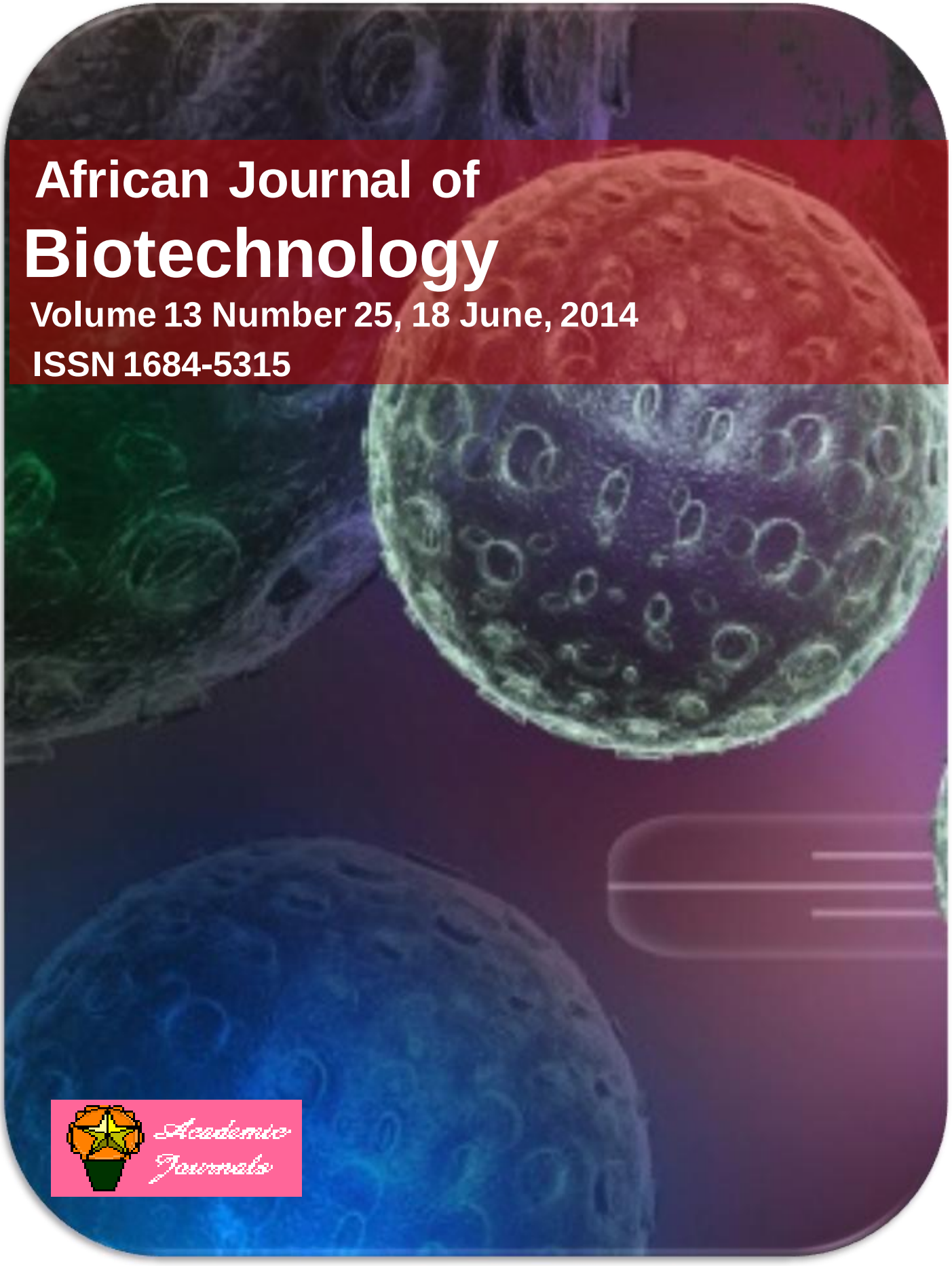

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Micrografting for fruit crop improvement

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Micrografting is an *in vitro* grafting technique which involves the placement of a meristem or shoot tip explant onto a decapitated rootstock that has been grown aseptically from seed or micropropagated cultures. Following early experiments of micrografting in ivy and chrysanthemum, the technique has been used in woody species, especially fruit trees. Major work was carried out in different *Citrus* species for the elimination of various viral diseases. *In vitro* micrografting has been used for improvement and multiplication of fruit trees as the technique has potential to combine the advantages of rapid *in vitro* multiplication with the increased productivity that results from grafting superior rootstock and scion combinations. Successful micrografting protocols have been developed for various fruit crops including almond, apple, cherry, chestnut, *Citrus*, grapes, mulberry, olive, peach, pear, pistacio, walnut, etc. Special techniques have been used for increasing the percentage of successful micrografts with the use of growth regulators, etiolation treatments, antioxidants, higher sucrose levels, silicon tubes, etc. The technique has great potential for improvement and large scale multiplication of fruit plants. It has been used on commercial scale for production of virus-free plants in fruit crops and viroid free plants in *Citrus.* Micrografting has also been used in prediction of incompatibility between the grafting partners, histological studies, disease indexing, production of disease-free plants particularly resistant to soil borne pathogens and multiplication of difficult to root plants.

Key words: Fruit crops, graft incompatibility, crop improvement, micrografting, propagation, shoot tip grafting.

INTRODUCTION

Micrografting is a relatively new technique for propagation of plants. According to Hartmann et al. (2002), micrografting is an *in vitro* grafting technique which involves the placement of a meristem or shoots tip explant onto a decapitated rootstock that has been grown aseptically from seed or micropropagated cultures.

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Abbreviations: PVP, Polyvinyl pyrrolidine; **DIECA,** sodium diethyl-dithiocarbamate; **NAA,** naphthalene acetic acid; **BAP,** benzylaminopurine; **MS,** Murashige and Skoog; **STG,** shoot tip grafting.

Following early experiments by Doorenbos in 1953 in ivy and later by Holmes (1956) on chrysanthemum, micro-grafting technique have been used in particular on woody species and especially on fruit trees, where work was carried out on different species of citrus with a view to eliminate viral diseases. The technique was modified and improved for increasing the graft success by Murashige et al. (1972) and Navarro et al. (1975). The technique has great potential for improvement and large scale multiplication of fruit plants. It has been used for the production of virus and viroid-free plants in fruit crops. Micrografting has also been used in prediction of incompatibility between the grafting partners, histological studies, virus indexing, production of disease-free plants particularly resistant to soil borne pathogens, safe germplasm exchange between countries and multi-plication of difficult to root plants. Reviews on micrografting have been published by Jonard et al. (1983), Jonard (1986), Roistacher et al. (1976), Parkinson et al. (1990) and Monteuuis (2012). The present review aims at examining the published literature related to micrografting to increase the application of this technique at commercial level for the improvement of fruit crops.

STAGES OF MICROGRAFTING

Micro-propagation protocol for scion as well as rootstock needs to be standardized separately before performing the micrografting operation under *in vitro* conditions. Thus, micrografting can be divided into three main stages:

Establishment and multiplication of scion

Shoot or meristem tips intended for grafting can be taken from actively growing shoots in greenhouse, chambers, field or *in vitro*. Generally, apical shoot tips or nodal cuttings are used as explants for the establishment of *in vitro* cultures. Following establishment, microshoots are transferred to shoot proliferation medium where shoot number increases by the development of new axillary shoots. Microshoots of desired thickness, age and length are used as scions for *in vitro* grafting operations.

Establishment and multiplication of rootstock

Rootstocks used for micrografting are *in vitro* or *in vivo* germinated seedlings and rooted or unrooted micropropagated shoots. When seedling rootstocks are used and all stages of grafting are conducted *in vitro*, seeds are surface sterilized and germinated aseptically in vessels containing nutrient salts. The seedlings may be supported on agar medium. Seedlings can also be on a porous substrate, such as sterile vermiculite, which allows the growth of a branched root system.

Preparation of rootstock and scion for micrografting

Micrografting is affected by cutting off the top of the seedling rootstocks usually just above the cotyledons or top of the micro-propagated shoot and placing small shoot apices of scion onto the exposed surface of decapitated rootstock in such a way that the cambium layer or vascular ring of the cut surfaces coincides with each other. This is called surface placement method. Wedge or cleft grafting is performed, incase thickness of rootstock and scion material is large enough to allow making of wedge on the scion material. Firm contact between rootstock and scion is extremely important at the graft junction for proper union of partners and callus formation (Canan et al., 2006). Several techniques have been developed for holding grafts together until fusion takes place such as translucent silicon tubing (Gebhardt and Goldbach, 1988), elastic strip (Jonard et al., 1983), filter paper bridge (Huang and Millikan, 1980), and glass tubing, nylon bands, aluminum foil tubes, dual layer apparatus of aluminum foil and absorbent paper (Obeidy and Smith, 1991). When grafts are successful, rootstock and scion grow together to produce a plant. It is usually necessary to examine freshly grafted seedlings on a regular basis and remove any adventitious shoot arising on or below the graft union.

GRAFTING SUCCESS DURING MICROGRAFTING

Grafting is a traditional method for production of composite plants but is season dependent. Failure of grafting means loss of one year for the production of grafted plants. This problem has been overcome through the use of micrografting which is done under controlled environmental conditions throughout the year and production can be planned according to market demand. Micrografting has particular utility in fruit tree production and protocol have been developed in many fruit crops including almond (Yıldırım et al., 2013; Isıkalan et al., 2011), apple (Huang and Millikan, 1980), apricot (Piagnani et al., 2006), avocado (Simon and Richard, 2005), cashew (Mneney and Mantell, 2001), cherry (Amiri, 2006, 2007), grapes (Tangolar et al., 2003; Aazami and Bagher, 2010), pear (Faggioli et al., 1997), pistachio (Abousalim and Mantell, 1992), walnut (Wang et al., 2010), etc. For commercialization of micrografting, protocol should be perfect to give higher percentage of successful micrografts. Good micrografting protocols have been developed for large scale production of micrografted plants in many fruit trees with high percentage of graft success (Table 1).

Table 1. Micrografting success in fruit crops.

S/N	Fruit crop	Scion cultivar	Rootstock	Successful micrografts (%)	Source
1	Pistachio	*Pistacia vera* cv. Siirt	Seedling raised rootstocks	79.25	Onay et al. (2004)
2	Pistachio	*Pistacia vera* cv. Mateur	Seedling raised rootstocks	94-100	Abousalim and Mantell (1992)
3	Mulberry	(*Morus alba*) cv. 707	Seedling raised rootstocks	>90	Fengtong et al. (1996)
4	Olive	*Olea europea* cv. Zard	*In vitro* raised seedlings of olive	45-83	Farah et al. (2011)
5	Grape	*Vitis venifera* cvs. Sahebi, Soltanin, Fakhri	41 B	50.1 -60.6	Aazami and Bagher (2010)
6	Grape	*Vitis venifera* cv. Early Cardinal	41 B, Salt Creek	71.4 - 80.0	Tangolar et al. (2003)
7	Apple	*Malus domestica* cv. Lal Ambri	M-9 rootstock	42.25	Khalid Mushtaq (2009)
8	Hazelnut	E-295-S (hazelnut)	G-029-N	72.00	Nas and Read (2003)
9	Chestnut	907 (chestnut)	711	80.00	Nas and Read (2003)
10	Walnut	*Juglans regia* cvs. Jinlong No 1, Xiangling	Seedling raised rootstock	56.70 - 73.30	Wang et al. (2010)
11	Almond	*Amygdalus Communis* cvs. Ferragnes, Ferraduel	*In vitro* germinated wild almond seedlings	90-100	Yildirim et al. (2010)
12	Almond	*Amygdalus Communis* cv. Nonpareil	*In vitro* germinated wild almond seedlings	90.00	Isıkalan et al. (2011)
13	Almond	*Prunus dulcis* cvs. Texas, Ferrastar, Nonpareil	*In vitro* germinated almond seedlings	83.33 - 100	Yıldırım et al. (2013)
14	Citrus	I: Kinnow mandarin II Succari sweet orange	*In vitro* germinated seedlings of rough lemon	I 36 II 33.3	Naz et al. (2007)
15	Pear	Pyrus communis cv. Le-Cont	*In vitro* shoots of *Pyrus betulaefolia*	83.00	Hassanen (2013)

IMPROVEMENT IN MICROGRAFTING TECHNIQUE

Micrografting procedures are difficult and generally results in a low rate of successful grafts, which makes it an expensive and time-consuming production technique. It is due to the fact that more technical expertise is required in preparing successful grafts on small-scale material and handling difficulties associated with preserving the delicate graft unions. In many experiments, failure rate for micrografts was higher than desired. *In vitro* grafts of fruit plants often fail due to incompatibility reaction, poor contact between stock and scion and phenolic browning of cut surfaces (Ramanayake and Kovoor, 1999). In order to alleviate some of these limitations, different techniques have been developed to make micrografting a successful and superior technology for the benefit of technicians, researchers, nursery operators and commercial tissue culture laboratories.

Browning and tissue blackening

Exudation of phenolic compounds from the cut surfaces and their oxidation by polyphenoloxidase and peroxidase enzymes cause discolouration of the tissues which results in poor micrografting (Martinez et al., 1979). Browning of the cut surfaces inhibits the growth and development of new cells and results in poor graft union. To block the oxidation phenomena and prevent tissue browning, various substances have been used which include thiourea, cysteine, chlorhydrate (Jonard, 1986), citric and ascorbic acid (He et al., 2005), Phytagel (Zhang and Luo, 2006), PVP (Rather et al., 2011), DIECA (Martinez et al., 1979). Tissue blackening, which commonly results in the death of very small scions, has been reduced by soaking explants in an anti-oxidant solution, and/or placing a drop of solution onto the severed rootstock immediately before inserting the scion (Jonard et al., 1990; Ramanayake and Kovoor, 1999).

Sucrose concentration of the medium

Sucrose concentration of nutrient medium had a significant effect on the percentage of successful grafts. Navarro et al. (1975) reported that sucrose concentration of medium of grafted plants played a significant role and that the highest rate of successful grafts in citrus species was obtained with 7.5% sucrose. Generally *in vitro*

growth and development increases with increased sugar concentration (Pierik, 1987). Naz et al. (2007) used 14 days old seedlings of rough lemon (*Citrus jambheri* Lash) grown under *in vitro* etiolated conditions as rootstock and microshoots of Kinnow mandarin/Succari sweet orange as scion. Micrograft success improved with increase in sugar levels in both cultivars from 20-22% with 3% sucrose to 36-38% with 7% sucrose. Hamaraie et al. (2003) also reported improvement in the micrograft success from 30 to 60% and scion growth from 8.7 to 13.8 mm with the increase in sucrose concentration from 2.5 to 7.5%, respectively during his studies with micrografting of grapefruit (*Citrus paradisi*) on sour orange seedlings germinated *in vitro*.

Light/dark incubation treatments

Significant variations have been reported in the percentage of successful grafts according to exposure of seedlings to light. Hamaraie et al. (2003) reported higher frequency of successful grafts (50%) in grapefruit (*Citrus paradisi*) cv. “Miami, when rootstock seedlings (sour orange) were obtained from seeds germinated under continuous darkness for two weeks as compared to only 5% successful grafts with seedlings which developed under light. Navarro et al. (1975) reported a very low frequency of successful grafts using Troyer citrange seedlings grown under continuous light as compared to seedlings grown in continuous darkness. Ewa and Monika (2006) found high percentage of successful micrografts in cherry under dark conditions.

Use of growth regulators

Usually growth regulators are not used in traditional grafting for increasing the graft success. However, under *in vitro* conditions, growth regulators particularly cytokinins and auxins have been found effective for improving the graft success rate. These growth regulators increase the rate of cell division and improve callus formation, which in turn help in increasing the percentage of successful graft unions. At the time of performing micrografting operation, prepared micro-scion is given a quick dip (5-10 s) in sterilized growth regulator solution of desired concentration and then inserted into or placed on the rootstock. Wang et al. (2010) found NAA effective in improving the micrograft success in walnut. Rafail and Mosleh (2010) reported increase in micrograft success from 30 to 90% in pear (cv. Aly-sur on Calleryana pear) and 40 to 90% in apple (cv. Anna on MM106) with increasing BAP concentration from 0-2.0 mg/L. Triatrniningsih et al. (1989) obtained a 24% increase in the frequency of successful grafts over untreated controls in *Citrus* by the use of BAP at 0.5 mg/L.

Nature of the supporting medium

Agar solidified medium and liquid medium have been used for the growth of micrograted plants. Rafail and Mosleh (2010) observed that number of successful micrografts increased from 10% in agar-solidified medium to 60% in apple and 70% in pear with use of liquid medium. There is usually more up take of nutrients and growth regulators by the microshoots in liquid media, which makes it more effective than solidified medium for micrografting success. MS liquid medium with vermiculite was found best for further development of the micrografts, because liquid medium alone or with agar forms asphyxic conditions, which prevents formation of lateral roots (Mosella-Chancel et al., 1979).

Preventing desiccation of the graft

Desiccation of graft or surfaces of the grafting partners is one of the major causes of graft union failure (Pliego and Murashige, 1987; George et al., 2008). To prevent this phenomenon, Pliego and Murashige (1987) applied a layer of moist nutrient agar gel to connect the grafting partners and obtained better graft success. Different chemicals have been tried to prevent graft desiccation so as to enhance the graft union. Rafail and Mosleh (2010) used an agar drop from the solidified culture medium and placed it on the cut area of the rootstock. Micrografts in which an agar drop was added to their grafted area were highly successful (70% in apple and 60% in pear) as compared to those without an agar drop (10%). Adding an agar drop usually prevents scion drying and makes the transport of different materials possible and holds the graft units together until the fusion takes place. Addition of agar drop supplemented with minerals and/or phytohormones further improved graft success. Amiri (2007) obtained 65% successful grafts in cherry using homoplastic grafting method (adding two drops of agar solution around the fitting site of micrograft) as compared to 41% through heteroplastic method (without application of agar drops).

Pretreatment of shoot apex

A technique which pretreats the apex allowing the selection of the viable apex and helping their development greatly improves the micrografting success. This is particularly effective when very small sized shoot apices are used. Excised apex is placed into a hemolyse tube on filter paper moistened by mineral solution of Murashige and Skoog (1962), supplemented with auxins and cytokinins. This treatment modified the physiological state of the excised apex and led to rapid development of leafy shoots even from smallest apices of 0.1 – 0.2 mm, the direct grafting of which is generally difficult and

ineffective (Jonard et al., 1983). Following proper development, the apex is micrografted on the rootstock. Mosella-Chancel et al. (1979) reported 64% successful micrografts in peach when pretreated with zeatin (0.1 mg/L) for 48 h as compared to 21.7% without any pretreatment.

Suitability of rootstock

Micrograft success varies with the rootstocks because of the compatibility reactions between the grafting partners**.** Evaluating the rootstocks for higher graft success with a particular scion will definitely help in commercializing micrografting technique for mass multiplication of fruit crops. Tangolar et al. (2003) studied micrografting success in two cultivars of grape (Early Cardinal and Yalova incisi) when grafted on four different rootstocks (Dogridge, Salt Creek, 1613 C and 41 B) and reported different rates of graft success which varied from 26.1% (Yalova incise on 1613 C)) to 80% (Early Cardinal on 41 B).

APPLICATIONS OF MICROGRAFTING

Micrografting has been used for the improvement and multiplication of various fruit crops and several papers have been published (Jonard et al., 1983; Jonard, 1986; Bhat et al., 2010). Some of the main applications of micrografting in fruit crops are discussed below:

Virus and viroid elimination

The production of high-quality plants which can be certified genetically and virus-free is considered problematic and very challenging. An innovative technique of micrografting was developed by Murashige et al. (1972) for production of uniform virus-free plants on commercial scale in a controlled environment. They grafted small apical shoot of citrus to the top of a decapitated seedling grown *in vitro*. A few of these grafted plants, when indexed, were found freed of exocortis and stubborn pathogens. Navarro et al. (1975) improved the technique by testing various media formulations, different ways of placing the scion tip on the decapitated epicotyl, different rootstocks, light intensities, different sources and sizes of the scion shoot-tip and reported maximum survival of micrografted plants when transplanted from the test tube to soil. Roistacher et al. (1976) used the shoot tip grafting (STG) technique for production of virus-free planting material in various selections of citrus including sweet orange, mandarin, grapefruit, lemon, lime, citron and tangor. Each source plant was infected with 1 or 2 viruses including virus of tristeza (TV), seedling yellows-tristeza (SYTV), psorosis-A (PSV-A), concave gum (CGV), yellow vein (WV), infectious variegation (IVV), cachexia, and tatterleaf (TLV), citrus exocortis viroid (CEV) and Spiro*plasma* citri (stubborn). Virus indexing of micrografted plants was carried out over a 2-year period using different indicator plants for different viruses. Out of 33 different cultivars of citrus, they were able to develop virus-free mother block of 31 cultivars through shoot tip grafting.

Since then, micrografting technique has been widely used for elimination of viruses, phytoplasma, systemic pathogens in fruit crops and a large number of fruit plants have been made virus-free (Jonard et al., 1983; Burger, 1985; Navarro et al., 1976, 1980, 1982; Navarro and Juarez, 1977; Deogratias et al., 1986; Navarro, 1988; Jarausch et al., 2000; Zilka et al., 2002). *In vitro* grafting was used in Spain to produce virus-free plants of citrus and is considered a major factor in improving the Spanish citrus industry (Navarro et al., 1975). The technique has been used since 1998 for elimination of *Citrus psorosis virus* (CPsV), Citrus cachexia viroid (CCaVd), *Citrus exocortis viroid* (CEVd) and other related viroids in the local Arakapas mandarin of Cyprus (Kapari-Isaia et al., 2002). Hartl-Musinov et al. (2006) succeeded in elimination of *Citrus tristeza* virus (CTV) in Satsuma mandarin in Croatia. Abbas et al. (2008) succeeded in producing 91-95% *C. tristeza virus* (CTV) free plants of Kinnow mandarin (*Citrus reticulata)* and sweet orange (*Citrus sinensis*) cultivars through this technique.

Micrografting exploits two concepts- meristems are relatively virus-free and meristems from mature plants retain the mature phase. Meristematic tissues in the shoot tips and axillary buds normally remain virus-free because the growth of the meristem is quicker than the systemic spread of the virus within the plant. Using micro shoot tips (less than 0.5 mm) as scions, STG produces plants that are virus-free and reproductively mature. Production of virus-free plants from nucellar seedlings or by thermotherapy has certain limitations. Although nucellar seedlings of citrus are both clonal and virus-free, the seedlings are juvenile and take many years to flower. In the case of thermotherapy, many viruses and viroids, such as exocortis viroid and stubborn virus, are difficult to clean up with this process (Roistacher, 2004). Thermotherapy has failed to eliminate citrus exocortis viroid, yellow vein virus (YVV), cachexia virus and Dweet mottle virus (Calavan et al., 1972; Roistacher and Calavan, 1972). These problems have been overcome through STG technique. High temperatures inactivate many viruses, thus *in vitro* propagation can be used in combination with heat treatment to produce virus-free material. A massive project was launched in Morocco and Israel to develop virus-free plants of commercial almond cultivars through *in vitro* micrografting in combination with thermotherapy during 1997-2001. The project resulted in successful sanitation of almonds and permitted recovery of virus-free plants from various varieties infected with PNRSV (Prunus necrotic ringspot virus), PDV (prune

dwarf virus, CLSV (chlorotic leafspot virus). Thermotherapy treatment at 30- 35°C for 14 days was applied to *in vitro* shoot cultures prior to excising shoot tips for performing shoot tip grafting. Size of shoot apex had a paramount influence on elimination of virus from the plants. Singh et al. (2008) reported low recovery of ICRSV-free plants (20%) from an infected plant of kinnow mandarin with shoot tip size of 0.3 mm through STG which increased to 100% with shoot tip size of 0.2 mm. Manganaris et al. (2003) developed an efficient micrografting protocol for production of nectarine plants free from PPV and PNRSV. Conejero et al. (2013) sucessfully used micrografting in stone fruits for elimination of not only graft-transmissible viruses but also viroids, like PLMVd affecting *Prunus* species worldwide. Once bud is obtained, micrografted plants were placed in a cold chamber at 4°C and then forced for 15 days at 35°C. This resulted in the elimination of not only viroids but also viruses in higher percentage than the previous protocols. In short, micrografting is the only technique to purify the horticultural crops from viral diseases without the spray of harmful pesticides.

Production of plants resistant to pests and diseases

Micrografting can be used as a means of elimination of pathogens in fruit crops. It has been successfully used in a wide range of horticultural plants as an effective method for the acquisition of plants resistant to soil borne pathogens. Grape phylloxera (*Daktulosphaira vitifoliae*) is considered as the most destructive insect pest of cultivated grapes worldwide, which feeds on the sap of grape roots, causing damage and often death of vines (Makee et al., 2004). An efficient and robust micrografting system was developed for production of phylloxera resistant plants in grapes by Kim et al. (2005) using pest resistant cultivars as rootstocks (Millardet et de Grasset 101-14, Couderc 3309, Rupestris du Lot and Kober 5 BB) and commercial favorable table grapes as scions (Kyoho, Campbell Early, Tamnara and Schuyler).

Assessment of graft incompatibility

The inability of two different plants when grafted together to produce a successful union and also to develop satisfactorily into one composited plant is termed as graft incompatibility. Graft incompatibility in fruit trees has been classified by Mosse (1962) into translocated incompatibility and localized incompatibility. Translocated incompatibility is often associated with the movement of some labile factors between the grafting partners and is not overcome by the insertion of a mutually compatible inter-stock. An example of this category is the combination of 'Nonpareli' almond on 'Mariana 2624' plum. Localized incompatibility depends upon actual contact between stock and scion. Separation of the components by insertion of a mutually compatible inter-stock overcomes the incompatibility. Bartlett pear grafted directly on quince rootstock shows this type of incompatibility. Another example is grafting of certain apricot cultivars with peach which is associated with a clear break of the trunk at the point of graft following strong winds even after several years of normal growth (Jonard et al., 1990).

Prediction of incompatible graft combinations is a very important area of study for preventing economic loss due to graft incompatibility. Signs of graft incompatibility are often detected after several years in the field but can be identified early using micrografting and *in vitro* callus fusion technique (Jonard et al., 1990; Errea et al., 2001). Micrografting has been used for assessment of graft compatibility/incompatibility between the grafting partners (Burger, 1985; Navarro, 1988). The technique facilitates early diagnosis of grafting incompatibilities and may provide a model for in-depth analysis of the incompatibility phenomenon (Chimot-Schall et al., 1986; Jonard, 1986; Jonard et al., 1990; Hossein et al., 2008; Errea et al., 1994; Espen et al., 2005). It has been used for studying histological, histochemical and physiological aspects of graft incompatibility between scions and rootstocks (Richardson et al., 1996; Ermel, 1999). Histological examination of the graft union revealed callus formation, cytodifferentiation and xylogenesis leading to the formation of vascular connections in successful micrografts (Gebhardt and Goldbach, 1988). Anatomical studies of incompatible grafts demonstrated a poor vascular connection, vascular discontinuity and phloem degeneration at the union area, which might be detected as early as few weeks after a graft establishment (Darikova et al., 2011).

In the case of incompatible associations, Martinez et al. (1979, 1981) used *in vitro* micrografting to analyse localized incompatibilities of apricot/myrobalan and translocated incompatibilities of peach/apricot or peach/myrobalan. In the case of localized incompatibility, the percentage of success was very good during first three weeks but from the 14th day, signs of incompatibility appeared around the graft. After 60 days, all the grafts perish leaving no visible plants. Translocated incompatibility also called delayed incompatibility resulted in the development of whole plants *in vitro*, but the early symptoms of incompatibility still appeared on the young plants in pots 2 months after grafting (Martinez et al., 1981). During this experiment, 80% homografts of peach/peach and apricot/apricot provided viable plants. However, the percentage of surviving plants after 60 days was very low under incompatible combination of *Prunus persica*/ *Prunus armeniaca* (6.0%) and *Prunus persica* / *Prunus cerasifera* (1.25%). Though *in vitro* grafting techniques did not give viable plants but gave a prediction of incompatibility. Signs of this type of incompatibility often develop 5-10 years later after branch

grafts are made in the orchard (Rodgers and Beakbane, 1957).

Micrografting was used to study the compatible and incompatible combinations of grape varieties, using survival rate as an index. The higher survival rate of grafting (>85%) was achieved in compatible combinations of RizamatV/Baixiangjiao and Canepubu/Muscat Hamburg. Under incompatible combinations of Canepubu/Baixiangjiao and Carignane/Baixiangjiao, the survival rates were only 3.33 and 13.33%. Both translocated and localized incompatibilities exist in the Canepubu/Baixiangjiao, while Carignane/Baixiangjiao had only translocated incompatibility. At the late stage of grafting union, necrotic layer (isolation layer) of compatible combinations became thinner and finally disappeared, conducting tissue of rootstock-scion connected and the graft plants survived. To incompatible combinations, the necrotic layer always existed or disappeared partly, and the grafting failed, vascular disconnection contributes to the failure of grafting (JiLing, 2001).

Improvement of plant regeneration

Micrografting provides an alternative production technique for mass multiplication of plants which are difficult to root (Preece et al., 1989) or propagation of difficult-to-root novel plants created in tissue cultures (Barros et al., 2005). This is done by micrografting micro shoots of difficult to-root plants/cultivars on seedling rootstocks grown *in vitro*. Micrografting has been used to rejuvenate cashew cultivars which were found difficult to root (Thimmappaiah et al., 2002). The technique has been successfully used to multiply difficult to root plants including walnut (Pei et al., 1998; Wang et al., 2010), pistachio (Onay et al., 2007; Abousalim and Mantell, 1992) cashew (Ramanayake and Kovoor, 1999) and almond (Martinez-gomez and Gradziel, 2001; Ghorbel et al., 1998; Channuntapipat et al., 2003).

Mass multiplication

Micrografting is a technique that potentially can combine the advantages of rapid *in vitro* multiplication with the increased productivity that results from grafting superior rootstock and scion combinations (Gebhardt and Goldbach, 1988). Mass production of superior plants through micrografting can be achieved throughout the year under controlled conditions in the tissue culture laboratories, by grafting elite scions onto desirable rootstocks. Generally, micro-propagation of woody trees is difficult due to low regeneration capacity, especially mature plant tissues. A major limitation is root regeneration rather than shoot multiplication (Hartmann et al., 2002). *In vitro* micro-grafting is often used where rooting capacity of micro-cuttings is poor. It has been used in the propagation of novel plants created in tissue cultures through transgenic or of novel plants created in tissue cultures that are difficult-to-root (Barros et al., 2005). Genetically transformed shoots of Avocado from somatic embryos were rescued by micrografting them onto the *in vitro* germinated rootstock seedlings with 70% success (Simon and Richard, 2003).

Indexing viral diseases

Grafting is used to determine the presence of latent (unseen) viral diseases in plants. A plant (the indicator plant) that is known to be susceptible to the disease of interest may be grafted onto the suspect plant. If the plant is in question is infected, typical symptoms induced by the specific virus are expressed on the indicators after the virus has been moved into the indicator plants. This type of testing is regularly carried out on plants imported to the country including grapes and roses. This test does not require the formation of a permanent, compatible graft union. Tanne et al. (1993) used micrografting system which increased the speed of viral detection. They reported the detection of corky-bark virus 8–12 weeks after grafting. Pathirana and McKenzie (2005) reported that micrografting of leaf roll infected scion material on to virus-free indicator rootstock (*Cabernet sauvignon*) resulted in the development of symptoms within 2-3 week. Valat et al. (2003) demonstrated that grapevine fan leaf virus is transmitted from infected rootstock to the uninfected indicator 41B variety used as scion within 45 days. Kapari-Isaia et al. (2002) used Madam Vinous or pineapple, sweet orange as indicator plants for indexing of CPsV in Local Arakapas Mandarin in Cyprus. This type of microindexing can be used for post-entry quarantine of imported materials (Sivapalan et al., 2001; MAF, 2004).

Safe germplasm exchange

Small micrografted trees are a convenient way to exchange germplasm between countries (Navarro et al., 1975). The exchange of fruit tree propagation material between countries is a major cause of spread of new pests and pathogens, particularly graft-transmissible viruses and viroids. The expansion of Prunus breeding worldwide, mainly in peach, has resulted in more than 20 new breeding programs producing hundreds of new varieties yearly. The associated exchange of plant material has increased notably the risk of introduction of new pathogens and pests (Llacer, 2009; Llacer et al., 2009). Imports of fruit budwood lacking effective phytosanitary control measures present the highest risks. More than 100 virus or virus-like diseases have been reported to affect Prunus species worldwide. For approxi-

mately half of these diseases, nothing is known about the causal agent except that it is graft-transmissible (Cambra et al., 2008). Moreover, traditional quarantine procedures are often ineffective, prompting the search for alternative procedures including those based on tissue culture techniques. An improved STG procedure based on the protocol described by Navarro et al. (1982) which is effective for virus and viroids elimination is a prerequisite for safe peach and Japanese plum budwood exchange (Conejero et al., 2013). It is a minimum risk method for importing plant material through quarantine.

CONCLUSION

Micrografting has great potential for improvement of fruit plants and has been used for the production of virus and viroid-free plants in horticultural crops without the application of harmful pesticides. Besides, it has also been used in prediction of incompatibility between the grafting partners, histological studies, virus indexing, production of disease-free plants particularly resistant to soil borne pathogens, safe germplasm exchange between countries and multiplication of difficult to root plants. It is a safe *in vitro* technique, which can be utilized for commercial production of virus-free grafted plants with desired cultivars and suitable rootstock throughout the year under controlled conditions.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Identification and cloning of two insecticidal protein genes from *Bacillus thuringiensis* strain S185

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Bacillus thuringiensis **(Bt) is the most widely applied type of microbial pesticide due to its high specificity and environmental safety. The activity of Bt is largely attributed to the insecticidal crystal protein encoded by the *cry* genes. Different insecticidal crystal proteins of Bt have different bioactivity against distinct agricultural insect pests, and combination of these proteins not only increases insecticidal activity, but also overcomes and delays development of resistance. A Bt strain, S185, was isolated from a soil sample collected in Songfeng Shan district, Heilongjiang Province, China. Bt S185 has highly specific insecticidal activity against Coleoptera, and was determined to contain *cry8*-type genes by peptide mess fingerprint (PMF) analysis. Application of polymerase chain reaction-restricted fragment length polymorphisms (PCR-RFLP) analysis further determined the genotype due to the high homology of *cry8Ea1* and *cry8Fa1* genes. Through the full-length primers design, two insecticidal crystal protein genes *cry8Ca* and *cry8Ea* were obtained. Using prokaryotic cloning vectors, the recombinant plasmids pEB-cry8Ca and pEB-cry8Ea were transferred into expression host strain *Escherichia coli* Rosetta, thus the two genes were successfully expressed in heterologous bacteria.**

Key words: *Bacillus thuringiensis*, peptide mess fingerprint, identification, clone, insecticidal crystal protein.

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions named cry or cyt during sporulation (Höfte and Whiteley, 1989). These inclusions consist of proteins with highly specific insecticidal activity (Aronson et al., 1986; Whiteley and Schnepf, 1986). The insecticidal spectrum varies within the 82 different serotypes reported (Lecadet et al., 1999), and affects insects primarily from the orders Lepidoptera, Diptera and Coleoptera (Rasko et al., 2005). There are also reports of Bt active against mosquitoes (Orduz et al., 1995).

Up to August 2013, 712 cry and 38 cyt proteins have been identified, of which 49 are cry8-type proteins, which consist of 1160 to 1210 amino acids and have molecular weights of 128 to 137 kDa (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/;

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Shu et al., 2007; Yu et al., 2006). The cry8-type protein has specific insecticidal activity against a variety of Coleopteran pests such as Scarabaeidae, Curculionidae and Chrysomelidae. Many kinds of Scarabaeidae are major pests of crops and trees, due to the difficulty of prevention and control of soil-dwelling pests. Their larvae (white grub) are one of the largest underground pest groups and cause great damage to many plant species. In China, every year about 16.47 million hectares is affected by the larvae, in a serious year reaching 52.72 million hectares, with yield losses of about 20 to 40%, 933 thousand hm^2 peanuts were only planted in 2002, and there were serious occurrence area of more than 40 hm^2; there was, economic losses of 600 million yuan (Wei et al., 1985; Wang et al., 2012). Therefore, it is of great importance to isolate and clone high-activity cry8-type genes for constructing engineered bacteria and genetically modified plants for prevention and control of Coleopteran pests.

In this context, it is essential to clone and express *cry8* genes from new Bt isolates. The present study describes the identification, cloning and expression of *cry8Ca* and *cry8Ea* from a new indigenous isolate of Bt S185 strain, which has high-virulence against *Holotrichia parallela*.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bt

The *Bt* strain S185 used in this study was isolated from soil obtained from Songfeng Shan district, Heilongjiang Province, China. The 1 g soil sample was suspended in 15 mL sterilized distilled water and shaken. After heat treatment at 80°C for 20 min, the appropriately diluted upper-layer of the suspension was incubated on ½ Luria Broth (LB) medium (0.5% tryptone, 0.25% yeast extract and 0.5% NaCl; pH 7.0) at 30°C for three days. Colonies were examined microscopically for the presence of parasporal crystals (Hastowo et al., 1992). *Bt* strains were incubated in LB medium for three days with shaking at 30°C at 250 rpm (Maniatis et al., 1982).

Escherichia coli

The Institute of Plant Protection, Chinese Academy of Agricultural Sciences supplied *E. coli* JM109 as the cloning host and *E. coli* Rosetta as the expression host. The strains were grown in LB medium at 37°C for 12-16 h.

Plasmids

pMD19-T (TaKaRa Biotechnology Company) was used as cloning vector. pEB vector, an improvement of pETblue-2 vector was used as the expression vector modified by CL Shu in 2009 (Changlong et al., 2009).

Microscopy

Optical microscopy observation

The tested strains were dyed with carbol fuchsin after cultivation on solid ½ LB medium at 30°C for 2 dayd, and then examined under an oil immersion objective (Gundersen et al., 1988).

Electron microscopy observation

Spores and crystals of *Bt* S185 were collected by centrifugation at 12 000 rpm for 10 min. The pellet was washed three times with distilled water, and suspended in phosphate buffer containing 3% glutaraldehyde, then dehydrated in dilute ethanol-propylene oxide series and embedded in an Epon resin mixture (Sangon Ltd. China). The sample was photographed by a New Bio-TEM electron microscope (Hitachi Ltd. Japan) operating at an accelerating voltage of 80 kV, after undergoing ultrathin sectioning by a Reichert ultramicrotome (Leika Aktiengesellschaft. Wien Austria) and staining with uranyl acetate and lead citrate.

Plasmid DNA extraction

Bt strain plasmid DNA extraction was as described by Song et al. (2003). The *E. coli* strain plasmid was prepared and further purified by a plasmid kit (Axygen Products), used according to the manufacturer's instructions. Plasmid profiles were determined for each strain by electrophoresis through 0.7% agarose gel.

Protein analysis

Bt S185 was grown with shaking at 180 rpm at 30°C in ½ LB medium until sporulation was complete, as determined by microscopy. The spores and crystals were harvested by isoelectric point deposition (Guo et al., 2005).

Recombinant *E. coli* cells were grown overnight at 37°C in LB containing ampicillin (amp). Bacterial cells were added to 200 ml LB/amp (200 µg/ml) medium and shaken at 180 rpm at 25°C to an OD600 of 0.6 to 0.7 (Srimonta et al., 2012). Expression was induced by adding 0.1 mM isopropyl-β-d-thiogalactoside (IPTG) at low temperature (20°C) for 6 h. The cells were gathered by centrifugation at 8 000 rpm for 10 min at 4°C. For experiments, the cells were resuspended in TE buffer (20 mM Tris, 1 mM EDTA, pH 8.0) and lysed using ultrasonic waves, repeated the 3 / 3 s intervals until the clock reached 10 min. The supernatant was collected and the precipitate was suspended in TE buffer; both stored at −20°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8% gel as described by Laemmli (1970). The molecular weights were estimated by comparison with the protein ladder (GenStar Biosolutions Co. Ltd).

Protein samples used for PMF were derived from SDS-PAGE. The enzymatic digestion was performed as described by Fernandez et al. (1998) and Gharahdaghi et al. (1999). Mass spectrometric analysis was done by the Beijing Genomics institution Co. Ltd.

Cloning and expressing of insecticidal crystal protein gene.

Total DNA of S185 was isolated as described by Iizuka et al. (1981). Primers were generated based on the published sequence of *cry8Ca* and *cry8Ea* genes. The primer sequences used in this study were as follows (SangonCo.Ltd):
cry8C5:5'ATGAGTCCAAATAATCAAAATG3';
cry8C3:5'TTACTCTTCTTCTAACACGAGTTC3';
cry8E5:5'ATGAGECCAAATAATCAAAATG3';
cry8E3:5'TTACTCTACGTCAACAATCAATTC3'.

PCR was carried out for 30 cycles under the following conditions: 94°C for 5 min, 94°C for 30 s, 52°C for 60 s, 72°C for 2 min and 72°C for 10 min. Restriction digestion and ligation were carried out as per the manufacturer's instructions (TaKaRa Biotechnology

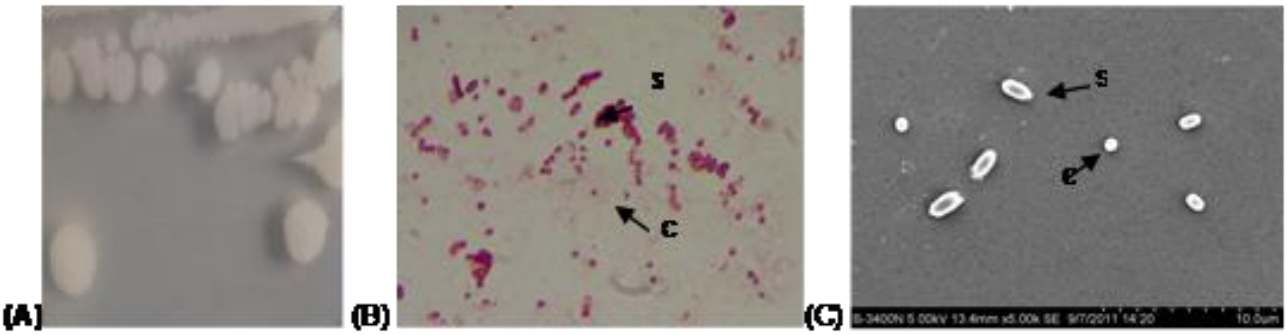

Figure 1. (A)The colony form of S185, Optical (B) and scanning electron (C) microscope image of *Bt* S185 strain. S, Spores; C, crystals.

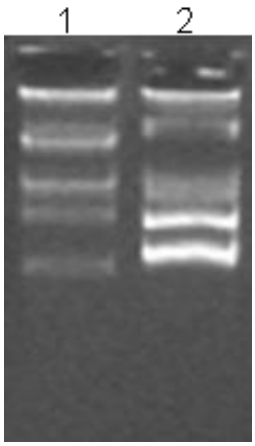

Figure 2. Plasmid DNA pattern of S185 and *Bt* subsp. *kurstaki* HD-73. Lane 1, *Bt* S185; lane 2, HD-73. The DNA fragments were electrophoresed on 0.7% agarose gel.

Company). Preparation of *E. coli* competent cells was as per the standard procedure (Sambrook et al., 1989). Vectors were inserted with approximately 3.7-kb target fragments, and then transformed into the *E. coli* host performed as described previously by Lenin et al. (2001). Positive recombinant-clones were selected by blue-white selection.

Insect toxicity assay

The toxicity of purified *Bt* S185 crystal was tested on larvae of *H. parallela, H. oblita* and *Anomala corpulenta* Motschulsky. Bioassays were performed in 24-well culture plates with 2-cm^2 wells. Purified crystal protein of S185 was suspended in distilled water to a final concentration of 10 g/L. Serial dilutions of crystals were tested by bioassay against *H. parallela* larvae. Bioassays were repeated three times. Larval mortality was recorded after 48 h, and concentrations causing 50% mortality were determined by Probit analysis. Lethal concentrations were expressed per cm^2 of surface.

GenBank accession number

The nucleotide sequence data published in this paper, *cry8Ca* and *cry8Ea*, were assigned GenBank accession numbers ADB54826 and JQ837282, respectively.

RESULTS

Optical and electron micrograph observation of *Bt* S185

After incubation for 48 h in ½ LB medium, *Bt* S185 formed a single milky colony with neat edges. The colony was of a thin circular or nearly circular disk somewhat thicker in the center (Figure 1A). The vegetative masses of the colony were long rod-shapes under the oil immersion objective. However, the spores were stick-like and the crystals were spherical (Figure 1B and C).

