With an interest Ozaki et al. (2003) further elucidated exceptional fruit set on few self-pollinated cultivars of *C. japonica* L. which showed 5-27% fruit set with scarce of perfect seeds. A report by Simura and Oosone (1956) mentioned fruiting rates of tea plant as 20 to 30% in crossed flowers and 3 to 10% in selfed flowers and time taken for double fertilization after pollination is 36-48 h and 62-72 h in crossed and selfed flowers, respectively. Tea fruit maturation requires 8 to 9 months after pollination and possesses two seeds/fruit on average with maximum of six seeds/fruit depending on parents (Ariyarathna et al., 2011). Indirectly, based on percentage of fruit set we can have a clear idea on percentage of pollination success of each cross combinations as described by Ariyarathna et al. (2011). Percentage of pollination success was defined as the percentage of fruit set per each of pollinated flower (Ariyarathna et al., 2011).

Thus, the merger of pollen tube growth observations with fruit set after self- and cross-pollination is most useful to determine the self-/cross-compatibility. In the present observations the pollen tube could be reached to ovary in self- and cross-pollinated cultivars. Regardless of *in-vivo* pollen tube growth the fruiting was conflicted between selfed and crossed pollinations. As selfed cross 'Yabukita' x 'Yabukita' failed in fruit set it proved that the fertilization was not occurred in that particular cross. Conversely, the cross pollinations eventually developed fruits owing to unbeaten fertilization due to successful pollen tube penetration into ovules. Therefore, we can presume that the self-pollen tubes of *C. sinensis* might have not entered ovules or they might have failed in fertilization after entered ovule. Thus, there might be post-zygotic barriers to overcome selfing rather than pre-zygotic barriers. Hence, the contemporary results further confirmed the late-acting self-incompatibility present in *C. sinensis*. Self-incompatibility of tea plant has been adequately appraised by numerous studies over the past decades. More recently, self-incompatibility in tea plant

has been comprehensively explored by Wachira and Kamunya (2005) and Chen et al. (2012) with aniline blue fluorescence assay and sturdily confirmed the self-incompatibility of tea plant as a late-acting self-incompatibility system or an ovarian sterility. This heritable reproductive phenomenon of tea plant has been further reviewed by Rogers (1975) and Fuchinoue (1979). Self-incompatibility of *Camellia* spp. contributes to huge genetic variation within the genus. The close intraspecific cross within *C. sinensis* var. *sinensis*, 'Yutakamidori' x 'Yabukita' showed positive responses in all examined criteria as discussed earlier and it confirmed out-crossing nature of tea plant. The close intraspecific hybridization in *C. sinensis* is extensively utilized and several hundred cultivars have been resulted from this hybridization technique in all tea growing countries (Takeda, 1990; Chen et al., 2012).

Last but not least, the successful pollen germination and pollen tube growth in pistils as far as to ovary of remote intraspecific cross combinations between *C. sinensis* var. *sinensis* and *C. sinensis var. assamica* with considerable fruit set did not indicate any obvious pre-zygotic barrier(s). The post-zygotic barriers have not been studied yet in the present intraspecific hybridization effort. The post-zygotic reproductive barriers, for instance hybrid embryo abortion in the genus *Camellia* have been recognized often in interspecific incompatibility comparing to pre-zygotic barriers (Ackerman, 1971; Hwang et al., 1992). Consequently, by insightful assessing of our research and out comes with previous reports it can be postulated that remote intraspecific hybridization might be feasible between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. Supplementary, the degree of compatibility between these hybridization species showed their genomic affinities to each other.

Conclusion

The present study revealed the effectiveness of remote intraspecific cross-compatibility between *C. sinensis* var. *sinensis* (China type) and *C. sinensis* var. *assamica* (Assam type) by means of *in-vivo* pollen tube growth and subsequent fruit set. This histological approach is known to be a reliable, rapid process to evaluate cross-compatibility of specific crosses. As intraspecific hybridization is highly renowned to breed superior tea cultivars the contemporary findings might be applicable in tea plant breeding programs. Additional experimental trials in several aspects on remote intraspecific hybridization between China type and Assam type tea are mandatory to compose a tangible statement on conclusion of this foremost attempt.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Physiological quality of habanero pepper (*Capisicum chinense*) seeds based on development and drying process