Plasmid and protein profile analysis of *Bt* S185

The plasmid profile of *Bt* S185 showed five bands, which was found to be clearly different from *Bt* subsp. *kurstaki* HD73 (Figure 2), especially in regard to the small plasmid bands. Protein profile analysis of *Bt* S185 showed a 130 kDa protein band (Figure 3).

Insect toxicity assays and the physiological and biochemical characteristics of S185

The corrected mortality rate of *Bt* S185 for larvae of *H. parallela*, *H. oblita* and *A. corpulenta* Motschulsky were all >50%. Analysis of the data showed that the highest mortality rate of different concentrations of S185 protein against larva of *H. parallela* was 85.1%. Purified S185 protein against *H. parallela* larvae had LC_{50} of 0.335 µg/ml, 95% limiteds (0.141-0.518 µg/ml).

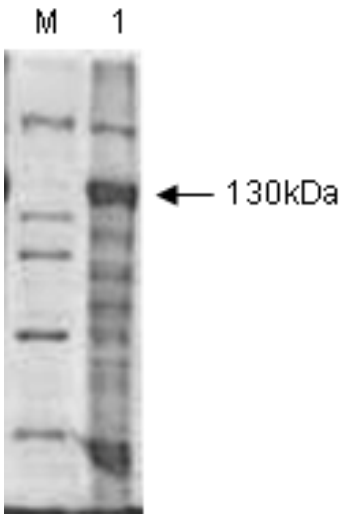

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of parasporal inclusion proteins from S185. Lane 1, S185 parasporal inclusion protein M: molecular weight marker. SDS was performed on an 8% gel. Spore crystal mixture obtained from the 20 ml culture was suspended in 200 ml of sterile distilled water. 5 µl of each sample was analyzed.

Peptide mass fingerprint and PCR-RFLP analysis of *cry*-type genes from *Bt* S185

With the homology comparison of the peptide fragment in the NCBI database, PMF of *Bt* S185 crystal protein preliminarily showed that the crystal protein produced by S185 strain contained *cry8Ca*-type and *cry8Ea*-type or *cry8Fa*-type genes (Figure 4). Using PCR-RFLP of *cry8*-type genes from S185, the primers for full-length genes were based on the encoding region of the known *cry8Ca* and *cry8Ea* genes in GenBank. With the strategy described in the Materials and Methods, a 3.7-kb fragment of *cry8Ca* and the same amount of *cry8Ea* were isolated from *Bt* S185. As *cry8Ea* has very high homology to *cry8Fa* and a close score was obtained with the PMF, PCR-RFLP was used to make a distinction. Analysis by DNAMAN showed that the full-length *cry8Ea* had two *Eco*RI site, 1148 and 2056 respectively. The completely digested *cry8Ea* fragments were 893, 908 and 1288 bp. However, the full-length *cry8Fa* had two *Eco*RI sites on 1572 and 2235, thus the completely digested fragments would be 663, 893 and 1572 bp. Therefore, enzyme action by *Eco*RI will distinguish *cry8Ea* from *cry8Fa*. There were obvious bands at 900 and 1200 bp on the restriction enzyme map instead of 663 bp (Figure 5), thus *cry8Fa* was not contained in the Bt S185 strain.

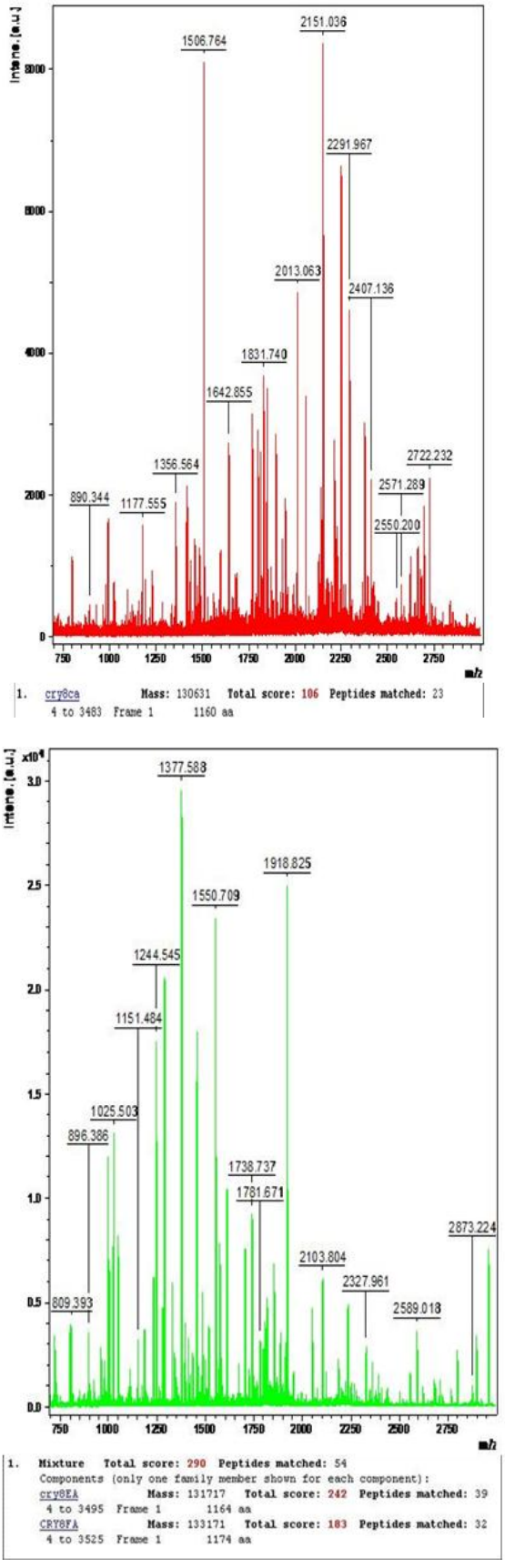

Figure 4. PMF analysis of S185 crystal protein.

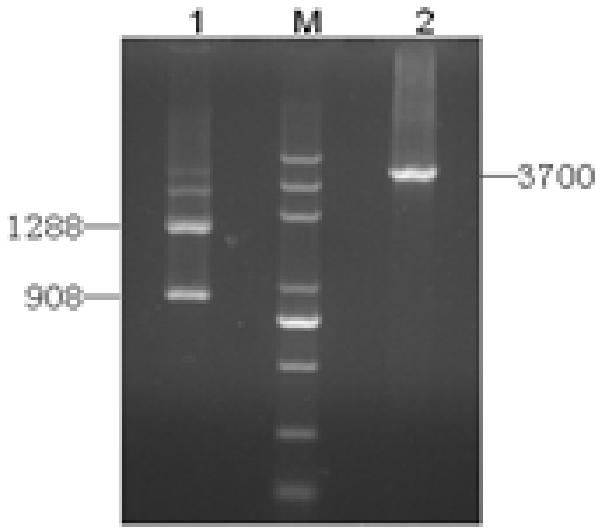

Figure 5. *B. thuringiensis* strain S185 after polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. S185 PCR products and PCR-RFLP patterns (using cry8Ea-specific primers) Lane 1, PCR-RFLP patterns of *cry8Ea*, digested with endonucleases *Eco*RI; lane 2, PCR products of *cry8Ea*.

Cloning and expression of the crystal protein genes

The recovered PCR product was separately selected and cloned into pEB vector as stated; termed the recombinant plasmids pEB-8Ca and pEB-8Ea. By sequencing the recombinant plasmids pEB-8Ca and pEB-8Ea, the overall length sequences *cry8Ca* and *cry8Ea* were obtained. The predicted amino acid sequence of the cloned gene was analyzed to identify any similarities to other known protein sequences of cry and cyt toxins on NCBI. The cry8Ca protein contained 1160 amino acids and had a molecular weight of 130.25 kDa. The cry8Ca protein was acidic, its isoelectric point was 4.85 and its amino acid sequence shared 99% similarity with that of three other cry8Ca proteins. This protein therefore belongs to a new class of *Bt* crystal proteins, named cry8Ca by the *B. thuringiensis* Delta-endotoxin Nomenclature Committee. In addition, the cry8Ea crystal protein showed 100% sequence identity to the cry8Ea1 crystal protein and contained 1140 amino acids with a molecular weight of 128.97 kDa. This protein was also acidic and its isoelectric point was 4.85. These two sequences are also assigned in the GenBank database.

The recombinant plasmids pEB-8Ca and pEB-8Ea were transformed into the expression host *E. coli* Rosetta, after being transformed into *E. coli* JM109 for propagation. Expression of the toxin proteins of *E. coli* were induced by addition of IPTG and were collected by centrifugation. SDS-PAGE showed that proteins of molecular mass of 130.25 and 128.97 kDa were highly expressed in *E. coli* by pEB vector (Figures 6 and 7).

DISCUSSION

In recent years, the serious damage caused by Coleopteran pests has increased attention on developing new insecticidal crystal proteins against them. As reported, cry8-type toxins are insecticidal to a number of Coleopteran pests, especially certain species of scarabs (Michaels et al., 1996; Ogiwara et al., 1995; Sato et al., 1994; Shin-ichiro et al., 2003; Yamaguchi et al., 2008). Various insecticidal crystal proteins against Coleoptera have been patented, such as cry8Aa1, cry8Ba1, cry8Ca2, cry8Da2, cry8Ea1, cry8Fa1, cry8Ga1 cry8Ha1 and cry8Na1 (Abad et al., 2002; Feng et al., 2008; Michaels et al., 1996; Song et al., 2007), and some of them have already been used for development of biological pesticides and research on transgenic plants (Feng et al., 2008; Bixby et al., 2007). Thus, new *cry8*-type genes have great application potential and broad application prospects.

Currently, peptide mass fingerprinting technology is used less in insecticidal gene identification. This method can quickly detect the insecticidal protein of a strain by directly comparing the similarity of peptides, and is an effective way to explore novel insecticidal protein genes with high reliability and ease of operation. However, because the current *Bt* insecticidal protein peptide database is incomplete, the peptide mass fingerprinting cannot distinguish high homology insecticidal proteins. In the present study, the peptide mass fingerprinting could not distinguish any cry8Ea or cry8Fa toxins that *Bt* S185 contained, so PCR-RFLP and sequencing were used for further identification of insecticidal protein genes. The combination of peptide mass fingerprinting, PCR-RFLP and sequencing technique successfully cloned and expressed two novel insecticidal protein genes from a high-activity insecticidal *Bt* strain.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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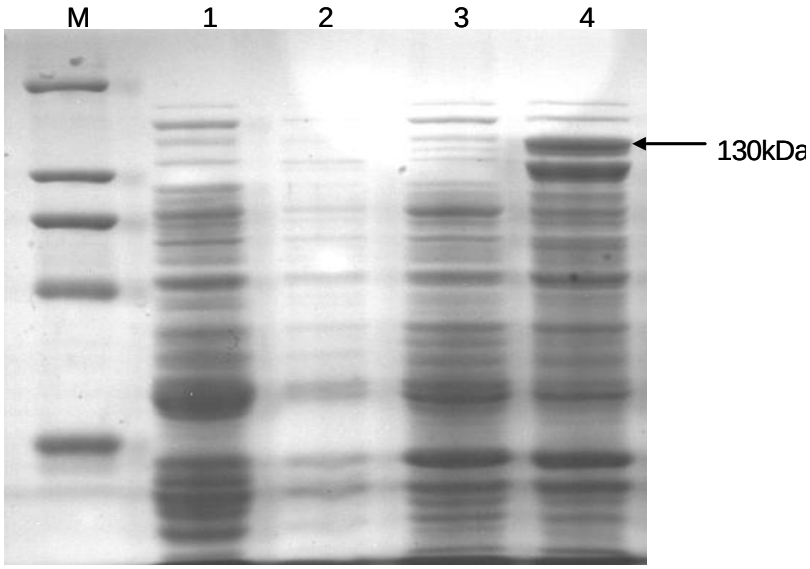

Figure 6. The SDS-PAGE profile of cry8Ea1 proteins from *E. coli* Rosetta. Lane 1, empty gsensor pEB; lane 2, induced empty gsensor pEB; lane 3, *cry8Ea1* in Rosetta without induced; lane 4, *cry8Ea1* in Rosetta with induced.

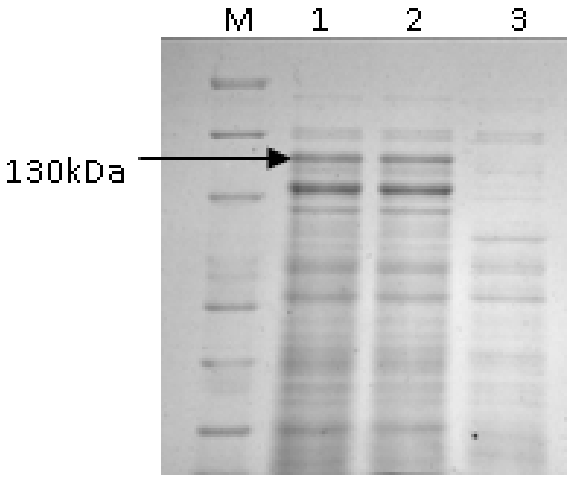

Figure 7. The SDS-PAGE profile of cry8Ca4 proteins from *E. coli* Rosetta. Lane 1, supernatant of *cry8Ca4* in Rosetta; Lane 2, sediment of *cry8Ca4* in Rosetta; Lane 3, empty gsensor pEB.

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Molecular characterization of African swine fever virus in apparently healthy domestic pigs in Uganda

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African swine fever (ASF) is a highly lethal and economically significant disease of domestic pigs in Uganda where outbreaks regularly occur. There is neither a vaccine nor treatment available for ASF control. Twenty two African swine fever virus (ASFV) genotypes (I - XXII) have been identified based on partial sequencing of the C-terminus of the major capsid protein p72 encoded by the *B646L* gene. The majority of previously characterized Ugandan ASFV strains belong to genotype IX. The major aim of the current study was to determine the ASFV genotypes among asymptomatic slaughter pigs at Wambizi slaughterhouse and in some parts of the country where surveillance was done. Three discrete regions of the ASFV were analysed in the genomes of viruses detected in asymptomatic domestic pigs. The analysis was conducted by genotyping based on sequence data from three single copy ASFV genes. The E183L gene encoding the structural protein P54 and part of the gene encoding the p72 protein were used to delineate genotypes, before intra-genotypic resolution of viral relationships by analysis of tetramer amino acid repeats within the hypervariable central variable region (CVR) of the B602L gene. All the ASF viruses obtained from this study clustered with previous viruses in genotype IX based on analysis of the *p72* and *P54* genes. Analysis of the *CVR* gene grouped the viruses in three different subgroups; 13, 23 and 25. Only one genotype is circulating in Uganda among asymptomatic domestic pigs and it is the same virus causing outbreaks in the country and parts of neighbouring Kenya.

Key words: African swine fever virus, asymptomatic, slaughterhouse, P54, p72, *CVR* gene, genotypes.

INTRODUCTION

African swine fever (ASF) is an important, highly contagious and lethal disease of domestic pigs caused by

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Abbreviations: ASF, African swine fever; **CVR,** central variable region; **ASFV,** African swine fever virus.

an icosahedral double stranded DNA virus that is presently the sole member of the *Asfivirus* genus within the family *Asfarviridae* (Dixon et al., 2000). Outbreaks of ASF have been sporadic in the different regions of Uganda (Atuhaire et al., 2013).

Epidemiology of ASF confirms that presence of the disease in one area has a potential risk for introduction and further spreading in any direction despite the natural and artificial borders and distance (Wieland et al., 2011). The viral genome comprises around 170 to 195 kb (depending on the isolate), which encode more than 150 different proteins.

Sequence analyses of virus genomes (Chapman et al., 2008; Chapman et al., 2011; de Villiers et al., 2010; Yanez et al., 1995) have established that the central region is relatively conserved but large length variations occur at the termini, particularly within 40 kbp of the left end of the genome, but also within 15 kbp from the right end of the genome.

Molecular epidemiology has been used to describe the heterogeneity and epidemiological links of ASFV (Bastos et al., 2003; Boshoff et al., 2007; Gallardo et al., 2011). Twenty two ASFV genotypes have been identified based on partial sequencing of the C-terminus of the major capsid protein p72 encoded by the *B646L* gene (Bastos et al., 2003; Boshoff et al., 2007; Lubisi et al., 2007). Previously characterized Ugandan ASF viruses have been placed in genotype IX (Atuhaire et al., 2013; Gallardo et al., 2011) and genotype X (Nix et al., 2006).

Previous studies have demonstrated the value of full P54 gene sequencing for providing additional, intermediate resolution when typing of ASFV viruses (Gallardo et al., 2009.

The ASFV P54 is an externally located viral structural protein of 25-27 kDa, encoded by the virus gene - the open reading frame (ORF) E183L (Rodriguez et al., 1996).

The ASFV protein P54 is involved in the adsorption of the virion on susceptible cells and the early steps of viral infection (Rodriguez et al., 2004).

In addition to p72 and P54 genotyping, higher resolution for viral discrimination has been achieved by use of the B602L central variable genome region (CVR) which contains 12-bp repeats which encode 4 amino acids that vary in number and sequence when genomes of different isolates are compared (Irusta et al., 1996; Nix et al., 2006). Therefore by combining p72, P54 and B602L, a high level resolution approach is achieved for viral discrimination (Gallardo et al., 2011; Lubisi et al., 2007).

The aims of the present study were to genotype ASFV in asymptomatic domestic pigs by p72, P54 and CVR sequencing and determine the relationship of these viruses from the abattoir and field surveillance, and viruses causing ASF outbreaks in Uganda (2010 to 2013) was obtained from GenBank.

MATERIALS AND METHODS

Ethical consideration

Full ethical clearance was obtained from the Uganda National Council for Science and Technology (UNCST) and the College of Veterinary Medicine, Animal Resources and Biosecurity, of Makerere University under reference number VAB/REC/11/110. Permission was obtained from the Wambizi slaughter house administrative authority. For collection of field samples, permission was obtained from area Veterinary Officers and farmers. All animals were handled humanely during sample collection.

Study design

The study design, study sites and sampling strategy were as described previously by Atuhaire et al. (2013).

DNA extraction

Viral DNA was extracted directly from 200 µl aliquots of blood collected in EDTA tubes using the DNeasy Blood and tissue kit (QIAGEN®, USA).

ASFV detection

A 278 bp region corresponding to the central portion of the p72 gene was amplified using the diagnostic primers, primer 1 (5'-ATGGATACCGAGGGAATAGC-3') and primer 2 (5'-CTTACCGATGAAAATGATAC-3') to confirm the presence of ASFV DNA (Wilkinson, 2000).

ASFV molecular characterization

Epidemiological primers which amplify the C-terminal region of the p72 gene (478 bp), p72-U (5'-GGCACAAGTTCGGACATGT-3') and p72-D (5'-GTACTGTAACGCAGCACAG-3') as described previously were used for p72 genotyping (Bastos et al., 2003). The complete gene encoding the P54 protein was amplified using the primers PPA722 (5'-CGAAGTGCATGTAATAAACGTC-3') and PPA89 (5'-TGTAATTTCATTGCGCCACAAC-3') flanking a 676 bp DNA fragment (Gallardo et al., 2009). The CVR located in the B602L gene was amplified using the primer pairs CVR-FL1 (5'-TCGGCCTGAAGCTCATTAG-3') and CVR-FL2 (5'-CAGGAAACTAATGATGTTCC-3') flanking a variable in size DNA fragment (Bastos et al., 2004). Conditions for PCR assays were as previously described (Gallardo et al., 2009) with slight modifications in the annealing temperature which was reduced from 55 to 50°C.

Sequencing and sequence analysis

Amplification products of the expected size were identified against a molecular weight marker, following electrophoresis on a 2% agarose gel. Bands of correct size were excised and purified by means of a Ron's Gel Extraction Kit (BIORON®, Germany) according to manufacturer specifications and sent to Macrogen Europe for sequencing. Analysis of sequence data was performed with Chromas (www.technelysium.com.au), BioEdit (www.mbio.ncsu.edu/ BioEdit/BioEdit.html) and ClustalX version 1.83 (www.clustal.org). For the tetrameric repeat sequences (TRS), analyses including that of the CVR sequences and deduced amino

acid sequences were manually aligned with gaps being inserted to optimize the alignment. Two datasets were generated for phylogenetic analyses conducted using MEGA version 5.0 (Kumar et al., 2001), the p72 and P54 gene data sets. Sequences generated in this study from the Ugandan domestic pig viruses were analysed together with homologous sequences of viruses that were representative of genotype X, IX, VII and II identified in previous studies. Neighbour joining (NJ) trees were constructed employing the p-distance nucleotide substitution model as implemented in the MEGA 5.0 program. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. To determine the degree of statistical support for each node in the resulting p72 and P54 trees, data were re-sampled 1000 times using the bootstrap method. Out of the 14 virus isolates analysed, 12 p72 and 11 P54 generated consensus sequences on alignment.

RESULTS

The origins of ASF viruses

Fourteen (14) ASF viruses obtained from the Wambizi slaughterhouse were selected for use in this study. The samples that tested positive by PCR were from Busoga sub region (Eastern region), Lango sub region (Northern region), Kalungu district, Nakasongola district, Sembabule district, and Nakaseke district all from the central region. These origins depended entirely on the information provided by the traders at the slaughterhouse. One ASF virus was obtained during field surveillance in Kibaale district in Western Uganda.

The *p72* gene phylogeny

The analysis of the p72 partial gene sequences from each of the 12 ASF viruses showed that they were almost identical at the nucleotide level with minor differences resulting from manual trimming of the aligned sequences. The phylogenetic analysis established that all the Ugandan viruses obtained in this study were placed in the p72 genotype IX together with some viruses isolated in previous studies in Uganda, Kenya, and Congo as shown in Figure 1. The 12 ASF viruses (marked with (●) in Figure 1) submitted to the GenBank and their accession numbers include; Uga12.Nakasongola-KF303310, Uga12.Kalungu1-KF303311, Uga12.Kalungu2-KF303312, Uga12.Kalungu3-KF303313, Uga12.Sembabule-KF303314, Uga12.Kibaale-KF303315, Uga12.Nakaseke-KF303316, Uga12.Busoga1-KF303317, Uga12.Busoga2-KF303318, Uga12.Lango1-KF303319, Uga12.Lango2-KF303320 and Uga12.Lango3-KF303321.

The *p54* gene phylogeny

Previous studies have confirmed P54 sequencing as a valuable additional genotyping method for molecular epidemiological studies of genotype IX ASF viruses (Gallardo et al., 2009; Nix et al., 2006). PCR amplification of the fragment containing the complete P54 gene from all of the Ugandan viruses in this study produced products of approximately 670 to 680 bp. The nucleotide sequence analysis of the P54 gene showed that all the isolates were identical. The sequences of the 11 Ugandan viruses were compared with 50 P54 ASFV sequences retrieved from GenBank. The phylogeny revealed that the Ugandan viruses obtained in this study cluster with the majority of the viruses from previous outbreaks in Uganda, Kenya and Congo (Figure 2). The P54 sequences of ASF viruses (marked with (●) in Figure 2) submitted to the GenBank and their accession numbers include; Uga12.Nakaseke-KF303302, Uga12.Busoga1-KF303303, Uga12.Lango1-KF303304, Uga12.Lango3-KF303305, Uga12.Nakasongola-KF303306, Uga12.Kalungu2-KF303307, Uga12.Kalungu3-KF303308, Uga12.Sembabule-KF303309.

Intra-genotypic resolution (CVR) of homogenous p72 genotype IX Ugandan viruses from asymptomatic pigs

In order to delineate the p72 genotype IX obtained in this study at a higher resolution, the CVR of the B602L gene was analysed. Amplification of the CVR gave products of varying sizes (400 to 600bp). The Ugandan viruses characterized in this study clustered with isolates from previous studies in Uganda and some from Kenya. However, differences were mainly observed in the number of tetrameric amino acid repeats of the viruses obtained in this study. The viruses obtained in this study clustered in three different subgroups; 13, 23 and 25 based on the analysis of the tetrameric amino acid sequences (TRS) (Figure 3). Viruses Uga12.Kibaale and Uga12.Kalungu1 had the same repeat sequences with an additional single internally located tetrameric repeat (CAST). Viruses Uga12.Busoga1, Uga12.Lango4, Uga12.Busoga3 and Uga12.Nakaseke clustered together and were different from the others in this study due to the absence of a single CAST repeat and presence of a CADI sequence instead of a CADT sequence. Virus Uga12.Nakasongola was unique due to the absence of 11 tetrameric repeat sequences. The CVR sequences of the ASF viruses (highlighted in Figure 3) submitted to the GenBank and their accession numbers include; Uga12.Nakasongola-KF303295, Uga12.Busoga1-KF303296, Uga12.Lango4-KF303297, Uga12.Busoga3-KF303298, Uga12.Kibaale-KF303299, Uga12.Kalungu1-KF303300, and Uga12.Nakaseke-KF303301.

When compared with sequences of viruses causing outbreaks in Uganda (2010 to 2013) obtained from the GenBank (Atuhaire et al., 2013), viruses Uga12.Busoga1, Uga12.Lango4, Uga12.Busoga3 and Uga12.Nakaseke

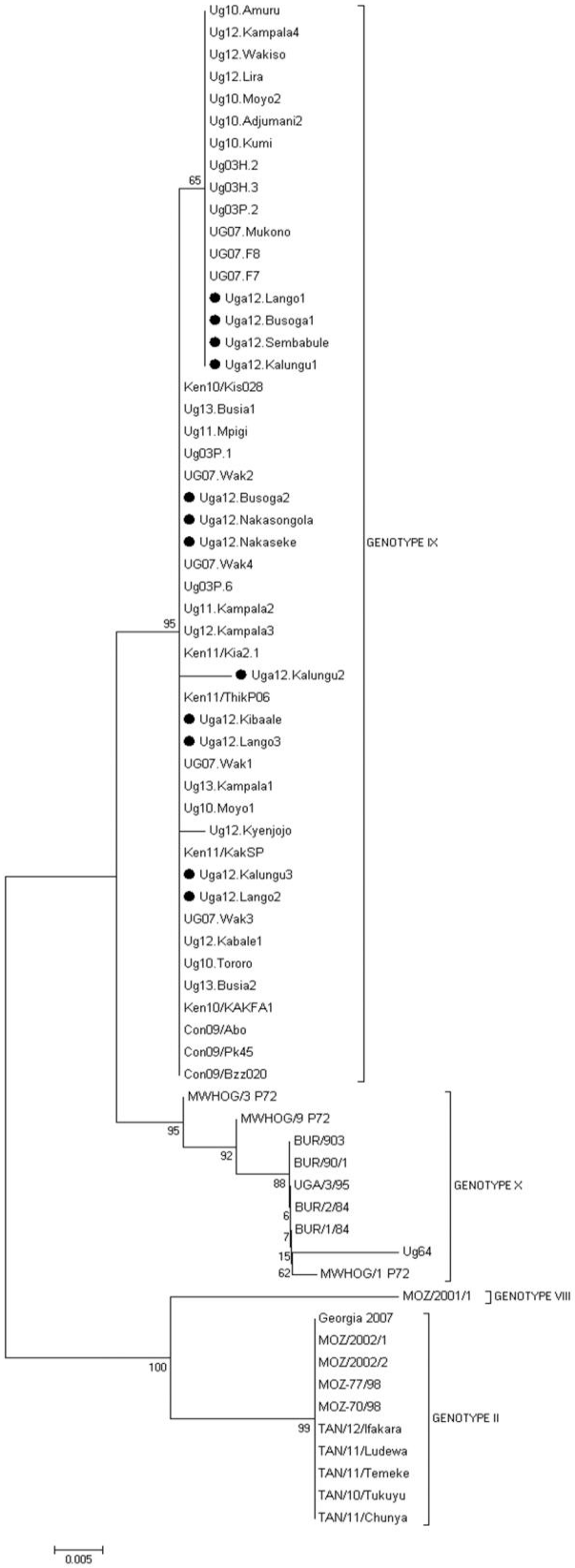

Figure 1. Evolutionary relationships of p72 genotypes: Neighbor-Joining tree of the p72 gene. The analysis involved 69 nucleotide sequences. The p72 sequences from this study are marked with ●. There were a total of 376 positions in the final dataset.

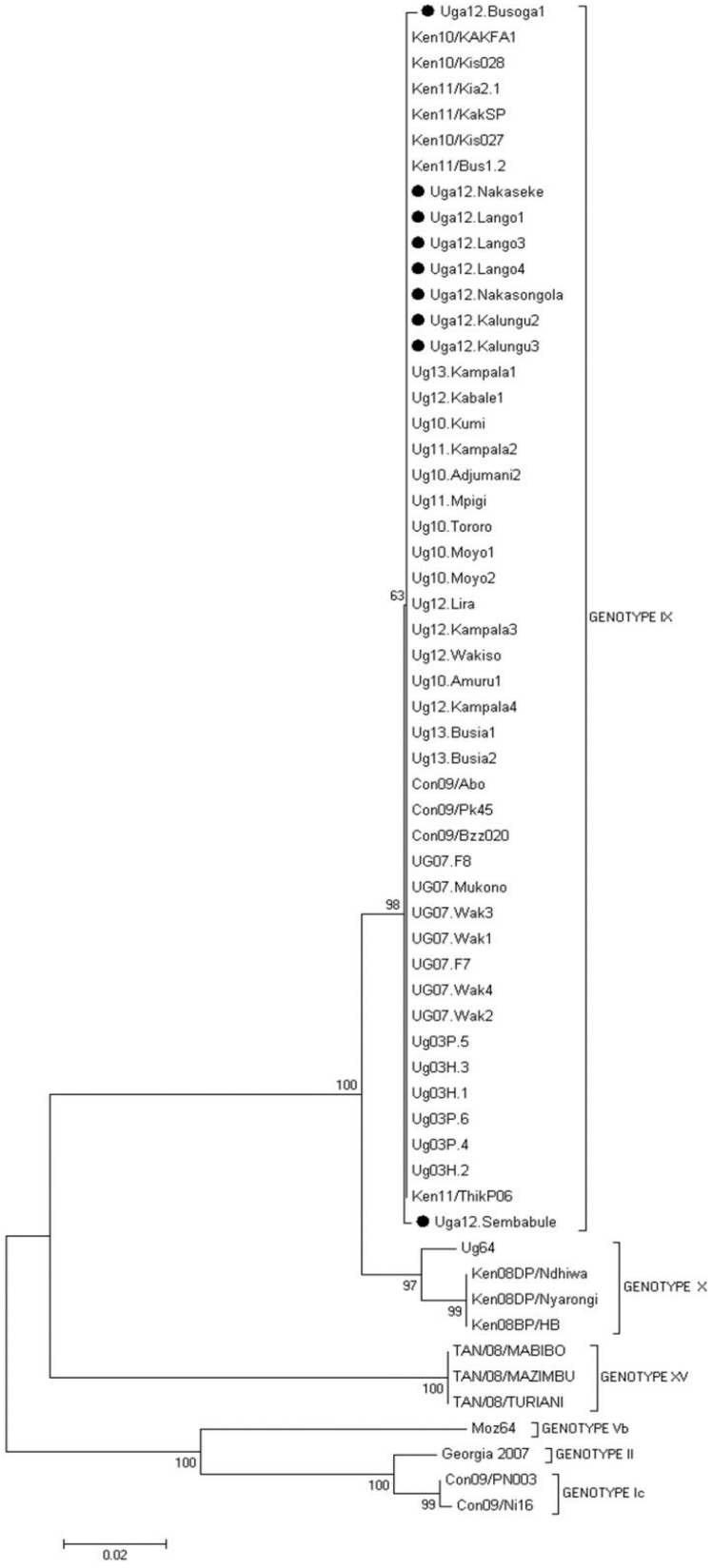

Figure 2. Evolutionary relationships of P54 genotypes: The Neighbor-Joining tree of the P54 gene. The analysis involved 61 nucleotide sequences. The P54 sequences from this study are marked with ●. There were a total of 535 positions in the final dataset.

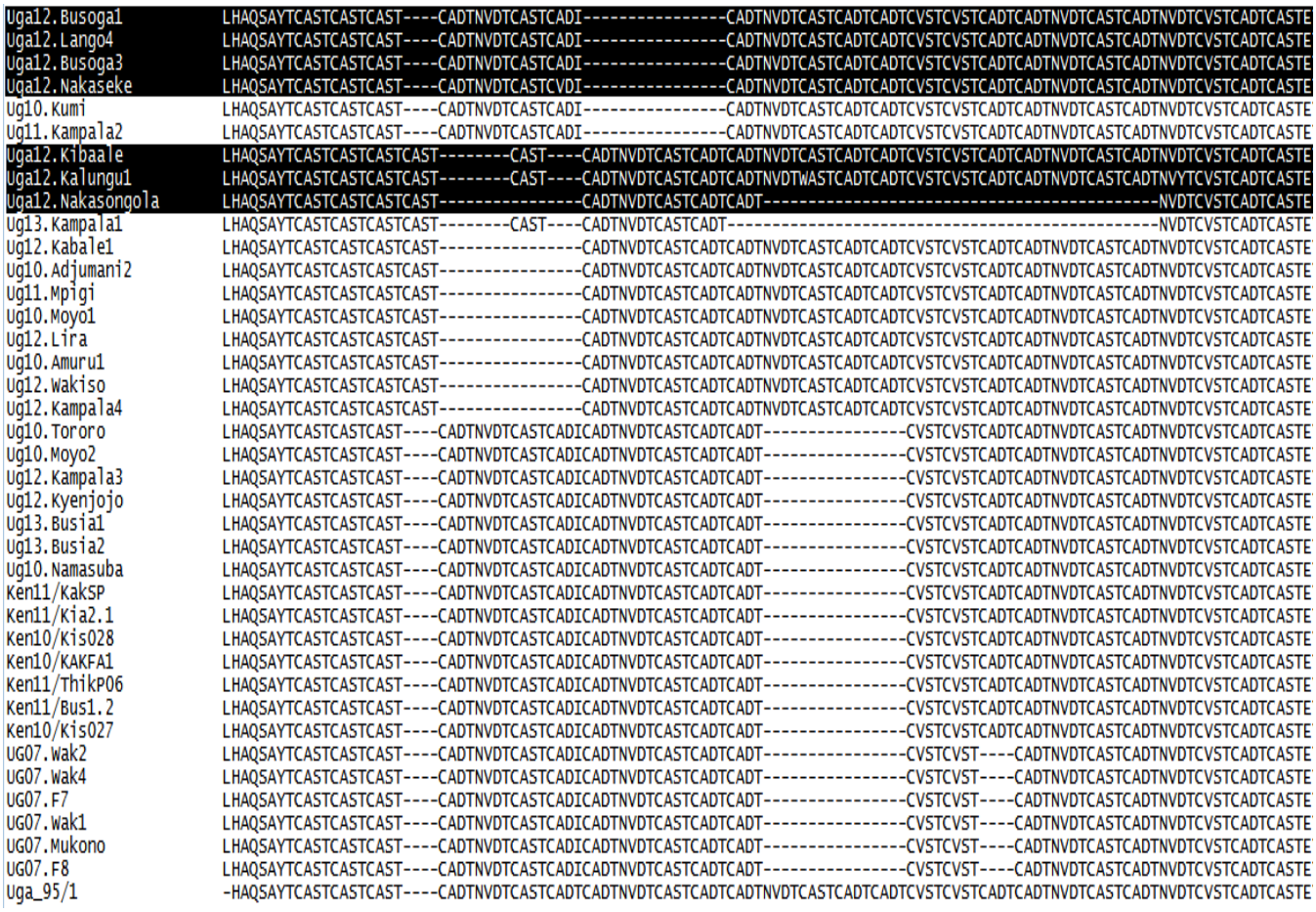

Figure 3. Amino acid sequence alignment of the tetrameric tandem repeats identified within the central variable region (CVR) of gene B602L from Ugandan viruses obtained during abattoir and field surveillance during 2012 (Uga12). The sequences obtained were compared with CVR sequences from isolates associated with outbreaks in Uganda between 2010 and 2013, viruses from an outbreak in Uganda in 2007 as well as UGA95/1 and sequences causing outbreaks in Kenya in 2010 and 2011.

had the same number of tetrameric repeats as Ug11.Kampala2, Ug12.Kampala3, Ug10.Namasuba, Ug10.Tororo, Ug13.Busia1, Ug13.Busia2, Ug10.Kumi, Ug10.Moyo2 and Ug12.Kyenjojo. These viruses were identical to viruses causing outbreaks in Kenya in 2010 and 2011 suggesting that the same virus is circulating between the two countries. Viruses Uga12.Kibaale and Uga12.Kalungu1 were similar to Ug12.Kampala4, Ug11.Mpigi, Ug12.Wakiso, Ug10.Adjumani2, Ug10.Moyo1, Ug12.Lira, Ug10.Amuru and Ug12.Kabale1 in that they had an extra CAST tetrameric repeat, however, they were different in the total number of amino acid tetrameric repeats (Table 1). Uga12.Nakasongola and Ug13.Kampala1 had 13 amino acid tetrameric repeats each (Table 1), with a difference in only one repeat sequence. Uga12.Nakasongola had a CADT sequence while Ug13.Kampala1 had a CAST sequence (Figure 3).

DISCUSSION

African swine fever continues to hamper the development of the pig industry in Uganda with outbreaks occurring sporadically throughout the year. The disease is endemic in the country (Atuhaire et al., 2013; OIE, 2010). The inability of ASFV to induce neutralizing antibodies has hampered the prevention and control of the disease by vaccination and to date there is no vaccine for ASF. In the absence of effective vaccines, control is based on rapid laboratory diagnosis and the enforcement of strict sanitary measures (Sánchez-Vizcaíno et al., 2009). The formulation of appropriate disease control strategies requires intensive molecular epidemiological investtigations not only during disease outbreaks but also field and abattoir surveillance. This would be of value in determining the nature of the viruses circulating in asymptomatic domestic pigs and comparing them with

Table 1. Amino acid sequence of the tetrameric repeats that constitute the CVR of the B602L gene identified in viruses belonging to p72 genotype IX.

Virus name	Country	p72 genotype	CVR amino acid sequence	No. of repeats	CVR GenBank accession no.	Reference
Uga12.Busoga1	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KF303296	This study
Uga12.Lango4	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KF303297	This study
Uga12.Busoga3	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KF303298	This study
Uga12.Nakaseke	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KF303301	This study
Ug10.Kumi	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990858	Atuhaire et al., 2013
Ug11.Kampala2	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990859	Atuhaire et al., 2013
Uga12.Kibaale	Uganda	IX	AAAAABNABBNABBaaBBNABNaBA	25	KF303299	This study
Uga12.Kalungu1	Uganda	IX	AAAAABNABBNABBaaBBNABNaBA	25	KF303300	This study
Uga12.Nakasongola	Uganda	IX	AAAABNABBNaBA	13	KF303295	This study
Ug13.Kampala1	Uganda	IX	AAAAABNABNaBA	13	KC990856	Atuhaire et al., 2013
Ug12.Kabale1	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990857	Atuhaire et al., 2013
Ug10.Adjumani2	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990860	Atuhaire et al., 2013
Ug11.Mpigi	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990861	Atuhaire et al., 2013
Ug10.Moyo1	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990863	Atuhaire et al., 2013
Ug12.Lira	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990865	Atuhaire et al., 2013
Ug10.Amuru	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990868	Atuhaire et al., 2013
Ug12.Wakiso	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990867	Atuhaire et al., 2013
Ug12.Kampala4	Uganda	IX	AAAABNABBNABBaaBBNABNaBA	24	KC990870	Atuhaire et al., 2013
Ug10.Tororo	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990862	Atuhaire et al., 2013
Ug10.Moyo2	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990864	Atuhaire et al., 2013
Ug12.Kampala3	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990866	Atuhaire et al., 2013
Ug12.Kyenjojo	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990869	Atuhaire et al., 2013
Ug13.Busia1	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990871	Atuhaire et al., 2013
Ug13.Busia2	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990872	Atuhaire et al., 2013
Ug10.Namasuba	Uganda	IX	AAABNABBNABBaaBBNABNaBA	23	KC990873	Atuhaire et al., 2013
Ken11/KakSP	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93414.1	Gallardo, 2012
Ken11/Kia2.1	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93412.1	Gallardo, 2012
Ken10/Kis028	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93410.1	Gallardo, 2012
Ken10/KAKFA1	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93408.1	Gallardo, 2012
Ken11/ThikP06	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93413.1	Gallardo, 2012
Ken11/Bus1.2	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93411.1	Gallardo, 2012
Ken10/Kis027	Kenya	IX	AAABNABBNABBaaBBNABNaBA	23	AGC93409.1	Gallardo, 2012
UG07.Wak2	Uganda	IX	AAABNABBNABBaaBNABNaBA	22	ACZ18202.1	Gallardo et al., 2011
UG07.Wak4	Uganda	IX	AAABNABBNABBaaBNABNaBA	22	ACZ18204.1	Gallardo et al., 2011
UG07.F7	Uganda	IX	AAABNABBNABBaaBNABNaBA	22	ACZ18206.1	Gallardo et al., 2011
UG07.Wak1	Uganda	IX	AAABNABBNABBaaBNABNaBA	22	ACZ18201.1	Gallardo et al., 2011
UG07.Mukono	Uganda	IX	AAABNABBNABBaaBNABNaBA	22	ACZ18205.1	Gallardo et al., 2011
UG07.F8	Uganda	IX	AAABNABBNABBaaBNABNaBA	22	ACZ18207.1	Gallardo et al., 2011
Uga_95/1	Uganda	IX	ABNABBNABBNABBaaBBNABNaBA	25	CAJ90783.1	Nix et al., 2006

A, CAST, WAST; a, CVST; B, CADT, CVDI, CADI; N, NVDT, NVYT.

viruses causing disease outbreaks. The major aim of the current study was to detect and characterize ASFV obtained during an abattoir surveillance and field surveillance in selected parts of the country. We used the combined p72, full length P54 and CVR approach to achieve optimal levels of discrimination of even the closely related viruses as previously described (Gallardo et al., 2011). The ability to delineate ASF viruses using the p72, P54 and CVR genes without the need to first isolate the viruses was explored in this study.

We used the OIE recommended diagnostic PCR with primers (Wilkinson, 2000) to confirm the presence of ASFV

DNA in blood samples collected from asymptomatic domestic pigs during abattoir and field surveillance. It could be that the pigs brought for slaughter are subclinical or chronic carriers of ASF. More so, our findings agree with a study carried out in Rakai district in Uganda where ASFV was detected in asymptomatic domestic pigs (Björnheden, 2011). A recent study in Uganda has also detected ASFV in apparently healthy domestic pigs in the same slaughter house (Atuhaire et al., 2013). A seroprevalence study in abattoirs in Mubende has also detected ASFV in domestic pigs (Muwonge et al., 2012). This emphasizes the role of sub-clinical and/or chronically infected carrier domestic pigs in the epidemiology of ASF and factors that lead to resurgence of the virus to cause active infection need to be investigated further.

In this study, phylogenetic analysis based on the p72 and P54 genes grouped all the Ugandan viruses into genotype IX. The results of this study agree with other previous studies in Uganda that grouped viruses causing outbreaks into the same genotype (Atuhaire et al., 2013; Gallardo et al., 2011). Our viruses are also similar to viruses causing outbreaks in neighbouring Kenya in 2010 and 2011 (Gallardo, 2012) emphasizing the role of neighbouring countries in the epidemiology of the disease.

Our findings suggest that there is no significant variation in the ASF viruses circulating in Uganda based on their p72 and P54 genome regions characterized at nucleotide level, confirming a remarkable genetic stability of these regions.

Although p72 and P54 genes are useful for identifying the major ASFV genotypes, higher discrimination of viruses enables more detailed dissection of the genotypes for epidemiological analysis and classification. The analysis of the B602L gene of the CVR revealed the presence of minor differences in the number of TRS placing the viruses into three clusters (subgroups). Uga12.Busoga1, Uga12.Lango4, Uga12.Busoga3 and Uga12.Nakaseke clustered together with 23 TRS. Viruses Uga12.Kibaale and Uga12.Kalungu1 clustered together and had 25 TRS. Uga12.Nakasongola had only 13 TRS which compared with an isolate Ug13.Kampala1 (Accession number GenBank: KC990856) from a previous study in Uganda (Atuhaire et al., 2013). Our findings confirm the value of the *CVR* gene as an additional marker for delineating ASFV in addition to p72 and P54 genotyping.