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The objective in this research was to study the physiological and biochemical alterations in habanero pepper during the development and after the natural and controlled drying. The experiments were conducted at Seeds Laboratory and in the experimental area of Agriculture Department at Universidade Federal de Lavras (UFLA). The fruits were harvested in four stages of development, being these E1-49, E2- 56, E3- 63 and E4- 70 days after anthesis. After harvest, parts of fruits were kept at rest for seven days. The seeds extracted from the fruits in different periods of harvest were submitted to two methods of drying: artificial drying at 35°C, and natural drying on shadow until 9% of water content. The performance of seeds under different treatments was evaluated through germination, emergence, electrical conductivity and water content tests and through the activity of the isoenzyme systems; esterase (EST), catalase (CAT), superoxide dismutase (SOD) and heat-resistant proteins. With the results, it was concluded that habanero pepper seeds from fruits harvested and submitted to post-harvest have higher physiological quality and less dormancy. Habanero pepper seeds, when harvested next to maturity points (E3 and E4) and dried in controlled conditions (35ºC) in constant air flux, induces the synthesis of heat-resistant proteins.

Key words: *Capsicum chinense* Jacquin, seeds formation, vigor of seeds.

INTRODUCTION

Habanero pepper is the most Brazilian between all the species being sourced in the amazon region and is extremely appreciated by the unmistakable flavor and spiciness. Like the other peppers species, the offer of quality seeds of habanero pepper is limited, mainly by lack of knowledge about the better seeds harvest stage and the utilization of adequate dry methods, which aim to increase the potential of storage and the establishment of plants in field (Queiroz, 2014) In the production process, the harvest and drying influences significantly on seeds quality should being performed in the adequate moment and following the technique recommendations to reduce

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at maximum the possible qualitative and quantitative losses (Faria et al., 2003). For the most part of species, the most appropriate time for seeds harvest is closest to the maturity physiological point, which corresponds to the time of greater weight, germination and vigor. When the harvest is realized in the optimum point, the preservation of physiological potential of seeds is favored, because when seeds are kept in field after the physiological maturity, occurs the process of deterioration (Abud et al., 2013).

In the case of fleshy fruits like habanero pepper, the maturity of seeds generally coincides with the beginning of coloration change, when the fruits present red color. Beyond the fruits coloration, other indicators of physiological maturity are the vigor, moisture content, mass and the size (Dias et al., 2006; Vidigal et al., 2011). Caixeta et al. (2014) reported that the better harvest time of habanero pepper for seeds production, varies between 60 to 67 days after anthesis, phase characterized by the changes of fruits coloration. However, researches have shown that even before the complete maturation of fruits, seeds already achieve the physiological maturity (Zanin, 1990).

It is known that the understanding about the changes that occurs in seeds during the different development stages when occurs loss of water, is important for the choose of the methodology which must be used in the drying process of seeds harvested with high water contents, once, with progressive water loss, seeds become tolerant to high temperatures of indicating that the events occurs together with the reduction of water content (Rosa et al., 2000).

Therefore, knowing the ideal moment of harvest and the adequate drying method for seeds is very important to guarantee the maximum quality and vigor in the field. With this, the objective of this work was to evaluate the effect of drying and harvest time of fruits on the quality of habanero peppers seeds, aimed at the maximum quality of these seeds.

MATERIALS AND METHODS

This research was conducted in the Central Laboratory of Seeds and in the experimental area from the Agricultural Department of Universidade Federal de Lavras (UFLA), in Lavras, MG. The city is located at southern region of Minas Gerais, coordinates 21°14'S of latitude and 40°17'W of longitude, and at 918.8 m of altitude. Also, varieties of yellow habanero pepper seeds (*Capsicum chinense* Jacquin) were used in this research.

Seeds were soaked in plastic trays with 72 cells with commercial substrate Plantmax, used for seedlings formation. These cells were transplanted to the experimental area after 45 days after soaking. The tests for the seeds production were conducted in the experimental area of the Agricultural Department at UFLA, in dark red latosol (LE), clayey texture and conventionally prepared. The transplanting of seedlings was realized in the second week of December. Each splot was composed by 2 lines of 10 m of length, with 12 plants. The fertilizations, as well as the others cultural practices, were realized according recommendations for this culture (Filgueira, 2003; Pinto et al., 2006). Was used the randomized

block design (DBC) with four replications, being the fruits harvested in four different period of harvest in each block: E1 (49 days after anthesis, fruits completely green), E2 (56 days after anthesis, fruits with first signals of yellowing), E3 (63 days after anthesis, yellow fruits with green signals) and E4 (70 days after anthesis, mature fruits characterized by the orange color). Part of the fruits was maintained in resting for 7days after the harvest.