In conclusion, only one genotype is circulating in Uganda among asymptomatic domestic pigs and it is the same virus genotype causing outbreaks in the country and parts of neighbouring Kenya based on molecular characteristics and genetic patterns of the analysed ASF viruses. The fact that ASFV was detected in asym-ptomatic domestic pigs emphasizes their role in the epidemiology of the virus.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

In vitro propagation of the elite species plant *Pluchea lanceolata:* Assessment of genetic stability by random amplified polymorphic DNA (RAPD) analysis

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An effective *in vitro* regeneration protocol was developed from nodal segment of *Pluchea lanceolata* (DC.) Oliver. & Hiern, a medicinally important plant used in ayurvedic system of medicine for curing diseases similar to rheumatoid arthritis. Nodal segments were cultured in MS medium supplemented with auxin and cytokinin and their combinations. The objective was to produce genetically identical plants, via multiple shoot induction from nodal segment. The culture medium consisted of Murashige and Skoog medium supplemented with one of 3 cytokinins [6-benzyladenine (BAP), Kinetin (Kn) and thidiazuron (TDZ)] at each of six different concentrations for shoot multiplication. The highest multiplication rate (24.57 shoots per explant) was obtained in the medium enriched with NAA. Shoots were successfully rooted in the half strength MS medium containing 0.1 µM NAA. *In vitro* produced plants were transferred to sterilized garden soil: compost (1:1) and then transferred to green house for hardening. Genetic stability of mother plant and the regenerants produced *in vitro* was assessed by random amplified polymorphic DNA (RAPD). In randomly selected plant material (mother plant) and its regenerants, 87 scorable bands were generated by four different primers, showing monomorphism with the mother plant. Thus, molecular analysis reveals that the micropropagation system described is a reliable method for propagation of *P. lanceolata*.

Key words: Conservation, genetic fidelity, micropropagation, RAPD, TDZ.

INTRODUCTION

Pluchea lanceolata (DC.) belongs to the family of Asteraceae. It is perennial, native, under shrub. There are 11 species in the genus but nine have been abundantly used in traditional uses (GRIN, 2011). *P.*

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Abbreviations: MS, Murashige and Skoog medium; **TDZ,** 1- phenyl-3-(1,2,3-thiadiazol-5-yl)-urea; **RAPD,** random amplified polymorphic DNA; **MP,** mother plant; **PCR,** polymerase chain reaction; **CTAB,** cetyltrimethylammonium bromide; **EDTA,** ethylenediaminetetraacetic acid; **BAP,** 6-benzyl aminopurine; **Kn,** Kinetin; **BM,** Basal culture medium; **NAA,** Naphthalenacetic acid; **IBA,** Indole butyric acid; **IAA,** Indole acetic acid; **PGR's,** Plant growth regulators.

lanceolata used in inflammation, bronchitis, psoriasis, cough and piles. It is also used as antipyretic, analgesic, laxative and uterine relaxant (Dwivedi et al., 1949; Nadkarni, 1954; Jadhav and Bhutani, 2005). In anemia and general debility it is salutary as a general tonic (Choudhary, 2012). *P. lanceolata* also exhibits anticancereous property and suppresses oxidative damage in Wistar rats (Jahangir and Sultana, 2006). *P. lanceolata* shows the immunosuppressive inhibition of Th1 cytokines (Bhagwat et al., 2010). Due to over collection and unscrupulous extraction of plants from the wild population for medicine and trade complexed with poor seed viability are the major factors for the continuous decline of its natural population in the country and the species has become vulnerable to extinction (Singh, 2004) and that is why the plant is listed in priority species by ministry health and family welfare, Govt. of India.

The plant is propagated from root but this method is time consuming and result in limited number of plants. Hence, development of an *in vitro* method for rapid micropropagation multiplication of *P. lanceolata* is warranted for providing uniform raw material for medicinal uses as well as its conservation. Production of genetically identical clones from axillary buds is achieved by various PGR's effectively. But cell mutations and genetic instability can happen because of many reasons such as the type of media used, PGR's and its concentration, the type of explant and number of subculture cycles (Bairu et al., 2011). So, it becomes necessary to check the genetic stability of the *in vitro* produced plants. In the present study, we aimed on (a) An efficient system of *in vitro* propagation from nodal segment of species and (b) Evaluation of genetic fidelity of *in vitro* regenerated plants.

MATERIALS AND METHODS

Plant material

Excised nodal segment from fresh twig were washed thoroughly under the running tap water for 30 min to remove the dust particles followed by treatment with 0.1% (w/v) Bavistin for 15 min along with 2% Tween-20 (v/v) for 10 min to remove the adhering particles and rinsed with sterilized distilled water. Explants were surface sterilized with 0.1% (w/v) $HgCl_2$ (Qualigens, India) for 60 s. This step was followed by rinsing 5 to 6 times with sterilized distilled water to remove the traces of sterilizant.

Culture media

The sterilized nodal explants were inoculated on basal culture medium (BM) consisting of MS medium (Murashige and Skoog, 1962), sucrose (20 gL^{-1}) and agar (8 gL^{-1}) with various concentration of PGR's viz., BAP (1.38 to 13.85 µM), Kn (1.45 to 14.5 µM) and TDZ (1.41 to 14.15 µM) alone or in combination. The pH of the medium was adjusted 5.8 before autoclaving for 20 min at 121˚C. The cultures were subcultured for 4 weeks until sufficient shoots were accumulated to enable shoot multiplication optimisation experiments.

Growth conditions for multiple shoot induction

Explants were inoculated with 50 ml of BM with different concentration of BAP, TDZ and Kn in culture tubes. The cytokinin treatment which gives the best result was tested in combination with NAA, IBA and IAA (0.1, 0.5 and 1.0 µM) for shoot multiplication. Cultures were incubated at 25±2˚C with a 16 h photoperiod (2000 to 2500 lux) with 40 W cool white fluorescent tubes. For rooting, the *in vitro* shoots were cultured on the inducing medium consisting of half strength MS with NAA, IAA and IBA for 4 weeks of culture. Then, *in vitro* rooted plantlets were transferred to a mixture containing sterilized garden soil and compost in 1:1 ratio and maintained in green house condition under natural light. After 4 weeks, acclimatized plants were transferred to earthen pots.

Statistical analysis

Three replicates (10 explants per replicate) were inoculated per treatment. Data were subjected to ANOVA (analysis of variance) treatment and means were determined by Duncan's multiple range test (DMRT) ($p \leq 0.05$) using SPSS 16.0 version, 2008.

DNA extraction and RAPD analysis

For genomic DNA isolation, young and fresh leaves were collected from the randomly selected cultures and also from field grown mother plants of *P. lanceolata* for comparison. Total genomic DNA was extracted from the frozen leaf material using standard protocol (Doyle and Doyle, 1990). RAPD profiles were produced through PCR amplifications. All the PCR reactions were carried out under optimized conditions in 0.2 ml polypropylene PCR tubes Bangalore Genei, India using Thermal cycler eppendrof. Each 25 µl reaction mixture contained 1x Taq buffer 100 mM Tris-Cl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% Gelatin, 2.5 mM $MgCl_2$, 100 µM dNTP mix Bangalore Genei, India and 50 ng of template DNA. All reactions were subjected to initial denaturation at 95°C for 5 min followed by 35 amplification cycles, each cycle consisting of 1 min at 43°C annealing step and 2 min at 72°C extension step with a final extension of 7 min at 72°C. Banding profiles generated by RAPD was compiled into a data binary matrix based on the presence (1) or absence (0) of the selected band. Only clear, unambiguous and reproducible bands amplified were considered for the scoring and data analysis. Data were analyzed using simqual route to generate Jaccard's similarity coefficient with NTSYS pc Version 2.02 k programme (Rohlf, 1998). Dendogram was prepared using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Sequential, Agglomerative, Hierarchical, and Nonoverlapping (SAHN) clustering for analysis of relationships.

RESULTS AND DISCUSSION

Multiple shoot induction

Establishment of the *in vitro* cultures in *P. lanceolata* posed considerable problem with contamination in primary cultures, and repeated subculture. The problem was overcome by treating the explants with 0.1% (w/v) Bavistin for 15 min and then with 0.1% $HgCl_2$ for 1 min. No organogenic changes were observed after 4 weeks, in the excised meristem cultured on BM devoid of PGR's. Presence of low and intermediate levels of TDZ produced maximum shoots per explant of the three cytokinins tested on *P. lanceolata*. There was a proportional

Table 1. Effect of various plant growth regulators on development of nodal explants in *P. lanceolata* after 8 weeks of culture.

Plant growth regulators (µM)						Mean number of shoots /explants	Mean shoot length
TDZ	BAP	Kn	IBA	IAA	NAA		
Control						1[a]	0
1.41						8.83 ± 0.59 [c]	2.00 ± 0.14[b]
2.83						16.48 ± 0.72[e]	2.60 ± 0.14[c]
5.66						12.90 ± 0.82[d]	1.84 ± 0.11[b]
8.49						7.93 ± 0.58[bc]	1.85 ± 0.11[b]
11.32						6.76 ± 0.89[c]	1.54 ± 0.20[b]
14.15						5.40 ± 0.47[b]	1.69 ± 0.15[b]
2.83			0.1			15.67 ± 1.05[cd]	3.28 ± 0.23[c]
2.83			0.5			14.97 ± 0.33[cd]	3.56 ± 0.32[c]
2.83			1			12.87 ± 0.88[c]	3.75 ± 0.24[c]
2.83				0.1		7.83 ± 0.15[b]	2.17 ± 0.32[b]
2.83				0.5		2.90 ± 0.24[a]	1.01 ± 0.43[a]
2.83				1		1.87 ± 0.24[a]	0.87 ± 0.58[a]
2.83					0.1	24.57 ± 0.37[e]	4.91 ± 0.27[d]
2.83					0.5	16.90 ± 0.46[d]	3.25 ± 0.28[c]
2.83					1	11.90 ± 0.38[c]	2.85 ± 0.31[bc]
	1.38					4.9 ± 0.66[a]	2.1 ± 0.05[a]
	2.77					6.33 ± 0.33[b]	2.3 ± 0.05[cd]
	5.54					8.66 ± 0.33[bc]	2.3 ± 0.0[d]
	8.31					10.08 ± 0.33[e]	2.5 ± 0.0[e]
	11.08					9.03 ± 0.47[bd]	2.2 ± 0.03[b]
	13.85					6.36 ± 0.53[a]	2.0 ± 0.03[a]
		1.45				3.66 ± 0.81[a]	2.0 ± 0.0[a]
		2.9				3.83 ± 0.88[a]	2.3 ± 0.0[d]
		5.8				5.03 ± 0.31[b]	2.5 ± 0.0[e]
		8.7				6.68 ± 0.34[cd]	2.5 ± 0.06[e]
		11.6				6.94 ± 0.35[cde]	2.6 ± 0.0[f]
		14.5				6.37 ± 0.85[cd]	2.2 ± 0.0[b]

Values represent mean ± S.E. means followed by the same letter are not significantly different at the level of confidence as per DMRT test (p≤0.05).

relationship between the increase in TDZ concentration up to the optimal level (2.83 µM) and the number of shoots per explants (Table 1). A maximum (16.48 ± 0.72) number of shoots developed within 8 weeks of culture (Figure 1a). Induced shoots at this stage attained a length of 2.60 ± 0.14 cm. Results obtained are in agreement with those of *Stevia rebaudiana* Bert (Lata et al., 2013). The maximum (24.57±0.37) number of shoots were obtained on BM supplemented with TDZ (2.83 µM) and NAA (0.1 µM) after 8 weeks of culture (Figure 1b). However, on IAA amended medium fewer shoots were produced as compared to IBA and NAA. The synergistic effect of TDZ in combination with other auxin has been demonstrated in the case of *Stemona hutanguriana* (Prathanturarug et al., 2012). By repeated sub-culturing on MS with TDZ (2.83 µM) and NAA (0.1 µM) number of shoots increased (45±1.33) after five times of sub cultures (each of 21 days) beyond which there is gradual decline in multiplication rate. This enhanced rate of shoot multiplication by subsequent subcultures substantiates with the earlier reports on *Ipomoea batatas* L (Sefasi et al., 2013) and *Andrographis neesiana* (Karuppusamy et al., 2010). Well developed microshoots of *P. lanceolata* were transferred to half strength MS medium containing IAA, IBA and NAA. The highest rooting frequency (100%) with maximum of (7.67±0.44) roots per shoot were achieved with 0.1 µM NAA (Figure 1c) where profuse rooting was observed. Similar results have also been achieved in *Arnebia hispidissima* (Shekhawat and Shekhawat, 2011) and regenerated plantlets were transferred to thermacoal cups consisted of sterilized garden soil and compost (1:1) as potting medium and maintained under controlled condition for two months prior to transfer to earthen pots containing garden soil the regenerated plant showed homogeneity with no phenotypic changes (Figure 1d). To confirm the genetic fidelity of the regenerated plants maintained in culture period of 6 months, RAPD analysis was carried out. The

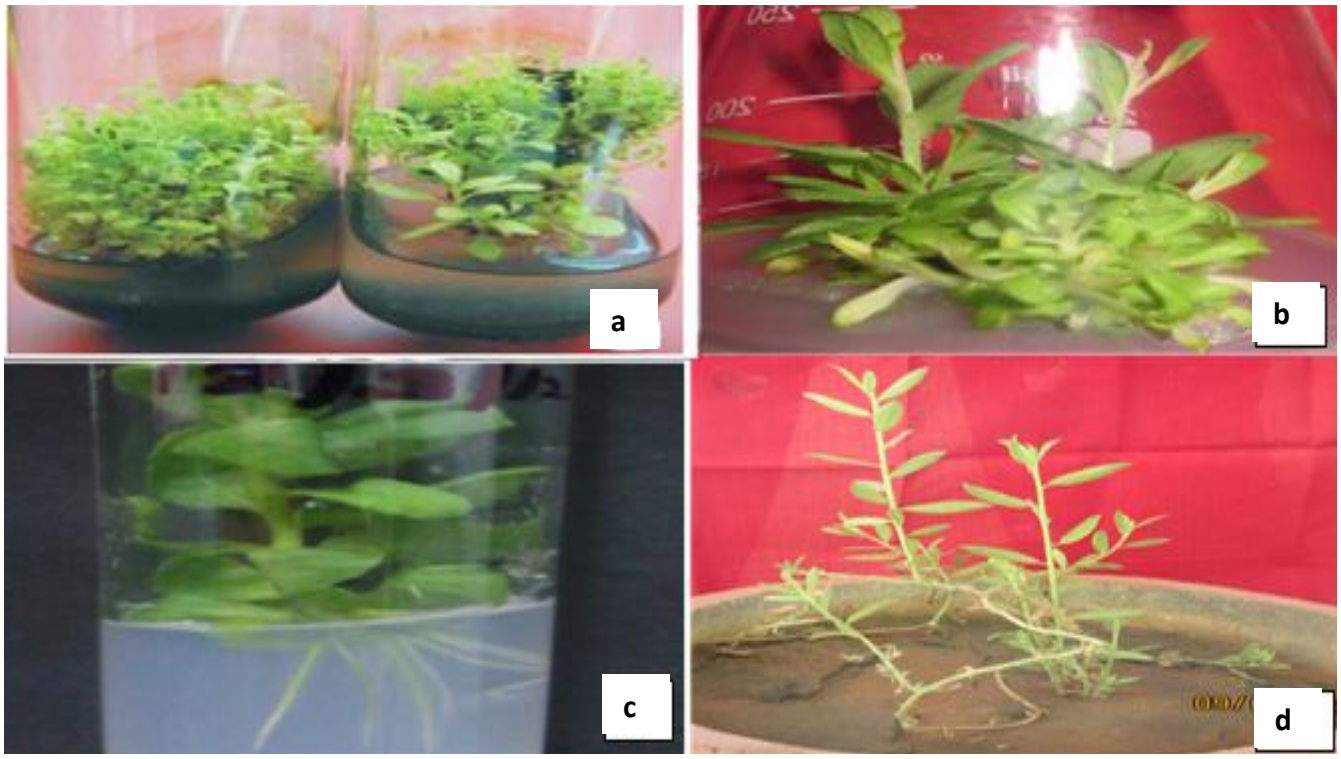

Figure 1. *In vitro* regeneration of *P. lanceolata*. **(a)** Multiple shoot induction from nodal segments cultured on BM + TDZ (2.83 µM); **(b)** Enhanced shoot multiplication and elongation on BM + TDZ (2.83 µM) + NAA (0.1 µM); **(c)** Rooted shoot plantlets on 1/2 strength MS with NAA (0.1 µM). **(d)** Acclimatized plants.

selected RAPD primers generated a total number of 87 bands, which were monomorphic for all the analyzed plants, including the control plant (Figure 2). Primer generated amplification products ranged in size from 226 to 1500 bp. A total of 87 bands were produced using 4 primers with 86 monomorphic and 1 polymorphic band (Table 2). Among treatments which were produced from different subcultures and mother plant, the range of similarity coefficients was from 0.93 to 1.00. Based on the dendrogram (Figure 3), 2 main clusters were obtained. Cluster 1 contained CC1, CC3, CC4, CC5 and CC6 which had similarity at the coefficient level of 1.00. Cluster 2 was occupied by CC2 corresponding to the first sub-culture which showed similarity at the coefficient level of 0.93 with cluster 1. The closest similarity with the mother plant was observed in the case of CC3, CC4, CC5 and CC6 which gave 100% similar RAPD profile to its mother plant. On the contrary, CC2 showed highest divergence with similarity value of 0.93 from MP (Table 3). The results are in contrast to inherited modifications in plants regenerated from tissue culture for *Dieffenbachia* cv. Camouflage (Shen et al., 2007), and in banana cultivar Valery, (Sheidai et al., 2008) and similar to *Tylophora indica* (Haque and Ghosh, 2013) where the genetic variation was induced with the time-period of the sub-culture. In the present study, it was found that minimal or no changes occurred between the MP and the sub culture produced and the variation which emerged after first sub culture reverted to the normal in the subsequent sub culturing.

Arya and Patni (2007) conducted a study on *P. lanceolata* using BAP (0.25 mgl^{-1}) and Kn (0.5 mgl^{-1}) for shoot induction on MS medium and obtained maximum number of shoots (30), while the present investigation give rise to quick and possibly less expensive method with more number of shoots at lower concentration of sucrose (2%) and lower concentration of growth regulator TDZ (2.83 µM) and the auxin used for rooting was at lower concentration NAA 0.1 µM and the rate of survival was 100% as compared to 70% from the previous study.

Conclusion

The study shows that plants regenerated through nodal culture *in vitro* could be successfully used for clonal propagation with very little risk of somaclonal variation and RAPD could be a good molecular marker to evaluate the genetic stability, to regenerates the *ex situ* conservation of this important medicinal plant.

Conflict of Interests

The author(s) have not declared any conflict of interests.

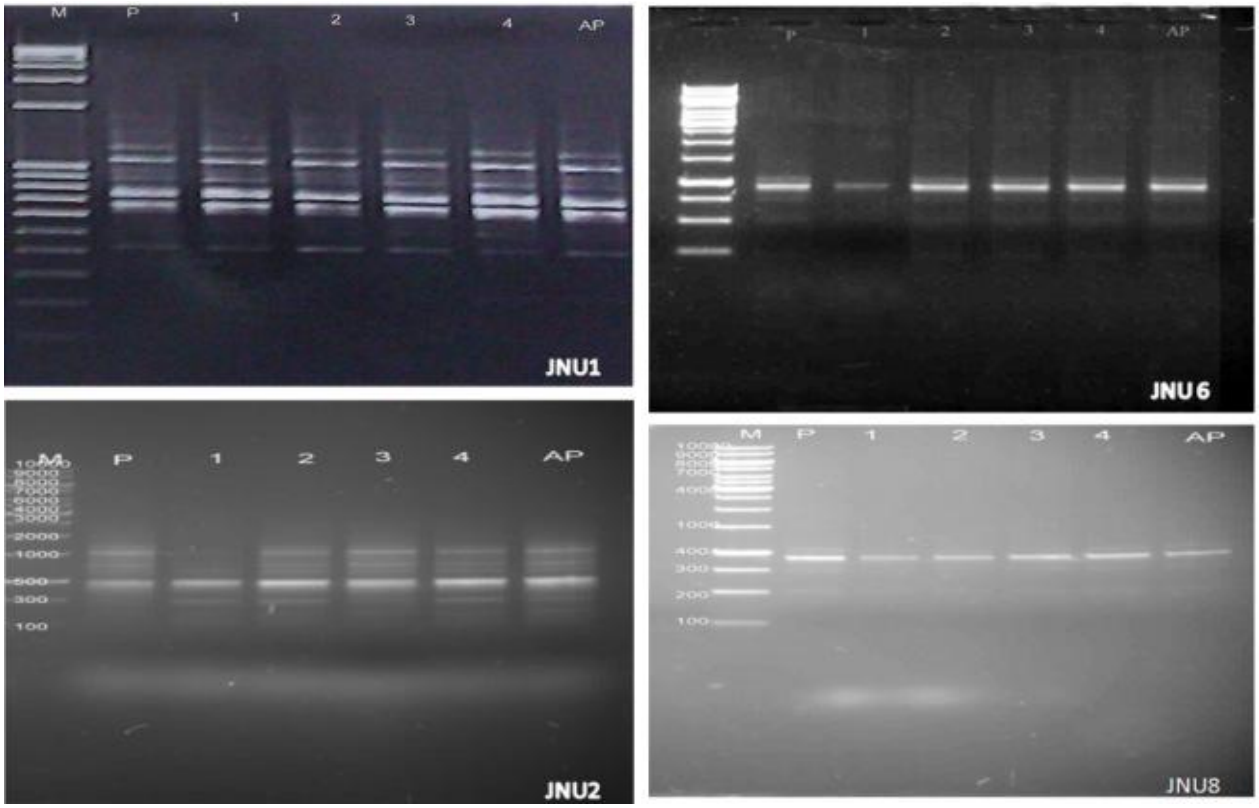

Figure 2. RAPD banding profile in *P. lanceolata* showing bands with JNU1, JNU2, JNU6 and JNU8.

Table 2. Total number and size range of amplified product and number of polymorphic bands generated by 4 (14mers) primer in micropropagated and mother plants of *P. lanceolata*.

Primers	Sequence 5'-3'	Scorable bands	Polymorphic bands	Monomorphic bands	Size Range	% Monomorphism	% Polymorphism
JNU1	CAGCCGCGGATCGT	36	0	36	1350-420	100	0
JNU2	CCCCCGGACCCAAA	27	0	27	412-200	100	0
JNU6	TTTCGGGGCCTTGG	11	1	10	390-400	100	9.09
JNU8	AGGGGCACGGATGC	12	0	12	410-380	100	0

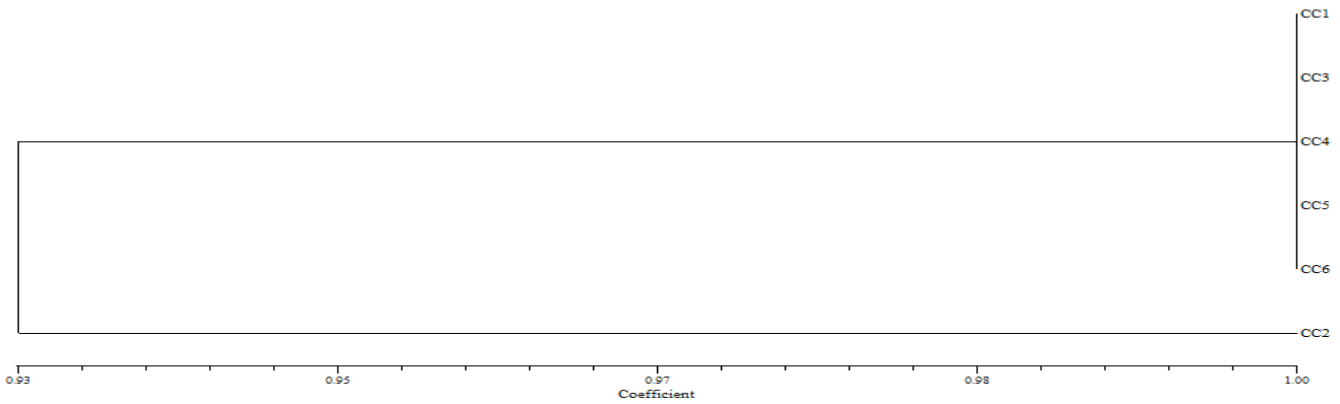

Figure 3. Dendogram of *P. lanceolata* showing genetic similarity among the *in vitro* generated plants and mother plant by UPGMA analysis based on single primers.

Table 3. Similarity matrix of the somaclonal variation between mother plant and different subcultures in *P. lanceolata*.

Cluster	CC1	CC2	CC3	CC4	CC5	CC6
CC1	1					
CC2	0.9333	1				
CC3	1	0.9333	1			
CC4	1	0.9333	1	1		
CC5	1	0.9333	1	1	1	
CC6	1	0.9333	1	1	1	1

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Conflict of Interests

There is no conflict of interest among the authors.

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Full Length Research Paper

Relative expression of genes related with cold tolerance in temperate rice at the seedling stage

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Low temperature is one of the main abiotic stresses affecting rice yield in Chile. Alterations in phenology and physiology of the crop are observed after a cold event. The objective of this work was to study the relative expression of genes related with cold stress in Chilean cultivars of rice. For this, we analyzed the expression of candidate genes using real-time polymerase chain reaction (rtPCR), after exposure to cold of the rice cultivars Diamante-INIA and Zafiro-INIA and one experimental line from INIA´s Rice Breeding Program in Chile, Quila 241701, with known high cold tolerance. For analysis, the Spanish cultivar, Susan, was used as check tolerance and Oryzica 1 as susceptible check. Oxidative stress was measured through lipid peroxidation. To find mechanisms of cold tolerance in Chilean cultivars, we determined the relative expression of genes related with oxidative stress, such as superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT). Lipid peroxidation allowed the measurement of the physiological stress level of the genotypes under study. The results indicate that lipid peroxidation in Oryzica 1 was higher than in the other genotypes. No differences were observed in expression levels of gene-encoding SOD and GR between genotypes. Contrary to expected results, high level of the gene-encoding CAT enzyme in Oryzica 1 after cold stress was observed. Future experiment, related with enzymatic activity and non-enzymatic antioxidant mechanism, are necessary to elucidate the relationship between cold stress and expression levels of gene-encoding antioxidant enzyme in Chilean rice germplasm.

Key words: Cold stress, reactive oxygen species, antioxidant enzymes.

INTRODUCTION

Chile is one of the coldest regions where rice is cultivated in the Southern hemisphere and therefore, low temperature is the most important abiotic stress affecting rice production. Low temperatures are responsible for up to 40% yield loss in rice production in Chile (Alvarado and Hernaiz, 2007). Similar results are observed in temperate

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areas such as Australia and Japan (Jacobs and Pearson, 1994; McDonald, 1994). The rice area in Chile has a Mediterranean climate and it is located between the Maule (35° S lat) to Biobío region (36º S lat). Rice cultivation occurs from October to March mainly in a flooding regime by using pre-germinated seeds (Alvarado and Hernaiz, 2007).

Lately, the direct sowing started to be used by the farmers. Rice plants are frequently exposed to cold temperatures at germination, seedling and the reproductive stage. Commonly, the minimum temperature is below 12°C, below the optimum (20-30°C) required for rice cultivation (Yoshida et al., 1996). In all cases, the level of damage associated to cold stress will be dependent on the development stage and the intensity of the cold event (Jacobs and Pearson, 1994). For example, at the germination stage, chilling can cause slow growth and severely reduce seedling vigour (Ali et al., 2006); at the vegetative stage may cause, lower tillering (Shimono et al., 2002), high mortality (Mackill and Lei, 1997; Andaya and Mackill, 2003; Baruah et al., 2009), non-uniform maturity of the crop (Shimono et al., 2004), and yellowing of the plants (Shimono et al., 2002; Ali et al., 2006; Baruah et al., 2009). Night temperatures at germination and seedling stages are below 10°C (Alvarado and Hernaiz, 2007). In this condition, Chilean rice cultivars such as Zafiro-INIA and Diamante-INIA have shown high cold tolerance, especially at the seedling stage (Pulver, 2002).

At molecular levels, cold stress can cause an increase of reactive oxygen species (ROS) causing oxidative stress (O'Kane et al., 1996; Mittler, 2002; Apel and Hirt, 2004). The oxidative stress occurs due to an imbalance between oxidative species formation and antioxidant defences of the cell. In abiotic stress the principal causes of oxidative imbalance are the absorption of more photons than can be used by the photosynthetic apparatus (chloroplast) and the over-reduction of electron transport chain (mitochondria) (Møller et al., 2007). Accordingly, levels of ROS in the cell increase rapidly (Tsugane et al., 1999). ROS is produced in the electron transport chains of chloroplasts and mitochondria, some peroxidases and oxidases and excited chlorophyll molecules (Cheeseman, 2007). In the mitochondrial electron transport chain, NAD dehydrogenase and cytochrome reductase generate the superoxide anion, which is reduced to H_2O_2 by dismutation. H_2O_2 can react with reduced Fe^{2+} and Cu^{+} to produce hydroxyl radicals ($HO^{\bullet}$) (Sweetlove and Foyer, 2004). In chloroplasts, ROS is generated by univalent oxygen reduction in the Mehler reaction in the electron transport chain (Asada, 1996; Edreva, 2005). Generated $O_2^{\bullet-}$ is protonated to $HO_2^{\bullet}$ and these molecules can initiate lipid peroxidation. On the external stromal membrane surface $O_2^{\bullet-}$ may be dismutated to H_2O_2 and O_2 (spontaneously or by Superoxide dismutase) (Grant and Loake, 2000). Through the Fenton reaction, H_2O_2 is transformed to $HO^{\bullet}$, the most reactive species. To avoid production of $HO^{\bullet}$, H_2O_2 is scavenged by the antioxidant machinery (Noctor and Foyer, 1998; Dat et al., 2000).

Another source of ROS is excited chlorophyll, inducing the production of superoxide ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) (Asada, 1996). ROS can cause damage to DNA, lipid membranes and proteins (Mittler, 2002; Apel and Hirt, 2004). Also, oxidative stress induces alterations in the redox status of proteins regulating protein functions and degradation (Møller et al., 2007).

Plants have developed numerous mechanisms to reduce the effects of the oxidative damage. Some of them are associated with enzymatic ROS scavenger systems, for example, superoxide dismutase (SOD), which catalyzes the conversion of superoxide to H_2O_2 and O_2, catalase (CAT), which convert H_2O_2 to water, glutathione reductase (GR) and the ascorbate peroxidase (APX), which eliminates the ascorbate H_2O_2 GSH cycle (Saruyama and Tanida, 1995; Hammond-Kosack and Jones, 1996; Huang and Guo, 2005; Ahmad et al, 2008).

Real time PCR is a powerful technique for evaluation gene expression based on the detection and quantification of the fluorescence emitted by PCR products accumulated through the amplification process (Higuchi et al., 1993). This technique can be used to quantify gene expression using two strategies: relative and absolute quantification. In relative quantification, gene expression of target gene is compared with one or more reference genes (Pfaffl, 2004). In the case of rice, the expression of the 18S rRNA gene is widely used as a reference gene (Kim et al., 2003; Welsch et al., 2008).

Tolerance to cold is a characteristic dependent on the genotype, which is controlled by more than one gene (Baruah et al., 2009). It has been demonstrated that cold tolerance is associated with capacity to remove or detoxify ROS (Malan et al., 1990). Therefore, the hypothesis of this research was that expression of genes associated with proteins that detoxify ROS (SOD, GR and CAT) is related to cold tolerant genotypes. The overall objective was to analyze gene transcription that encodes antioxidant enzymes in 3 cultivars and one cold tolerant experimental line, at the seedling stage.

MATERIALS AND METHODS

Plant and growth conditions

Seeds of rice genotypes Quila 241701 (Experimental line), Diamante-INIA, Zafiro-INIA, Susan (tolerant control) and Oryzica1 (susceptible control) were germinated on absorbent paper with a solution of the fungicide Benlate (2 ppm) at 28° C in the dark for 3 days. After, germination seedlings were transplanted into plastic pots of 500 mL with clay soil (Vertisol), fertilized with 100mg of Urea, 55 mg of potassium chloride and 45 mg of triple superphosphate (45% of P_2O_5).

Plants were grown in a greenhouse with a photoperiod of 14 h of light and 10 h of dark, at 28°C. The plants were illuminated with artificial light using metal halide lamps with a radiation of 300 µmol

Table 1. Primer sequences used for real-time PCR analysis.

Gen name	Primer sequence	Amplicon size (bp)
OsSODCu/Zn P93407	5' ACCATTGTTGATAAGCAGATTCC 3' 5' GTCGCCCACCAGCATTTC 3'	139
OsCAT Q0E4K1	5' AACTACCTCTCCCAGTGTGATGAG 3' 5' AGTTTCTTCACATGCTTGGCTTCA 3'	82
OsGR Q8S5T1	5' ACAGCAAACTTCAGGCCACTTAGG 3' 5' TGTCAGCCCAGCTTTAACAGCA 3'	171
18S rRNA*	5' ATGGTGGTGACGGGTGAC 3' 5' CAGACACTAAAGCGCCCGGTA 3'	159

* Primers developed by Todaka et al., 2012.

photons m^{-2} s^{-1}. Seedlings with 3 to 4 expanded leaves were treated at 5°C in a cold chamber in darkness (Baruah et al., 2009) for 72 h. Finally, three plants per pot were sampled and frozen in liquid nitrogen and stored at 80°C until analysis.

Lipid peroxidation

The protocol described by Dionisio-Sese and Tobita (1998), with modifications, was used to determine lipid peroxidation; the main purpose was to measure malondialdehyde (MDA) concentration. The leaves of the seedlings were ground into a fine powder with liquid nitrogen and weighed. A volume of 500 µL of 50 mM potassium phosphate buffer at pH 7 was added to the sample that was stirred in a Vortex and homogenized with a homogenizer (Heidolph, Diax 900). The extract was centrifuged at 16,000 *g* for 30 min at 4°C. This process resulted in a 300 µL aliquot of extract that was mixed with 300 µL 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). Subsequently, the mixture was heated at 95°C for 30 min and reaction was stopped by placing tubes on ice. After, the samples were centrifuged at 10,000 *g* for 10 min. Absorbance of 532 and 600 nm was determined in the supernatant with spectrophotometer. The MDA concentration was determined by the extinction coefficient, which is equal to 155 $mM^{-1}cm^{-1}$ using the standard equation for weight in grams for each of the samples:

$MDA = [(A532 - A600)/ 155\ mM^{-1}cm^{-1}]$ [nmol g-1 FW]

FW = fresh weight tissue (g)

Analysis of genes by real- time PCR

Total RNA was extracted from 0.1 g of ground tissue using RNA-Solv Reagent (Omega Bio-tek). For removal of genomic DNA, DNase I (Applied Biosystems) treatment was applied and cDNA was retro-transcribed from 2 µg of RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real time PCR was performed with a Stratagene Mx3000p system using GoTaq Green Master Mix (Promega). Expression was calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) with 18S rRNAas -the Housekeeping gene (Table 1). For amplification, GoTaq Mix from Promega was used according to manufacturer's instructions. For this, 3 ng of cDNA was used for amplification of 18S rRNA, 12.5 ng of cDNA for amplification of SOD, and GR, and 25 ng of cDNA for CAT amplification. Furthermore, dissociation curves were performed in each case to determine the absence of primer dimers and/or nonspecific products. All primers are described in Table 1.

Experimental design and statistical analysis

A completely random experimental design was used with three biological replicates. Also, for all Real time PCR analysis, three technical replicates were used. Two way ANOVA (treatment and genotype as factor of variations), was used for the statistical analysis of the MDA concentration. One way ANOVA was used for the analysis of the relative expression of each gene studied. All data were analyzed using Infostat® software (Di Rienzo et al., 2012).

RESULTS AND DISCUSSION

Lipid peroxidation showed values between 2.94 and 5.57 nmol g^{-1}PF in the analyzed genotypes (Figure 1). This result was similar to that found by Cabas (2012), in which lipid peroxidation of different rice genotypes showed variations between 1.39 to 5.56 nmol g^{-1}. Analysis of variance for MDA concentration showed that seedlings of Oryzica 1 were 1.86 times higher than Susan and 1.37 times higher than Diamante-INIA and 1.71 times higher than the control condition. Also, levels of MDA for Diamante-INIA exposed to cold was 1.28 times greater than the control plants and Quila 241701 exposed to cold.

No differences between Susan and Zafiro-INIA seedlings were found in control conditions and cold. Similar results were also observed by Kim and Tai (2011) and Wang et al. (2013), who found differences in MDA levels between tolerant and susceptible seedlings after cold treatment at 9°C for 14 days and at 5°C for three days, respectively. This suggests that cold treatment was enough to cause oxidative stress in the susceptible check, Oryzica 1 (Yun et al., 2010).

Expression levels of the gene-encoding the SOD enzyme in Susan (cold-tolerant genotype) was 2.7 times higher than Quila 241701, but no differences were

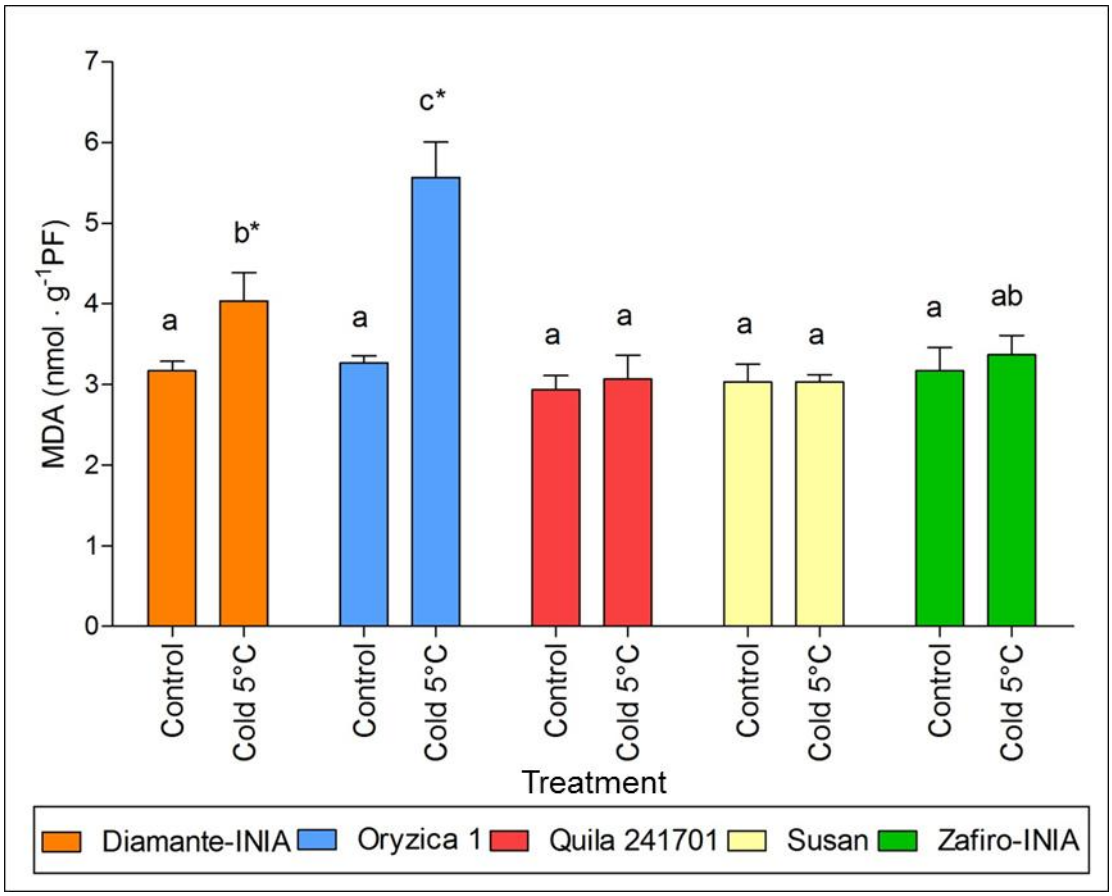

Figure 1. Lipid peroxidation after cold stress in rice seedlings. Letter and asterisk (*), represent differences between genotypes and treatment, respectively (Fisher LSD test, P≤0.05). Error bars represent the standard error of 3 biological replicates.

observed between other genotypes (Figure 2). The enzymatic activity of SOD showed that cold-tolerant genotypes increase enzyme activity with respect to a susceptible genotype (Wang et al., 2013). SOD is an intracellular enzyme that catalyzes the dismutation of O_2^- into H_2O_2 and O_2. This enzyme represents the first defense mechanism against ROS in comparison with GR and CAT that act in detoxifying H_2O_2. Some studies have revealed that tolerant genotypes express a higher level of antioxidant enzyme activity than sensitive genotypes (Guo et al., 2006).

No differences were observed between genotypes in the analysis of expression levels of the gene-encoding GR. This finding is not in agreement with other results that showed that the activity of the GR increases in the cold tolerant-genotypes (Wang et al., 2013).

The analysis of the level of the expression of the gene-encoding the CAT enzyme indicated that Oryzica 1 showed 4 to 10 times greater levels of expression than in the other genotypes (Figure 2). This high expression level of the gene-encoding CAT in Oryzica 1 is in agreement with other studies in rice (Wang et al., 2013), where a significant increase in expression levels of this enzyme was observed in a susceptible genotype.

Several mechanisms can contribute to the reduction or prevention of oxidative stress and its deleterious effects (Bonnecarrère et al., 2011). The first is related to prevention of ROS formation by the dissipation of excess energy via xanthophylls cycle involving synthesis of zeatin (Z) and antheraxanthin (A) from violaxanthin (V) (Demming-Adams and Adams, 1992).

A second mechanism is the detoxification of ROS by carotenoids or by the action of antioxidant enzymes (Saruyama and Tanida, 1995; Huang and Guo, 2005). For example, carotenoids such as lutein and neoxanthin have been associated with the process of elimination of ROS (Dall'Osto et al., 2007). Other studies have compared the contribution of enzymatic and non-enzymatic detoxification of ROS in cold susceptible genotypes (*Oryza sativa* L. spp. *indica*) and cold tolerant genotypes (*Oryza sativa* L. spp. *japonica*). However, the results have shown that there exist a complexity pattern, where enzymes increase or decrease their activity after cold stress (Bonecarreré et al., 2011).

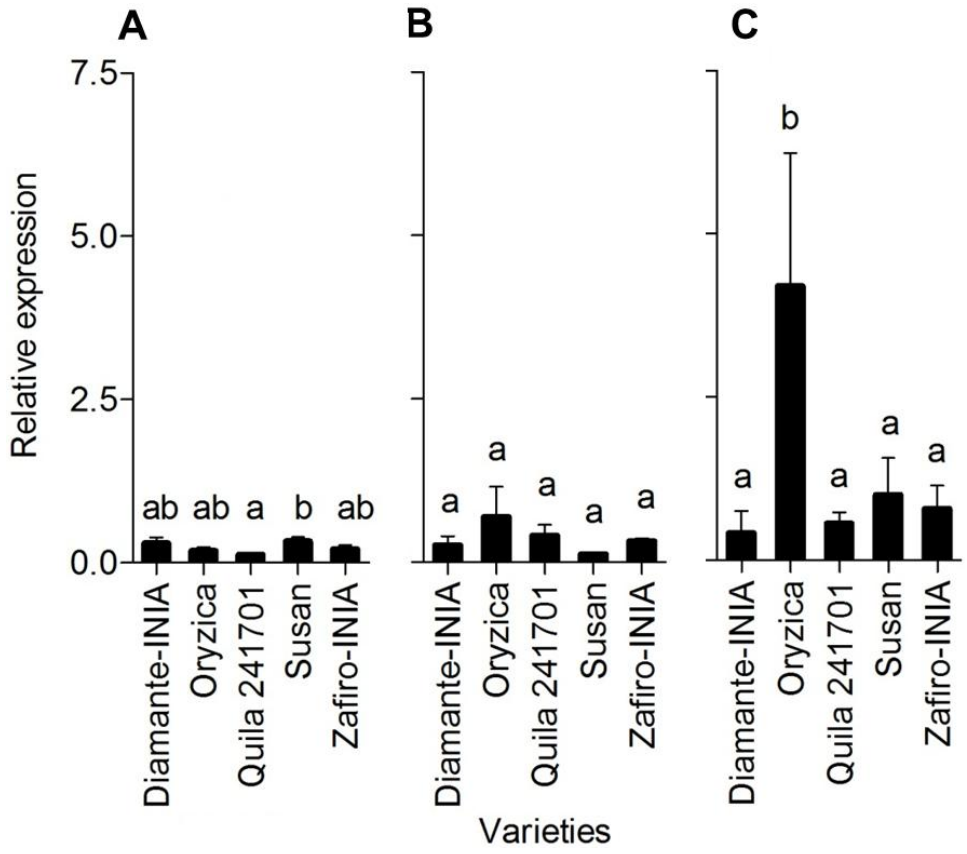

Figure 2. Relative expression levels of gene-encoding SOD (A), GR (B) y CAT (C). Different letters represent differences in expression levels between genotypes. Error bars represent the standard error of 3 biological replicates.