Seeds were manually extracted with the aim of stylus. After the extraction, seeds were disinfected with solution of 1% of sodium hypochlorite for one minute. Following, were done tests to evaluate the seeds quality.

Seeds extracted from fruits in different periods of harvest with and without resting for seven days, were submitted to two methods of drying: M1 (artificial drying at 35°C, until 9% of water content) and M2 (natural drying at shadow, until 9% of water content). After drying, the quality of seeds was evaluated by the germination, emergence and electric conductivity tests.

The water content of seeds was evaluated in greenhouse, at 105 ± 3°C during 24 h, using two subsamples for each treatment, according the Regras para Análise de Sementes – RAS (Brasil, 2009). The results were expressed in medium percentage by treatment.

In the germination test, the sowing was realized in gerboxes with two papers moistened with water, in the proportion of three times the weight of the dry substrate. The boxes were maintained in chamber germination under alternate temperature and light (20°C/16 h in dark and 30°C/8 h in light presence). At 7 and 14 days, was realized the evaluation according to Brasil (2009). Each treatment was composed of four subsamples of 50 seeds. The results were expressed in percentage of normal seedlings.

Seeds which not germinated were submitted to tetrazolium test. These seeds were immersed for coloration in solution of 2,3,5 triphenyl tetrazolium chloride at 0.075%, during 3 h in dark at 35°C. After this period, seeds were washed in current water and submersed in cold water until the evaluation. In the second phase, the embryos were individually analyzed, after opening lengthwise with stylus, verifying their external and internal parts. The interpretation was made with aim of magnifying glass with fluorescent lighting, to verify if the seeds were dead.

In emergence test, the sowing was realized in multicellular trays, containing commercial substrate (Plantmax®). The trays were kept in greenhouse with intermittent nebulization system, at temperature of 28°C. For each treatment were used four subsamples of 50 seeds. Were realized daily evaluations from the beginning of emergence, computing the number of emerged plants until the stabilization on the stand. Was computed the percentage of normal seedlings at 21 days.

For the electrical conductivity were used four subsamples of 50 seeds with known masses, immersed in 25 mL of distillated water and kept in BOD chamber, at 25°C for 24 h (Vidigal et al., 2008). After this period, the electric conductivity of each solution was determined in conductivimeter, and the results were expressed in $\mu S.cm^{-1}.g^{-1}$ of seeds.

For the enzymes analyses, two samples of 100 seeds of each treatment were macerated in recipient with liquid nitrogen. Were removed subsamples of 100 mg, which were added extraction buffer (Tris HCl 0.2 M, pH 8) in the quantity of 2.5 times the weight of each sample and 0.1% of β-mercaptoethanol. The material was homogenized in vortex and kept in refrigerator during 12 h followed by the centrifugation at 14000 rpm for 30 min at 4°C and them, applied in polyacrilamide gel. The electrophoretic run was realized in a discontinuous polyacrylamide gel system at 7.5% (separating gel) and 4.5% (concentrating gel) using Tris-glycine pH 8.9 as standard buffer in the gel electrode system. In each gel channel, was applied 50 µL of the sample supernatant and the running was performed at 150 V for 5 h. At the end of running, the gels were revealed for the enzymes superoxide dismutase (SOD-EC.1.15.1.1.), catalase (CAT- EC.1.11.1.6.) and esterase (EST- EC

Table 1. Germination percentage in function of development stages (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).

Development stages	Drying			
	Without resting		With resting	
	Natural	Controlled	Natural	Controlled
49	23[dA]	3[bB]	43[cA]	30[bA]
56	48[cB]	73[aA]	80[aA]	78[aB]
63	64[bA]	60[aA]	69[bA]	72[aA]
70	72[aA]	67[aA]	80[aB]	85[aA]
CV(%)		19.39		

Means followed by the same lower.

3.1.1.1.) according the protocols established by Alfenas (2006).

For extraction of heat-resistant proteins, 100 seeds of each treatment were macerated in appropriate recipient with liquid nitrogen. Were separated 100 mg in microtubes for the application of 1000 µL of buffer solution (Tris-HCl (pH 7.5); 500 mM NaCl; 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The homogenate was centrifuged at 14,000 rpm, at 4°C, for 30 min. and supernatant was incubated in water bath at 85°C, for 15 min and again centrifuged by 30 min like above related. The supernatant was poured into microtubes and pellet was discarded. Before the application to gel, the tubes with samples containing 70 µl of protein seed extract + 40 µl of sample buffer solution, composed by 5 ml of glycerol; 2.5 mL of buffer solution of concentrating gel, 2.5 mg of Bromophenol Blue (BPB) completing the volume to 25 mL of distillated water, were placed into boiling water bath for 5 min. Subsequently, on each groove of polyacrylamide SDS-PAGE gel at 12.5% (separating gel) and 6% (stacking gel) were applied 50 µl of the extract containing the heat resistant proteins + the sample buffer solution.