In general, the increase in expression levels of enzymes related with detoxification of ROS, can cause a decrease of lipid peroxidation. For example, the reduction of lipid peroxidation in rice plants after cold acclimation is related with the increase of antioxidant defense system as SOD, GR, and CAT (Kuk et al., 2003; Moradi and Ismail, 2007; Prasad, 1996). However, a high level of CAT transcript did not result in lower levels of lipid peroxidation in Oryzica 1.

The low correlation between mRNA, protein levels and enzyme activity could be explained mainly due to alternative splicing of mRNA precursors and post-translational modifications of proteins (Gygi et al., 1999; Washburn et al., 2003).The low increase in MDA concentration observed in Susan, Quila 241701 and Zafiro-INIA was not associated with the expression levels of genes-encoding antioxidant enzymes. Therefore, high levels of enzyme activities, non-enzymatic or other mechanisms could be related with cold tolerance of these genotypes. The high level of lipid peroxidation in the susceptible genotype Oryzica 1 could be the cause of the increase in the transcript levels of the gene that encodes the CAT enzyme. However, inefficient protein translation or low activity of CAT enzyme could be also explained by the low level of protection against ROS under cold stress in Oryzica 1.

Conclusions

The high level of lipid peroxidation observed in the genotype Oryzica 1 was associated with the susceptibility to cold stress. On the contrary, the low levels of MDA observed in Susan, Zafiro-INIA and Quila 241701 may indicate that these genotypes have a good mechanism to avoid lipid peroxidation after cold treatment. Unexpectedly, no relationship between lipid peroxidation and SOD and GR enzyme was observed. Also, contrary to expected results, high levels of the gene-encoding CAT enzyme in Oryzica 1 were not related to avoid lipid peroxidation. Future experiment related with enzymatic activity and analyses of non-enzymatic mechanism are necessary to elucidate the mechanism of protection against ROS in Chilean rice germplasm.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effect of plant growth regulators on regeneration of the endangered medicinal plant *Calligonum comosum* L. Henry in the Kingdom of Bahrain

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The use of *in vitro* techniques for conserving plant biodiversity and protecting rare and endangered multipurpose plant species is considered as one of the most important *ex-situ* conservation policies. Development of an efficient *in vitro* regeneration protocol of *Calligonum comosum* is important and that has achieved to protect the endangered multipurpose medicinally important desert plant in the Kingdom of Bahrain. Nodal segments were used as explants source and the effect of various plant growth regulators (PGRs) were studied for responses and to regenerate the whole plants in modified Murashige and Skoog (MS) media through direct and indirect organogenesis via callus induction. 50% explants of *C. comosum* responded to initiate shoot in presence of 4.44 µM BAP with 2.68 µM NAA after four weeks of culture while 40% shoot initiation response was the highest value in presence of 9.29 µM KI with 5.37 µM NAA after 4 weeks of culture among the treatments of KI with NAA. The highest callus induction rate of 100% was found in media containing 9.29 µM KI and 5.37 µM NAA after four weeks. Multiple initial shoots those originated from nodal segments develop calli and showed organogenic differentiation of shoots in presence of BAP and IAA. The highest shoot multiplication frequency of 15 was observed while the shoots initiated in media contained 4.44 µM BAP with 2.85 µM IAA and were transferred to 8.56 µM IAA with 2.22 µM BAP. Shoot multiplication and shoot regeneration capacity was compared in different media and the highest performance of 234 shoots /explants after second multiplication was observed while shoots initiated in presence of 13.3 µM BAP and 5.71 µM IAA. As a precautionary approach to conserve the endangered medicinal plant species in the Kingdom of Bahrain, the application of *in vitro* culture is considered as an important alternative method in *ex situ* conservation strategy in the present study.

Key words: *Calligonum comosum,* endangered plants, *ex situ* conservation, *in situ* conservation, organogenesis, plant regeneration, tissue culture.

INTRODUCTION

The Kingdom of Bahrain is an aggregate of a group of scattered islands forming an archipelago lying almost in the middle of the Arabian Gulf. The temperature is generally high with an average of 35°C, ranging from 14 to 41°C. The average rainfall is 74 mm/year, varying from 39 to 128 mm/ year. Bahrain was relatively rich in

biodiversity with 323 species of flora, those were important in relation to environment protection and national interest (El-Oqlah and Abbas, 1994). Among them, 25% of plants are used as medicinal herb by Bahrain and also by others in the Arabian Peninsula or neighboring countries (Jameel et al., 2010). The unprecedented socio-economic development in the Kingdom of Bahrain has caused part of habitat destruction, widespread ecological modification and biodiversity degradation. Hence, many plant species has became threatened and some have been considered as endangered in Bahrain (Al-Eisawi, 2003). Preserving global biodiversity is a priority project in strategic conservation plan that was designed to engage public policy and concerns affecting local, regional and global scales of communities, ecosystems and cultures (Gascon et al., 2007). *In situ* and *ex situ* conservation is applied according to the need of preservation process. *In situ* conservation is the most valuable and effective method, relies upon the conservation of plants in their living forms, particularly in their natural conditions and habitats. Considering the importance of conserving biodiversity, Al-Areen Wildlife Reserve in the Kingdom of Bahrain established in 1976 (Khanna, 2012). Under certain specific conditions, *ex situ* methods in conservation of some endangered plant species using plant tissue culture has gained a lot of interest in different aspect (Paunescu, 2006). The use of *in-vitro* techniques increase the chances of recovery of endangered species and has reduced the risk of extinction (Nadeem et al., 2000; Anburaj et al., 2011). Similarly, *in vitro* culture commonly known as tissue culture (TC) not only offer the possibilities of faster multiplication of clones of endangered plant species for conservation of genotypes, but also help to preserve some specific cells, tissues, organs in a special way for future use (Benson, 2003; Khan et al., 2012). The stimulation of endogenous growth substances by the addition of exogenous growth regulators to the nutrient media promote cell division, cell growth and differentiation of plant organs (Aboel-nil, 1997). The induction of somatic embryogenesis and organogenesis help to develop large number of regenerated plants within a short period of time by the differentiation of different types of tissues (Tripathi and Tripathi, 2003). *In vitro* plant regeneration via organogenic response of explants either direct or indirect way through axillary and / adventitious shoot regeneration is a well recognized method in obtaining large number of plants (Li et al., 2002; Fatima et al., 2009; Saini et al., 2011).

Calligonum comosum L. Henry, belongs to the family of Polygonaceae, under the local name “arta” (Muschler, 1912) and is considered as one of the endangered multipurpose desert medicinal plants in the Kingdom of Bahrain. It is perennial, small leafless woody shrub, strictly psammophil, grows in arid, sandy ecosystem which may attain a height of 2 to 3 m. Flowers are small, petals are white in color with red anthers during early spring (March and April) and young fruits are red spiny (soft) capsules (Karim, 2003). The plant is a rich source of several secondary metabolites such as flavonoids, alkaloids and phenols. It is traditionally used in folk medicine to treat rural population from microbial infections. Anthraquinones and terpenoids of *Calligonum* showed high antimicrobial potentiality (Zaki et al., 1984), anti-listerial activity (Riadh et al., 2011), anti-inflammatory as well as antiulcer activity (Liu et al., 2001). Rapid urbanization and industrialization, uncontrolled exploittation factors are correlated with loss of natural genetic resources in Bahrain (Al-Eisawi, 2003). The application of tissue culture as a biotechnological tool for the production of large number of plants within short time period particularly in the conservation of threatened medicinally important multipurpose plants has gained huge interest in the last two decades. In spite of extensive advertisement in world-wide research interests on *in vitro* multiplication method to conserve biodiversity as well as to preserve endangered medicinally important plants and pharmaceutically important compounds, no strategic ways have been used in Bahrain. Considering the above points, the objective of the present study was to investigate the role of plant growth regulators (PGRs) in developing and optimizing the protocol of regeneration of endangered medicinal plant *C. comosum*. It is the first approach in Bahrain of using *in vitro* method to propagate endangered multipurpose desert plants.

MATERIALS AND METHODS

Collection of plant materials

Stem segments of *C. comosum* L. Henry (Figure 1A) were collected from plants growing in the southern part of protected reserve forest area in Al-Areen Wild Life Park of Kingdom of Bahrain (Figure 1B). Stem segments (20 to 30 cm) were collected during March to April in 2012 for tissue culture purpose with special permission from the Park authority. Collected stem parts were cut into pieces, stored at 4ºC in closed plastic bags and were used as source of explants for culture initiation after surface sterilization.

Surface sterilization of explants

The stem segments were surface sterilized following modified

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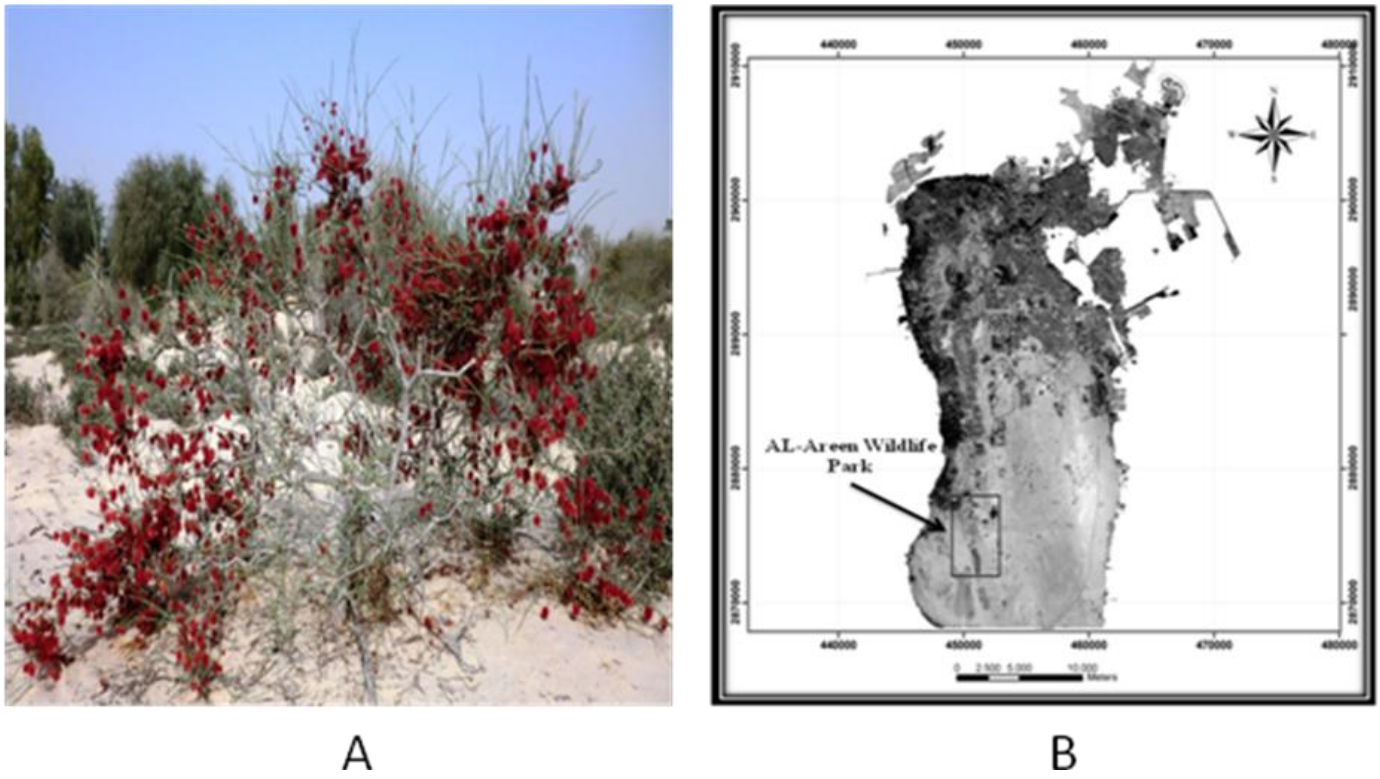

Figure 1. **A.** *Calligonum comosum* L. Henry growing in protected reserve forest area in Al-Areen Wild Life Park of Kingdom of Bahrain. **B.** Al-Areen Wildlife Park (Earth, Google, 2013).

method of Pathak and Hamzah (2008), in running tap water for 5 min to remove dust particles. Then the stem segments were kept for 5 min successively in 1% of lux solution, 0.5% of mercuric chloride solution with few drops of tween 20, 0.1% of copral solution, while the respective solutions were discarded in each step by thorough washing (5 to 6 times) with autoclaved water. Later on, 50% (v/v) Clorox solution (containing 2.625% hypochlorite) was used for 15 min and washed (5 to 6 times) with autoclaved Millipore water to remove hypochlorite nicely. Final washing in 70% ethanol for 30 s was performed before transfer in autoclaved Millipore water until use. The stem segments were sliced into smaller pieces (1.5 to 2 cm), each containing one node used as explants for culture initiation.

Culture media and conditions

The media used throughout culture were modified Murashige and Skoog (MS) media (1962) containing 0.3% of casein hydrolysate, 3% of sucrose, 1 mg/L Nicotinic acid, 1 mg/L Pyridoxine HCl, and 10 mg/L Thiamine HCl. The pH of the medium was adjusted to 5.8. The media were solidified with 0.8 to 0.9% agar and autoclaved at 121°C, for 20 min at 15 psi. Filter (0.22 µm) sterilized plant growth regulators (PGRs) of different combinations and concentrations were added in autoclaved modified MS media and poured in Magenta vessel (30 ml). The cultures were maintained at 16/8 h (light/dark) cycles with cool, white, fluorescent light intensity of 2000 to 2500 LUX, temperature of 24 ± 2 °C, 70 to 80% humidity in culture room.

Treatments for shoot and callus induction, shoot multiplication and rooting

Nodal segments collected from plants (Figure 1A) were used as explants and were cultured *in vitro* in (1) MS media and MS media supplemented (2) 4.44 µM BAP + 2.85 µM IAA; (3) 6.66 µM BAP + 2.85 µM IAA; (4) 8.88 µM BAP + 5.71 µM IAA; (5) 13.32 µM BAP + 5.71 µM IAA; (6) 4.44 µM BAP + 2.68 µM NAA; (7) 6.66 µM BAP + 2.68 µM NAA; (8) 8.88 µM BAP + 5.37 µM NAA; (9) 13.3 µM BAP + 5.37 µM NAA; (10) 4.65 µM KI + 2.85 µM IAA; (11) 6.97 µM KI + 2.85 µM IAA; (12) 9.29 µM KI + 5.71 µM IAA; (13) 13.9 µM KI + 5.71 µM IAA; (14) 4.65 µM KI + 2.68 µM NAA; (15) 6.97 µM KI + 2.68 µM NAA; (16) 9.29 µM KI + 5.37 µM NAA; (17) 13.9 µM KI + 5.37 µM NAA. Based on morphological responses, initially developed shoots in different media were sub-cultured two times for multiplication and regeneration of shoots in modified MS media supplemented with (4) 8.88 µM BAP + 5.71 µM IAA; (12) 9.29 µM KI + 5.71 µM IAA; (22) 8.88 µM BAP + 1.14 µM IAA; (25) 1% charcoal + 4.92 µM IBA; (26) 1% charcoal + 7.4 µM IBA; (27) 1% charcoal + 9.84 µM IBA; (28) 0.93 µM KI + 5.71 µM IAA; (29) 2.32 µM KI + 8.56 µM IAA; (30) 2.85 µM IAA; (32) 0.89 µM BAP + 2.85 µM IAA; (33) 0.89 µM BAP + 5.71 µM IAA; (34) 2.22 µM BAP + 8.56 µM IAA; (35) 2.22 µM BAP + 11.2 µM IAA; (36) 4.44 µM BAP + 2.27 µM TZN + 1.14 µM IAA; (37) 8.88 µM BAP + 4.54 µM TZN; (41) 3% sorbitol + 0.49 µM IBA.

Newly formed microshoots measuring 1-2 cm were cultured in rooting media (1) MS; (4) 8.88 µM BAP + 5.71 µM IAA; (29) 8.56 µM IAA + 2.32 µM KI; (31) 2.85 µM IAA in ½ MS. Rooted plantlets were taken away from culture media, washed nicely in autoclaved

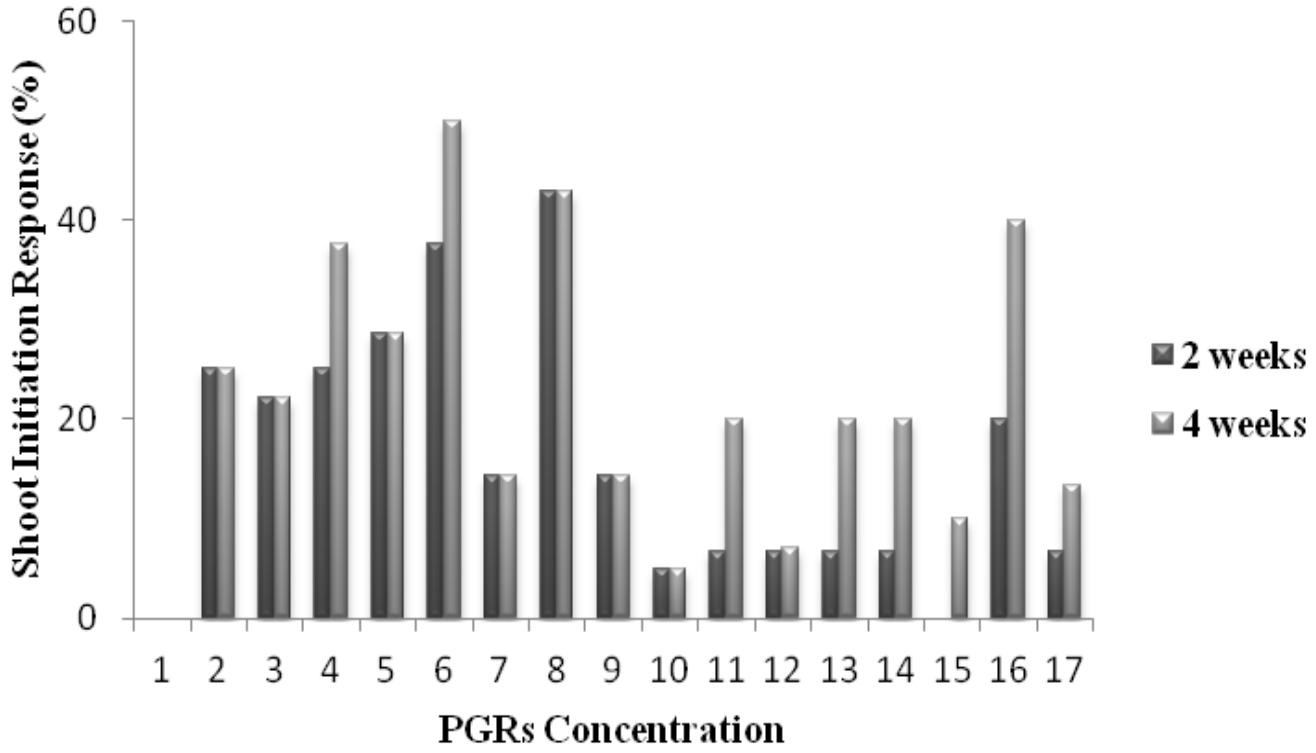

Figure 2. Effect of different plant growth regulators (PGRs) supplemented to modified MS media for shoot initiation response from nodal explants of *C. comosum* after 2 and 4 weeks of culture. Results are percentage of shoot initiation response.

distilled water to remove media from their rooting surface. The plantlets were transplanted in plastic pots containing autoclaved compost soil (1:1 mixture of peat-substrates and potting soil) and were kept in transparent small covered chamber to keep moist. The plants were acclimatized in room conditions at 25±3°C, 16/8 hs photoperiod and watered regularly at 3 days interval. Based on morphological responses of explants, percentage of shoot and callus initiation response, frequencies, percentage of shoot proliferation in first and second transfer, shoot multiplication frequency from developed callus and plant regeneration capacity per explants were calculated. The shoot initiation frequency was measured by calculating the number of shoots initiated/explants. Plant regeneration capacity was calculated by counting mean number of shoots initiated in each step based on shoot initiation frequency of explants multiplied by two cycles of mean proliferation frequencies where shoots initiated direct and indirect way of organogenic response of calli.

Experimental design and statistical analysis

The experiments were carried out by using completely randomized design (CRD) with three replications of each experiment and 3 to 5 explants per replications. Data were analyzed using SPSS (SPSS Inc. Version 17.0), statistical package. Means of 3 replicates per treatment were compared with control as well as multiple treatment groups. Mean comparisons were performed at $P \leq 0.05$ level of significance using one way analysis of variance (ANOVA) according to Duncan's multiple range test (DMRT) using JMP (version 9) statistical software.

RESULTS

Shoot and callus initiation response

Nodal segments (Figure 6A) were cultured on MS media fortified with different concentrations and combinations of cytokinins (BAP and KI) and auxins (IAA and NAA) to study the initial responses. The nodal explants responded by shoot and callus initiation response (Figure 6B and 6C). The percentage of shoot initiation response was scored after two and four weeks of culture (Figure 2). MS medium devoid of PGRs showed no shoot initiation response, explants remained fresh for one to two week then turned necrotic and died. The highest shoot initiation response of 50% was observed in media containing 4.44 µM BAP with 2.68 µM NAA after 4 weeks of culture among the all treatments. While 40% shoot initiation response was the highest value in presence of 9.29 µM KI with 5.37 µM NAA after 4 weeks of culture among the treatments of KI with NAA. Similarly, the highest shoot initiation frequencies of 37.5 and 25% were noticed in media containing 8.8 µM BAP with 5.71 µM IAA and 6.97 µM KI with 2.85 µM IAA after 4 weeks of culture respectively among the treatments of IAA with BAP and

Table 1. Effect of various growth regulators supplemented to modified MS media on *in vitro* shoot initiation response from nodal explants of *Calligonum comosum* (Arta) after 4 weeks of culture.

PGRs combinations	Mean calli number
4.44 µM BAP + 2.68 µM NAA	2.66^{A}
8.88 µM BAP + 5.37 µM NAA	2.66^{A}
8.88 µM BAP + 5.71 µM IAA	1.66^{AB}
13.3 µM BAP + 5.71 µM IAA	1.66^{AB}
4.44 µM BAP + 2.85 µM IAA	1.33^{BC}
6.66 µM BAP + 2.85 µM IAA	1.33^{BC}
6.66 µM BAP + 2.68 µM NAA	1.33^{BC}
9.29 µM KI + 5.37 µM NAA	1.33^{BC}
13.3 µM BAP + 5.37 µM NAA	1^{CD}
4.65 µM KI + 2.85 µM IAA	1^{CD}
6.97 µM KI + 2.85 µM IAA	1^{CD}
13.9 µM KI + 5.71 µM IAA	1^{CD}
13.9 µM KI + 5.37 µM NAA	1^{CD}
9.29 µM KI + 5.71 µM IAA	0.66^{DE}
4.65 µM KI + 2.68 µM NAA	0.33^{EF}
6.97 µM KI + 2.68 µM NAA	0^{G}
Media without PGRs	0^{G}

Results are means of shoots developed per explant of 3 sets of individual experiments. Means followed by the same letter are not significantly different at P ≤ 0.05

KI (Figure 2). The shoot initiation response of 39% was observed in presence of 4.44 µM BAP with 2.68 µM NAA after two weeks of culture, but after 4 weeks it reached to 50%. The highest response of shoot initiation of 28.6% was observed in media supplemented with 13.3 µM BAP and 5.71 µM IAA after two week of culture initiation, while in 4 weeks old culture it reached to 37.5 (Figure 2) among the treatments of BAP with IAA. Similarly, the shoot initiation response increased with time of culture in different media which is a common trend as observed in all treatments. The effect of KI and NAA were less in compare BAP with NAA in shoot initiation response. The highest shoot initiation frequency was 2.66 in presence of (4.44, 8.88) µM BAP with (2.68, 5.37) µM NAA respectively after 4 weeks of culture. Analysis of data showed interactive effects of different culture media containing different concentrations and combinations of PGRs in inducing initial shoots (mean) were statistically significantly different at P ≤ 0.05 level (Table 1). The induction of adventitious shoot from nodal part and induction of callus were observed in all the PGRs treatment, presented in Figure 6B and 6C. Analysis of variance of shoot initiation response with PGRs showed the prominent effect of BAP with NAA and IAA than KI with NAA and IAA to induce initial axiliary shoots. Figure 3 shows the percentage of callus initiation response in presence of different concentrations and combinations of PGRs as presented in different media after 2 and 4 weeks of culture. The callus was initiated from the cut end of the explants which touched the media. The highest callus induction rate of 40 and 100% was found in media containing 9.29 µM KI and 5.37 µM NAA after 2 and 4 weeks of culture of nodal segments respectively among all the treatments. The callus was compact, white to off white in color. The highest response of callus initiation of 60% was found in media supplemented with KI (6.97, 13.9 µM) and IAA (2.85, 5.71 µM) after 4 weeks of culture among all the treatments of IAA with KI. Under similar condition, the same medium constituents showed only 20% callus initiation after 2 weeks of culture. Among all the treatments of BAP and IAA, the highest callus induction response of 87.5% was observed while media was supplemented with 8.88 µM BAP and 5.71 µM IAA after 2 and 4 weeks. The callus was white in color initially but turned brown with time. The highest callus induction rate of 50% was observed in presence of 4.44 µM BAP and 2.68 µM NAA after 4 weeks, while 42.8% response was found in media containing (8.88, 13.3) µM BAP with 5.37 µM NAA after 2 weeks. The callus was initially compact and white in color, later on became friable and loose. There was no response for callus in media without PGRs after 2 and 4 weeks. The highest callus initiation frequency of 7 ± 0.57 was observed on medium supplemented with 8.88 µM BAP and 5.71 µM IAA after 4 weeks (Table 2). Different treatments of PGRs were compared in callus initiation response with control and multiple treatment groups and their means were compared and analyzed by analysis of variance (ANOVA) using Duncan's multiple range test at (P ≤ 0.05) level and showed significant differences. Interactive effects of culture media on development of mean number of calli per explants were statistically significant (P ≤ 0.05) presented in Table 2.

Shoot multiplication and plant regeneration

Initially, developed shoots from different media were transferred in media containing various combinations and concentrations of PGRs for first time multiplication. The effects of BAP, IAA, NAA, KI, TZN and IBA on first time multiplication of initially developed shoots and the response of shoot associated callus were presented in Table 3. The percentage of proliferated shoot from initially developed shoots, callus, shoot multiplication frequency with associated callus growth were compared in different media. The initially developed shoots were transferred in various media and 100% shoot and callus proliferation were noticed mostly. The highest shoot multiplication frequency of 15 was observed while the shoots initiated in media contained 4.44 µM BAP with 2.85 µM IAA and were transferred to 8.56 µM IAA with 2.22 µM BAP. The lowest shoot multiplication frequency

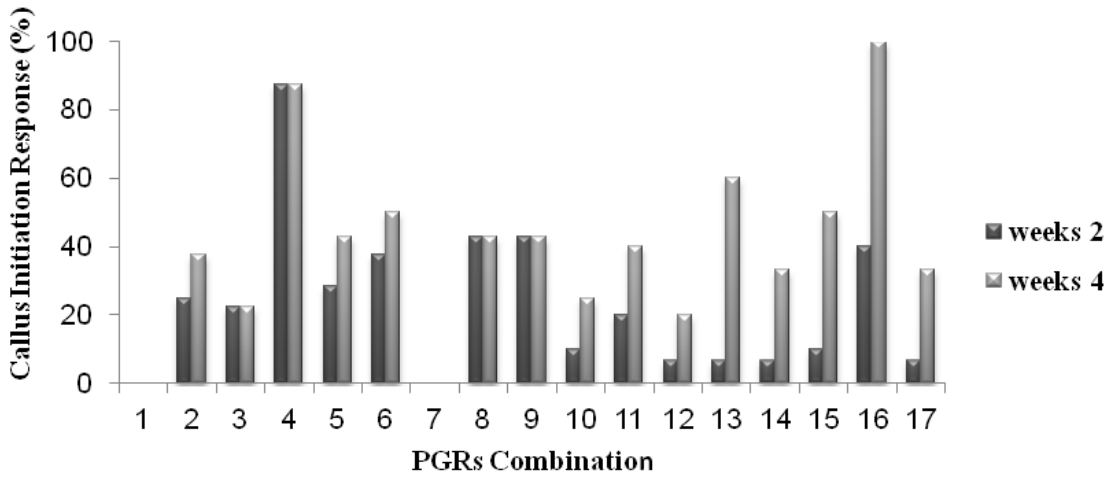

Figure 3. Effect of PGRs supplemented to modified MS media for callus initiation response of *C. comosum* after 2 and 4 weeks of culture. Results are percentage of callus initiation response.

Table 2. Effect of various growth regulators supplemented to modified MS media on *in vitro* callus initiation response from nodal explants of *Calligonum comosum* (Arta) after 4 weeks of culture.

PGRs combinations	Mean calli number
8.88 µM BAP + 5.71 µM IAA	7^{A}
4.44 µM BAP + 2.68 µM NAA	2.66^{B}
4.44 µM BAP + 2.85 µM IAA	2^{BC}
13.3 µM BAP + 5.71 µM IAA	2^{BC}
8.88 µM BAP + 5.37 µM NAA	2^{BC}
6.97 µM KI + 2.85 µM IAA	2^{BC}
6.66 µM BAP + 2.85 µM IAA	1.66^{BCD}
4.65 µM KI + 2.85 µM IAA	1.66^{BCD}
9.29 µM KI + 5.37 µM NAA	1.66^{BCD}
13.3 µM BAP + 5.37 µM NAA	1.33^{CDE}
9.29 µM KI + 5.71 µM IAA	1^{DE}
6.97 µM KI + 2.68 µM NAA	1^{DE}
13.9 µM KI + 5.37 µM NAA	0.66^{EF}
13.9 µM KI + 5.71 µM IAA	0.33^{FG}
4.65 µM KI + 2.68 µM NAA	0.33^{FG}
6.66 µM BAP + 2.68 µM NAA	0^{G}
Media without PGRs	0^{G}

Results are means of calli developed per explant of 3 sets of individual experiments. Means followed by the same letter are not significantly different at $P \leq 0.05$.

of 1 was observed while shoot initiated in presence of 13.94 µM KI with 5.71 µM IAA and 9.29 µM KI with 5.71 µM IAA, and were transferred to media containing 11.22 µM IAA with 2.22 µM BAP and 4.44 µM BAP, 2.27 µM TZN with 1.14 µM IAA. Shoot multiplication frequencies after first and second time proliferation were compared and the maximum shoot multiplication was observed in shoot initially developed in media containing 13.32 µM BAP with 5.71 µM IAA (Figure 4). The initially developed shoots in media 2, 4, 5 after transfer to different media (4, 12, 32, 33, 34, 35) developed callus which showed organogenic response efficiently by differentiating a large number of shoots. The callus showed differentiation of shoots were granular, compact, white to green in color (Figure 6D) but became brown to red in color, more compact, nodular and more embryogenic nature in subsequent culture (Figure 6E). The initially developed callus from explants being compact in nature did not differentiated into shoots in different media after (data not shown), but the calli developed after transfer of the newly differentiated shoots, were more compact and nodular, embryogenic nature and finally differentiation into large number of shoots (Figure 6F, G, H). Clustered shoots were separated and sub-cultured in the same media for multiplication. The highest shoot regeneration capacity of 234 shoot/explants were observed while initial shoots developed in media 5, were transferred to media 4 and 41 subsequently for shoot differentiation and multiplication (Figure 5). The callus developed from newly developed shoots in different media showed multiplication of shoots and differentiation of calli into new shoots too (Figure 6F, G, H). The highly defined organogenic response of calli in second multiplication cycle was noticed (Figure 6I, J). Multiplied shoots were isolated and further cultured in different rooting media (Figure 6K, L, M) and regenerated plants (Figure 6N) were transferred to soil (Figure 6O).

DISCUSSION

In the present study, both callus initiation and shoot

Table 3. Effect of various growth regulators on first time multiplication of *C. comosum* shoots those were initiated on modified MS medium supplemented with different concentration and combination of PGRs.

Media	% of proliferated shoot	% of proliferated callus (mean)	Shoot multiplication frequency	Callus growth
2 → 12	50	100	2.5	+ +
2 → 32	50	75	4	+ +
2 → 34	100	100	15	+ +
4 → 32	100	100	10	+ +
4 → 33	75	100	8.12	+ +
4 → 34	66.6	100	5	+
5 → 4	50	100	7.5	+ +
5 → 32	100	100	8.5	+
5 → 35	100	100	12.5	+
7 → 22	100	100	10	+ +
7 → 25	100	100	4	+ +
11 → 22	100	100	10	+ +
11 → 36	100	100	2	+ +
13 → 35	100	100	1	+
13 → 37	100	100	5	+ +
14 → 36	100	100	2	+ +
14 → 37	100	100	2	+
15 → 22	100	100	2	+ +
15 → 26	100	100	10	+
16 → 36	100	100	1	+ +
16 → 26	100	100	3	+
17 → 37	100	100	2	+ +
17 → 27	100	100	3	+ +

Results are percentage of proliferated shoots, calli, shoot multiplication frequency and callus growth data of three sets of individual experiments.

initiation response varied in MS medium supplemented with different PGRs. It was evident from this study that the optimum concentration of BAP with NAA was very effective in shoot initiation. Analysis of variance of shoot initiation response with PGRs showed the prominent effect of BAP with NAA and IAA than KI with NAA and IAA to induce initial axillary shoots. Similar effect of BAP with NAA for shoot induction was reported earlier in Bermuda grass (Jain et al., 2005), *Vigna unguiculata* (Muhammad et al., 2008), *Pteris vittata* (Shukla and Khare, 2012). Tripepi (1997) reported that various concentrations of BAP help to induce larger number of adventitious shoot than other PGRs in micropropagation of several ornamental plants. Similarly, in several studies BAP was more effective for axillary shoot initiation from nodal segments of *Melia azedarach* (Sen et al., 2010) and *Bacapa monneiri* (Gurnani et al., 2012). While Nodal segments of *Melissa officinalis* L. showed *in vitro* plantlet development in presence of different concentration of BAP with IAA (Mohebalipour et al., 2012). He also reported that genotypes and culture media differentially responded in organ differentiation and plant regeneration. PGRs concentrations and combinations particularly BAP with NAA and IAA both play important role in shoot initiation response in different degrees. The comparative effect of different PGRs in stimulating shoot multiplication response showed more effectiveness of BAP with NAA than BAP with IAA and it is the fact that total hormonal response has been gathered from initial culture to subsequent cultures. The ratio of auxin and cytokinin 1: 2-1:3 was more effective in organogenic response and adventitious shoot induction was reported in several studies (Makunga and Staden, 2008; Sen et al., 2009).

The highest response of callus initiation of 60% was found in media supplemented with KI (6.97, 13.9 µM) and IAA (2.85, 5.71 µM) after 4 weeks of culture. The callus developed around the cut surface of explants were sub-cultured in MS media supplemented with different concentration and combinations of BAP, KI, IAA and NAA but there was no organogenic response of calli either root or shoot initiation except its own growth. Initially, white to off-white, light yellow, less compact callus developed. Different treatments of PGRs showed significant differences in callus induction frequencies in different

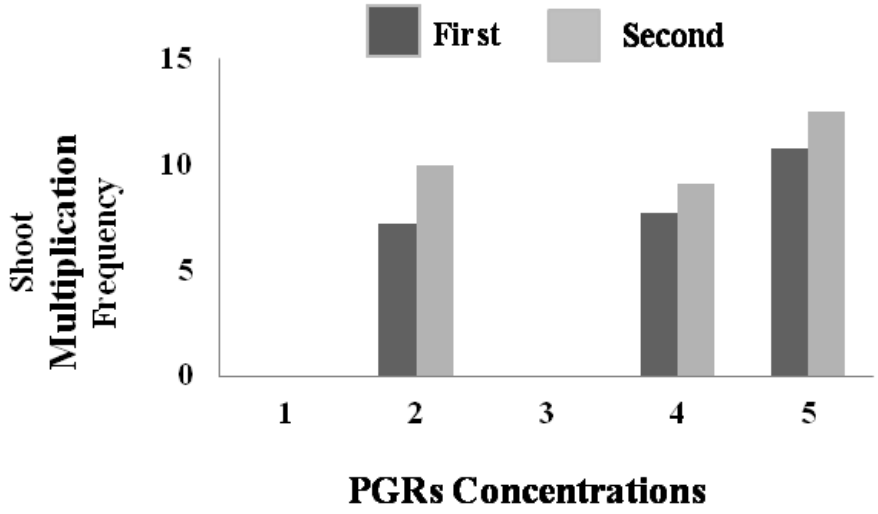

Figure 4. Effect of PGRs on shoot multiplication frequencies of *C. comosum* after 1st and 2nd transfer.

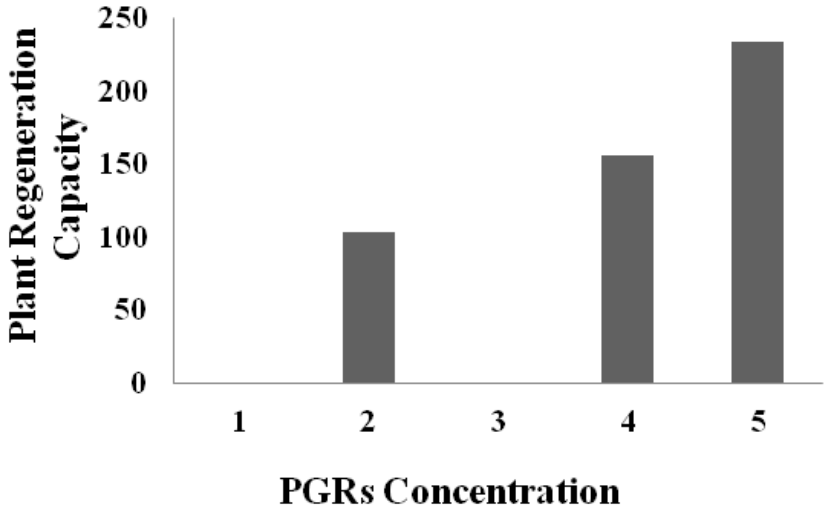

Figure 5. Effect of PGRs in shoot regeneration capacity of *C. comosum* after 2nd transfer.

media but the nature of the initially developed calli were nonembryogenic in all the media. In general, the nature of callus mass was loose, friable and watery in appearance, but with gradual exposure to BAP with IAA in two successive transfers developed compact, hard and more organized calli mass. Moreover, with increased time of culture, calli mass changed its nature, which was explained by Skoog and Miller (1957). They proposed that the type and the concentration of hormone present in medium directly affect the nature, color and biochemical composition of callus. Callus with slow growth rate, friable and watery in appearance has been considered non embryogenic and less regenerative potential (Moris and De-Macan, 1994), this was observed in our experiments also.

Newly differentaiated axillary shoots that developed in presence of 4.4 µM BAP with 2.85 µM IAA, 8.88 µM BAP with 5.71 µM IAA, 13.32 µM BAP with 5.71 µM IAA showed good shoot regeneration capacity during subculture in media containing 8.56 µM IAA with 2.22 µM

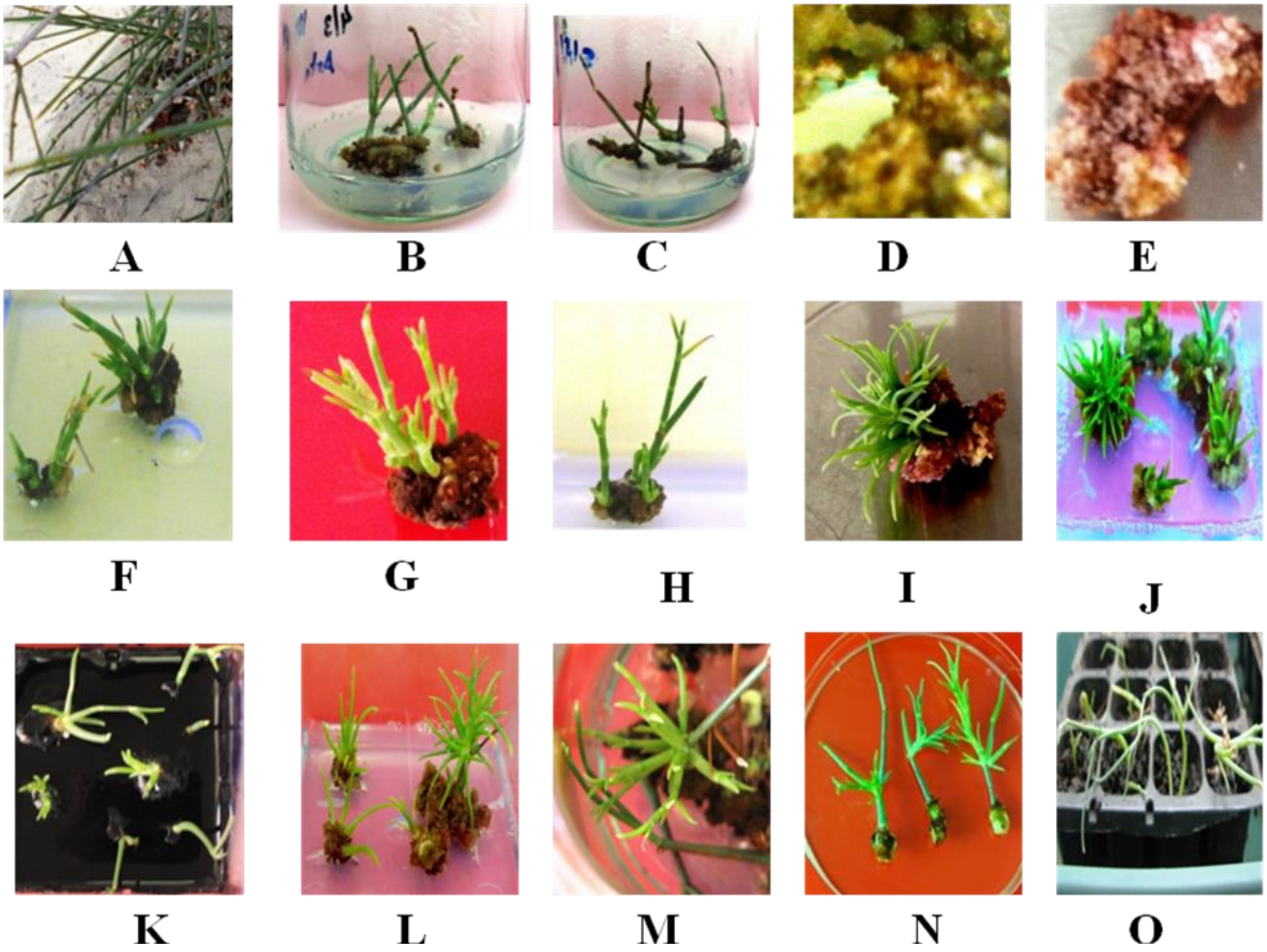

Figure 6. Stages of organogenic response of *C. comosum* in plant regeneration from nodal segments. **A.** Explants were collected from plants growing in Al Areen Wildlife Park in the Kingdom of Bahrain. **B, C.** Stem segments with nodal part in culture media showing shoot and callus initiation response. **D, E.** Callus of *C.comosm* which showed differentiation of shoots. **F, G, H.** Initial shoots showing multiplication of shoots in large number after first transfer in different media. **I, J.** Multiplied shoots were transferred to in different media for proliferation in different media. **K, L, M.** Proliferated shoot were transferred to rooting media. **N.** Micro-shoots showing initial root development. **O.** Plantlets growing in soil pots.

BAP, 2.85 µM IAA with 0.89 µM BAP, 11.22 µM IAA with 2.22 µM BAP, respectively. During this subculture of shoot, calli developed around the new shoot showed differentiation and large number of shoots developed by indirect embryogenesis from calli. The interacting effect of BAP with IAA was more prominent in differentiation of shoot as well as shoot regeneration from calli we observed here. Similar effect of BAP and IAA on shoot regeneration was observed in melon genotypes (Melara and Arias, 2009), and peanut (Iqbal et al., 2011). BAP and IAA are effective to increase the regeneration potential of peanut calli (Iqbal et al., 2011). The highest shoot regeneration capacity of 234 shoots per explant was observed, while shoots were initiated in presence of BAP with IAA, and was subsequently cultured in media containing the same hormones but different concentrations. The synergistic effect of BAP and IAA is more compare to other PGRs in regenerating large number of shoots in this plant. According to Coenen and Lomax (1997), auxins are known to exhibit synergistic, antagonistic and additive interactions with cytokinins at multiple levels depending on the plant species and tissue types in regulating responses. Similarly, Mohebalipour et al. (2012) observed that addition of BAP and IAA to MS medium is more effective than other hormones which significantly increased the number of shoot induction per

explants in most of the genotypes of *M. officinalis* L. The plant regeneration capacity of 2000 to 3000 plants from one shoot after long subculture of *Salvia africana-lutea* L. was reported (Makunga and Staden, 2008) where BA and IAA were used for subcultures. Plant regeneration ability in the culture medium is usually enhanced by the addition of auxins and cytokinins which is very common (Varshney et al., 1996; Fatima et al., 2009). Similarly, the effect of BAP as the most reliable, useful PGR for shoot proliferation, for breaking dormancy in several medicinal plant species were also reported (Tavares et al., 1996; Meszaros et al., 1999; Da Silva et al., 2005; Ghiorghita et al., 2005). The well developed regenerated shoots of *C. comosum* were transferred for rapid multiplication as well as for root development and finally rooted plants were transferred in soil. The current studies highlighted differential response of auxin and cytokinin on direct and indirect shoot regeneration of desert shrub. The success of this protocol offers a high efficient method for micropropagation of *C. comosum* which would be beneficial for the plant tissue culturist, pharmaceutical and nursery industries where regular supply of plants is important part. Moreover, protocol will be helpful in further research in biodiversity conservation of desert plants in this Arab region.

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Rock phosphate solubilization by the ectomycorrhizal fungus *Laccaria fraterna* and its associated mycorrhizal helper bacterial strains

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Phosphorus (P) is mostly present in the form of insoluble phosphates in soil and so plants cannot utilize it. Plant roots are often associated with mycorrhizal fungi and other microorganisms that effectively can absorb P and other elements from the soil. In the present study, few mycorrhizal helper bacterial (MHB) strains such as *Pseudomonas putida*, *Erwinia herbicola* and *Bacillus subtilis* were isolated and identified from surface sterilized basidiomata and mycorrhizosphere of an ectomycorrhizal fungus *Laccaria fraterna* association with *Eucalyptus globulus*. To evaluate phosphate solubilization of the fungus and its MHB strains, they were grown on rock phosphate (RP) and tricalcium phosphate as sole P sources in Pikovskaya's liquid medium. The fungus and its MHB showed varying levels of phosphate solubilization activity in the presence of different carbon and nitrogen sources. These fungus and MHB had the potential to solubilize these phosphates by decreasing the pH and confirmed that phosphate solubilization is accompanied by acid production. Thus, the evidence that fungus and MHB strains do convert insoluble phosphates such as RP into soluble forms, may not only replace expensive phosphorous fertilizer in agriculture field but also lead to better mobilization of the fertilizers added to soils.

Key words: Ectomycorrhizal fungi, *Laccaria fraterna*, mycorrhizal helper bacteria, phosphate solubilization, rock phosphate.

INTRODUCTION

Phosphorus (P) is the second major nutrient next to nitrogen required by plants. However, many soils throughout the world are P deficient while nearly 95 to 99% of soil P is present in the form of insoluble phosphates (Vassileva et al., 1998) and only 1 to 5% is available in the form of soluble, plant-available form (Molla et al., 1984). To overcome the deficiency, large amount of chemical P fertilizers is being applied to soil. Despite this, a large proportion (75 to 90%) of fertilizer P is quickly transformed into the insoluble form (Omar, 1998), thus making a continuous application necessary (Abd Alla, 1994). Moreover, widespread phosphorus

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deficiencies throughout the world's soil make use of phosphorus fertilizers an expensive necessity for agricultural crops worldwide.

Many soil fungi and bacteria are known to solubilize inorganic phosphates (Asea et al., 1998). Phosphates solubilized by these microorganisms are absorbed more efficiently by plants through mycorrhizal-association that occurs between the plant roots and surrounding soil (Landeweert et al., 2001). Ectomycorrhizal fungi (EMF) that live in symbiotic association with root of most terrestrial plants play an important role in forest ecosystems. Ectomycorrhizal symbioses are mostly formed by basidiomycetes and some by ascomycetes. The extramatrical hyphae of the fungus explore the soil, travelling long distances beyond the nutrient depletion zone. Minerals like N, P, K, Ca, S, Zn, Cu and Sr are absorbed from soils and translocated to the host plant. In turn, the plant supplies the fungus with carbohydrate necessary for its growth (Chellappan et al., 2002). The EMF *Laccaria fraterna* is widespread throughout the world, where *Eucalyptus* and other ectomycorrhizal associated plants are grown (AnithaChristy et al., 2014). *L. fraterna* is an early colonizing fungus that produces large number of basidiomes and dominates disturbed land and farm sites (Tommerup et al., 1991). Studies showed that many bacterial strains were able to promote ectomycorrhizal symbioses and these bacterial strains have been called as mycorrhizal helper bacteria (MHB) (Duponnois, 2006).

In EMF, mostly basidiomycetes positively interact with MHB except in EM ascomycete fungi *Tuber melanosporum*, where Mamoun and Olivier (1992) reported the presence of soil pseudomonads. The MHB strains that have been identified thus far belong to bacteria of the gram-negative and gram positive groups such as *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia* beside some *actinomycetes*. Application of these microorganisms along with EMF enhances the availability of scarce nutrients such as N, P and micronutrients through nitrogen fixation, phosphate solubilization or as biological disease suppression. Thus, they might be reducing the use of more P fertilizer and increased crop yield (Aspray et al., 2006). MHB interactions in *L. fraterna* have not been adequately explored. So in the present study, the RP solubilization ability of MHB and the ectomycorrhizal fungus *L. fraterna* were investigated.

MATERIALS AND METHODS

Microorganisms

The ectomycorrhizal fungus *L. fraterna* was isolated from the basidiomata associated with *Eucalyptus globulus* plantations in the Nilgiri hills of the state of Tamilnadu in Southern India. The sporocarp and mantle of *L. fraterna* were surface sterilized using sterile distilled water, chopped, homogenized in 10 ml sterile distilled water using a homogenizer. A 1.0 ml aliquot of this was then plated onto Tryptic Soy Agar containing cycloheximide (75 mg/L) and incubated at 25°C for three to five days.

For isolation of bacteria, soil samples each weighing 1.0 g were taken from the mycorrhizosphere of the *E. globulus* roots. The soil samples were suspended in one-quarter strength Ringer's solution. The suspension was then diluted to 10^{-4} dilutions and 200 µL of diluted soil samples were spread on Tryptic-Soy agar medium (TSA) for estimation of total number of bacterial colonies (Kirchner et al., 1993) and on P1 medium (Katoh and Itoh, 1983) for fluorescent pseudomonads. Plates were incubated at 25°C. Bacterial colony forming units (cfu) were counted after 3 days for the fast growing bacterial colonies and after 5 days for the slower-growing colonies.

Bacterial strains were identified as *P. putida*, *E. herbicola* and *B. subtilis* using phenotypic and physiological tests. They were further confirmed using partial 16S rRNA gene amplication (data not shown) (Bending et al., 2002). These strains were maintained on TB agar medium as well as in glycerol stock (Sambrook et al., 1989).

Media and growth conditions

Fungal cultures were maintained on Modified Melin-Norkrans (MMN) (Marx, 1969) medium at 25 ± 2°C in the dark. Fungal mycelial discs of 1.0 mm were grown on MMN medium for 10 days (Marx and Bryan, 1975), then transferred to Pikovskaya's (PKS) medium (Pikovskaya, 1948) with tricalcium phosphate (Ca_3 $(PO_4)_5$) (TCP) as sole P source.

Single colony of each bacterium was picked up and suspended into 10% TS broth. After 48 h at 25°C, culture was centrifuged (3 300 *g*, 25°C, 10 min), washed once, and resuspended in deionized water to obtain an OD of ~ 0.7 at A600 nm. Flasks inoculated with 10 days old mycelia discs of the fungus and 50 µL of each bacterial suspension, were incubated at 25 ± 2°C in the dark for 20 days (Deveau et al., 2007). Phosphate solubilizing ability of the fungus and MHB strains were tested on six different liquid media with 0.5% TCP as sole P source after 20 days (Table 1). Further experiments were performed on PKS medium with rock phosphate (RP-140) as sole P source.

Effect of different carbon sources such as cellulose, cellobiose, carboxyl methyl cellulose (CMC), lactose, maltose and fructose instead of glucose (Table 2) were also checked for P solubilization. For determining the effect of different nitrogen sources on P solubilization, $(NH_4)_2SO_4$ was replaced with 0.05% of different nitrogen salts such as Urea, KNO_3, $NaNO_3$, NH_4Cl, $NaNO_2$ (Figure 2). Phospholytic activity of fungus along with MHB strains without any carbon and nitrogen sources acted as control and measurements were made after 20 days at 25 ± 2°C in the dark

Solubilization of phosphorus from rock phosphate

The RP -140 samples which contained ~18.9% of P_2O_5 was used for all the experiments. Quantitative estimation of RP solubilization was carried out in PKS medium amended with 0.25% (w/v) of RP as sole P source for duration of 20 days.

Estimation of phosphorus

Cultures were checked after 20 days to see the drop in pH and increase in concentration of P released in the medium. After centrifugation at 12 000 rpm for 20 min, the pH of the culture medium was measured with a pH meter. Dissolved phosphate concentration in the culture filtrate was measured using Murphy and Riley's (1962) method. Measurement of phosphate was expressed as µg/ml of phosphorus released into culture medium.

Table 1. Composition of different media used for this study.

Component g/L	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6
Glucose	20	10	100	10	10	10
$(NH_4)_2SO_4$	1.0	-	-	0.5	0.5	0.1
$MgSO_4.7H_2O$	0.5	0.4	0.2	0.1	0.1	0.25
Yeast extract	0.2	0.5	-	0.5	-	0.5
KCL	-	-	-	0.2	0.2	0.2
NaCl	-	1.0	-	0.2	0.2	-
$FeCl_3$	Trace	-	-	-	-	-
$FeSO_4.7H_2O$	-	-	-	0.002	0.002	-
$MnSO_4.H_2O$	Trace	-	-	0.002	0.002	-
$MgCl_2.6H_2O$	-	-	-	-	-	5.0
$CaCl_2$	-	0.2	-	-	-	-
NH_4NO_3	-	1.5	0.5	-	-	-
$ZnSO_4$	-	-	0.004	-	-	-
$Ca_3(PO_4)_5$	5.0	5.0	5.0	5.0	5.0	5.0
pH	6.8	7.0	5.0	7.0	7.0	7.0

Medium 1, AYG: Halder et al. (1991); medium 2, Kim et al. (1997); medium 3, Vassilev et al. (1998); medium 4, PKS; Pikovskaya (1948); medium 5 and 6, NBRIY; Nautiyal (1999).

Table 2. Effect of different Carbon and Nitrogen sources.

Carbon source	P concentration (µg/ml)	Final pH	Nitrogen sources	P concentration (µg/ml)	Final pH
Glucose	528	4.8	$(NH_4)_2SO_4$	622	4.2
Fructose	399	4.7	Urea	484	6.5
Lactose	380	4.65	KNO_3	300	5.08
Cellobiose	482	4.8	$NaNO_3$	382	4.5
Maltose	280	5.4	NH_4Cl	398	4.3
Cellulose	228	5.3	$NaNO_2$	322	5.4
Carboxyl Methyl Cellulose	200	5.3	Control	82	6.18
Starch	180	5.35			
Control	85	6.11			

Statistical analysis

All experiments have been conducted in triplicates and average mean values were estimated.

RESULTS AND DISCUSSION

Solubilization of insoluble phosphates

To check the phosphorus solubilizing ability of the fungus *L. fraterna* and all MHB strains such as *P. putida*, *E. herbicola* and *B. subtilis*, we used TCP as sole P source and used six different media (Table 1) to figure out which medium supported best results. We noticed among six different media (Figure 1), that PKS medium and AYG medium (Halder et al., 1991) showed maximum solubilization. PKS medium showed maximum P solubilization at the rate of 722 µg/ml of P from 0.5% TCP with decrease in pH from 7.00 to 4.53 when used with 10% glucose and 0.5% $(NH_4)_2SO_4$. While AYG medium with 20% glucose and 1% $(NH_4)_2SO_4$ released 916 µg/ml of P from 0.5% TCP with decrease in pH from 6.88 to 4.05 was observed in culture filtrate after 20 days and in remaining other three media low level of P solubilization was observed.

Among these six different media, PKS medium proved to be most cost effective based upon the amount of glucose utilization and corresponding efficacy of P solubilization (Pradhan and Shukla, 2005). Recent results with *L. fraterna* (AnithaChristy et al., 2014) and other fungi (Pradhan and Shukla, 2005) have also confirmed that PKS medium was most cost effective on the basis of P solubilization efficacy. Therefore for further studies PKS medium was used.

Phosphate solubilization was usually accompanied by a decrease in the pH of the medium by all these strains.

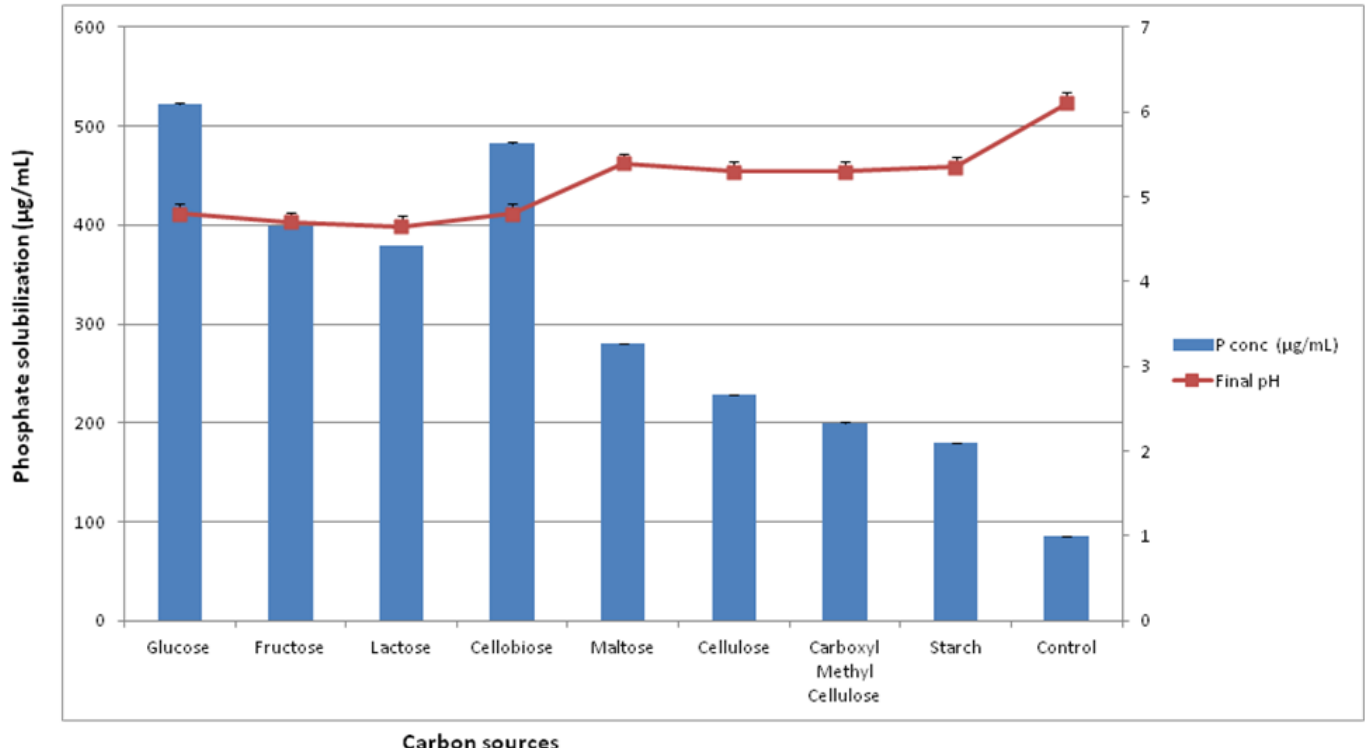

Figure 1. Effect of carbon sources on 'P' solubilization using *L. fraternal* and its MHB strains.

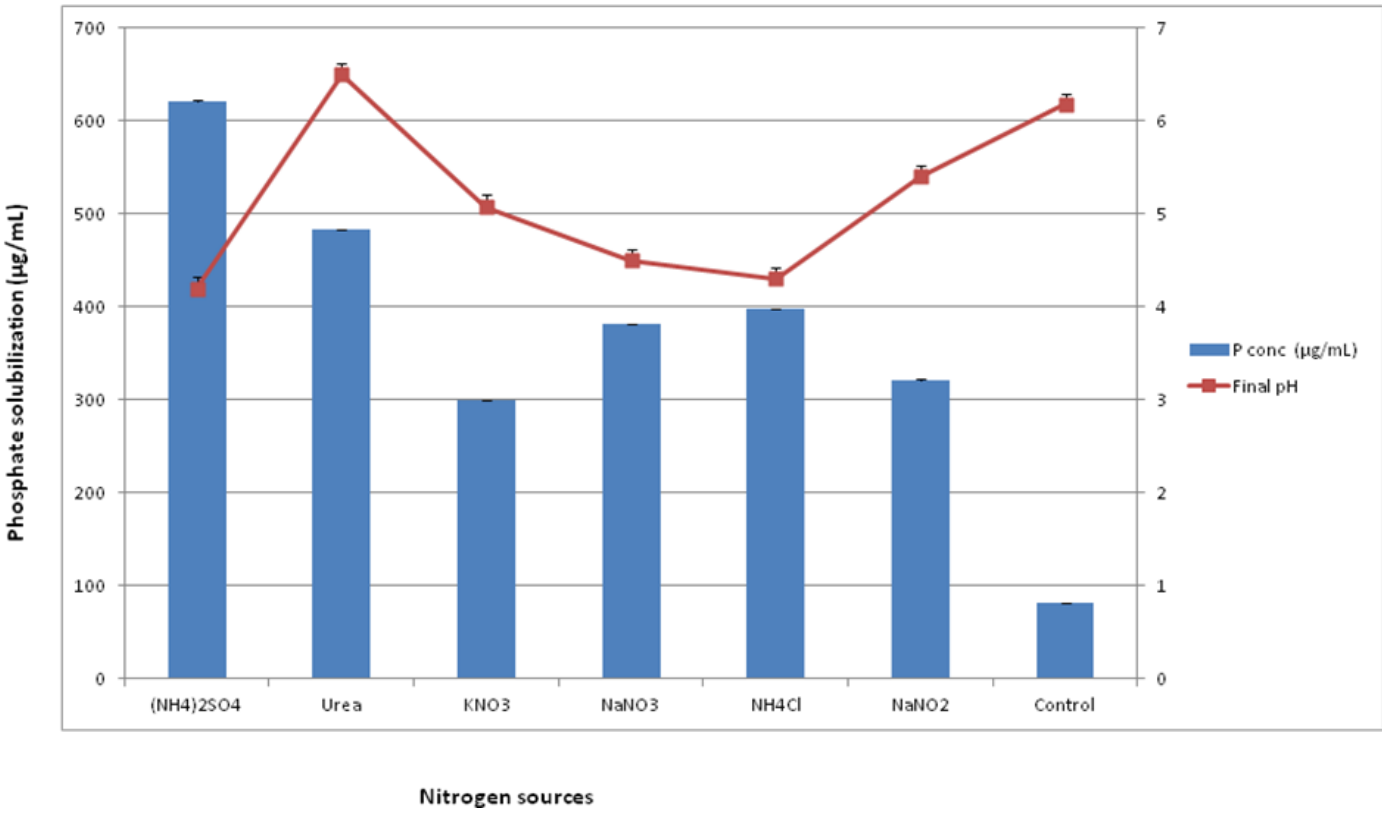

Figure 2. Effect of nitrogen sources on 'P' solubilization using *L. fraternal* and its MHB strains.

L. fraterna solubilized 116 µg/ml of phosphorus from 0.5% TCP and caused a decrease in pH from 7.0 to 4.2 in 20 days. When RP was used as sole P source, *L. fraterna* released 85 µg/ml of P in PKS medium after 20 days of incubation. In comparison, an *Aspergillus sp.* isolated from rice field soils of Orissa, India released 58 µg/mL of P after seven days of incubation in PKS medium with RP as sole P source (Pradhan and Shukla, 2005). *L. fraterna* and the other MHB strains solubilized 928 µg/ml of phosphorus in 20 days with a reduction in pH reduced from 7.0 to 4.16. It was lower when compared to TCP as sole P source because of the complexity of RP's structure. Similar finding was also observed by Pradhan and Shukla (2005).

Phosphorus solubilizing microorganisms mostly dissolved insoluble phosphates by the production of organic acids (Khan et al., 2010) such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate and others to decrease the pH. These organic acids solubilized RP using acidification, chelation, and exchange reactions (Bashan et al., 2013b, Omar, 1998). The reduction in pH and the extent of soluble phosphate formed was negatively correlated.

Earlier reports states that the soluble or insoluble phosphates are dissolved using acidification while showing some phosphorus solubilizing activity. AnithaChristy et al. (2014) also observed that oxalic acid was secreted by *L. fraterna* to solubilize RP. In most soils, proton substitution reactions are driven by microbial production of organic acids, which is represented generically by the following equation:

$$(Ca^{2}+)\ m\ (PO4^{3-})\ n + (HA) = (H^{+})\ (PO4^{3-}) + (Ca^{2+})\ (A^{-})$$

There is no stoichiometry in the equation, because of the complexity of CaP chemistry, release of microbial produced organic acids (HAs) or differing numbers of dissociable protons released to their surroundings (Goldstein, 1986). The organic acids secreted directly dissolve the mineral phosphate either as a result of anion exchange of PO_4^{2-} by acid anion or by chelating both Fe and Al ions associated with phosphate molecule (Gyaneshwar et al., 2002).

Factors affecting solubilization of phosphorus

The role of carbon source is important in mineral phosphate solubilization as the carbon source determined the type of organic acid to be secreted which played an important role in solubilization (Di Simine, 1998). Thus production of acid greatly influenced phosphate solubilization activity. Nature of acid produced during solubilization is more important than the quantity of the acid (Agnihorti, 1970). Phosphate solubilization activity of fungus and MHB strains was tested in the presence of five different carbon and seven different nitrogen sources by replacing glucose and $(NH_4)_2SO_4$, respectively of the PVK medium. The fungus and MHB strains showed different amounts of phosphate solubilization activity in the presence of different carbon sources and nitrogen sources. The fungus and MHB strains showed varying amounts of phosphate solubilization activity in the presence of various carbon sources such cellulose, cellobiose, carboxyl methyl cellulose (CMC), lactose, maltose and fructose. Glucose, cellobiose, fructose, lactose decreased the pH of the medium to a maximum extent and caused highest solubilization of phosphate, followed by maltose, cellulose, CMC and starch (Table 2).

The growth of *L. fraterna* on PKS medium with glucose as carbon source also supported maximum phosphate solubilizing activity (AnithaChristy et al., 2014). This observation is also in line with other earlier reports. *A. aculeatus* and *Aspergillus sp.,* also showed maximum phosphate solubilizing activity on glucose (Narsian and Patel, 2000; Pradhan and Shukla, 2005). Fasim et al. (2002) have reported that most bacterial isolates solubilized P well in presence of glucose, while other researchers have showed varying degrees of utilization and solubilization in presence of a wide range of carbon sources. Our results correlated with results of Nautiyal et al. (1999). Control flask even without the addition of a carbon source showed some growth due to the presence of yeast extract in the medium. It also showed a small drop in pH and P solubilization

Nitrogen salts having either an ammonium or nitrate group or both were used as nitrogen sources for this study. It is known that nitrogen sources such as ammonium nitrogen or nitrate nitrogen significantly influenced phosphate solubilization by ectomycorrhizal fungi (Lapeyrie et al., 1991). In our experiment, we observed that $(NH_4)_2SO_4$ was used efficiently in reducing the pH of the medium to 4.2 while showing also maximum solubilization of 622 µg/ml of P (Table 2). Here also the control flask showed a drop in pH and P solubilization due to the presence of yeast extract and glucose. When compared to $(NH_4)_2SO_4$ the remaining nitrogen sources showed only moderate increase in P solubilization. This finding was also confirmed by the effects of different media on P solubilization (Table 1). AYG and PKS media confirmed that *L. fraterna* and MHB strains used $(NH_4)_2SO_4$ as nitrogen source showed maximum P solubilization. Low levels of P solubilization were observed in other media containing NH_4NO_3 (Kim et al., 1997; Vassileva et al., 1998) and medium with lower concentration of $(NH_4)_2SO_4$ (NBRIY medium) as nitrogen sources (Figure 2). Previous reports on phosphorus solubilizing microorganisms (Whitelaw, 2000; Pradhan and Shukla, 2005; Khan et al., 2010; AnithaChristy et al., 2014) have also confirmed that phosphate solubilization depends upon the presence of ammonium as the nitrogen source. It has also been reported that in the presence of ammonium nitrogen, calcium phytate and calcium phosphate were easily solubilized, due to acidification (Salsac et al., 1982).

Nitrogen sources such as ammonium nitrogen or nitrate nitrogen significantly influenced phosphate solubilization which uses different mechanisms to generate of acidity in the culture (Lapeyrie et al., 1991; AnithaChristy et al., 2014). Our observation was similar. However, there is no significant correlation between the amount of phosphate solubilization and drop in pH. Pradhan and Shukla (2005) noticed that when urea was used as a nitrogen source, the pH decreased from 7.0 to 5.23. However in our study, urea decreased the pH only to 6.5 without compromising phosphate solubilization. Besides acid production there are many factors that also influenced phosphate solubilization (Whitelaw, 2000).

MHB strains and their association with mycorrhizal fungi deserve special attention because of their practical importance in crop production and their potential applications in agriculture, horticulture and forestry for nutrient mobilization from soil minerals, fixation of atmospheric nitrogen, and protection of plants against root pathogens (Frey-Klett et al., 2007). MHB strains involved in RP solubilization along with *L. fraterna* were identified as *P. putida*, *E. herbicola* and *B. subtilis*. Frey-Klett and Garbaye (2005) showed that, in a forest nursery, the proportion of phosphorus-solubilizing fluorescent pseudomonads was much higher in Douglas-fir-*L. bicolor* ectomycorrhizas than in the surrounding root-free soil. Similarly, Calvaruso et al. (2007) also observed that the oak (*Quercus sessiliflora*)-*Scleroderma citrinum* mycorrhizosphere significantly selected very efficient strains for phosphorus mobilization from the organo mineral horizon of a forest composed of various fungal and bacterial strains. When associated with plant roots, mycorrhizal fungi received up to 30% of the total carbon fixed and frequently transformed it into trehalose, a disaccharide that has been proposed to act as a carbon sink (Lopez et al., 2007). Reports showed that this trehalose was responsible for the selection of specific bacterial communities in the mycorrhizospheres of tree roots (Uroz et al., 2007). Dunstan et al. (1998) showed that the sporocarp of *L. fraterna* associated with *E. diversicolor* showed colonization with *Bacillus sp.* and *Pseudomonas sp.* increased mycorrhizal formation. They increased the formation of first order ectomycorrhizal roots (Bending et al., 2002). Most of the *Laccaria sp.* showed an association with *Pseudomonas* sp. This *Pseudomonas sp.* protects the mycorrhizal fungi against virulence of other plant-pathogenic fungal isolates (Schelkle and Peterson, 1997). It has been showed some EMF and arbuscular mycorrhizas are associated with *E. herbicola*. *E.herbicola* produced indole 3 acetic acid (IAA) (Hamil et al., 1993) an auxin required for organogenesis in EMF when secreted at lower concentrations (Kaska et al., 1999).

Conclusions

The results of the present study show the effective utilization of TCP and RP by *L. fraterna* and its MHB strains under different conditions. Thus, the use of these organisms could present a better strategy to improve plant growth than the use of expensive phosphorous fertilizers.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Effects of wall materials and lyophilization on the viability of *Weissella confusa*

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The effects of wall materials and encapsulation by lyophilization on the viability of *Weissella confusa* were evaluated. Aloe vera gel, sodium casein at 5 and 15% p/v, sodium alginate at 2% p/v, buffer phosphate, and a mixture (Aloe vera gel, sodium casein, and sodium alginate) as wall materials, were used. Bacteria without encapsulation *W. confuse* as control were used. Encapsulated bacteria were freeze dried for 48 h, in order to determine their viability in the freezing and sublimation-drying stages. Results indicate that bacteria without encapsulation, showed greater loss of viability in the sublimation-drying stage. All the wall materials evaluated, may be used for encapsulation of bacteria, because at the end of the freeze-drying process, the encapsulated bacteria showed higher viability percentages than non-encapsulated bacteria, with significant statistical difference ($p<0.05$). The protective effect of wall materials was higher in the sublimation-drying stage, compared to freezing stage.

Key word: Aloe, *Weissella*, probiotic, encapsulation.

INTRODUCTION

Probiotic lactic acid bacteria (LAB) are useful for the dairy and nutraceutical industry, due to their applications to human and animal health (Reddy et al., 2009). Several studies have demonstrated the probiotic potential of *Weissella confusa*, its antimicrobial activity against pathogenic microorganisms, including *Helicobacter pylori* (Nam et al., 2002), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella pneumonic* (Serna-Cock et al., 2012), and its ability to adhere itself to the vaginal and intestinal epitheliums (Ayeni et al., 2011; Lee et al., 2012). In the food, probiotics processing is important, the concentration of probiotic bacteria (WHO / FAO, 2006), and the techniques are used to maintain their viability (Carvalho et al., 2004). Encapsulation techniques have been developed and successfully used in the preservation and protection of probiotic LAB. In encapsulation, the material used to trap the substance or microorganism to be encapsulated is called encapsulation material, cover membrane, shell, vehicle, wall material, or external phase matrix (Serna-Cock and Vallejo-Castillo, 2013). Encapsulation of LAB reduces damage caused by

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Abbreviation: LAB, Lactic acid bacteria.

external factors such as storage conditions (time, temperature, moisture, oxygen) (Burgain et al., 2011), also, the encapsulation of LAB, decreases the degradation of bacteria in the human gastrointestinal tract, where the pH is less than 2.00 (Kailasapathy, 2006). Lyophilization (freeze drying), is an encapsulation technique consisting in the elimination of water of a product, by means of sublimation of free water in the solid stage, (previous freezing of the product), followed by vacuum pressure application (Abdelwahed et al., 2006). Sublimation occurs when vapor pressure and ice surface temperature are below water triple point (Song et al., 2005). Lyophilization is one of the best methods to conserve the properties of biological products (Shui et al., 2006). Lyiophilization-encapsulated probiotics are more stable along storing, especially at low temperatures and inert atmospheres (nitrogen or vacuum) (Manojlović et al., 2010).

Khoramnia et al. (2011), used skimmed milk, sucrose, and lactose as wall materials in the lyophilization-encapsulation of *Lactobacillus reuteri*; these authors report viabilities of 96.4 and 73.8%, for 6-month storage at 4 and 30°C, respectively. Carvalho et al. (2003) evaluated the effect of sorbitol and monosodic glutamate in a skimmed milk solution at 11% on the vialibility of stored of *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Enterococcus durans,* and *Enterococcus faecalis,* using encapsulation by lyophilization. The findings show that sorbitol and monosodic glutamate maintain the viability of the strains along prolonged storage, with no significant differences between the viability of encapsulated and free cells. Chan et al. (2011), obtained cell viabilities of 5%, using liofilization and sodium alginate (2%) and sodium caseinate (10%) as wall materials, for stabilizing the viability of encapsulated cells. *Lactobacillus paracasei ssp. paracasei F19 (Lactobacillus F19)* and *Bifidobacterium lactis Bb12* in sodium caseinate (15% w/w) were microencapsulated, and remained the cell viability in 16 and 43%, respectively (Heidebach et al. (2010). Sodium caseinate offer suitable physical and functional properties for microencapsulation, due to its amphiphilic character and emulsifying characteristics (Hogan et al., 2001). Studies show improving in viability when different types of wall materials as polysaccharides and proteins were included. Hence, cell viability during the lyophization encapsulation process is affected by the type of strains, the parameters of the lyophilization process, the physiological cell state, and the use of cryoprotectors (Abadias et al., 2001). Thus, it is necessary to carry out specific encapsulation studies for each type of strain.

The main causes leading to cell viability loss during lyophilization are, ice formation and high osmolarity, (resulting from high internal solutes concentration) which causes cell membrane damage, macromolecular denaturalization, and water loss (Huang et al., 2006). The choice of a cryoprotector is important in maintaining the viability of LAB during the dehydration and storage stages (Carvalho et al., 2004). The most commonly used cryoprotectors include skimmed milk, glycerol, manitol, sorbitol, trealhose, sucrose, maltose, fructose, and proteins (Abadias et al., 2001; Carvalho et al., 2004; Gbassi et al., 2009; Huang et al., 2006). However, the most important selection criterion of an encapsulation material is its functionality in respect to the probiotic (Nedovic et al., 2011). At present, there is a high interest in Aloe vera for the food industry, thus being used as functional nutrient in drinks and ice cream (Martínez-Romero et al., 2006). Spite of its functionality, Aloe vera has not been used as wall material in encapsulation.

A mixture between carbohydrates and proteins can improve the effectiveness of encapsulated probiotics. Therefore, the aim of this present study was to evaluate the effects of wall materials and the lyophilization on the viability of *W. confusa*. The wall materials were Aloe vera gel, sodium casein at 5 and 15% p/v, sodium alginate at 2% p/v, buffer phosphate pH 7.5, and a mixture of pure Aloe vera, casein at 10% and alginate at 2% p/v. During the lyophilization process, the freezing and sublimation-drying stages were evaluated.

MATERIALS AND METHODS

Microorganism culture conditions

A cryoconserved strain, biochemically identified as *W. confuse*, isolated by Serna et al. (2010) was used. *W. confusa* strain was replicated for three generations using MRS commercial substrate (De Man et al., 1960; Scharlau, Spain) (24 h at 37 ± 0.2°C). For its growing, batch fermentation were used, following the methodology of Serna et al. (2010). After fermentation, *W. confuse* was separate from its metabolites, using centrifugation for 30 min at 5000 rpm (Eppendor Centrifuge 5804R, Germany). The bacteria were washed using 1 mL of NaCl at 0.9% and then centrifuged for 5 min at 5000 rpm. Finally, the supernatant was discarded (Picot and Lacroix, 2004).

Wall materials (encapsulation materials)

Sodium casein of 92.7% (alanate 180, Fonterra, New Zealandd), Sodium alginate (Sigma-Aldrich Co. USA), buffer phosphate, and Aloe vera (Aloe barbadensis Miller), were used as wall materials. In addition, a mixture of these materials was used. The phosphate buffer was selected, because this compound is used for the release of encapsulated cells. Aloe vera was obtained from an experimental plantation at Universidad Nacional de Colombia—Palmira, located 1,100 m above sea level. Sodium casein was used in aqueous solutions at 5% p/v (C5) and 15% p/v (C15). The solutions were shaken for 12 h (Heidebach et al., 2010). Sodium alginate was used at 2 % p/v (AG) (Kailasapathy, 2006). The buffer phosphate (BP) solution was used at pH 7.5. In order to produce Aloe vera gel, acibar (a yellow color liquid) was extracted by cutting the base of the leaf and leaving it drain for 1 h (Miranda et al., 2010). Then, the crystals from leaf epidermis were isolated and processed in a juice extractor (Black & Decker JE2200B, USA), under aseptic conditions. The frozen Aloe vera gel (A) was stored at 5°C for 12 h and used undiluted. Additionally, a mixture (MZ) of Aloe vera,

Table 1. Viability percentage of *Weissella confusa* in different wall materials, during freezing and sublimation-drying.

Time (h)	AG	A	BP	C5	C15	FC	MZ
0				100.00 ± 0.00^{a}			
12	41.93 ± 2.03^{Ab}	97.57 ± 1.98^{Da}	92.71 ± 2.38^{Da}	$90.33 \pm 1.03C^{Dab}$	90.14 ± 4.47^{CDa}	79.84 ± 0.12^{Bb}	83.11 ± 1.39^{BCb}
24	41.52 ± 2.46^{Ab}	96.58 ± 1.93^{Da}	91.00 ± 4.20^{CDa}	82.79 ± 6.47^{BCb}	88.65 ± 2.90^{CDa}	71.77 ± 0.16^{Bc}	81.85 ± 0.81^{BCb}
36	41.18 ± 1.24^{Ab}	88.10 ± 0.58^{Bb}	47.20 ± 4.57^{Ab}	80.67 ± 2.77^{Bb}	85.91 ± 6.39^{Ba}	45.16 ± 0.31^{Ad}	79.74 ± 0.84^{Bb}
48	40.87 ± 1.23^{CDb}	81.70 ± 0.50^{Fc}	46.77 ± 4.57^{Db}	36.51 ± 1.29^{BCc}	31.37 ± 0.43^{Bb}	0.02 ± 0.00^{Ae}	69.19 ± 0.96^{Ec}

The values correspond to mean ± SD. FC = bacteria without encapsulation; C5 sodium casein at 5% p/v; C15 = casein at 15% p/v; AG = sodium alginate; A = Aloe vera; BP = buffer phosphate; MZ = mixture of Aloe vera; sodium casein at 10% and sodium alginate at 2 % p/v. Different letters in the superscripts in the same column or row indicate significant differences; according to Tukey's comparison ($p < 0.05$). Capital letters indicate significant differences ($p < 0.05$) between different treatments (row). Lowercase letters indicate significant differences ($p < 0.05$) for the same treatment during the time evaluated (column).

sodium casein at 10%, and sodium alginate at 2 % p/v was used (this mixture was evaluated in previous experiments).

Encapsulation process by freeze-drying

Bacteria without encapsulation (FC) in concentration of 10.344 ± 0.038 $Log_{10}UFC\ g^{-1}$ were separately mixed with the corresponding wall materials, using a 1:4 ratio between the bacteria and the wall material (Brinques and Ayub, 2011). Encapsulated and non-encapsulated bacteria were freeze-dried (frozen at -20°C, vacuum pressure 0.120 mbar and condensing temperature -50°C) (Labconco, England). During the freezing process, cell count was made at 0, 12 and 24 h, and during the sublimation-drying process, cell count was made at 24, 26 and 48 h. The cell count made at 24 h in freezing process, corresponded to initial conditions of the sublimation-drying stage. FC treatment corresponded to treatment control. FC was freeze-dried to the same conditions described above (Doherty et al., 2010; Kailasapathy, 2006).

Quantification of living bacteria

For liberation of bacteria, the encapsulated bacteria were dissolved (1:10, v/v) in buffer phosphate (pH 7.5), and were centrifuged for 2 min at 5000 rpm (Eppendorf Centrifuge-5804R, Germany). Quantification of cell viability was done on spread-plate (agar MRS, 48 h, and 37°C ± 0, 2°C). Afterward, plates containing 30 to 300 colonies were enumerated, expressing the counting in $UFCg^{-1}$ (Doherty et al., 2010; 2011).

Viability of the probiotic strain

Viability was assessed after freezing and sublimation-drying, in accordance with Doherty et al. (2010), using Equation 1:

$$\% \text{ Viability} = (100 \times N/N_o) \quad (1)$$

Where, N is the number of viable cells after freezing in UFC g^{-1}, and N_o is the number of viable cells before freezing in en UFC g^{-1}.

During the sublimation-drying process, the percentage of viable cells was calculated using Equation 2:

$$\% \text{ Viability} = (100 \times N_t^*/N_o^*) \quad (2)$$

Where, N_t^* is the number of viable encapsulated cells in UFC g^{-1}, each time, along sublimation-drying and N_o^* is the number of viable encapsulated cells before freezing in en $UFCg^{-1}$ (Semyonov et al., 2010).

Statistical analysis

A univariate design with 7 levels, FC, C5, C15, AG, A, BP and MZ was used. The response variable was the percentage of viability. Response variable was evaluated during the freezing and drying-sublimation processes, at the time t0 = 0 h, t1= 12 h, t2 = 24 h, t3 = 36 h and t4 =48 h. Results were presented as mean ± standard deviation (SD) of three replicates. The results were analyzed using SPSS 15.0 for Windows (SPSS Inc, Chicago IL, USA). The comparison between averages was made using Tukey, with a probability of $p < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the means of percentages of viability of *W. confusa* in different wall materials, during freezing and sublimation-drying times. Percent viability of FC, presented statistically significant decrease during the freezing and sublimation-drying (Table 1). At the end of the freezing process (24 h), the viability of FC decreased to 28.23%, however its viability percentage was statistically equal to treatments C5 and MZ (Table 1). This indicates that C5 and MZ have no effect on cell cryoprotectant in the freezing step. At the end of the process of sublimation-drying (48 h), FC had the lowest percentage of viability compared to all treatments, with statistical significance of $p<0.05$ (Table 1). Figure 1 shows that after 36 h of process, the cell concentration, measured as $Log_{10}CFUg^{-1}$, decreased significantly reaching 6.722 ± 0.033 $Log_{10}CFUg^{-1}$ after 48 h (In Figure 1, axis Y was divided for including information of FC treatment). For all treatments, the behavior of the cell concentration during the sublimation drying process was similar to the behavior of % viability, since this percentage was calculated from the cell concentration. This may be caused by cell stress produced by the formation of ice crystals during freezing and during the sublimation-drying stage in lyophilization (Otero et al., 2007). When bacteria are lyophilized, some membrane regions may be negatively affected, mainly because of water crystals in the freezing stage, as well as changes in membrane permeability and protein denaturalization (De Giulio et al., 2005; Yang et al., 2012).

At the end of sublimation-drying process (Table 1), the

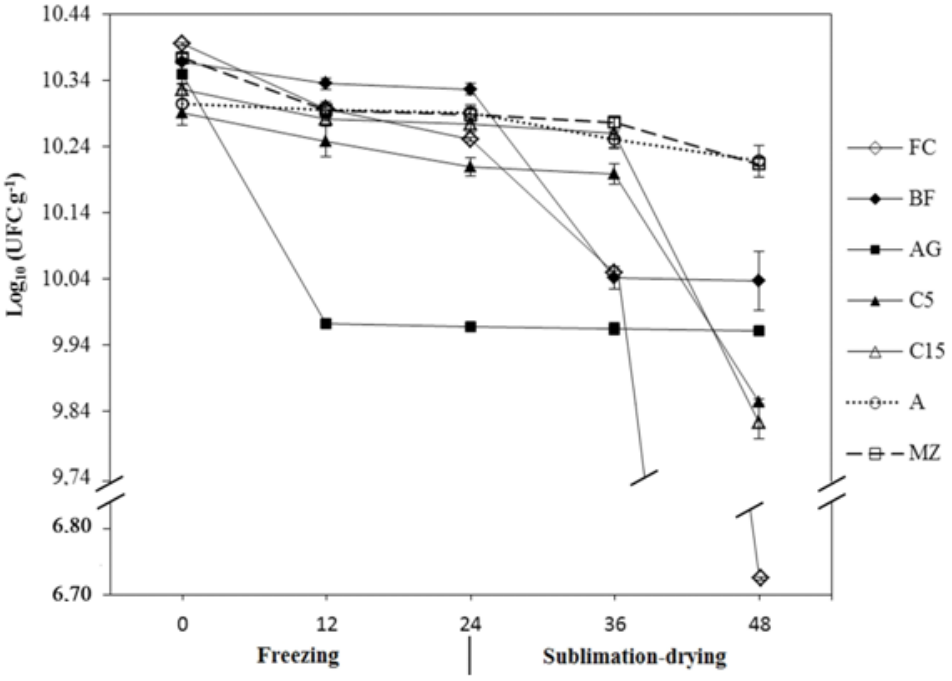

Figure 1. Cell count of *Weissella confusa* for different encapsulation materials during the freezing-sublimation stage of lyophilization. FC = bacteria without encapsulation, C5 sodium casein at 5% p/v, C15 = casein at 15% p/v, AG = sodium alginate, A = Aloe vera, BP = buffer phosphate, MZ = mixture of Aloe vera, sodium casein at 10% and sodium alginate at 2 % p/v.

encapsulated bacteria with wall material A, showed the highest percentage of viability (81.70 ± 0.50%) ($p < 0.05$), however, from 36 h of processing, there was a significant decrease in the viability percentage ($p < 0.05$), which shows that there was an adverse effect on the stage of sublimation-drying (Table 1); referring to the cell concentration at the end of the sublimation-drying process that was obtained (10.217±0.019 $\log_{10}CFUg^{-1}$), this being the highest value for all treatments. At the end of the freezing process, the encapsulated bacteria with the wall material A, showed no statistically significant differences with BP and C15 treatments (Table 1). Kanmani et al. (2011), used glucose and galactose at 35% concentration as protecting substances for the probiotic *Enterococcus faecium* MC13, obtaining viabilities of 85.6 and 84.7%, after encapsulation by lyophilization, respectively. Zayed and Roos (2004) also evaluated a mixture of sucrose and trehalose for lyophilization encapsulation of *Lactobacillus salivarius*, obtaining 80% viability of the strain. The viability percentage obtained with Aloe vera gel as cryoprotector, is due to its high content of polysaccharides, such as mannose, glucose, and galactose (Chang et al., 2011). The use of sugar-based cryoprotectors (mono and disaccharides) produces high rates of viability (Chávez and Ledeboer, 2007; Huang et al., 2006; Khoramnia et al., 2011; Semyonov et al., 2010). This is attributed to the protection that exerted the sugars on the functionality of cell proteins. The sugars create a glassy matrix during the lyophilization stage, which presents high viscosity and low mobility. Additionally, the increase in cell viability is attributed to the fixation of solutes to cell proteins, because the solutes behave as a substitute for water, when the zones of protein hidration, are altered as a result of drying (Carvalho et al., 2004). Aloe vera has high glass transition temperature (Tg=70°C in lyophilization) (Nindo et al., 2010), and this gives additional protection to the cells, compared to other plysaccharides such as sucrose. The drying temperature in this study was 25°C; when a biological product is stored below the glass transition temperature, the chemical reactions such as oxidation of free radicals is slowed and the cellular damage is limited (Fu and Chen, 2011). The main bioactive compound of Aloe vera is acemannan, a water-soluble carbohydrate which consists of glucose and manose monomers linked by glucosíde β-(1,4) bonds (Femenia et al., 2003; Reynolds and Dweck, 1999). Aloe vera pulp contains 93% in dry base of polysaccharide, of which 62.9% is mannose, 13.1% is glucose and 1.5 % is galactose (Ni et al., 2004).