Following the methodology described by Alfenas (2006), electrophoretic run was performed at 150 V, for 12 h and subsequently gels were stained with solution of coomassie brilliant blue at 0.05% and discolored with 10% acetic acid solution.

Completely randomized experimental design was used in a factorial scheme of (4x2x2), being the factors; periods of harvest (E1, E2, E3 and E4), conditions of the fruits (with and without resting for seven days) and dry methods (M1 and M2).

Analyses of variances for all the tests were realized with the aid of SISVAR® statistical program (Ferreira, 2011). For the comparison between the averages, was used the Scott-Knott test at 5% of probability. The evaluation of the enzymatic patterns was made according to the intensity of the bands, using a surface of a transilluminator.

RESULTS AND DISCUSSION

The effect of fruits harvest time and the dry conditions was significant ($p < 0.05$), by the F test, in relation to the germination and vigor of habanero pepper seeds.

The values of water content in seeds harvested in different times after drying varied from 8.8 to 9.1%. The percentage of seeds germination harvested after 70 days after anthesis (DAA) and which were kept inside the fruits by seven days were higher than those harvested at 70 DAA, but that were not kept in resting inside the fruits. Seeds harvested in initial time of development, at 49, 56

and 63 DAA, presented low germination when compared to the time of 70 DAA (Table 1). In different stages were possible to verify variations in germination taxes before and after drying, showing that the pepper seeds can be included in a group described by Kermode and Bewley (1989), for which the desiccation represents a signal which diverts the development program for a germination program, since the drying was not harmful to the germination even in the stage of 56 DAA, where the factors like high content of ABA and resistance of tissues, which surround the embryo, could affect the germination and cause that, can confuse the degenerative effects of drying (Marcos, 2005).

The resting after harvest caused an increase of seeds germination, being the maximum value (85%) found in the treatment where seeds were dried with temperature control and at 70 DAA.

These results are in agreement with the results found by Sanchez et al. (1993) which, studying the resting after harvest of bell pepper, concluded that the resting showed to be beneficial to seeds harvested in the stage 1 (green) that initially presented germination of 60% and, after nine days of resting, achieve around 90% of germination.

According with Santos et al. (2015), also working with habanero pepper seeds, the period of fruits resting of seven days promotes the germination, with higher potential germinative was obtained when the period of fruits resting was superior to nine days.

This result comes to reinforce the necessity of post-harvest resting of pepper fruits above 56 DAA, when the germination achieves 60%, once that during the post-harvest resting period, seeds not yet fully mature would complete their maturation stage, while those already mature have their quality preserved for keep in osmotical equilibrium inside the fruit.

In Table 2, the percentage of viable seeds evaluated by the tetrazolium test is shown. Accordingly, the results revealed that the drying temperature of 35°C promoted lower values of dormant seeds at 70 DAA, with resting of fruits. The vigor evaluated by the first count of germination increased during the post-harvest resting in treatments where the fruits were harvested at 56, 63 and 70 DAA

Table 2. Average results (%) of viable seeds evaluated by the tetrazolium test in habanero pepper seeds in function of development stages (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).

Development stages	Drying			
	Without resting		With resting	
	Natural	Controlled	Natural	Controlled
49	18^{aA}	23^{aA}	16^{aA}	15^{aA}
56	12^{bA}	08^{cB}	05^{bB}	07^{bA}
63	18^{aA}	20^{aA}	14^{aB}	15^{aB}
70	10^{bB}	12^{bA}	05^{bA}	02^{cB}
CV(%)	4.13			

Means followed by the same lower case letter in the column and capital letter in line do not statistically differ by the Scott-Knott test at 5% significance.

Table 3. Percentage of normal seedlings in the first count of germination in function of development stages (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).

Development stages	Drying			
	Without resting		With resting	
	Natural	Controlled	Natural	Controlled
49	9^{bA}	3^{aA}	6^{cA}	5^{cA}
56	2^{bA}	4^{aA}	32^{aA}	22^{bB}
63	6^{bA}	4^{aA}	23^{bA}	6^{cB}
70	21^{aA}	9^{aB}	25^{bB}	53^{aA}
CV(%)	29. 54			

Means followed by the same lower case letter in the column and capital letter in line do not statistically differ by the Scott-Knott test at 5% significance.