Carvalho et al. (2004), evaluated the influence of the addition of different sugars on the survival of *L. bulgaricus*, during freeze-drying. The study showed that the presence of mannose produced higher rate of survival of the bacteria, after freeze-drying. Abadias et al. (2001), evaluated during freeze-drying, the viability of

Candida sake, when it was coated with different protective materials. They obtained viabilities of 0.2% when concentration of glucose and fructose of 1% were used and viabilities of 1% when galactose solution to 1% was used. The acemannan plays an important role in the healing of wounds, due to inhibiting bacterial growth and promoting macrophage activity (Djeraba and Quere, 2000) therefore, Aloe vera provides functional advantages, compared with other wall materials.

The MZ treatment viability decreased in the first 12 h of processing and at the end of the sublimation-drying process. The viability showed statistically different values, compared to the other treatments. These findings are in accordance with those reported by Nanasombat and Sriwong (2007); they used mixtures of skimmed milk, lactose, sucrose, and trehalosa in different combinations, for the lyophilization-encapsulation of *Lactococcus lactis* 13IS3 and *Lactobacillus sakei* 13IS4, and they obtained viabilities of 61 and 75% for *L. lactis* and 64 and 74% for *L. sakei*. The viability percentages when a mixture of materials was used, were higher compared with not mixed materials. Collagen, trehalosa, L-cisteín and glycerol in the encapsulation by lyophilization of *Bifidobacterium longum* BIOMA 5920 were used. Viabilities of 83% using mixtures of wall materials were obtained, and viabilities of 53.22% using no-mixture wall materials were obtained (Yang et al., 2012). Gbassi et al. (2009) used lyophilization, and sodium alginate (20 g/L) in combination with whey protein (10 g/L), to encapsulate *Lactobacillus plantarum* 299v, *L. plantarum* 800 and *L. plantarum* CIP A159 strains. The researchers concluded that the combination of polysaccharides and proteins is a feasible alternative, since it improves cell viability. Furthermore, they assessed the viability of the strains in gastric and intestinal simulated juices, the results showed that encapsulated strains had higher viability in gastric juices than no-encapsulated cells. In intestinal juice only encapsulated bacteria maintained viability.

In buffer treatment, viability did not differ significantly with AG treatment. The buffer is used to release the cells from the capsules, and subsequently obtaining a cell count (Doherty et al., 2010; 2011). AG treatment presented at 12 h of processing is the largest decrease in the percentage of viability. Values were statistically different compared to the other treatments (Table 1). After 12 h of the process, the percentage viability was statistically unchanged until the end of sublimation-drying process. This behavior was similar to that found in Chan et al. (2011); they indicated that the sodium alginate no protects cells during lyophilization, due to physical properties of sodium alginate cause cellular stress. Between all encapsulation materials, C5 and C15 treatments had the lowest percentage of viability at the end of sublimation-drying process (cell concentration of 9.854 ± 0.003 and 9.823 ± 0.003 $Log_{10}CFUg^{-1}$, respectively) (Figure 1). In these two treatments after 36 h, the greatest decrease in the percentage of viability was observed. C5 treatment did not differ significantly with treatments AG and C15 to 48 h of processing. These findings are similar to those reported by Heidebach et al. (2010), they used lyophilization with sodium casein at 15% to encapsulate *Bifidobacterium* Bb12 and *Lactobacillus* F19, obtaining viabilities of 40 and 30%, respectively.

The low viability percentages found in this study can be attributed to the ability of bacteria to survive in different ways under the same adverse or comfort conditions (Carvalho et al., 2004; Meng et al., 2008). Therefore, for each strain, there should be an evaluation of different encapsulation materials, in order to find the most convenient (Carvalho et al., 2003; Otero et al., 2007). Further research is necessary to test new wall materials and determine other variables such as viscosity, molecular weight, gelification, composition, *Tg*, and other properties that can be useful in technical applications and materials optimization, in order to enhance viability.

Conclusions

Using wall materials, the life of the bacteria was protected in higher percentage in the sublimation-drying stage. Bacteria without-encapsulation showed higher viability decrease in the sublimation-drying stage (24 to 48 h). All wall materials evaluated in this study, have potential in the encapsulation of lactic acid bacteria, due to cell counts at the end of the process, these are found within the ranges accepted by several countries, for probiotics foods (at least 7 to 9 Log 10 probiotic cultures per serving of product). Thus, *W. confusa* encapsulated can be used in the formulation of probiotics. Aloe vera gel is a promising material for the encapsulation of active compounds because it improves the functionality of the material to be encapsulated. In this study, Aloe vera was the only one wall material that maintained the viability of *W. confusa* above 80%. Likewise, buffer phosphate was found to be a cheap material that could be used as a complement in the formulation of wall materials. Encapsulation of *W. confusa* expands the application horizons of this lactic acid bacteria to the food industry, including foods with probiotic effects, as application in milk fermented, desserts, ice cream, and powdered starter culture (for fermentation process).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Optimization of mutanase production by *Trichoderma harzianum*

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The present paper describes optimization of fermentation conditions in shaken flasks and scale-up of fermentor production up to 115 L. The response surface methodology (RSM) has been successfully applied in standardization of mutanase production by *Trichoderma harzianum* CCM F-340. The model was very well fitted to the experimental data and explained more than 96% of the whole variation of the response (adjusted R^2 = 0.962). In order to confirm the adequacy of the regression model based on the experimental data, validation cultures were grown in conditions created through optimization. The highest enzyme activity (0.747 U/mL) was reached in shaken flask cultures on Mandels' medium in a volume of 140 mL modified in terms of carbon (cell wall preparation from the polypore fungus *Laetiporus sulphureus* 8.08 g/L) and nitrogen (soybean peptone 1.38 g/L) sources, under culture conditions 30°C, pH 5.3, agitation 270 rpm. The scale-up of the culture in the bioreactors with a working volume of 5 and 115 L resulted in a slight decrease in the mutanase activity (0.734 and 0.682 U/mL, respectively). The validation experiment showed a 70.6% increase in the production of mutanase compared with the culture before optimization. The results proved that the cultures could be scaled-up successfully from shaken flasks to the bioreactor scale. Our results indicate that in optimal conditions, *T. harzianum* could be a highly effective extracellular mutanase source. This report is the first to deal with optimization of mutanase biosynthesis using a mathematical model and scale-up of enzyme production in controlled fermentors with a view to facilitate application thereof in industry.

Key words: Mutanase, *Trichoderma harzianum*, response surface methodology (RSM), bioreactors, submerged culture.

INTRODUCTION

Mutanases (α-(1→3)-glucan 3-glucanohydrolases) hydrolyze the α-(1→3)-glycosidic bonds of streptococcal mutan - water-insoluble, alkali-soluble α-glucan found in oral biofilms. Currently, the most promising application of mutan-

degrading enzymes is prevention of dental caries (Inoue et al., 1990; Pleszczyńska et al., 2011). Mutanases could be used as an active additive in preparations intended for oral hygiene, such as mouthwashes, toothpastes, and dental gels, and for washing and storage of prosthesis and prosthetic devices for removal of denture plaque located on their acrylic surfaces. As active ingredients, mutanases could become useful supplements to mechanical cleaning of teeth and dentures with a toothbrush, dental sticks, and dental floss.

In addition to their potential usefulness in dentistry as oral therapeutic agents, α-(1→3)-glucanases might be applicable in investigations of α-(1→3)-glycosidic linkages found in microbial cell-wall structures and glucans of certain higher plants. Mutanases obtained in a pure form are invaluable tools for studying the chemical structure of carbohydrates. Analyses of the products of mutan hydrolysis by these enzymes provided important structural information (Hare et al., 1978). Moreover, various preparations of mutanases have also been successfully used for obtaining fungal protoplasts (Balasubramanian et al., 2003).

The most common source of extracellular mutanases are filamentous fungi, such as *Trichoderma* spp., *Aspergillus* spp. and *Penicillum* spp., which exhibit higher enzyme activity than bacteria and yeast (Fuglsang et al., 2000; Guggenheim et al., 1972). However, to our knowledge, there are few reports on detailed characteristics of production of fungal mutanases. In our previous studies, we described the effects of nutrients and culture conditions on mutanase production by various *T. harzianum* strains in flask cultures and batch fermentation using "one-variable-at-a-time" experiments (Wiater et al., 2005b). The "one-variable-at-a-time" approach is time consuming and does not account for the interactions among the medium components. The statistical approach for medium optimization is believed to be a better alternative to the classical approach because of the utilization of fundamental principles of statistics, randomization, replication, and duplication (Singh et al., 2008). A combination of the factorial design and response surface optimization are used to identify the factors and their levels for obtaining an optimal response. In this report, we have applied the response surface methodology (RSM) to a biological model. This technique involves primary screening of variables by application of one of the screening methods, for instance the most popular Plackett-Burman design or the "one-variable-at-a-time" method or (Preetha et al., 2007; Xin et al., 2005) an experiment following the design for fitting the chosen model (Myers and Montgomery, 1995), as well as analysis of the experimental results and the response optimization process (Waśko et al., 2010). The most popular approach is based on the full factorial central composite design (CCD), which enables estimation of the coefficients in a second-order model.
The RSM is usually applied in engineering or industrial chemistry; however, this approach has limited use for biological systems.

In our previous pre-clinical and clinical studies, we demonstrated that mutanase produced by *T. harzianum* CCM F-340 degraded streptococcal biofilm *in vitro* and dental plaque on the teeth surface in humans quickly and effectively (Pleszczyńska et al., 2011; Wiater et al., 2008). However, the possibility of the practical application of mutanase is dependent on the costs and availability of the enzyme preparation. Thirty to forty percent of the production costs of industrial enzymes are estimated to be the cost of the growth medium (Joo et al., 2002). Therefore, it is important to optimize the conditions for cost-efficient enzyme production. Hence, our report is the first to deal with optimization of mutanase biosynthesis using a mathematical model and scale-up of enzyme production in controlled fermentors with a view to facilitate application thereof in industry.

The present paper describes production of mutanase from *T. harzianum* CCM F-340 using a sequential study of the factorial Plackett–Burman design followed by CCD. The factorial design of Plackett–Burman was used to screen the most significant factors affecting enzyme production. The CCD was used to identify the optimum levels of significant variables to generate an optimal response. This paper also describes the transfer of the results from the shaken flask culture to a laboratory 5 L fermenter, and finally to a 150 L pilot-scale fermenter.

MATERIALS AND METHODS

Microorganism

T. harzianum strain CCM F-340 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a starting culture for mutanase production.

Stationary culture conditions

Stock cultures of *T. harzianum* maintained at 4°C on potato dextrose agar slants were used for inoculation. Liquid medium A (pH 5.3), as described by Mandels et al. (1962), supported by 0.3% (unless otherwise stated) cell wall material from *Laetiporus sulphureus* (CWP) as a mutanase inducer, 0.05% peptone (unless otherwise stated), and 0.1% Tween 80 were used for mutanase production. Shaken cultures were conducted in 500 mL conical

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Table 1. Placket-Burman experimental design for evaluating factors influencing mutanase biosynthesis by *T. harzianum*.

Run	Bacto-peptone (g/L)		Soybean peptone (g/L)		CWP (g/L)		pH		Age of inoculums (h)		Volume of inoculums (%)		Volume of medium (mL)	
	C	A	C	A	C	A	C	A	C	A	C	A	C	A
1	-1	0	-1	0	-1	5	1	5.3	1	48	1	48	-1	15
2	1	0.5	-1	0	-1	5	-1	4	-1	12	-1	12	1	75
3	-1	0	1	0.5	-1	5	-1	4	1	48	1	48	1	75
4	1	0.5	1	0.5	-1	5	1	5.3	-1	12	-1	12	-1	15
5	-1	0	-1	0	1	10	1	5.3	-1	12	-1	12	1	75
6	1	0.5	-1	0	1	10	-1	4	1	48	1	48	-1	15
7	-1	0	1	0.5	1	10	-1	4	-1	12	-1	12	-1	15
8	1	0.5	1	0.5	1	10	1	5.3	1	48	1	48	1	75

C: Factors in coded values, A: factors in actual values.

flasks containing 15 - 200 mL of sterile medium depending on the experimental model. Unless otherwise stated, the flasks were seeded with conidia to a final concentration of about 2×10^5 conidia/mL and placed on an orbital rotary shaker (Inforce, Switzerland) at 270 rpm and 30°C for 3 days. Samples of the culture media were withdrawn periodically from shaken flasks and analyzed for mutanase activity.

L. sulphureus cell wall preparation (CWP)

The fruiting bodies of *L. sulphureus* (Bull.: Fr.) Murrill representing a combination of young and old basidiocarps were collected from deciduous trees growing in Lublin and its surroundings, Poland. The preparation of the cell wall from the *L. sulphureus* fruiting bodies was performed according to the procedure described by Wiater et al. (2008). The lyophilized fungal material was milled and the resulting powder was treated with water at 121°C for 1.5 h (×3). The wall material was removed by centrifugation (17001 × *g* for 30 min) and freeze-dried (Cell Wall Preparation, CWP).

Screening of important nutrient components using the Placket-Burman design

The Plackett-Burman design based on the first order model (Plackett and Burman, 1946) was chosen for primary screening of the seven factors at two levels; maximum (1) and minimum (-1). The experimental design and levels of each variable are presented in Table 1. The experiment was conducted in three replications in order to compute the variability of measurements within each unique combination of factor levels and estimate the pure error in the experiment. The results are presented in the form of a Pareto chart, where the absolute values of the ANOVA effect estimates are sorted from the largest to the smallest one. The *p*-value line shows the statistical significance of the influence of the factors on the feature studied (mutanase production).

Optimizing the concentration of the selected medium components with the use of the response surface methodology

A rotatable three-factor central composite design (CCD) with five replications of central points, axial points for α=1.68, and with nine replications of all combinations of the factors (including replications of the central points) was used to determine the unknown regression coefficient of the second-degree polynomial equation (Elibol, 2004):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response variable (mutanase activity), β_0 is the interception, β_i are linear coefficients, β_{ii} are quadratic coefficients, and β_{ij} are coefficients of the interaction. X_i and X_j represent factors chosen on the basis of the results of Plackett-Burman design analysis. A total number of 20 experiments were employed for optimizing medium components and culture conditions for mutanase production (Table 2). The factorial points represent variance of the optimal design for a first-order model or a first-order two-factor interaction type model. The center points provide information about the existence of curvature in the system. If the curvature is found in the system, the axial points allow efficient estimation of the pure quadratic terms (Park et al., 2008). In the present study, the range of response values, Y, was very wide (Table 2). Generally, a ratio greater than 10 indicates higher likelihood that the transformation of the response value may improve the polynomial model. Therefore, the procedure described by Box and Cox (1964) was used in order to identify the power transformation for describing data in an appropriate form for statistical analysis. Application of the Box–Cox procedure indicated that the mutanase activity should be described by a square root transformation. The accuracy and general ability of the above polynomial model were evaluated using the adjusted coefficient of determination $Adj\text{-}R^2$ and the model *p*-value. The relationships between values predicted on the basis of this model and the chosen independent factors were represented in the form of 3D response surface plots. The calculations were performed using the Experimental Design unit of the STATISTICA software system (Stat Soft, 2007).

Validation of the experimental model and process scale-up

The statistical model was validated with respect to mutanase biosynthesis under the conditions predicted by the model in shaken flasks and in bioreactor conditions. The reference laboratory-scale fermentations were performed in a 5 L stirred tank bioreactor

Table 2. Experimental plan for optimization of mutanase biosynthesis using the central composite design.

Run	CWP (g/L)	Soybean peptone (g/L)	Volume of medium (mL)	Mutanase activity (U/mL)	Std Dev
1	3	0.2	80	0.358	0.014
2	3	0.2	200	0.341	0.018
3	3	1.8	80	0.019	0.008
4	3	1.8	200	0.021	0.006
5	9	0.2	80	0.049	0.009
6	9	0.2	200	0.030	0.008
7	9	1.8	80	0.654	0.021
8	9	1.8	200	0.643	0.009
9	0.96	1	140	0.041	0.005
10	11.04	1	140	0.173	0.008
11	6	0	140	0.060	0.011
12	6	2.344	140	0.009	0.006
13	6	1	39.2	0.562	0.011
14	6	1	240.8	0.584	0.019
15	6	1	140	0.558	0.029
16	6	1	140	0.580	0.025
17	6	1	140	0.560	0.025
18	6	1	140	0.587	0.017
19	6	1	140	0.578	0.020
20	6	1	140	0.554	0.050

Biostat B Plus (Sartorius Stedim, Germany). The larger scale process was performed in a pilot-scale bioreactor (BioFlo Pro, New Brunswick Scientific, USA) with a total volume of 150 L. The specifications of the bioreactors are summarized in Table 3. The fermentation temperature was controlled at 30°C. The pH of the medium was adjusted to 5.3 before autoclaving and was not controlled during fermentation. Dissolved oxygen (DO) was calibrated after autoclaving to 0 by nitrogen input and to 100% by air to its saturation point. During fermentation, dissolved oxygen was not controlled. Foaming was controlled by adding Antifoam 204 (Sigma Chemical Co., USA) before autoclaving at a concentration of 50 mg/L. Samples of the culture were withdrawn at 24 h intervals and examined for mutanase activity.

The main task of the microbial process scale-up is to transfer the biological processes effectively from the laboratory to production scale. Mutanase synthesis scale-up was performed on the basis of the working conditions determined for the 5 L bioreactor. However, the optima of aeration and agitation intensities in the 5 L bioreactor were set up based on the data presented by Bhattacharyya et al. (2008). They also demonstrated operation strategies for enzyme production by fungal strains. In the case of filamentous fungi, a constant impeller tip speed is often used as a scale-up criterion, because of the shear sensitivity of the microorganisms used (Amanullah et al., 2004). Here, the impeller tip speed (U_{Tip}) in the 5 L bioreactor has been calculated in a form involving the impeller agitation rate (N) and the diameter of the impeller (D):

$$U_{Tip} = \pi ND \tag{2}$$

An equal tip speed results when the small-scale stirrer speed N_1 is multiplied by the inverse geometric ratio of the impeller diameters D_1/D_2 to get the large-scale stirrer speed N_2:

$$N_2 = N_1\left(\frac{D_1}{D_2}\right) \tag{3}$$

However, the geometric dimensional similarities must be kept in the large-scale bioreactor in order to apply this scale-up criterion. The geometric dimensional similarity is expressed as follows:

$$\left(\frac{D_{T2}}{D_{T1}}\right) = \left(\frac{V_{L2}}{V_{L1}}\right)^{\frac{1}{3}} \tag{4}$$

where D_T is the tank diameter and V_L is the actual liquid volume. This assumes reasonably constant impeller geometry (impeller diameter (D_I) and number of impellers). As shown in Table 3, the geometric similarity parameter calculated for the actual working volume of the pilot scale bioreactor decreased as the scale increased. In order to bring the geometric similarity parameters of the larger bioreactors to that of the 5 L vessel, the actual working volumes of 150 L scale were decreased to 115 L. As a result, all the values of liquid height ratios for all the bioreactors used were comparable (Table 3).

Determination of the physical properties of fermentation broth

Viscosity measurements were carried out in a rotational rheometer Rheo Stress 1 (Haake, Germany) equipped with a temperature control unit DC 30 (Haake, Germany) and concentric cylinder geometry Z20 DIN Ti (Haake, Germany). Equipment control and

Table 3. Scale-up of bioreactors based on geometric similarity and constant tip speed

Parameter	Scale (L)	
	5	150
Nominal volume (m^3)	0.0066	0.15
Working volume according to the manufacturer (m^3)	0.005	0.120
Actual working volume (m^3)	0.005	0.115
Liquid height H_L (m)	0.249	0.721
Cross section area of bioreactor (m^2)	0.020	0.159
Bottom geometry	Spherical	Spherical
Tank diameter D_T (m)	0.160	0.450
Tank height H_T (m)	0.345	0.980
Impeller type	Rushton	Rushton
Impeller diameter D_I (m)	0.064	0.150
Number of impellers	2	2
H_T/D_T	2.156	2.178
D_I/D_T	0.400	0.333
H_L/D_T	1.555	1.602
Aeration rate (vvm)	1	1
Tip speed (m/s)	1.005	1.005
Stirrer speed (rpm)	300	128
Geometric similarity at V_L ($D_T/(V_L)^{1/3}$)	1	0.926

data handling was performed using RheoWin software version 3.40 (Haake, Germany). The shear stress was measured at the shear rate of 100 s^{-1}. The density of the fermentation broth was estimated by glass pycnometry at 30°C using water as reference.

Determination of the volumetric oxygen transfer coefficient (K_La)

The K_La was determined by the method described by Jin-Ho et al. (2007). In this method, the airflow into the bioreactor is temporarily stopped and the dissolved oxygen is stripped by nitrogen flux. After the DO (dissolved oxygen) concentration had been reduced to 0, air flowed into the fermenter at 1.0 vvm. The K_La could be then deduced, since it corresponded to the slope of the dissolved oxygen concentration rate as a function of the dissolved oxygen concentration:

$$\frac{dC_L}{dt} = K_L a\left(C^* - C_L\right) \quad (5)$$

Where, K_L and a are the oxygen transfer coefficient in the liquid phase and the specific surface of air bubbles. C_L and C^* are the dissolved oxygen concentration and its saturation value.

Mutanase assay

The standard mutanase assay mixture contained 0.5 mL of 0.2% (w/v) dextranase-pretreated mutan (DTM) in 0.2 M sodium acetate buffer (pH 5.5) and 0.5 mL of a suitably diluted enzyme solution. After 1 h incubation at 45 °C, the reducing sugars released were quantified by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1945). One unit of mutanase activity (U) was defined as the amount of enzyme hydrolyzing mutan to yield reducing sugars equivalent to 1 µmol of glucose/min and expressed as units per ml of culture (U/mL). 1 U corresponds to 16.67 nkat.

Preparation of dextranase-pretreated mutan (DTM)

Dextranase-pretreated mutan (DTM) was prepared (50 U of dextranase/mg of native mutan, pH 6.0, 37°C, 3 × 24 h) as a substrate for mutanase activity. Native mutan was synthesized from sucrose with the use of a mixture of crude glucosyltransferases of cariogenic *S. sobrinus*/*downei* CCUG 21020 as described previously (Wiater et al., 2005a). Dextranase of *Penicillium* sp. with an enzyme activity of 12.9 U/mg preparation was purchased from Sigma-Aldrich (St. Louis, USA). The linkage structure of the native and the dextranase-pretreated mutan determined by ^{1}H NMR showed that they were mixed-linkage α-(1→3) and α-(1→6) biopolymers with a greater proportion of α-(1→3) to α-(1→6) linkages, namely, 59.1 and 40.9 mol % for native mutan and 79.8 and 20.2 mol % for DTM, respectively.

RESULTS AND DISCUSSION

Placket-Burman design

The Pareto Chart illustrates the order of significance of the variables affecting mutanase production by *T. harzianum* (Figure 1). All the examined features, except for the age of the inoculum, significantly influenced mutanase production. The first three factors in the rank of the absolute value of estimators were chosen for the

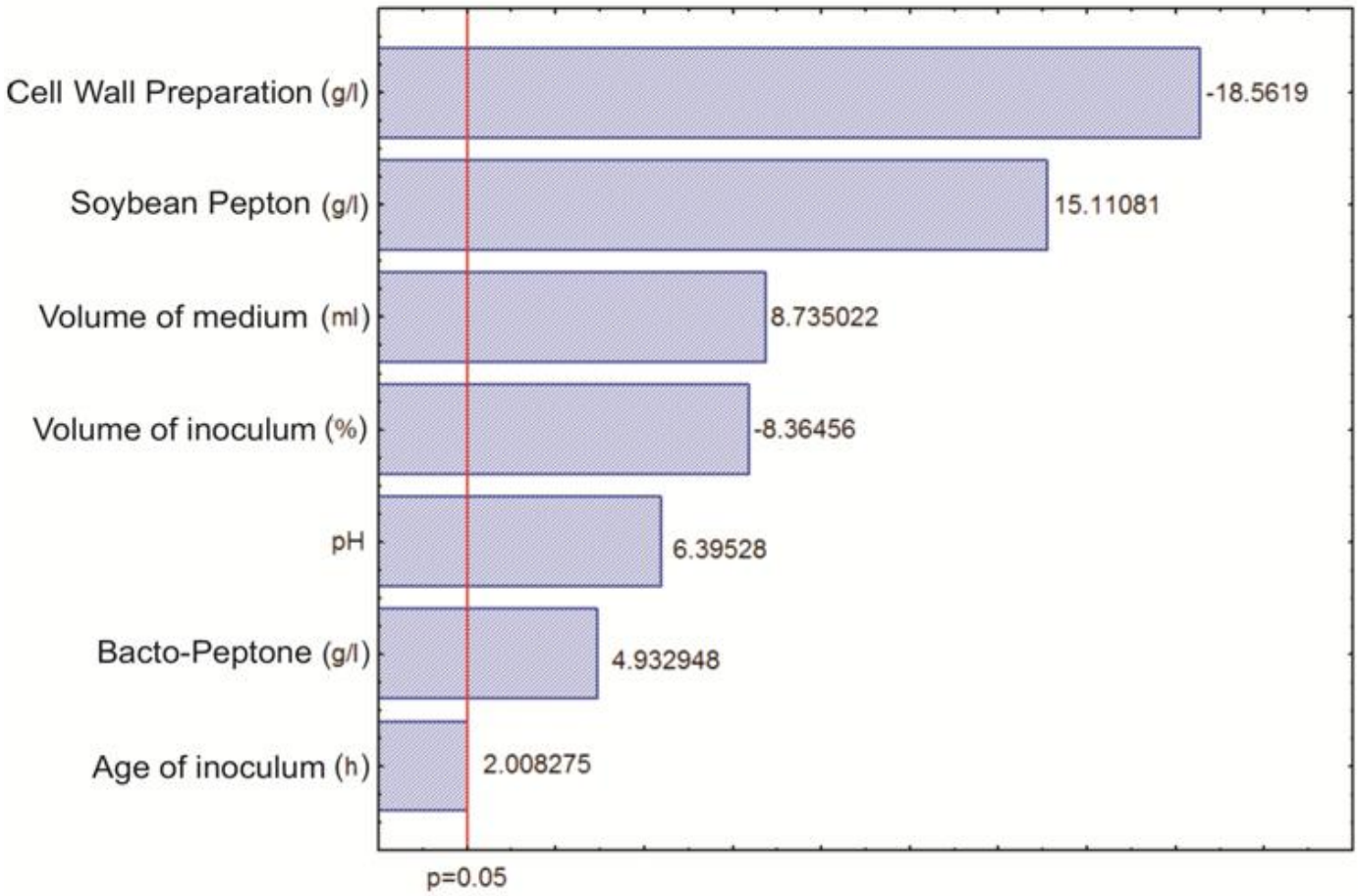

Figure 1. Pareto chart for the Plackett-Burman design for mutanase biosynthesis by *T. harzianum.*

further steps of the analysis (CWP, soybean peptone, and volume of the medium). The cell wall preparation from *L. sulphureus* (CWP) is rich in α-(1→3)-glucans; it effectively induces mutanase activity and fully substitutes other inducers when used as a sole carbon source in the culture medium (Wiater et al., 2008). CWP used as a mutanase inducer had the highest absolute value; however, its influence on the enzyme production was negative (-18.5619), which indicates that an excessive concentration of this substrate can reduce production of the enzyme. This finding is in agreement with our previous study, which indicated that an increase in the CWP concentration in the culture medium above 0.5 % markedly decreased the mutanolytic activity, and at the CWP dose of 2 %, the mutanase yield declined by 81 % in comparison with that obtained with 0.4% CWP (Wiater et al., 2008). Soybean peptone had the highest positive effect (15.1108) on mutanase production. It provided a source of nitrogen for fungal growth and enzyme production, which resulted in a higher yield than that reported for other organic nitrogen sources (Wiater et al., 2005b). An additional advantage of the use of this nitrogen source is the fact that soybean peptone is free of allergens and animal pathogens (Hemmer et al., 2011). This may prove significant for mutanase production on the industrial scale. The third important variable for mutanase production is the volume of the growth medium (8.735), which is connected with the aeration of the culture. *Trichoderma harzianum* is an aerobic filamentous fungus, which absolutely needs oxygen as a substrate.

Therefore, the ratio of the flask size and volume of the medium becomes a restricting element in the liquid state culture of the aerobic mycelium. Due to the morphology of the mycelium, oxygen transfer is not easily built up (Jin-Ho et al., 2007). Additionally, increasing the agitation rate can improve mycelium aeration.

The magnitude of the effects indicates the level of significance of the variables with respect to mutanase production. Consequently, based on the results of the experiment, statistically significant variables, i.e. CWP, soybean peptone, and the medium volume, which had a positive effect on mutanase production were further investigated with a central composite design to find the optimal range of these variables.

`**Table 4.** Analysis of variance for the current regression model.

Source of variation	SS	df	MS	*F*-value	*p*-value
Model	1.439	5	0.288	97.4	< 0.0001
X_1-CWP	0.059	1	0.059	20.1	0.0005
X_2-Soybean Peptone	0.019	1	0.019	6.5	0.0236
$X_1 \times X_2$	0.559	1	0.559	189.5	< 0.0001
X_1^2-CWP^2	0.279	1	0.279	94.5	< 0.0001
X_2^2-Soybean Peptone^2	0.565	1	0.565	191.5	< 0.0001
Residual	0.041	14	0.003		

SS - sum of squares, df – degree of freedom, MS - mean square.

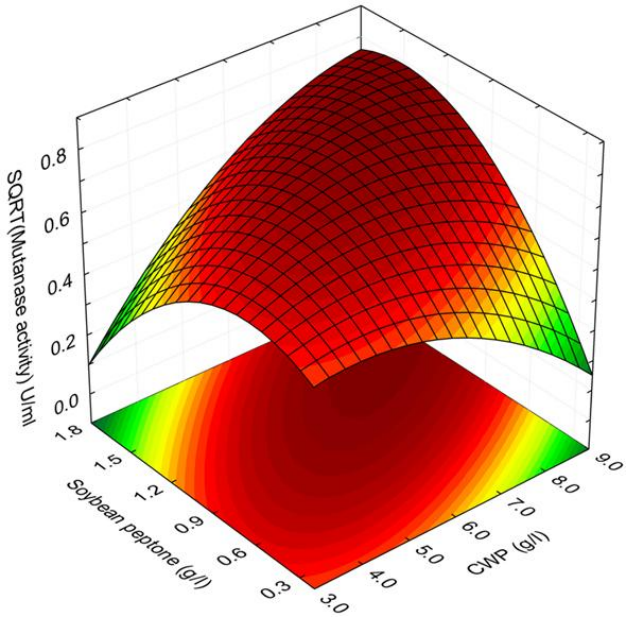

Figure 2. Three dimensional response surface plot for the effect of CWP (X_1), soybean peptone (X_2) on mutanase production by *T. harzianum.*

Central composite design

To examine the combined effect of the selected factors, a full-factorial central composite design of RSM (Response Surface Methodology) containing six central points and star points was applied to maximize the mutanase production. Table 2 presents the design with the level of variables and the values of mutanase activity observed. Run 7 shows maximum mutanase production reaching 0.654 U/mL (CWP - 0.9%, soybean peptone - 0.18%, medium volume - 80 mL). The regression analysis of mutanase activity showed that X_1 and X_2 in linear and quadratic terms was highly significant, giving an overall curvilinear effect. The changes in the medium volume (X_3) were found to be insignificant at the probability level of 95%; hence, they were removed from the model by the backward elimination procedure of ANOVA. Besides the above-mentioned factors, the interaction effect between the X_1 and X_2 was also found to be significant ($p < 0.0001$). Based on the results of CCD design analysis, the following quadratic regression function for the original factor values was determined:

$$\sqrt{Y} = 0.321 + 0.096X_1 - 0.0153X_1^2 + 0.124X_2 - 0.367X_2^2 + 0.110X_1X_2 \quad (6)$$

The model was also very well fitted to the experimental data and explained more than 96% of the whole variation of the response (adjusted R^2 = 0.962). The results of ANOVA indicate statistical significance of the model (Table 4).

The 3D response surface plots show graphical representations of the equation (6). The changes in the response value are represented by different colors from green, describing the minimum, to red, describing the maximum of the predicted value (Figure 2).

The final goal of the present study was to find operating conditions (CWP and soybean peptone) that maximize mutanase activity. In this work, the statistical CCD model made it possible to design a scale-up optimization experiment. It was evident that mutanase production was mainly influenced by the CWP concentration of 4 - 8 g/L and the soybean peptone concentration of 0.6 - 1.4 g/L. Using the model, the optimal condition for mutanase production was obtained when CWP was at the concentration of 8.08 g/L, soybean peptone 1.38 g/L, and medium volume 140 mL. Under optimum conditions, the maximum predicted response of mutanase activity was 0.631 U/mL.

Validation of the experimental model and process scale-up

In order to confirm the adequacy of the regression model set on the basis of the experimental data obtained,

validation cultures were grown in conditions created on the basis of optimization. The cultures were grown in shaken flasks and bioreactors with working volumes 5 and 115 L. The highest mutanase activity (0.747 U/mL) was observed in the flask cultures (data not shown). Compared with the mutanase activity from the culture in non-optimized conditions, a 5% increase was observed (Wiater et al., 2008). The activity obtained during the validation experiment was higher by 33% from the maximum value recorded during the tests within the central composite design. On the other hand, the maximum activity of the enzyme in the 5 L bioreactor was 0.734 U/mL. As shown in Figure 3, after the third day of the culture in the 5 L bioreactor, 90% of maximum mutanase activity was achieved and the highest value was recorded on the seventh day. These data indicate a 70.6% increase in mutanase production, in comparison with the enzyme activity from the culture in the 5 L bioreactor before the optimisation experiment (data not shown). The increase in the process scale to the volume of 115 L caused a slight decrease in mutanase activity. The highest activity, that is, 0.682 U/mL, recorded on the fourth day of the process, was approx. 7% lower than the activity of mutanase observed in the case of the 5 L bioreactor culture. However, all the above results at each scale showed that within the measurement variations for the biological system, the pH, viscosity, K_La, and mutanase activity across the scales appeared to be comparable. The presented data clearly indicate that the culture could be scaled-up successfully from shaken flasks to the bioreactor scale.

The comparison of process performance across the scales revealed that the shaken flask process exhibited slightly higher mutanase activity. One may speculate that this outcome may be to some extent determined by the negative impact of shearing forces generated as a result of agitation, which causes unfavourable changes in cell morphology. According to Amanullah et al. (2004), in fungal fermentation, engineering variables such as agitation conditions require attention due to their effect on the morphology, which in some cases can affect productivity. A decrease in productivity resulting from the impact of shearing forces on the cells was observed in the case of synthesis of penicillin by *Penicillium chrysogenum* (Smith and Lilly, 1990) and pigments by *Monascus* (Kim et al., 2002). In the case of studies presented by Kim et al. (2002), increasing the stirrer rotation speed from 350 to 550 rpm resulted in a 10-fold increase in pigment production efficiency. However, the pigment yields at 700 rpm were reduced to approximately 55% of the yield at 500 rpm. A study by Li et al. (2002) documented a similar effect in large-scale (80 m^3), fed-batch *Aspergillus oryzae* fermentations. It must be noted that fungal cultivation processes are challenging due to the presence of a variety of interconnected factors. Agitation conditions require attention also due to their effect on the oxygen transfer and dissolved oxygen tension, which in many cases can affect productivity. This hypothesis has also been substantiated by the investigations of Amanullah et al. (2002, 2004), and Cui et al. (1998). In general, under the conditions of strong agitation, small pellets and short mycelia are formed. If the cells in the culture broth exist in the form of mycelia with short branches or compact pellets, the viscosity of the culture broth can be maintained at low levels, resulting in high K_La values (Kim et al., 2002). Data presented in Figure 3 allow a conclusion that the limited oxygen transport related to the increased viscosity of the medium is a probable cause of minor differences in mutanase activity (Figure 4). The viscosity was inversely proportional to the volumetric mass transfer coefficient, K_La (Figure 3). The correlation coefficient between these parameters in the case of the 5 L and 115 L reactors amounted to -0.81 ($p=0.015$) and -0.79 ($p=0.021$) respectively. Air bubbles were more likely to be clumped together rather than dispersed, even with agitation, leading to the reduction of the K_La value. The presented results suggest that further studies should aim at optimization of conditions in terms of not only agitation speed but also bioreactor construction. The hydrodynamic conditions during fermentation are also important due to the medium composition and particularly due to the presence of Triton X100. This substance can be positive because it disperses CWP and is conducive to intense foam formation during aeration and agitation. During the study, we observed that a layer of foam containing both cells and CWP was formed even though a small amount of anti-foaming agent was added. This is an unfavourable phenomenon because it results in decreasing the inductor concentration in the medium, which causes a drop in mutanase production. Foam reduction can be performed with the use of mechanical foam breakers or addition of antifoams. However, excessive addition of chemical antifoams may markedly decrease the value of K_La and thus product formation (Martin et al., 1994). Hence, in the present study a small amount of anti-foaming agent was added. Probably, the mechanical foam breakers should be used in foam control to avoid the drawbacks associated with the use of chemical antifoams. Further work is still needed to resolve this problem.

Conclusion

Using the RSM optimization of mutanase production by *T. harzianum* strain CCM F-340, the highest enzyme activity was achieved at the Mandels medium modified in terms of carbon and nitrogen sources: CWP 8.08 g/L, and soybean peptone 1.38 g/L and under the following culture conditions: temperature 30°C, pH 5.3, agitation 270 rpm, and medium volume 140 mL. To sum up, mutanase activity in a shaken flask CCD experiment was

5% higher than before optimization. However, the validation experiment in bioreactor scale showed a 70.6% increase in production of mutanase compared to the culture in the bioreactor before optimization. This indicates that *T. harzianum* in optimal conditions in large culture volume could be a highly effective extracellular mutanase source. However, our experimentally observed mutanase activity can still be further improved *via* optimization of fermentation conditions. This involves development of a novel fermentation mode (that is, fed-batch vs. continuous) and the type of bioreactor construction (i.e. airlift bioreactor).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Characterization of effective bio-control agent *Bacillus* sp. SRB 27 with high salt tolerance and thermostability isolated from forest soil sample

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A potential antagonist *Bacillus* sp. SRB 27, against *Fusarium oxysporum*, *Rhizoctonia solani* and *Alternaria solani* was obtained from forest soil sample by carrying out *in vitro* and *in vivo* screening techniques. This study reports the identification and characterization of a *Bacillus* sp. SRB 27 that may be used as a bio-control agent against the plant diseases in crop plants. It was identified as *Bacillus* sp. SRB 27 based on 16S rDNA sequence analysis and biochemical tests. The isolate showed a wide range of antifungal activity *in vitro* against a number of phytopathogens such as *F. oxysporum*, *Alternaria* and *Rhizoctonia* in terms of percentage of growth inhibition which were 76.78, 78.57 and 77.55%, respectively. Strain SRB 27 was tolerant to high salt concentration up to 13%, was phosphate solubilizer, proteolytic and amylase positive and coagulase negative. It had compatibility with broad spectrum of fungicides with field recommended dose. Apart from the antagonistic activities, it showed a positive effect on the growth of the castor and cotton plants (both under seed and soil treatments) in comparison with control (non-inoculated). The growth parameters of the test plant surpassed the control in all the cases.

Key words: *Bacillus* sp. SRB 27, biological control, *Fusarium*, *Rhizoctonia*, *Alternaria*, 16S rRNA gene sequence.

INTRODUCTION

Microorganisms can colonize the tissues of healthy plants. Such endophytic bacteria have been reported to prevent disease development by controlling the spread of plant pathogens or by enhancing plant resistance (Stein, 2005; Ryan et al., 2008). Therefore, there is a considerable potential for finding new and beneficial endophytic bacteria that can serve as bio-control agents. *Bacillus* sp. were considered as potential bio-control agents due to their high spore production ability, resistance and ability to survive desiccation, heat, ultra-violet (UV) irradiation and organic solvents (Romero et al., 2007). *Bacillus* sp. (Gram positive) form biofilms on

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root surfaces, which are multicellular matrixes of bacteria surrounded by extra cellular polysaccharides called a glycocalyx. The glycocalyx acts as a physical barrier and is strongly anionic, thereby can protect the microcolony from external agents (Jeyasekaran and Karunasagar, 2000). Recent studies suggested that the biofilm mode is important for the bacteria's ability to act as bio-control agents (Bais et al., 2004).

According to Backman et al. (1997), the effectiveness of endophytes as biological control agents (BCAs) is dependent on many factors. These factors include: host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissue, and the ability to induce systemic resistance. For example, *Pseudomonas* sp. strain *PsJN*, an onion endophyte, inhibited *Botrytis cinera* pers. *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 reduced cucumber mosaic virus in tomatoes and cucumbers (Raupach et al., 1996) as well as anthracnose and Fusarium wilt in cucumber (Liu et al., 1995). Jetiyanon (1994) established that cabbage colonized by endophytes in the green house had season-long reduced black rot in the field due to induction of defense mechanisms. Non-treated cabbage plants reached the economic threshold (symptoms of systemic disease) approximately 33 days after inoculation with *Xanthomonas campestris* pv. *campestris.*

Research concluded that *Bacillus mycoides* isolate *BacJ* (Bargabus et al., 2002) and *Bacillus pumilis* isolate 203-7 (Bargabus et al., 2004) suppressed *Cercospora* isolates BT8, and BP24 has successfully been used to experimentally manage diseases on several crop species including tomato, potato and pecan.

The objective of present study was to investigate the antagonistic properties, salt and heat tolerance, phosphate solubilization, compatibility with broad spectrum of fungicides and growth promoting ability of *Bacillus* sp. SRB 27.

MATERIALS AND METHODS

Isolation

The forest soil sample was collected in air tight polythene bags from Nallamala forest located at Munnanoor in Mahabubnagar district, Andhra Pradesh, India. The processed soil sample was serially diluted, spread plated on full strength nutrient agar and incubated at 28°C for 48 h. A total of 46 different colonies were isolated on nutrient agar (NA). Pure cultures were developed with repeated culturing which were maintained in 20% glycerol at -20°C. A potential isolate was screened and selected based on the antagonistic properties, phosphate solubilization and salt tolerance. Further, the isolate was identified based on phenotypic and 16S rDNA sequence.

Antagonistic ability

The *in vitro* antagonistic assay was performed using dual culture method on potatoes dextrose agar (PDA) medium. Seven days old culture agar discs (5 mm) of *Fusarium oxysporum* f.sp. *ricini, Rhizoctonia solani* and *Alternaria solani* were disposed at the center of Petri dishes and the bacterial strain was streaked in a square form around the agar disc at 4 cm distance. The antagonistic activity of the studied bacterial strain was estimated by the inhibition of the fungal growth monitored by measuring the diameter in millimeter of the colony until seven days at 28°C in biochemical oxygen demand (BOD) incubator. The experiment was replicated thrice. The percentage of growth inhibition of the fungus was calculated according to the formula given by Whipps (1987).

Per cent growth inhibition = $((R_1-R_2)/R_1) \times 100$

Where, R_1 is the farthest radial distance (measured in millimeter grown by the fungus after seven days of incubation in the direction of the antagonist (a control value). R_2 is the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist. All *in vitro* antagonism assays were replicated thrice. The bacterial strains that showed obvious inhibition to *F. oxysporum, R. solani* and *A. solani* were selected for further evaluation and stored in 20% glycerol at -20°C.

Fungicide susceptibility test by agar well diffusion method

Agar well diffusion assay is the key process used to evaluate the antifungal potential of fungicides. In the present study, six systemic fungicides; that is, carbendazim, tebuconazole + trifloxystrobin, hexaconazole, propiconazole, azoxystrobin and benomyl were used. Petri dishes (100 mm) containing 20 ml of Mueller Hinton agar (MHA) were seeded with approximately 100 µl inoculums of bacterial strains (inoculums size was adjusted so as to deliver a final inoculum of approximately 10^8 CFU/ml). After solidification of the media, wells of 6 mm diameter were cut into media using a sterilized cork borer. 100 µl of each fungicide was poured into respective well and the plates were incubated at 32°C overnight. The experiment was replicated thrice under strict aseptic conditions to ensure consistency of all findings. Fungicidal activity on inoculums was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced at the end of the incubation period.

Phosphate solubilization

The isolate was screened for phosphate solubilization as per the methodology described by Gupta et al. (1994) on modified Pikovskaya agar (Glucose - 10 g, $Ca_3(PO_4)_2$ - 5 g, KCl - 0.2 g, $MgSO_4$ - 0.1 g, $MnSO_4$ - trace, $FeSO_4$ - trace, Yeast extract 0.5 g, Agar - 15 g, Distilled water- 1 L, pH - 7.0) and the plates were incubated at 30±1°C for 48-96 h. Phosphate solubilization is indicated by the formation of a solubilization or a clear zone around the bacterial colony. A loop full of SRB 27 culture was placed on the center of agar plates and incubated at 30±1°C for 5 days. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone. Halo surrounding the colonies were measured and the solubilizing efficiency (SE) was calculated by the following formula: SE= (Solubilization diameter (S)/Growth diameter (G)) x 100.

Stress tolerance study

Salt tolerance

The bacterial isolates were inoculated separately on specific agar medium containing NaCl at 4 to 13% concentration. Four replications of the plates for each isolate and concentration were maintained along with control. After 48 h of incubation, observations

Table 1. Antifungal activity shown by the isolate *Bacillus* sp. SRB 27.

Fungi	Control (R_1)	SRB (R_2)	Percentage of growth inhibition $((R_1-R_2)/ R_1) \times 100$
Fusarium oxysporum	5.6	1.3	76.78
Rhizoctonia solani	4.2	0.9	78.57
Alternaria solani	4.9	1.1	77.55

for survival and growth of inoculum started (Benson, 1990). The test was repeated for the promising isolates for the salt tolerance confirmation.

Temperature tolerance study

SRB 27 culture was streaked on nutrient agar plates and incubated at different temperature (40, 50 and 60°C) for 24 h with four replications. Control was also maintained for comparison. All the inoculated Petri plates were incubated in BOD incubator at 28±2°C up to 2 to 15 days (Benson, 1990). Observation was recorded for survival and growth of inoculum.

Bacterial identification and characterization

The isolate was subsequently differentiated by gram reaction, microscopic observation, biochemical tests ortho-nitrophenyl-β-galactoside (ONPG), lysine utilization, ornithine utilization, urease, phenylalanine deamination, nitrate reduction, H_2S production, citrate utilization, voges proskauer's, methyl red, indole, malonate utilization, esculin hydrolysis, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose, lactose, oxidase, catalase, coagulase, amylase, protease (casein hydrolysis), lipase (Tween 20), HCN and gelatinase (using Hi-25 Kit, Himedia, Mumbai).

Amplification, sequencing and phylogentic analysis of 16S rRNA gene

The cells of isolate SRB 27 were harvested after incubation in liquid Luria Bertani (LB) medium for 24 h. After centrifugation at 10,000 rpm for 5 min, cells were collected and washed several times by Tris-HCI-EDTA (TE) (pH 8.0) buffer. Genomic DNA was extracted by X-pert bacterial genomic DNA extraction teaching kit (solution based Hi-media kit). Amplification of 16S rRNA gene of the isolate was carried out in PCR cycler using universal primers FD1 (5′-AGA-GTT-TGA-TCC-TGG-CTC-AG-3′) and RP2 (5′-ACG-GCT-ACC-TTG-TTA-CGA-CTT-3′). Amplification reactions were performed in a total volume of 50 µl of 10 X PCR buffer, 5 µl of 25 mmol/l, $MgCl_2$, 5 µl of 2 mmole/l dNTPs mixture, 2 µl of primer FD1, and 2 µl of RP2, 1 µl of Taq DNA polymerase (5 U/µl) and 2 µl of template DNA. Thermo cycling procedure was as follow: an initial keeping at 94°C for 45 s, 55°C for 45 s and 72°C for 90 s, then an extension in the last cycle at 72°C for 5 min. The PCR product was purified using X-Pert gel extraction teaching kit (solution based Hi-media gel extraction kit). The PCR product was sequenced at Bioserve Pvt. Ltd, Hyderabad.

Coagulase test (method for tube test)

The plasma was diluted 1 in 10 in physiological saline (mix 0.2 ml of plasma with 1.8 ml of saline). In this test, three small test tubes were labeled as follows T = test organisms, Pos = positive control (*Staphylococcus aureus* broth culture), neg = negative control (sterile broth). To each tube, 0.5 ml of the diluted plasma was added and later five drops (about 0.1 ml) of the test organism culture was added to the tube labeled 'T'. To the tube labeled 'pos', five drops of the *S. aureus* culture was added. Later, five drops of sterile broth was added to the tube labeled 'neg' after mixing gently, after which the three tubes were incubated at 35-37°C. The test tubes were examined for clotting after 1 h and if no clotting occurred, examined at 30 min intervals for up to 6 h; each tube was gently tilted upon observing the clotting.

Protease production

Bacterial isolates were tested for production of protease by growing them on skim milk agar (SKM) (Chantawannkul et al., 2002). An ability to clear the skim milk suspension in the agar was taken as evidence for the secretion of protease. Petri plates with no bacterial inoculation were used as the control.

Green house experiments

Growth promotion efficacy

Castor and cotton seeds were treated with *Bacillus* sp. SRB 27. For control treatments, seeds were not treated. Treated and controlled seeds were sown in pots. Root length, shoot length, root dry weight and shoot dry weight were measured 30 days after sowing. In another experiment, soil was treated with the test bacteria.

RESULTS AND DISCUSSION

In the present investigation, an attempt was made to isolate the potential bio-control agent with high salt tolerance and thermo-stability properties for which the bacteria was isolated. The isolate was characterized and identified using morphological, biochemical and 16S rDNA sequence analyses. Further, its effect on growth parameters of test plants under green house conditions was evaluated.

Assessment of *in vitro* antifungal activity

The isolate showing bio-control property was identified as *Bacillus* sp. SRB 27 by preliminary morphological and biochemical observations. The data presented in Table 1 explicates the antifungal activity shown by the *Bacillus* sp. SRB 27 against the fungi *F. oxysporum*, *R. solani*, *A. solani* (Figure 1). The *Bacillus* sp. SRB 27 showed the antagonistic activity in terms of growth inhibition in the

Figure 1. Inhibition of the growth of *Fusarium* sp. and *Alternaria* sp by *Bacillus* sp. SRB 27. Control plate without *Bacillus* sp. SRB 27 shows a luxuriant growth of fungi.

Table 2. Effect of fungicides on *Bacillus* sp. SRB 27**.**

Fungicide	Concentration of the fungicide in ppm			
	1000	2000	3000	4000
Carbendazim	R	R	11 mm	14 mm
Tebuconazole + Trifloxystrobin	R	R	10 mm	18 mm
Hexaconazole	R	R	28 mm	35 mm
Propiconazole	R	R	25 mm	31 mm
Azoxystrobin	R	R	R	R
Benomyl	R	R	R	R

R= Resistant; susceptibility indicated by the halo zone (mm)

above mentioned fungi. The growth size of the *F. oxysporum*, *R. solani* and *A. solani* in the control plate was found to be 5.6, 4.2 and 4.9 mm, respectively whereas their growth was restricted to 1.3 (*F. oxysporum*), 0.9 (*R. solani*) and 1.1 mm (*A. solani*) in presence of the test organism. The growth inhibition was found to be 76.78, 78.57 and 77.55% for *F. oxysporum*, *R. solani*, and *A. solani,* respectively.

Assessment of anti fungicide susceptibility of the *Bacillus* sp. SRB 27

In agriculture, the bio-control agents are supposed to be mixed with the fungicides for seed/soil treatment. Such kind of treatments may enhance the effective elimination of fungal pathogens. In light of the above, the *Bacillus* sp. SRB 27 was assayed for the fungicide susceptibility. The data pertaining to Table 2 describes effect of fungicides namely: carbendazim, tebuconazole + trifloxystrobin, hexaconazole, propiconazole, azoxystrobin and benomyl on *Bacillus* sp. SRB 27. All the fungicides were applied at different concentrations; that is, 1000, 2000, 3000 and 4000 ppm. The fungicidal activity was recorded in terms of the mean of diameter of zone of inhibition (in mm) produced at the end of the incubation period. The test organism was completely resistant to the two concentrations that is, 1000 and 2000 ppm for all the

Table 3. *Bacillus* sp. SRB 27 showing tolerance to NaCl (4 to 13 %).

Salt concentration (%)	Level of tolerance to NaCl
4	+++
5	+++
6	++
7	++
8	++
9	+
10	+
11	+
12	+
13	+

+++ = High tolerance; ++ = moderate tolerance; + = low tolerance.

Table 4. Temperature tolerance shown by the *Bacillus* sp. SRB 27 at the range of 30 to 50°C**.**

Temperature (°C)	Level of growth
30	++
35	+++
40	+++
45	++
50	+
55	+
60	-
70	-

+++ = High tolerance; ++ = Moderate tolerance; + = Low tolerance; - = No tolerance.

Table 5. Phenotypic characteristics of strain *Bacillus* sp. SRB 27.

Characteristic	*Bacillus* sp. SRB 27
Colony size	Big
Surface	Mucoid
Colony color	White
Margin	Irregular edge
Bacterial cell shape	Bacilli
Endospore formation	Present (Sub terminal)
Elevation	Flat
Growth in liquid medium	Pellicle
Motility	+
Growth optimum temperature	40
Growth pH	7

whereas it was resistant for azoxystrobin and benomyl at concentrations 3000 and 4000 ppm. *Bacillus* sp. SRB 27 showed susceptibility reaction for tebuconazole + trifloxystrobin, hexaconazole, propiconazole, azoxystrobin and benomyl at 3000 and 4000 ppm.

Evaluation of NaCl and temperature tolerance shown by *Bacillus* sp. SRB 27

The data pertaining to Tables 3 and 4 describes tolerance levels shown by *Bacillus* sp. SRB 27 towards high NaCl concentrations and temperatures, respectively. The tolerance range was designated in connection with growth of the bacteria (in terms of optical density) in the broth at various concentrations. The isolate was tolerant for a wide range of NaCl concentrations (4 to 13%). High tolerance (+++) was shown at 4 and 5% whereas moderate tolerance (++) at 6 to 8% and low tolerance at 9 to 13% (Table 3). The test organism tolerated a wide range of temperatures (45 to 60°C) and exhibited high tolerance at 45°C (+++); whereas moderate tolerance (++) at 50°C and low tolerance at 55 and 60°C (Table 4).

Phenotypic and biochemical characterization

In light of the results obtained for the assessment of fungicide susceptibility, high NaCl concentrations and temperatures tolerance shown by the test organism, the phenotypic and biochemical characterization of the bacteria was carried out and the results given in Tables 5 and 6. The isolate was found to be Gram positive, aerobic and motile aerobic rod that produced a big size colony with mucoid surface in white colour with irregular margin. The bacteria produce endospores (sub terminal) and form a pellicle in liquid medium. It grows better at temperature 40°C and pH 7.

The results pertaining to the biochemical tests performed are presented in Table 6 which explicates that, the *Bacillus* sp. SRB 27 utilized ornithine as the nitrogen source and did not utilized ONPG, lysine and urea. It showed positive response for nitrate reduction whereas negative for phenylalanine deamination and H_2S production. Response for citrate, voges proskauer's and indole tests was positive for the bacteria. The *Bacillius* sp. SRB 27 utilized some carbon sources (arabinose, cellobiose, saccharose, trehalose, glucose and lactose) and rejected the other (xylose, adonitol, rhamnose, melibiose and raffinose). The test organism showed positive activity for oxidase, amylase, protease, lipase and gelatinase whereas negative for catalase, coagulase and HCN production.

Phylogentic analysis of 16S rRNA gene and deposition in NCBI

The PCR amplified product was gel electrophoresed and showed the band with 1.5 kb on the gel (Figure 2). The 16S rDNA gene sequencing of the isolate was amplified

Table 6. Biochemical tests carried out for the isolate *Bacillus* sp. SRB 27.

Test	SRB
ONPG	-
Lysine utilization	-
Ornithine utilization	+
Urease	-
Phenylalanine deamination	-
Nitrate reduction	+
H_2S production	-
Citrate utilization	+
Voges Proskauer's	+
Methyl red	-
Indole	+
Malonate utilization	+
Esculin hydrolysis	-
Arabinose	+
Xylose	-
Adonitol	-
Rhamnose	-
Cellobiose	+
Melibiose	-
Saccharose	+
Raffinose	-
Trehalose	+
Glucose	+
Lactose	+
Oxidase	+
Catalse	-
Coagulase	-
Amylase	+
Protease (Casein hydrolysis)	+
Lipase (Tween 20)	+
HCN	-
Gelatinase	+
IAA	+

+ = Positive; - = negative

analysed (NCBI Accession no. JX276739). The data pertaining to the basic local alignment search tool (BLAST) analysis is shown in Table 7. Neighbor-joining phylogenetic tree based on the alignment of the nearly complete 16S rDNA gene sequence of the *Bacillus* sp. SRB 27 with the 16S rDNA sequences of the seven described *Bacillus* type strains available in GenBank and NCBI databases is shown in Figure 3. BLAST analysis and a neighbor-joining dendrogram constructed using MEGA revealed that the bacterium belongs to the genus *Bacillus* and as closely clustered together with *Bacillus amyloliquefaciens.* The amplified 16S rRNA gene sequence of isolate *Bacillus* sp. SRB 27 was most closely

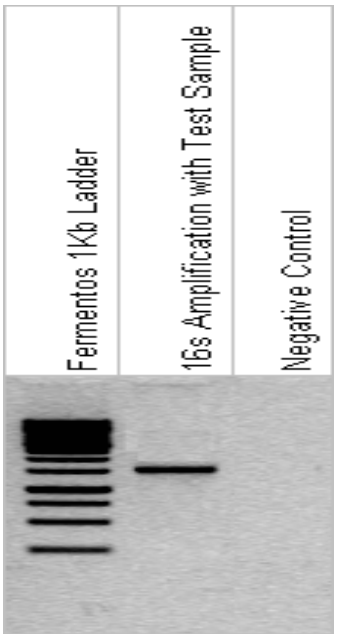

Figure 2. Gel image showing the PCR product (1.5kb) of the 16SrRNA gene.

related to that of *Bacillus* sp. HNR03 (GenBank accession number, EU373340.1) and showed 99% identity with the sequence from *Bacillus* sp. HNR03. The sequence showed 99% identity with the sequences obtained from most of the *B. amyloliquefaciens* strains and *Bacillus subtilis* strains. On the basis of the results of the classical bacteriological tests and the analysis of the 16S rRNA gene, it was concluded that the isolate was a strain of *B. amyloliquefaciens* and *Bacillus* sp. SRB 27.

Phosphate solubilization

The *Bacillius* sp. SRB 27 has the ability of phosphate solubilization (Table 8) the solubilizing efficiency (SE) of the bacteria was found to be 163.63%.

Effect of the isolate on growth of castor and cotton plants in soil and seed treatments under green house conditions

The results pertaining to the effect of the *Bacillus* sp. SRB 27 for growth promotion of castor and cotton when treated to soil and seed under green house condition are mentioned in Table 9. The test organisms was able to influence the growth of castor and cotton in terms of the shoot length (LS), root length (LR), dry weight of shoot (DWS) and dry weight of root in connection with the seed and soil treatment when compared with control at 30 days after sowing. The shoot length was recorded as 25.5±0.2 and 28.4±0.6 cm for castor and cotton,

Table 7. Strain types of *Bacillus* as reference for 16s rDNA sequence determination.

Species number	Gene bank accession
Bacillus sp. TPR06 16s ribosomal RNA gene	EU373402.1
Bacillus sp. TPL08 16S ribosomal RNA gene	EU373378.1
Bacillus sp. HNR03 16S ribosomal RNA gene	EU373340.1
Bacillus subtilis Strain AQ1 16S ribosomal gene	FJ644629.1
Bacillus amyloliquefaciens subsp. Plantanum CAUB946 complete	HE617159.1
Bacilis subtilis strains Aj080718IA-25 16s ribosomal RNA gene	HQ727971.1
Bacillus subtilis strain BL4 16S ribosomal RNA gene HQ	GU826160.1
Bacillus amyloliquefaciens strain SB 3200 16S ribosomal RNA	GU191911.1
Bacillus amyloliquefaciens strain SB 3195 16S ribosomal RNA	GU191910.1
Bacillus subtilis strain En7 16S ribosomal RNA gene	GU258545.1

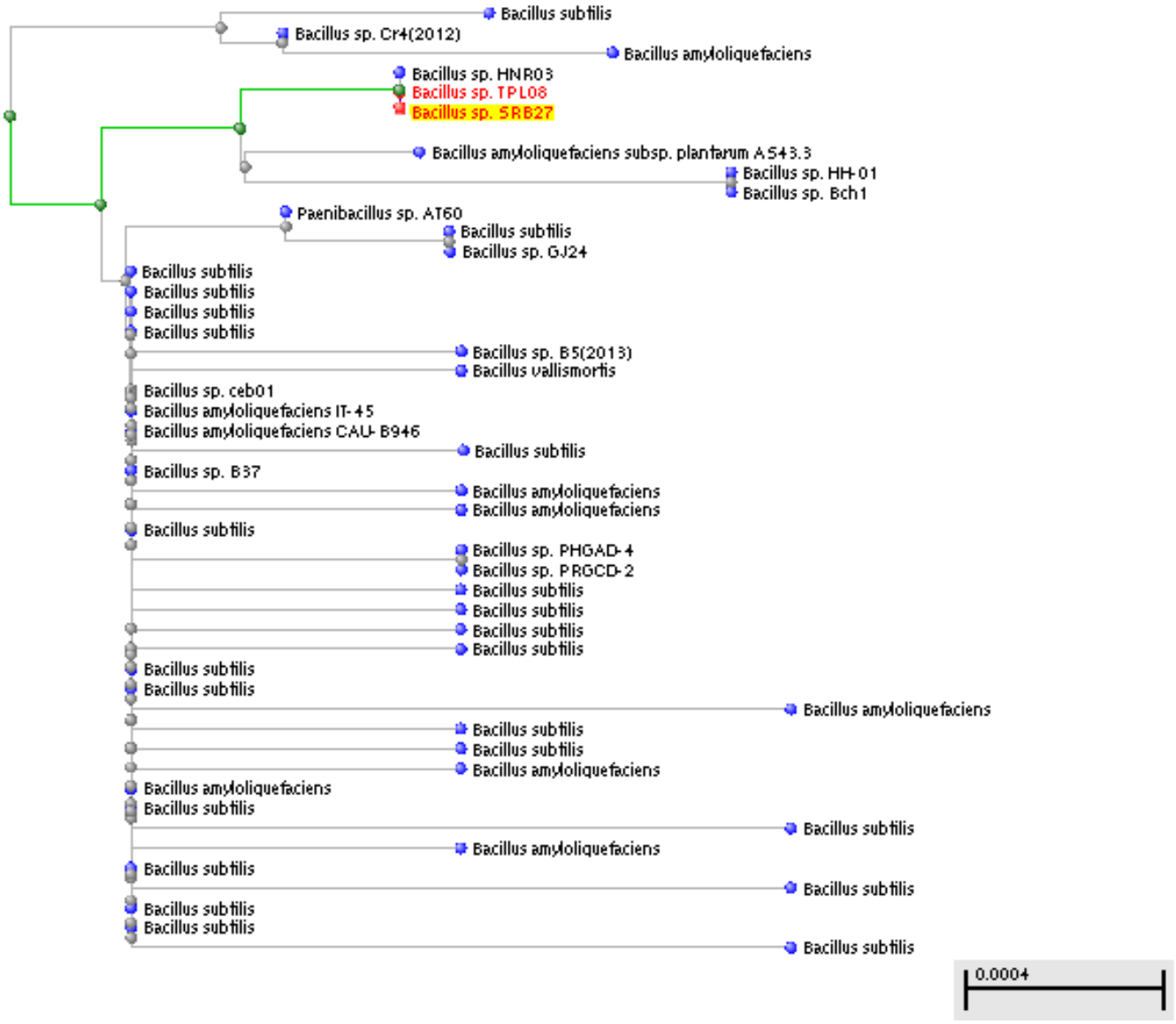

Figure 3. The evolutionary history of *Bacillus* sp. using the neighbor-Joining method. The optimal tree is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Table 8. Phosphate solubilizing ability of *Bacillus* sp. SRB.

Organism	Growth diameter (mm)	Solubilization diameter (mm)	Solubilizing efficiency (SE)
SRB	11±0.12*	180.32	163.63%

*Standard error (SE).

Table 9. The effects of bacterial isolates on growth parameters of plants in soil and seed treatments under greenhouse conditions.

Growth parameter	Castor		Cotton	
	Control	*Bacillus* sp. SRB 27	Control	*Bacillus* sp. SRB 27
Seed treatment				
LS (cm)	18.3 ± 0.3*	25.5 ± 0.2	24.5 ± 0.32	28.4 ± 0.6
LR (cm)	4.7 ± 0.1	7.6 ± 0.4	8.2 ± 0.23	12.6 ± 0.45
DWS (g)	1.4 ± 0.21	2.5 ± 0.11	1.05 ± 0.51	1.8 ± 0.36
DWR (g)	0.7 ± 0.32	1.5 ± 0.3	0.54 ± 0.11	0.9 ± 0.5
Soil treatment				
LS (cm)	18.3 ± 0.22	26.2 ± 0.4	24.5 ± 0.14	30.2 ± 0.4
LR (cm)	4.7 ± 0.15	8.1 ± 0.13	8.2 ± 0.32	14.6 ± 0.1
DWS (g)	1.4 ± 0.54	3.2 ± 0.52	1.05 ± 0.15	2.3 ± 0.42
DWR (g)	0.7 ± 0.4	1.9 ± 0.1	0.54 ± 0.33	1.2 ± 0.54

*Standard error (SE).

respectively under seed treatment whereas it was recorded as 26.2±0.4 and 30.2±0.4 cm for castor and cotton, respectively under soil treatment similarity; the root length was recorded as 7.6±0.4 (castor), 12.6±0.45 cm (cotton) under seed treatment and 8.1±0.13 (castor), 14.6±0.1 cm (cotton) under soil treatment. In case of dry weight of shoot, 2.5±0.11 (castor) and 1.8±0.36 g (cotton) under seed treatment and 3.2±0.52 (castor) and 2.3±0.12 g (cotton) under soil treatment was recorded. The dry weight of castor and cotton was recorded as 1.5±0.3 and 0.9±0.5 g, respectively; whereas for soil treatment 1.9±0.1 and 1.2±0.54, respectively.

The results obtained in the present investigation are found to be similar with studies of Stein (2005); Romero et al. (2007) in which, the species of the genus *Bacillus*, particularly *B. amyloliquefaciens* and *B. subtilis*, have been shown to produce a range of antimicrobial dipeptides or cyclic lipopeptides. Some of the metabolites are strain-specific and may be associated with certain species and subspecies of *B. amyloliquefaciens* and *B. subtilis*. In the studies reported by Earl et al. (2008) and Rosas-Garcia (2009), it was revealed that the *in vitro* prescreening test of dual culture selected four *B. subtilis* strains, PLC1605, PCL1608, PCL1610 and PCL1612 with noticeable antifungal activity against *R. necatrix* and other soil-borne phytopathogenic fungi. *B. subtilis* possess several characteristics that enhance its survival in the rhizosphere and thus its effectiveness as a biopesticide. Compant et al. (2005) noticed that many root associated bacteria have a direct positive influence on plant growth and can indirectly stimulate plant health. Plant bacterial endophytic populations correlate to a certain extent with plant growth performance (Sessitsch et al., 2004). Studies conducted by Yun et al. (2011*)* revealed that *B. amyloliquefaciens* PEBA20 showed antimicrobial activity against a wide range of fungi and bacteria. The efficacy of the bacterial suspension in inhibiting fungal growth *in vitro* ranged from 38.44 to 89.37%, while that of the fermentation filtrate ranged from 19.2 to 82.62%. The diameter of the inhibition zone after treatment with bacterial suspension and fermentation filtrate of PEBA20 ranged from 9.97 to 19.14 mm and from 5.1 (very weak effect) to 19.05 mm. Nitrogen-fixing *Bacillus* sp. isolated by Jadhav et al. *(*2010) has 15% salt-tolerance, indicating they are new novel strains qualified by adaptation to environment and thereby acquiring additional traits. *B. subtilis* subsp. Subtilis NCIB 3610T and *Bacillus sonorensis* NRRL B-23154T isolated by Jadhav et al. (2010) tolerated very high salt concentration (10 and 15%, respectively). The inhibitory potential and antibiotic production of *B. subtilis* was investigated *in vitro* against phytopathogen fungus and bacteria (Földes et al., 2000).

Touré et al. (2004) was attributed to lipopeptides produced by one isolate of *Bacillus subtilis*, the antagonistic action over mycelia growth, which they obtained over many phytopatogenic fungi from soil, as *Fusarium*, *Pythium ultimum*, *Rhizoctonia solani* and

Rhizopus. Mariana et al. (2009) observed the differences in susceptibility for each fungal isolate, in relation to the antagonist action of *Bacillus* E164 which may be related to variations in the production of inhibitory metabolites by the bacterial strains. The phosphate solubilization of *Bacillus cereus* [0.32+0.05] and *B. subtilis* [0.38+0.01] were analyzed using various parameters such as pH [7 to 9], temperature (30 to 45°C], and nutrient supplementation. The role of phosphorus in increasing the yield and improving the quality of *Bacillus* sp. is well known. Phosphorous, next to nitrogen is a vital nutrient for plant and microorganisms.

In conclusion, the isolated bacterial species was identified as *Bacillus* sp. based on the phenotypic, biochemical and phylogenetic characterization. The *Bacillus* sp. SRB 27 was found to be tolerant to high temperatures and high salt conditions which is a positive aspect of the strain to grow better for the crops that grow under high saline conditions and withstand high temperatures. Apart from that, the *Bacillus* sp. SRB 27 has the superior quality of antagonistic activity against phytopathogenic fungal genera like *Fusarium* sp., *Rhizoctonia* sp. and *Alternaria* sp. The phosphate solubilization efficiency was found to be very high which helps in better uptake of the phosphorous under field conditions. Moreover, *Bacillus* sp. SRB 27 has shown proven effect on the growth of castor and cotton in specific and many plant species in general.

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Isolation and identification of antimicrobial-producing lactic acid bacteria from fermented cucumber

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Lactic acid bacteria (LAB) responsible for spontaneous fermentation of cucumber were isolated and their antimicrobial producing potentials were screened against 10 indicator strains. 65% of the isolated LAB produced antimicrobial activities against at least two indicator strains. The indicator strains used were: *Escherichia coli*, *Bacillus licheniformis*, *B. cereus*, *Proteus* species, *Staphylococcus aureus*, *Salmonella* species, *Pseudomonas flourescence*, *P. aeruginosa*, *Serratia* species and *Pediococcus acidilactici*. Of the 42 antimicrobial producing isolates characterized, 16, 12, 6 and 8 were identified as *Lactobacillus plantarum, L. fermentum*, *L. acidophilus* and *Leuconostoc mesenteroides*, respectively. Highest lactic acid producers DW7, DT6, DH13 and DF12 were selected for further investigations and were identified as *L. plantarum*. None of the selected *L. plantarum* isolates had antagonistic activity against *S. aureus*, *Salmolnella* species and *P. acidilactici*. The effect of pH and temperature on the antimicrobial activity of selected isolates showed that pH 5.5 and temperature 30°C were the optimum pH and temperature respectively. Thus, the selected *L. plantarum* isolates are good producers of antimicrobial compounds and could be suitable for application in food industry in preservation of vegetables so as to increase their shelf life.

Key words: Cucumber, fermentation, antimicrobial activity, indicator strains, lactic acid bacteria.

INTRODUCTION

Lactic acid bacteria (LAB) are common fermentation microorganisms because of their mechanisms for survival in acidic, high salt environments and the end products they produce through metabolism (Hutkins, 2006). They are intrinsically present in vegetables, plant materials and gastro-intestinal tract of human and animals and dairy products among other foods and have generally recognized as safe (GRAS) status. Consequently, LAB may be added to minimally processed vegetable products intended for consumption (Caplice and Fitzgerald, 1999).

LAB is natural colonizer of fresh vegetables and have been previously described as good antagonist of several bacteria and fungi in different food products (Stiles and Holzapfel, 1997). According to Ogunbanwo et al. (2004), LAB has the potential to inhibit the growth of pathogenic and spoilage bacteria and possibility exist for using them

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to improve the shelf life of different foods.

LAB strains showing functional properties such as degradation of phytic acid in vegetable products were reported to have antimicrobial activities including production of bacteriocin in fermented olive (Rubia-Soria et al., 2006), sauerkraut (Tolonen et al., 2004), fermented carrots, fermented cucumbers and organic leafy vegetables (Ponce et al., 2008). Researchers have shown that LAB can decrease the pathogen numbers in vegetable products and they can develop the immune system of the hosts (Irkin and Songun, 2012).

Lactic acid bacteria (LAB) are generally recognized as safe (GRAS microorganisms) and play an important role in food and feed fermentation and preservation either as the natural microflora or as starter cultures added under controlled conditions. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid) which result in lowered pHs (Daeschel, 1989). LAB also produce antimicrobial compounds including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas et al., 2001). Since the isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products (Ibourahema et al., 2008), this work therefore aimed at isolation of antimicrobial producing lactic acid bacteria from spontaneously fermenting cucumber and identifying the highest antimicrobial producing isolates.

MATERIALS AND METHODS

Sample collection and processing

Cucumber was obtained from a vegetable Market in Ibadan, Oyo state, Nigeria. It was fermented spontaneously for 96 h and sample was taken at 24 h interval for microbial analysis and pH measurement. Analysis of samples was carried out in triplicate.

Microbial analysis

Isolation was by serial dilution using pour plate method (Harrigan and McCance, 1966). 1 ml of appropriate dilutions was mixed with molten de Mann Rogosa Sharpe agar (Oxoid, Basingstoke, UK) which was aseptically poured into sterile petri dishes. They were incubated at 35±2°C for 48 h under anaerobic condition. Distinct colonies were streaked out in order to obtain pure cultures. Gram positive, catalase negative, non-spore forming rods were selected for further tests. The selected microbes were identified based on their biochemical and physiological characteristics (Kandler and Weiss, 1986).

Screening of lactic acid bacteria (LAB) isolates for antimicrobial activity

Antimicrobial activity of LAB isolates was determined by agar well-diffusion method (Tagg and McGiven, 1971). Ten bacterial strains used as indicators to evaluate the antimicrobial activity of LAB included: *E. coli*, *B. licheniformis*, *B. cereus*, *Proteus* sp., *S. aureus*, *Salmonella* sp., *P. flourescence*, *P. aeruginosa*, *Serratia* sp. and *P. acidilactici*. The cell-free supernatants (CFS) of 48 h old LAB culture in MRS broth were tested. All indicator strains were grown in Nutrient broth at 37°C. Mueller Hinton agar (LAB M, Heywood, UK) plates were overlaid with 5 mL of soft agar (0.75%) containing 50 µL of freshly grown culture of indicator organisms. The wells were made in agar and filled with 100 µL of the tested strain CFS. After incubation at 37°C for 24 h, the diameter of the inhibition zones was measured. All antimicrobial tests were performed in triplicate.

Quantitative estimation of lactic acid

Sodium hydroxide (0.1 N) was titrated against 25 ml of broth culture of test organism with 2 drops of phenolphthalein as indicator. Each millilitre of sodium hydroxide (NaOH) is equivalent to 90.08 mg of lactic acid. Titratable acidity of lactic acid was calculated according to AOAC (1980).

Quantitative estimation of hydrogen peroxide

Hydrogen peroxide production was determined by measuring 25 ml of broth cultures of the test organisms into 100 ml flask. To this was added 25 ml of dilute H_2SO_4. This was then titrated with 0.1N potassium permanganate ($KMnO_4$). Each millilitre of 0.1N $KMnO_4$ is equivalent to 1.701 mg of H_2O_2. A decolourization of the sample was regarded as the end point. The volume of H_2O_2 produced was then calculated (AOAC, 1980).

Quantitative estimation of diacetyl

Diacetyl production was determined by transferring 25 ml of broth cultures of test organisms into 100 ml Erlenmeyer flasks. Hydroxylamine solution (7.5 ml) of 1 molar was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1M HCl to a greenish yellow end point using bromophenol blue as indicator. The equivalence factor of HCl is 21.52 mg. The concentration of diacetyl produced was calculated using the following formula (AOAC, 1980).

Effects of different pH on antimicrobial activity of the isolates

The pH of the medium (Mueller Hinton agar) was adjusted to initial pH of 4.5, 5.0, 5.5, 6.0, 6.5 (Hernandez et al., 2005). 24 h culture of indicator organisms ($\times 10^6$ cfu/ml) was seeded into the molten agar, allowed to set and 10 mm well were made in agar with sterile cork borer. About 100 µL of cell free supernatant of 24 h broth of LAB isolates were then poured into the well (Schillinger and Lucke, 1989) and incubated for 24 h. The antimicrobial activity of isolate was determined by the diameter of inhibition zones around indicator strain.

Effects of different temperature on the antimicrobial activity of the isolates

Indicator organisms ($\times 10^6$ cfu/ml) cultured for 24 h were seeded into molten agar of Mueller Hinton (LAB M, Heywood, UK) in which 10 mm agar well were bored after it has set. 24 h culture of cell free supernatant of test isolate's broth (100 µL) was poured into the well. It was then incubated at temperature of 30, 35, 40 and 45°C. The antimicrobial activity of isolate was determined by the diameter of inhibition zones around indicator strain.

Table 1. Total microbial counts and acidity of fermenting cucumber.

Time (h)	LAB isolate count (cfu/ml)	pH	Total titratable acidity
0	9.2×10^5	6.07	3.2
24	2.1×10^7	5.64	3.5
48	1.4×10^8	4.82	3.6
72	1.8×10^8	4.33	4.1

RESULTS

Table 1 shows the total microbial count, pH and total titratable acidity of fermenting cucumber. The microbial counts, total titratable acidity increased with increase in fermentation period whereas, pH decreased with increase in fermentation time. A total of 42 antimicrobial producing LAB was isolated, characterized based on morphological, biochemical and physiological characteristics and identified as *L. plantarum* (38.10%), *L. acidophilus* (14.29%), *L. fermentum* (28.57%) and *L. mesenteroides* (19.05%) with their respective percentage of occurrences.

The quantity of antimicrobial produced by all the LAB isolates increased within 48 h of incubation period and decreased by 72 h (Table 2). The highest lactic acid production of 4.9 g/l was recorded in isolate DF12 followed by 4.5, 4.4 and 3.7g/L by isolates DW7, DT6 and DH13, respectively. Higher lactic acid production was observed in other isolates like DW6, DW11 and DW13 (3.7g/L), DT4 (4.3g/L), DH6 and DH14 (3.8g/L), DF4 (3.8g/L), and DF13 (4.2g/L). Among the 24 h isolates, DW6 and DW12 had the highest diacetyl production while isolates DT5 (48 h), DH6 (72 h) and DF23 (96 h) had highest diacetyl production in their respective groups. The hydrogen peroxide production ranged from 0.02 to 0.13 g/L by all the isolates, with isolate DT5 having the highest (0.13 g/L) at 48 h. Isolates DW6 and DW12 had the highest hydrogen peroxide production among 24 h isolates, DT5 among 48 h isolates, DH6 among 72 h isolates, and DF6 and DF23 among 96 h isolates.

The morphological, biochemical and physiological characterization of the isolates revealed that all the isolates that produced highest lactic acid among each group are *L. plantarum* (DW7, DT6, DH13 and DF12), while the highest producers of diacetyl (DW6, DW12, DT5, DH6 and DF23) and hydrogen peroxide are identified as *L. mesenteroides*.

Figures 1A to E showed the antagonistic activity of four isolates with the highest lactic acid contents among the LAB isolates against indicator organisms at different pH. The diameter of zones of inhibition ranged between 0 and 21 mm. Antagonistic activity of selected LAB isolates at pH 4.5 is shown in Figure 1A. Isolate DF12 had highest antimicrobial ability against most of the indicator organisms however; DT6 had antagonistic activity against *Proteus* species which no other isolate did. Isolate DW7 and DF12 had the highest antagonistic activity (16 mm) against *B. licheniformis*. Isolate DH13 recorded highest antimicrobial ability (7 mm) against *P. fluorescence* compared to other LAB isolates. None of the LAB isolates recorded antagonistic activity against *S. aureus*, *Salmonella* sp. and *P. acidilactici* at pH of 4.5.

At pH 5.0, all the LAB isolates showed non-antagonistic activity against *Proteus* sp., *S. aureus*, *Salmonella* sp. and *P. acidilactici* except isolate DT6 which had antagonistic activity against *Proteus* sp. and no activity against *Serratia* sp., *P. fluorescence* and *P. aeruginosa*. The highest antagonistic activity against *B. licheniformis* (17 mm) was recorded by isolates DW7 and DF12.

Figure 1C showed the antagonistic activity of LAB isolates at pH 5.5, from the Figure, all the isolates had antagonistic activity against most of the indicator organism. Isolate DT6 was the only isolate which had activity against *Proteus* sp. but had no activity against *S. aureus*, *Salmonella* sp., *P. fluorescence* and *P. acidilactici*. Highest antagonistic activity against all the indicator organisms was observed with isolate DF12. None of the LAB isolates had activity against *S. aureus*, *Salmonella* sp. and *P. acidilactici*.

The effect of adjusting the medium pH to 6 on the antagonistic activity of the cell free supernatant of the selected LAB isolates is as shown in Figure 1D. Higher antimicrobial activity was observed in all LAB isolates against the indicator organisms except *S. aureus*, *Salmonella* sp. and *P. acidilactici* which were resistant. Only isolate DT6 has antagonistic effect on *Proteus* sp. at this pH and no activity on *P. fluorescence* and *P. aeruginosa*.

At pH 6.5, none of the LAB isolate had inhibitory effect on *S. aureus, Salmonella* sp., *P. aeroginosa* and *P. acidilactici* (Figure 1E). Isolate DT6 had no antimicrobial activity against *P. flourescence*. Generally, at all tested pH, highest antimicrobial activity was recorded by isolate DF12 while only isolate DT6 had inhibitory effect on *Proteus* species.

The effect of varying incubation temperature on the antimicrobial abilities of isolate DW7 on selected indicator organisms is as shown in Figure 2A. Increase in temperature from 30 to 45°C resulted in a decreased antimicrobial activity with the least activity observed at 40ºC and the best at 30°C

The result of the antimicrobial potentials of isolate DT6 at different incubation temperature showed that the

Table 2. Quantitative Determination (g/l) of Antimicrobial production.

Isolates code	Lactic acid (g/l)			Diacetyl (g/l)			Hydrogen peroxide (g/l)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
DW1	3.1	3.6	3.4	2.4	2.9	2.6	0.02	0.05	0.03
DW2	2.5	2.9	2.6	2.5	3.3	2.7	0.03	0.07	0.04
DW3	3.1	3.5	3.3	2.2	2.8	2.4	0.04	0.09	0.05
DW4	2.4	2.9	2.6	2.1	2.7	2.4	0.03	0.08	0.05
DW5	2.9	2.5	3.1	2.2	2.7	2.5	0.03	0.09	0.07
DW6	3.1	3.7	3.4	2.8	3.4	3.1	0.07	0.12	0.09
DW7	3.8	4.5	4.2	2.4	2.9	2.7	0.02	0.05	0.04
DW8	2.0	2.6	2.5	2.2	2.7	2.6	0.03	0.08	0.04
DW9	2.1	2.8	2.4	2.5	2.9	2.6	0.03	0.09	0.05
DW10	2.2	2.7	2.5	2.4	3.0	2.8	0.03	0.08	0.05
DW11	3.1	3.7	3.5	2.2	2.8	2.4	0.02	0.06	0.04
DW12	3.0	3.6	3.5	2.7	3.5	2.9	0.07	0.12	0.09
DW13	3.0	3.7	3.4	2.6	3.4	2.8	0.07	0.11	0.09
DW14	2.9	3.5	3.4	2.2	2.7	2.6	0.02	0.05	0.04
DT3	1.9	2.5	2.2	2.3	2.9	2.8	0.03	0.08	0.05
DT4	3.7	4.3	3.9	2.4	3.2	2.9	0.02	0.05	0.03
DT5	2.5	3.2	2.7	2.7	3.6	3.2	0.07	0.13	0.09
DT6	3.8	4.4	3.0	2.2	2.9	2.4	0.02	0.05	0.03
DT7	2.9	3.5	3.1	2.2	2.8	2.4	0.05	0.09	0.07
DT8	2.4	2.9	2.6	2.3	3.0	2.5	0.03	0.08	0.05
DH4	2.6	3.2	2.8	2.5	3.2	2.7	0.07	0.12	0.08
DH5	1.9	2.5	2.2	2.2	2.8	2.4	0.03	0.07	0.04
DH6	3.0	3.8	3.4	2.8	3.6	3.2	0.07	0.12	0.09
DH9	2.4	2.9	2.7	2.1	2.7	2.5	0.04	0.09	0.05
DH10	1.8	2.2	2.0	2.2	2.8	2.5	0.03	0.09	0.05
DH13	3.3	3.7	3.5	2.4	3.2	2.9	0.02	0.06	0.04
DH14	3.0	3.8	3.5	2.2	2.8	2.4	0.02	0.07	0.04
DF2	3.0	3.6	3.5	2.4	3.2	2.9	0.02	0.06	0.05
DF3	1.8	2.5	2.1	2.2	2.8	2.4	0.03	0.09	0.06
DF4	3.0	3.8	3.5	2.0	2.6	2.3	0.02	0.07	0.04
DF5	2.9	3.4	3.1	2.1	2.7	2.4	0.04	0.09	0.06
DF6	2.5	3.0	2.7	2.4	2.9	2.6	0.07	0.12	0.09
DF9	2.1	2.8	2.4	2.1	2.7	2.5	0.04	0.08	0.07
DF10	2.4	3.0	2.8	2.2	2.8	2.7	0.03	0.08	0.06
DF11	2.4	2.9	2.6	2.2	2.9	2.6	0.03	0.08	0.05
DF12	4.2	4.9	4.4	2.4	3.1	2.7	0.03	0.08	0.06
DF13	3.5	4.2	3.8	2.2	2.9	2.5	0.04	0.09	0.07
DF15	2.9	3.5	3.1	2.2	2.9	2.6	0.04	0.08	0.06
DF17	2.7	3.4	2.9	2.1	2.6	2.5	0.04	0.09	0.07
DF19	3.0	3.7	3.4	2.4	3.0	2.8	0.02	0.06	0.05
DF22	2.0	2.7	2.5	2.2	2.8	2.7	0.03	0.07	0.05
DF23	3.1	3.6	3.5	2.6	3.2	2.9	0.07	0.12	0.09

L. plantarum, DW1, DW3, DW7, DW11, DW14, DT4, DT6, DH13, DH14, DF2, DF4, DF12, DF15, DF13, DF19, DF22; *L. fermentum,* DW2, DW4, DW8, DW9, DW10, DT3, DT8, DH5, DH10, DF3, DF10, DF11; *L. acidophilus,* DW5, DT7, DH9, DF5, DF9, DF17; *Leuconostoc mesenteroide,* DW6, DW12, DW13, DT5, DH4, DH6, DF6, DF23.

optimum antimicrobial activity is at 30°C (Figure 2B). As the incubation temperature increased, antimicrobial activity decreased with no activity on most of the indicator organisms at 45°C. Increasing temperature has drastic

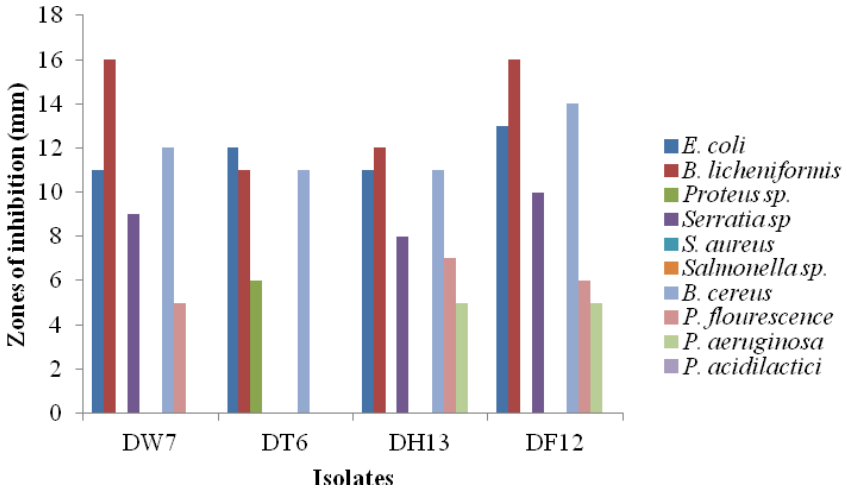

Figure 1A. Antagonistic activity of LAB isolate at pH 4.5.