(Table 3).

The treatment of 70 DAA differs of the others with germination values superior in the first count, what can infer that for be the treatment harvested in the stage where the fruit is completely yellow, implies that the seeds were with complete formation when compared to the fruits harvested in other stages.

These results are in agreement with studies realized by Teixeira et al. (2005) with bell pepper cv. 'Tico' indicating that the fruits harvest of bell pepper for extraction of seeds can be realized when these fruits achieve the orange coloration, being recommended the post-harvest resting of seven days.

The percentage of seedlings emergence in trays (Table 4) presented, in general, lower values than the percentage of germination. This difference could be cause in BOD chamber, the temperatures is alternate of 20 to 30ºC and the trays are kept in controlled temperature of 25ºC. It is known that between the environmental conditions which affect the germinative process, the temperature is one of the most influent factors (Mayer and Poljakoff-Mayber, 1989).

For the most part of species adapted to tropical climate,

the optimum temperature is around 20 to 30°C (Marcos, 2005). According this same author, there are species for which the alternate temperature have more significant effects than the constant temperature. This behavior, associated to species that have dormant seeds, like example of habanero pepper seeds, could favor the germination.

It is important to emphasize that the emergence was evaluated at 21 days, because at 14 days after sowing, none plant had emerged. Many authors have been observed in habanero pepper seeds that the seedlings emergence is slow and irregular, even in favorable conditions (Lakshmanan and Berke, 1998).

Based on data presented in Tables 5 and 6, is possible to observe a values reduction of electric conductivity for seeds in their different stages of development and drying methods. Higher values of conductivity in seeds harvested at 49 DAA was also observed, as well as what can be justified for the incomplete formation of the membranes of these seeds. With the advance of maturation process occurs the development and the structural organization of cellular membrane systems, along with an explanation on the reduction in values of electric

Table 4. Percentage of seedlings emergence in function of development stages (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).

Development stages	Drying			
	Without resting		With resting	
	Natural	Controlled	Natural	Controlled
49	0[bA]	0[bA]	7[dA]	6[dA]
56	5[bA]	5[bA]	22[bB]	30[bA]
63	0[bB]	4[bA]	14[cA]	16[cA]
70	32[aA]	22[aB]	39[aB]	63[aA]
CV(%)		25.79		

Means followed by the same lower case letter in the column and capital letter in line do not statistically differ by the Scott-Knott test at 5% significance.

Table 5. Values of electric conductivity in habanero pepper seeds in function of development stages (49, 56, 63 and 70 days after anthesis) and drying methods (natural and controlled).

Development stages	Drying	
	Natural	Controlled
49	1207.1[aA]	1268.3[aA]
56	919.9[bA]	1055.8[bA]
63	944.2[bA]	729.0[cB]
70	791.5[bA]	725.3[cA]
CV (%)		10.50

Means followed by the same lower case letter in the column and capital letter in line do not statistically differ by the Scott-Knott test at 5% significance.

conductivity. As seeds harvested in maturation stage became more advanced, the protection mechanisms of membranes to tolerate the desiccation are presented, promoting a decrease of solutes leaching after the drying, indicating a better structuring of membranes, which can be favored by the synthesis of heat-resistant proteins (Sun and Leopold, 1993).

These results are in agreement with those presented by the germination test and clearly demonstrate that the drying promotes damages to the seeds cellular membrane systems, which are differentiated in relation to the development stage of seeds, being in the stage of 49 DAA, seeds are more susceptible to these damages than in the stages of 56, 63 and 70 DAA, successively indicating that the membrane protection mechanism is developed during the seed development. A lot of seeds suffer a quick transition of one intolerance phase, approximately in the half of their period of development, predating or coinciding with their reserves deposition (Hong and Ellis, 1992).

The enzymes superoxide dismutase (SOD) and catalase constitute an efficient mechanism for detoxification, acting in the removal of free radicals. Analyzing the activity of these enzymes was possible to observe that there was alteration in the superoxide dismutase electrophoretic pattern over the time (Figure 1). The stress caused in seeds when not submitted to the resting can be the cause of oxidative process and free radicals production, being observed higher activity of SOD in treatments in this condition. For the treatments where the seeds were submitted to resting, it was possible to observe lower activity of this enzyme. However, it is important to highlight that the seeds which were dried in controlled conditions, presented pattern for this enzyme lower than the others treatments.

The catalase enzyme has the function of to eliminate the H_2O_2 produced in the photorespiration and in the β-oxidation of fatty acids. During the oxidative stress the peroxides increases in quantity and thus higher quantity of CAT is available to combat the increase in reactive oxygen species production (Mittler, 2002). It is possible to observe that seeds from fruits which were in resting for seven days, have a reduction in the activity of the CAT enzyme. This can be directly related to the condition of rest, which allows a restructuration of seeds enzymatic complex.

When the drying pattern was observed, it was possible to observe a lower activity of catalase when seeds were dried in controlled conditions (Figure 2), which is a possible implication that the process of natural drying
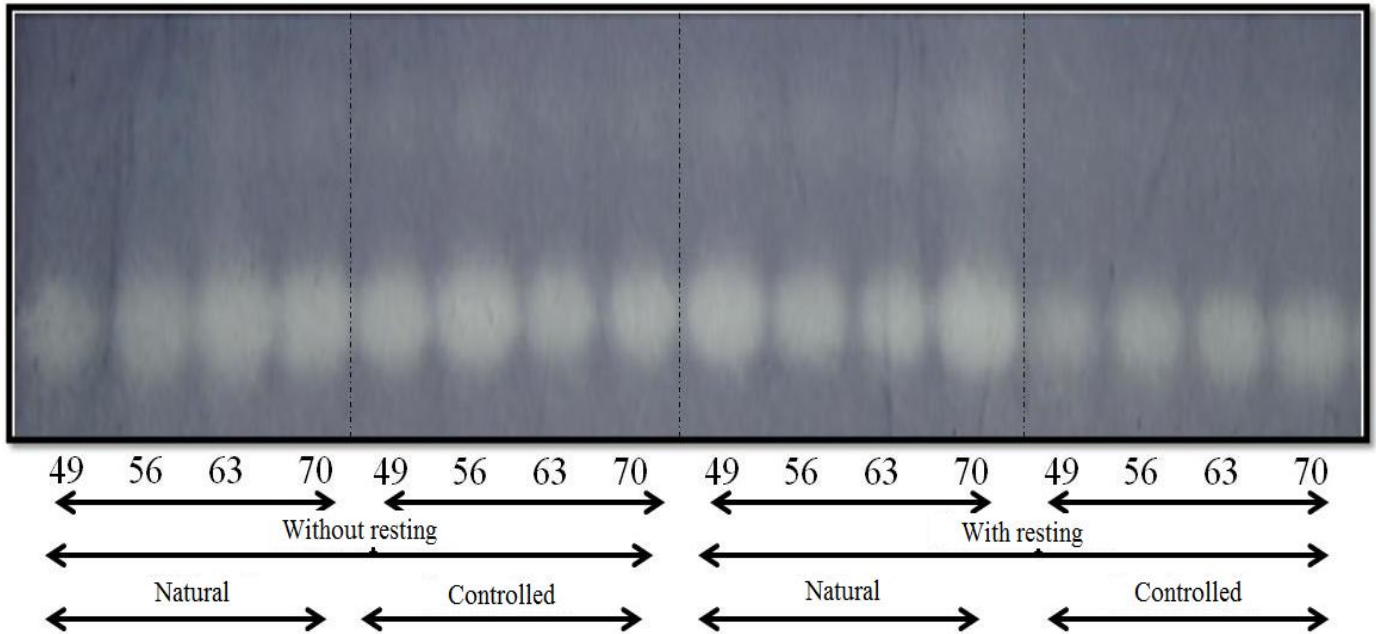
Figure 1. Electrophoretic pattern of superoxide dismutase (SOD) extracted of habanero pepper seeds in different stages of development (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).
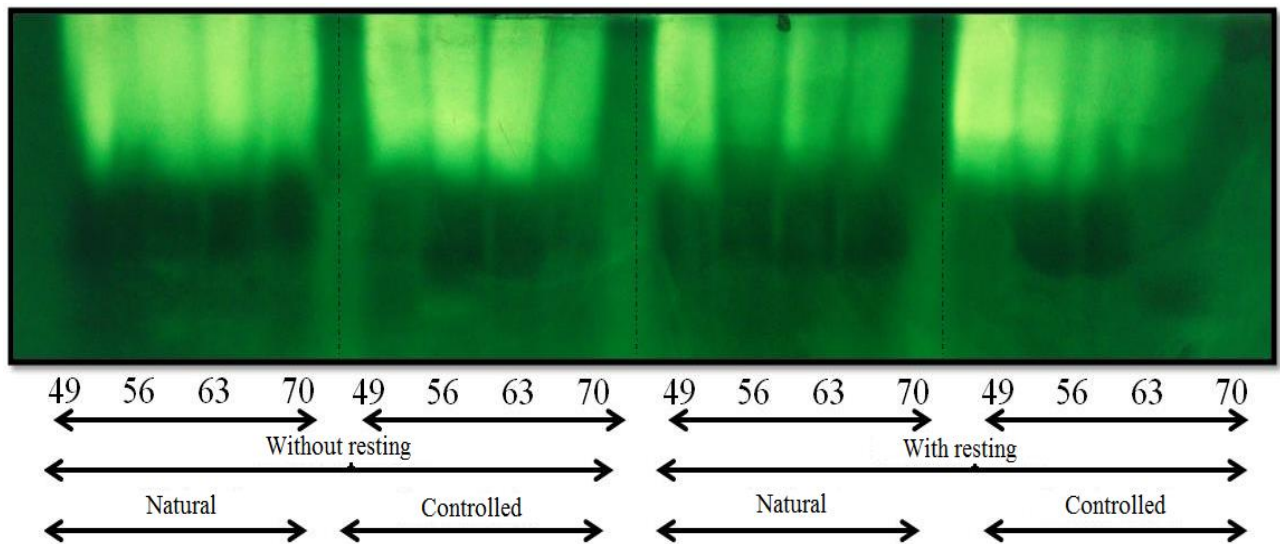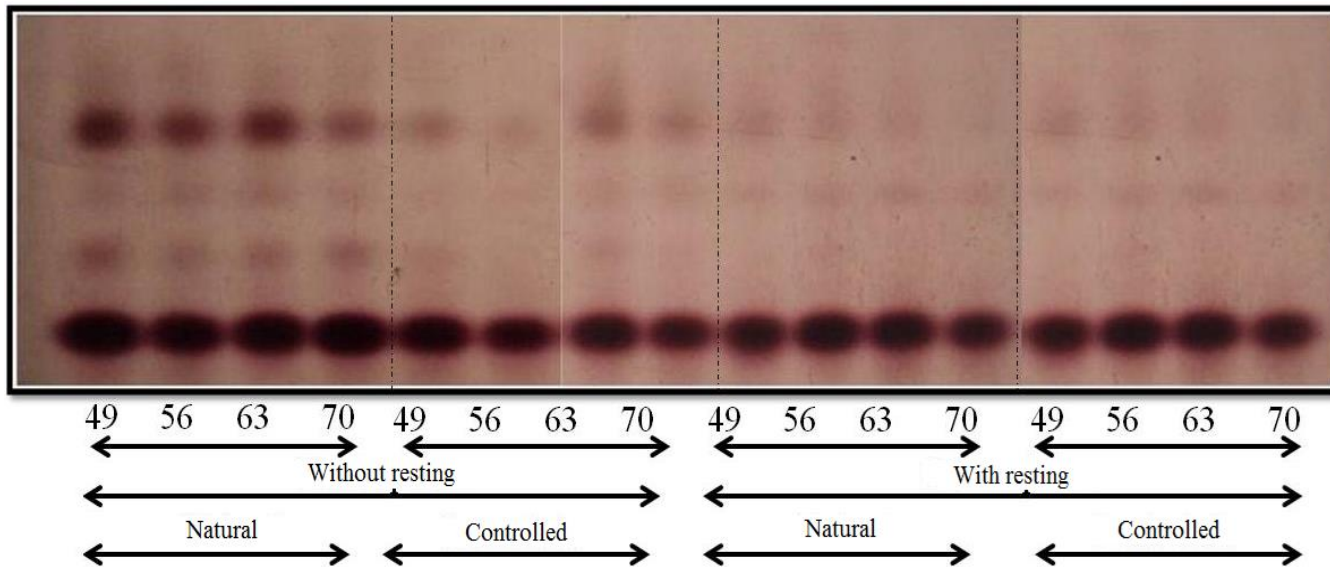

Figure 2. Electrophoretic pattern of catalase enzyme (CAT) extracted of habanero pepper seeds in different stages of development (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).

infers in the enzymatic arrangement of seeds for the production of enzymes capable to remove fatty acids, accelerating the production of these enzymes to keep the quality.

The esterase (Figure 3) had behavior similar to those seeds dried at environmental temperature and at 35ºC. It is known that esterase, besides characterizing seeds in

deterioration, could assist in the germinative process. The esterases are the most important group of enzymes in germination of oleaginous seeds. This big group of hydrolytic enzymes releases fatty acids of lipids, which are used in the β-oxidation, like energy source for the germinative process. Being this, the higher activity of this enzyme in the initial stages is due to the physiological

Figure 3. Electrophoretic pattern of esterase enzyme (EST) extracted of habanero pepper seeds in different stages of development (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).
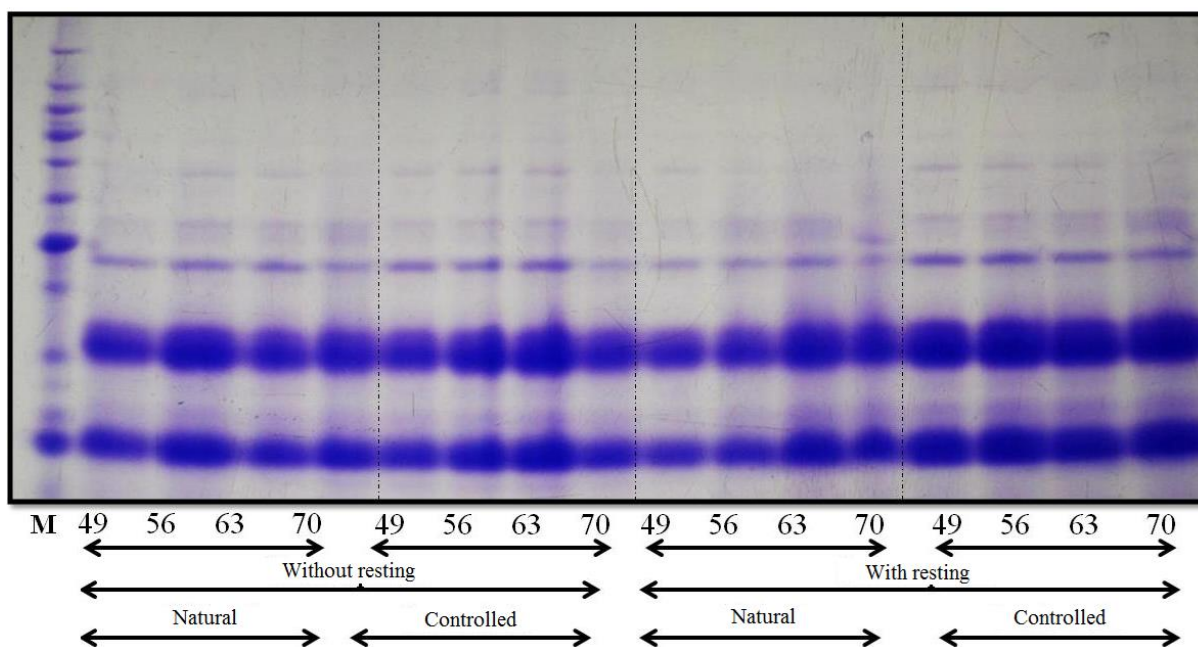
Figure 4. Electrophoretic pattern of heat resistant proteins (LEA) extracted of habanero pepper seeds in different stages of development (49, 56, 63 and 70 days after anthesis), conditions of resting (with and without resting) and drying methods (natural and controlled).

immaturity of these seeds, also as the damages caused by the drying inside the fruits.

The electrophoretic pattern of the heat-resistant proteins presented in Figure 4, revels the presence of heat-resistant proteins in all development stages evaluated, independently of the drying method and the resting

condition of fruits. However, there was evidence of the absence of some bands in seeds without drying. These results are in agreement with the tendency observed in the others studies, which were verified a behavior of desiccation tolerance, by the maintenance of the quality demonstrated in the most part of evaluations of seeds

submitted to drying. These results are in agreement with the tendency observed in the others studies, which were verified a behavior of desiccation tolerance, by the maintenance of the quality demonstrated in the most part of evaluations of seeds submitted to drying (Vidigal et al., 2009).

Blackman et al. (1991) point out that these proteins are accumulated during the drying in the maturation phase and their stability, hydrophilicity and abundance in organisms tolerant to desiccation, and suggests a paper associated with tolerance to drying.

Conclusions

Habanero pepper seeds from fruits harvested and submitted to resting have high physiological quality and lower dormancy. Habanero pepper seeds, when harvested near to the maturity point and dried in controlled conditions (35ºC) in constant air flow, induces to the synthesis of heat resistant proteins.

Conflict of Interests

The authors have not declared any conflict of interests.

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