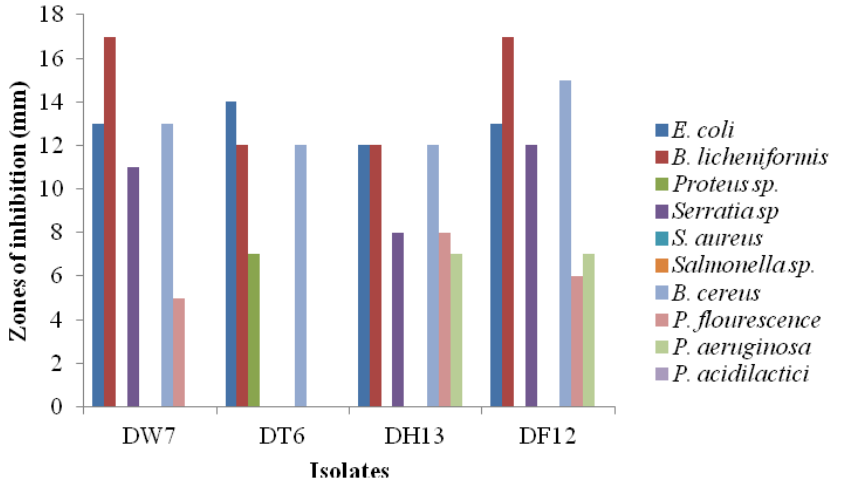

Figure 1B. Antagonistic activity of LAB isolates at pH 5.0.

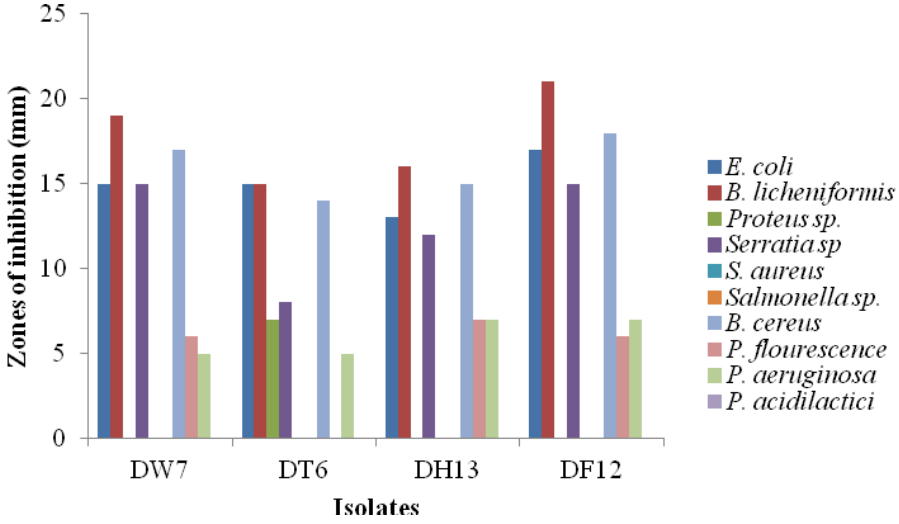

Figure 1C. Antagonistic activity of LAB isolates at pH 5.5.

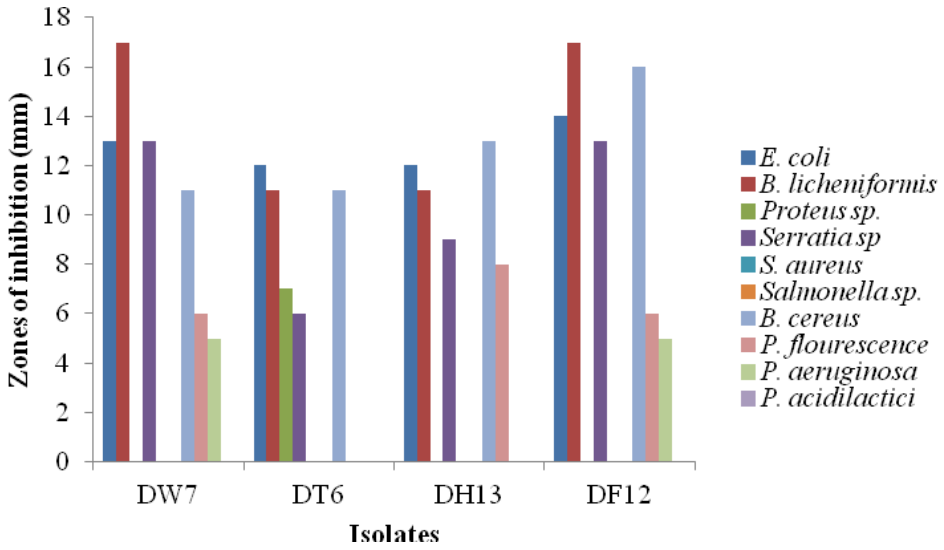

Figure 1D. Antagonistic activity of LAB isolates at pH 6.0.

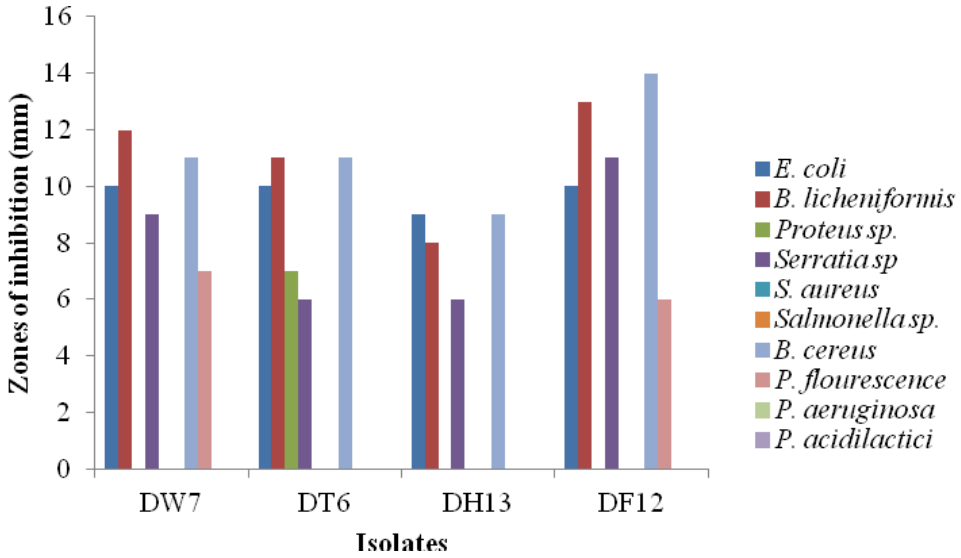

Figure 1E. Antagonistic activity of LAB isolates at pH 6.5.

drastic effect on the antimicrobial abilities of isolate DT6.

Figure 2C shows the antagonistic spectrum of LAB isolate DH13 at different incubation temperature. The antagonistic activity of the organism decreased with increase in incubation temperature. The optimum and least antimicrobial activity were recorded in 30 and 45°C. It had wide range of antimicrobial activities against indicator organisms at all incubation period.

The antagonistic spectrum of LAB isolate DF12 at different incubation temperature is shown in Figure 2D. Antagonistic properties also decreased with increase in incubation temperature. The best antimicrobial ability was recorded at 30°C. The highest antimicrobial activity (21 mm) was recorded against *B. licheniformis* at 30°C.

DISCUSSION

The lactic acid bacteria were identified according to Kandler and Weiss (1986). *L. plantarum* was found to have a dominant role in cucumber fermentations due to its tolerance for high acidity (Fleming and McFeeters, 1981) which is in support of this work. *L. plantarum* isolated in this work is in line with the work of Lu et al.

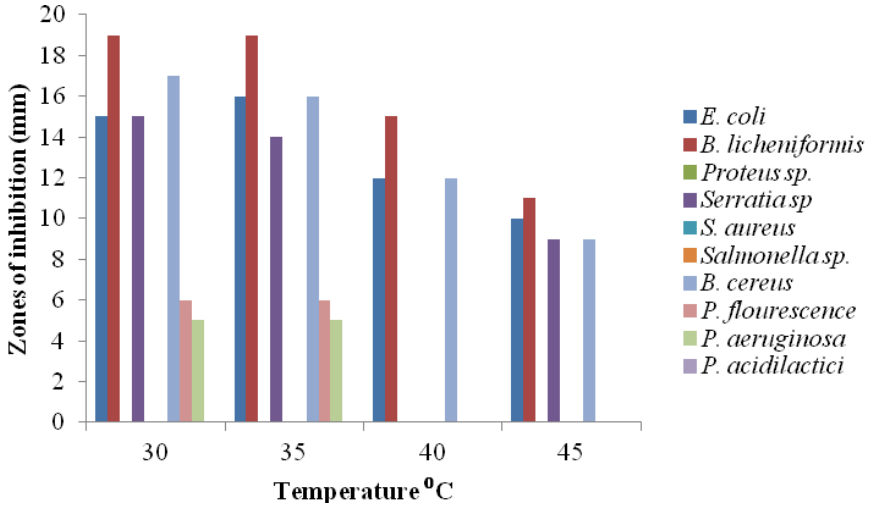

Figure 2A. Antagonistic effect of LAB isolate DW7 at different incubation temperature

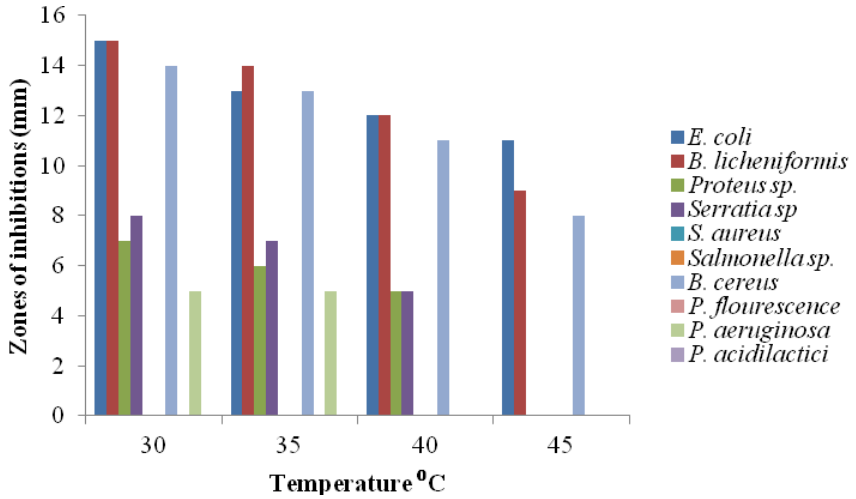

Figure 2B. Antagonistic effect of LAB isolate DT6 at different incubation temperature

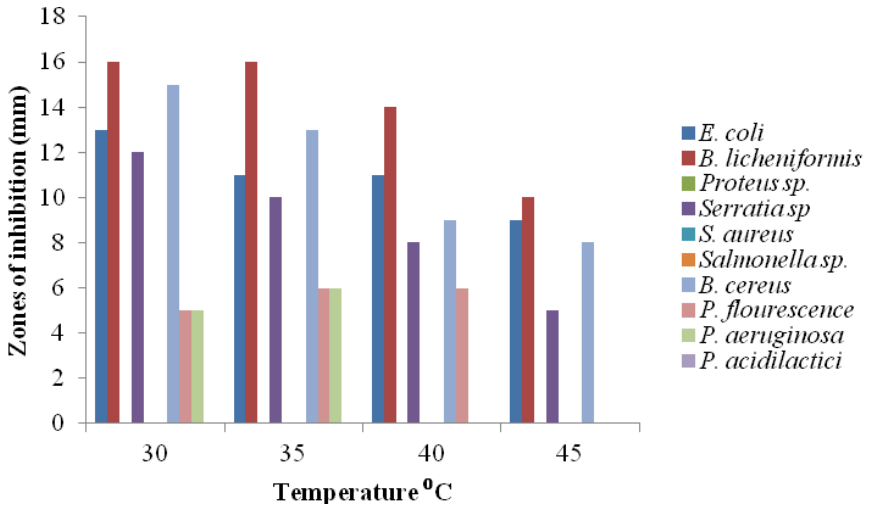

Figure 2C. Antagonistic effect of LAB isolate DH13 at different incubation temperature.

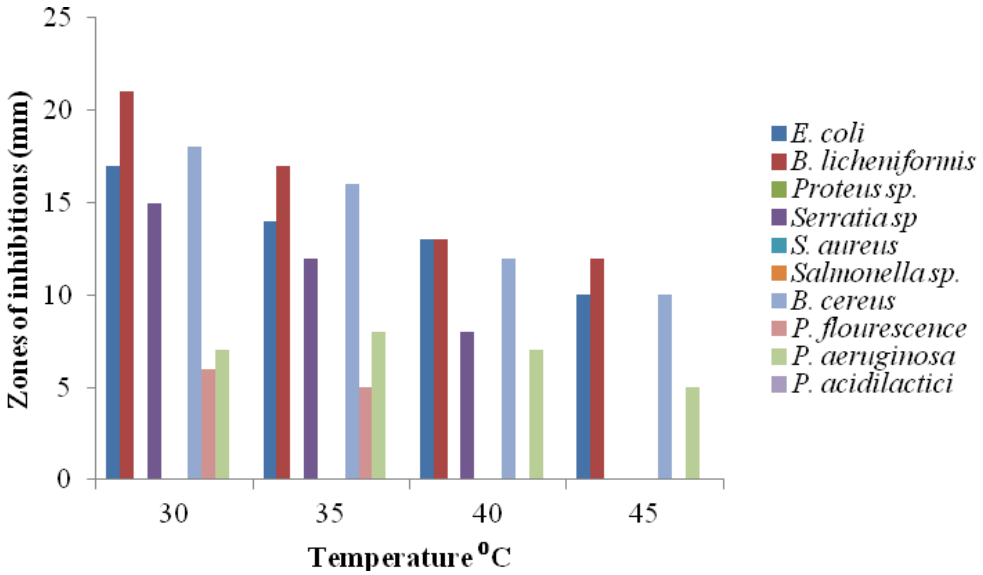

Figure 2D. Antagonistic effect of LAB isolate DF12 at different incubation temperature.

(2003) who isolated *L. plantarum* from commercial cucumber fermentation.

The ability of some species of LAB particularly *L. plantarum* in acidification of the substrates is significant in food preservation (Ammor and Mayo, 2007). It was found that some LAB strains isolated from fermented bamboo products were able to lower the pH to 3.8 (Tamang and Sarkar, 1996). LAB cultures were used effectively against Gram positive pathogens, coliforms, *Aeromonas hydrophila*, *S. aureus*, *Salmonella typhi* and *Listeria monocytogenes* in ready to use vegetables (Vescovo et al., 1995).

It is well documented that lactic acid bacteria (LAB) were involved in vegetable fermentation, so this genera had been extensively studied with the aim of using them as starter culture (Leal-Sanchez et al., 2002). Of the 42 LAB isolates screened, all exhibited a broad activity against indicator culture. Similar results have been reported by Geis et al. (1983) and Klaenhammer (1988) who detected activity only in agar medium. Other authors have also found that cell-free culture supernatant were inhibitory (Schillinger and Lucke 1989; Stiles and Holzapfel, 1997).

The isolated and identified *Lactobacillus* strains exhibited different levels of antimicrobial activity against the selected indicator strains. Among the isolates, *L. plantarum* (DW7, DT6, DH13 and DF12) recorded higher inhibitory activity compared to the others, suggesting that those strain could be used as starter culture especially because of the high inhibitory effect of these strains on the pathogen bacteria such as *Bacillus* species and *E. coli* and this can increase their importance for the industrial applications. Gagiu et al. (2013) showed that *L. plantarum* can be used to develop a wheat sourdough with inhibitory activity against bread spoilage fungi. Bamidele et al. (2013) reported that LAB isolated from vegetable exhibited varied spectra of inhibition against the test MRSA which was resistant to cloxacillin, augmentin, streptomycin, tetracycline and cotrimozaxole.

L. plantarum produced high lactic acid content in this study because under anaerobic conditions, *L. plantarum* had homofermentative pathway, thus produce only lactic acid (Holzer et al., 2003) which may be the reason for their being suggested as antagonistic starter culture for their high lactic acid production and various inhibitory metabolites (Caplice and Fitzgerald, 1999). The ability of a rapid and high acid production has been demanded for lactic cultures to be used as starter in the vegetable fermentation technology (Buckenhuskes, 1993).

From the work carried out by Trias et al. (2008) LAB isolates like *L. plantarum* isolated from ready to eat vegetables, inhibit the growth of food borne pathogens like *Listeria monocytogenes*, *Salmonella typhii* and *E. coli* but in the present study the isolate did not inhibit *Salmonella* sp. Okereke et al. (2012) also reported that the LAB isolated in their study inhibited the growth of *S. aureus*, *E. coli* and *B. cereus* however; the LAB isolated in this research work was not able to inhibit *S. aureus* but was able to inhibit both *E. coli* and *B. cereus*.

Effect of varying pH on antimicrobial ability was observed in this study which showed that pH had significant effect on antimicrobial activities. The optimum pH recorded was 5.5. This is in line with the work of Oskay (2011) who reported that varying pH had significant effect on antimicrobial activities of microorganisms; however, his optimum pH (7.5) was contrary to this work.

It was observed in this work that changed in incubation

temperature had drastic effect on antimicrobial activities. The antimicrobial ability decreased with increase in incubation temperature. The optimum antimicrobial activity was recorded at 30°C. This is in relation to the work of Oskay (2011) who reported that temperature had a significant effect on antimicrobial ability. He also reported 30°C as the best temperature for antimicrobial activity, below and above which there will be reduction in microbial ability of the microbe.

In conclusion, antimicrobial producing LAB can be isolated from fermenting cucumber and best activity can be achieved with *L. plantarum* at pH 5.5 and temperature 30°C. This shows the possible use as potential starter culture in vegetable product preservation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Acceptability of prebiotic fiber-treated whey drink fermented with *Lactobacillus acidophilus*

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This work aimed to develop a fermented drink by diversifying the quantities of *Lactobacillus acidophilus* inoculum and prebiotic fiber in the form of inulin and using the total dry extract of whey and sucrose. After fermentation, the following measurements were made after 0, 7, 14, 21 and 28 days of storage at 6°C: titratable acidity (according to the Dornic method), pH, moisture content, fat content, ash content, protein content, lactose content and the probiotic cell count. After 28 days of storage, the viable *L. acidophilus* cell counts had decreased for all six treatments but were still above the minimum count of 7 log CFU/mL recommended by the Brazilian legislation. All samples presented satisfactory acceptability with the exception of treatment 6, in which the inulin was decanted, thereby altering the color and causing a decrease in acceptance.

Key words: Fermented milk, whey, probiotic microorganism, prebiotic, sensory evaluation.

INTRODUCTION

Fermented dairy products containing probiotic bacteria have received increasing attention in recent decades, including the expansion of the market for functional foods and research into the development of probiotic foods (Karimi et al., 2011).

Fermentation is the chemical transformation of organic substances into simpler compounds through the action of enzymes, complex organic catalysts produced by microorganisms such as molds, yeasts or bacteria (Jafarei and Ebrahimi, 2011). These bacteria produce lactic acid as a result of carbohydrate fermentation and are widely used in the production of fermented foods, from dairy products to fruit and vegetable products. The reasons for the widespread use of lactic acid bacteria

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(LAB) include increasing the shelf life; improving the safety, flavor, appearance and texture and enhancing the physiological and hygienic value due to the presence of viable cells and valuable LAB metabolites (Semjonovs et al., 2008; Kaboosi, 2011; Paraschiv et al., 2011).

The mechanisms of probiotics include the remodeling of microbial communities, the suppression of pathogens, immunomodulation via the up-regulation of anti-inflammatory factors, enhancement of immunity, effects on epithelial cell differentiation and proliferation, promotion of the intestinal barrier function (Preidis and Versalovic, 2009), reduction of serum cholesterol, vitamin synthesis, anti-carcinogenic activity and anti-bacterial activity (Belviso et al., 2009; Ibrahim et al., 2010; Lourens-Hattingh and Viljoen, 2001; Robinson and Samona, 1992; Songisepp et al., 2004; Arunachalam, 1999; Blanchette et al., 1995; Gomes and Malcata, 1999). The beneficial effects attributed to probiotic bacteria also include the alleviation of lactose-intolerance symptoms and constipation, a reduction in serum cholesterol, the prevention of drug-induced colitis and efficacy against a number of other conditions including ulcerative colitis, pouchitis, radiation colitis, atopic eczema and diarrhea (Fotiadis et al., 2008). Many studies have reported the effects of probiotics on gut function as well as visceral sensitivity (Thomas et al., 2012; Preidis et al., 2012), such as reductions in visceral nociceptive reflex responses in rodents and abdominal discomfort in humans (Tillisch et al., 2013).

Probiotics are defined as a dietary supplementation of beneficial bacteria, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Tannock, 1995; Fuller and Gibson, 1997; Macfarlane and Cummings, 1999; Rolfe, 2000; Sanders, 2000; Dunne, 2001; Ishibashi and Yamazaki, 2001; Marteau et al., 2001), which are consumed to antagonize pathogenic bacteria that can invade the human intestine and cause gastrointestinal diseases (Oliveira et al., 2011). The survival and permanence of these microorganisms in the intestinal tract depend on their ability to survive in the gastric medium and utilize the available nutrients (Barrangou et al., 2006).

Products obtained by LAB fermentation processes are therefore of special importance for functional foods such as probiotics (Semjonovs et al., 2008). In addition to dairy products, there are many other commercial probiotic products based on *L. acidophilus*. This bacterium is thought to produce healthy byproducts that protect the stomach, the gut and the reproductive area from harmful bacteria. *Lactobacillus acidophilus* is the best-known species of this Lactobacillus complex in the LAB group and is naturally present in the gastrointestinal tracts of humans and animals. In fermented food, the metabolic activity of this microorganism results in the production of flavor and aromas that produce the organoleptic properties of fermented foods and inhibit food spoilage (Parvaneh and Ebrahimi, 2011). Commercially, the most Important probiotic strains are lactic acid bacteria (Oliveira et al., 2011).

Prebiotic foods contain certain types of dietary fibers, that is, non-digestible carbohydrates with a molecular configuration that makes them resistant to enzyme action. Examples of efficient and commercially available prebiotics are fructooligosaccharides (FOS), inulin and galactooligosaccharides (Tuohy et al., 2003; Barrangou et al., 2003). Inulin is an oligomeric fructose-based carbohydrate that can be easily dispersed in water (Hoppert et al., 2013). The concept of synbiotics represents the combination of probiotics and prebiotics (Shukla et al., 2011; Rastall and Maitin, 2002; Tuohy et al., 2003; Holzapfel and Schillenger, 2002).

Considering the environmental impacts caused by the high chemical oxygen demand (COD) of whey, which liberates 50 KgO_2/ton permeate from 9 kg of whey produced for every kilogram of cheese manufactured, different uses for this waste material have been devised (Martin-Diana et al., 2006). In recent years, due to the need to minimize environmental pollution and the use of available nutrients to attenuate the demand, whey has become an eminent requirement (Ammar et al., 2011) because its proteins have a higher biological value than other proteins such as those of egg, soy and even milk caseins (Smithers, 2008).

Thus, the aim of the present study was to evaluate the fermentation of total dry whey extract and sucrose using a lactic starter, such as pure *L. acidophilus*, and varying the formulations with respect to the size of the inoculums and the amount of prebiotic such as inulin.

MATERIALS AND METHODS

Whey fermentation

This study was divided into three stages, with the first stage consisting of whey fermentation using varying amounts of inoculum (1 -2%) and prebiotic fiber (0, 2 and 4%). The primary purpose of this stage was to determine the best technology to ferment whey when reconstituted to 7% of the total solids and to obtain the highest viability of probiotic microorganisms. A concentrated freeze-dried probiotic starter for direct use was used, which was composed of *L. acidophilus* (*LA3*) donated by SACCO® (a dairy products company).

According to the specific experiment, the whey powder was reconstituted in water to approximately 7% of the total solids, and then sugar (5% w/v) was added with vigorous stirring, followed by the addition of different percentages of inulin. The inulin prebiotic consisted of Raftiline®GR (92% inulin and 8% glucose/-fructose/sucrose) and was donated by CLARIANT® (a company that represents ORAFTI®, Tienen-Belgium in Brazil). This mixture was heated to 85°C and maintained at this temperature for 20 min in a thermostatic bath. The mixture was then cooled to 37°C in an ice-water bath to

obtain the probiotic lactic culture under aseptic conditions. The product was then incubated at 37°C, and the fermentation time of the milk drink, as calculated from the inoculation, was used to obtain an acid value close to 60° Dornic. This temperature is within the optimum temperature range (37 to 40°C) for the growth of *L. acidophilus* (Ahmed et al., 2006). After fermentation was complete, the product was initially cooled to approximately 20°C, and the clots were broken by manual shaking for 30 s. Then, a final cooling step was performed in an ice-water bath, followed by the addition of fruit salad pulp (10% w/v) to enhance the flavor and mask the bitter taste of the whey. The beverage was filled into plastic cups and stored in a refrigerator at a temperature of approximately 6°C. In this stage, six samples were developed: T_1= Fermented drink with 1% inoculum and the addition of fruit salad pulp; T_2= Fermented drink with 1% inoculum and the addition of fruit salad pulp and 2% inulin; T_3= Fermented drink with 1% inoculum and the addition of fruit salad pulp and 4% inulin; T_4=Fermented drink with 2% inoculum and the addition of fruit salad pulp; T_5= Fermented drink with 2% inoculum and the addition of fruit salad pulp and 2% inulin; and T_6= Fermented drink with 2% inoculum and the addition of fruit salad pulp and 4% inulin.

Chemical, physicochemical and microbiological assessment

The second stage was related to the chemical, physicochemical and microbiological assessment. The following measurements were obtained: acidity as lactic acid (° Dornic), pH, moisture, fat, ash, protein, lactose (AOAC, 1997), and microbiological quality as required by law (Brazil, 2000). After fermentation, the physic-chemical analysis was carried out in triplicate. The pH values were determined using a digital potentiometer (DIGIMED) calibrated with pH 7.0 and 4.0 buffer solutions. The total acidity was determined by measuring the lactic acid content of 100 g of sample. In particular, 5-ml aliquots of the samples were titrated with 0.1 N NaOH in the presence of the indicator phenolphthalein, according to the technique described by Instituto Adolfo Lutz (2008).

Probiotic cell count and probiotic microorganism count

Microbiological analyses of the probiotic microorganisms under study were carried out in triplicate after 0, 7, 14, 21 and 28 days of storage, representing the third stage of the experiment. All samples were collected under aseptic conditions and were immediately taken to the laboratory.

Serial decimal dilutions were prepared by aseptically transferring 10 ml of sample into a sterile conical flask containing 90 mL of sterile 0.1% distilled peptone water. This solution was then homogenized, and dilutions were made.

Each sample was serially diluted from 10^{-1} to 10^{-15} in peptone water. One milliliter of each dilution was inoculated into triplicate plates containing MRS agar (De Man et al., 1960). The analyses were conducted each week over a 4-week storage period at 4°C. The probiotic bacteria (*L. acidophilus*) were counted in MRS agar, followed by 72-h incubation at 37°C under anaerobic conditions. Formulations with *L. acidophilus* were counted on MRS agar containing maltose using the spread plate method, followed by 72-h incubation at 37°C under aerobic conditions. Identification of the lactic acid bacteria was performed using the catalase test and Gram staining, according to the methods of Holt et al. (1994).

Sensory analysis

The sensory evaluation was performed in a single step and after the assessment of microbiological parameters to ensure the food safety of the volunteer participants. A nine-point hedonic scale was applied (Meilgaard et al., 1999) using an untrained panel of 50 teachers and students, between 19 and 50 years of age, who represented consumers at a higher education level from the Federal University of Technology-UTFPR. Statistical tests were performed using an analysis of variance (ANOVA) with test comparison by means of Tukey's test at 5% significance (Barbetta, 2002). The statistical analysis was performed using the software *Statistica 6.0*.

Ethical procedures

The volunteers provided free and informed consent according to standard procedures, and the Ethics Committee for Research with Human Beings of the Federal University of Santa Catarina approved the research project under approbation number 380/05.

RESULTS AND DISCUSSION

Acidity and pH parameters

Table 1 shows the evolution of the parameters of acidity and pH during fermentation for the six treatments. The values obtained during fermentation showed that as the lactic acid content increased, the pH value decreased, which is consistent with data obtained by previous researchers (Lourens-Hattingh and Viljoen, 2001). When the values obtained for these parameters were compared to those in Resolution nº 5 of November 13th, 2000 (Brasil, 2000) for the manufacturing of fermented milk, only treatment 1 presented values in agreement with this

Table 1. Values obtained for acidity and pH during fermentation.

*Treatment		Time (h)									
		0	1	2	3	4	5	6	7	8	9
1	Titratable acidity (ºD)	13	19	39	45	51	56	58	60	-	-
	pH	6.30	4.97	4.75	4.50	4.31	4.29	4.22	4.10	-	-
2	Titratable acidity (ºD)	13	22	27	32	37	41	46	50	51	-
	pH	6.30	5.56	5.26	4.93	4.87	4.62	4.50	4.39	4.31	
3	Titratable acidity (ºD)	13	19	25	31	36	42	46	50	54	59
	pH	6.30	5.46	5.09	4.86	4.62	4.51	4.42	4.36	4.31	4.29
4	Titratable acidity (ºD)	13	16	23	26	30	33	39	46	48	50
	pH	6.30	5.92	5.59	5.36	5.12	5.00	4.88	4.75	4.71	4.50
5	Titratable acidity (ºD)	13	17	22	25	27	34	39	43	47	50
	pH	6.30	5.88	5.49	5.17	4.90	4.85	4.81	4.76	4.52	4.48
6	Titratable acidity (ºD)	13	16	21	26	28	33	37	41	46	48
	pH	6.30	5.83	5.47	5.15	4.98	4.87	4.65	4.51	4.47	4.41

*Data represent the evolution of the acidity and pH parameters during fermentation for the six treatments.

resolution at the end of the fermentation process (minimum of 60° Dornic). However, there is no quality standard for fermented beverages made with whey.

We found that the fermentation time was longer and the evolution of acidity (° Dornic) was slower as a function of the low multiplication rate of the probiotic cultures in relation to the traditional lactic bacteria, in accordance with the results of Lourens-Hattingh and Viljoen (2001). It was also observed that the time spent for fermentation was greater and the acidity evolution (° Dornic) was slower, due to the low rate of multiplication of probiotic cultures, in comparison to traditional lactic acid bacteria. These results are also consistent with those of Gomes and Malcata (1999).

In comparison to the values reported in Brasil (2000), the values for acidity and pH in Treatment 1 indicated that this formulation (with 1% starter and no inulin) is likely the most technologically and economically viable formulation for production on an industrial scale.

Physicochemical results

Table 2 shows the results obtained for the three replicates of the physicochemical analysis, considering acidity and pH.

It was observed that during storage, the pH values for all treatments decreased to below 4.5, a desirable value for preventing the growth of pathogenic contaminants (Micanel et al., 1997).

As mentioned by Gomes and Malcata (1999), the probiotic species of Bifidobacterium and Lactobacillus, particularly *L. acidophilus*, in addition to the benefits they provide in terms of nutrition and health, have the advantage of promoting reduced acidification (° Dornic) during storage, as confirmed in the six treatments evaluated in this study. This reduced acidification is important because it helps maintain the level of viable probiotic bacteria in drinks (Dave and Shah, 1997).

As shown in Table 2, at the end of twenty-eight days of storage, the values for acidity (° Dornic) and pH of the six treatments were close to the minimum values required by Resolution No. 5, November 13th, 2000 (Brasil, 2000) for fermented milks (that is, 60° Dornic).

The pH values obtained in this study at the beginning of the storage period were similar to those obtained in the formulation of acidophilus milk (pH 4.68) by Zacarchenco and Massaguer-Roig (2004), although the values for acidity were lower than those reported by these authors (77° Dornic).

This difference is likely related to our use of whey, whereas the aforementioned study used cow's milk, which may have contributed to better fermentation, thereby increasing the acidity of the drink.

Considering the values obtained for pH, moisture, fat, total carbohydrates, lactose, ash and total solids (Table 3), a significant difference was observed between the treatments (p-value <0.05). Only the protein content (p-value> 0.05) showed no significant difference among the six treatments, which supports the finding of Klaver et al. (1993) that probiotic bacteria grow slowly in milk and have low proteolytic activity.

The varied percentages of inoculum and prebiotic used in each of the six treatments could have been the cause of the different values obtained for pH. However, these values were similar (pH 4.68) when compared to those of the acidophilus milk produced in the study of Zacarchenco and Massaguer-Roig (2004).

As expected, there was a significant difference (p-value < 0.05) in the values for moisture content between

Table 2. Values obtained for acidity and pH during storage.

Sample		Storage period (days)				
		0	**7**	**14**	**21**	**28**
1	Acidity (ºD)	60	70	73	77	79
	pH*	4.10 ± 0.02	3.98 ± 0.02	3.96 ± 0.01	3.81 ± 0.02	3.75 ± 0.02
2	Acidity (ºD)	51	56	58	63	66
	pH*	4.31 ± 0.01	3.87 ± 0.02	3.81 ± 0.01	3.67 ± 0.01	3.62 ± 0.01
3	Acidity(ºD)	59	61	64	69	71
	pH*	4.29 ± 0.01	3.97 ± 0.01	3.88 ± 0.01	3.73 ± 0.02	3.70 ± 0.01
4	Acidity (ºD)	48	50	52	55	56
	pH*	4.50 ± 0.02	4.15 ± 0.01	4.09 ± 0.02	4.00 ± 0.01	3.88 ± 0.01
5	Acidity (ºD)	50	52	54	55	57
	pH*	4.48 ± 0.02	4.08 ± 0.01	3.93 ± 0.01	3.88 ± 0.02	3.84 ± 0.02
6	Acidity (ºD)	48	50	54	57	57
	pH*	4.41 ± 0.02	4.19 ± 0.02	3.97 ± 0.02	3.82 ± 0.02	3.80 ± 0.01

*Data represent the means ± S.D of three replicates. T1 = fermented drink with 1% starter and fruit saladflavor; T2 = fermented drink with 1% starter, fruit salad flavor, and supplementation with 2% inulin; T3 = fermented drink with 1% starter, fruit salad flavor and the addition of 4% inulin; T4 = fermented drink with 2% starter and fruit salad flavor; T5 = fermented drink with 2% starter, fruit salad flavor and supplementation with 2% inulin; T6 = fermented drink with 2% starter, fruit salad flavor and the addition of 4% inulin.

Table 3. Values for the physical and chemical determinations of the flavored fermented beverages obtained from the various treatments.

Parameter	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	F Test
pH	4.100 ± 0.0000^{f}	4.307 ± 0.0058^{d}	4.290 ± 0.0000^{e}	4.510 ± 0.0100^{a}	4.477 ± 0.0058^{b}	4.413 ± 0.0058^{c}	P<0.0001
Moisture content (%)	77.300 ± 0.2500^{a}	75.500 ± 0.3606^{b}	73.800 ± 0.2000^{c}	77.600 ± 0.2646^{a}	75.650 ± 0.1323^{b}	73.600 ± 0.2646^{c}	P<0.0001
Proteín (%)	0.890 ± 0.0100^{a}	0.800 ± 0.0700^{a}	0.820 ± 0.0436^{a}	0.850 ± 0.0265^{a}	0.840 ± 0.0700^{a}	0.830 ± 0.0721^{a}	P=0.4830
Fat (%)	0.300 ± 0.0000^{a}	0.200 ± 0.0000^{b}	0.200 ± 0.0000^{b}	0.300 ± 0.0000^{a}	0.200 ± 0.0000^{b}	0.200 ± 0.0000^{b}	P<0.0001
Carbohydrates (%)	21.210 ± 0.0000^{f}	22.940 ± 0.0000^{c}	24.680 ± 0.0000^{b}	21.020 ± 0.0000^{f}	22.840 ± 0.0000^{d}	24.830 ± 0.0000^{a}	P<0.0001
Lactose (%)	5.340 ± 0.0265^{a}	5.340 ± 0.0624^{a}	5.250 ± 0.0265^{ab}	5.150 ± 0.0500^{b}	4.800 ± 0.0458^{c}	5.150 ± 0.0300^{b}	P<0.0001
Fixed min. res. (%)	0.300 ± 0.0361^{b}	0.460 ± 0.0173^{a}	0.500 ± 0.0529^{a}	0.230 ± 0.0656^{b}	0.470 ± 0.0458^{a}	0.540 ± 0.0557^{a}	P<0.0001
Total solids content (%)	22.700 ± 0.0436^{c}	24.400 ± 0.2646^{b}	26.200 ± 0.1732^{a}	22.400 ± 0.1000^{c}	24.350 ± 0.0458^{b}	26.400 ± 0.0173^{a}	P<0.0001

*Data represent the means ± S.D of three replicates. *A p-value<0.01 indicates a significant difference between the formulations. Means sharing the same letter within a column are not significantly different at the 5% significance level.

treatments with different amounts of prebiotic fiber, which followed the changes in the percentage of total solids between the beverages. The fat content only showed a significant difference in those treatments with no added prebiotic fiber. When compared to the parameters of Normative Instruction n° 51 (Brasil, 2002), the fermented beverage in the present study was classified as skimmed because it showed a maximum total milk fat content below 0.5%. As expected, the carbohydrate contents showed no significant difference (p-value < 0.05) between the treatments with no added prebiotic fiber (treatments 1 and 4).

The lactose content was determined post-fermentation to verify whether variation in the size of the initial inoculum influenced the consumption of lactose by the probiotic microorganisms. According to Fuller (1999), probiotics are characterized by their ability to decrease the residual lactose level in the final product. This concept was confirmed in our study, as an increase in the amount of probiotic starter inoculated generally led to an increase in the consumption of lactose by the micro-organism inoculated.

Measurement of the fixed mineral residue and total dry extract levels demonstrated that the values obtained were only significantly different (p-value < 0.05) when the percent of added inulin (prebiotic fiber) was increased, as this modification altered the total solids content of the treatments.

Table 4. Results obtained in the analyses of the microbiological quality of the fermented beverage samples.

Microorganism	*Acceptance criteria	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Coliforms at 35°C (MPN/mL)	10^2	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Coliforms at 45°C (MPN/mL)	10^1	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Yeasts and molds (CFU/mL)	2×10^2	<10	$2x10^1$	<10	<10	<10	<10

*According to Brazilian Legislation, Resolution nº5 of November 13th, 2000.

Table 5. Acceptability of the six samples of fermented whey beverage.

Sample	*Average scores for the attributes			
	Color	Flavor	Aroma	Consistency
(T_1)	7.54 ± 1.46^a	7.34 ± 1.56^a	7.44 ± 1.51^a	6.9 ± 1.73^a
(T_2)	6.86 ± 1.60^a	6.76 ± 1.72^a	6.68 ± 1.65^b	6.68 ± 1.68^a
(T_3)	6.96 ± 1.52^a	7.04 ± 1.61^a	7.08 ± 1.35^a	7 ± 1.61^a
(T_4)	7.54 ± 1.47^a	7.16 ± 1.62^a	7.32 ± 1.22^a	7 ± 1.47^a
(T_5)	6.9 ± 1.46^a	7.04 ± 1.58^a	7.14 ± 1.37^a	7.08 ± 1.54^a
(T_6)	5.84 ± 2.31^b	6.9 ± 1.85^a	7.02 ± 1.57^a	6.9 ± 1.66^a
**HSD	0.72	0.76	0.60	0.60

*Average scores of the 50 consumers. Hedonic score: (9) *Like extremely*; (8) *Like very much*; (7) *Like moderately*; (6) *Like slightly*; (5) *Neither like nor dislike*; (4) *Dislike slightly*; (3) *Dislike moderately*; (2) *Dislike very much*; (1) *Dislike extremely*.** HSD: *Tukey´s* significant difference at the 5% level.[a, b, c, d] Scores (average and standard deviation) followed by the same letter (same column) do not differ from each other. T1 = fermented drink with 1% starter and fruit salad flavor; T2 = fermented drink with 1% starter, fruit salad flavor, and supplementation with 2% inulin; T3 = fermented drink with 1% starter, fruit salad flavor and the addition of 4% inulin; T4 = fermented drink with 2% starter and fruit salad flavor; T5 = fermented drink with 2% starter, fruit salad flavor and supplementation with 2% inulin; T6 = fermented drink with 2% starter, fruit salad flavor and the addition of 4% inulin.

Microbological quality of the fermented beverage samples

Table 4 shows the results obtained in the analysis of the quality of the flavored fermented beverages. It was found that the counts obtained for coliforms at 35 and 45°C (MPN/mL) and those for yeasts and molds (CFU/mL) were below the maximum acceptable numbers established by Resolution n° 5 of November 13th, 2000 (Brasil, 2000) for fermented milk. This result assured that the beverages were microbiologically safe for consumption.

Acceptance of the fermented beverage

Table 5 demonstrates the results for acceptability of the six samples of fermented beverages. The analysis of variance (ANOVA) showed there was a significant difference ($p<0.05$) between treatments regarding the attributes of color and aroma, whereas there was no significant difference ($p>0.05$) for the attributes of flavor and consistency. It was observed that sample T_1 (Fermented drink with 1% inoculum plus added fruit salad pulp) and sample T_4 (fermented drink with 2% starter and fruit salad flavor) presented greater acceptability, as they were both classified in the category “Like moderately”, and this finding indicates that these two beverages presented good acceptability. In addition, sample T_6 (fermented drink with 2% starter, fruit salad flavor and the addition of 4% inulin) was scored between the categories “Neither like nor dislike” and “Like slightly”, which suggests that improvement could be made in relation to the attribute of color.

Probiotic bacterial cell count

Table 6 shows the results obtained for the probiotic bacterial counts for the six treatments during the storage period. Significant differences in probiotic bacterial counts were observed between the treatments (p value <0.05) throughout the storage period (Table 6). Thamer and Penna (2005) developed a fermented beverage with different amounts of whey and sugar fructooligosaccharides and assessed the growth of the probiotics and their physicochemical characteristics. The highest counts for the probiotic microorganisms corresponded to the treatment that showed low acidity and high levels of solids, as in treatments 4, 5 and 6 of this study, which showed the greatest initial growth of *L. acidophilus* due to the low level of acidity.

Various studies on the survival of probiotic

Table 6. *L. acidophilus* counts during storage at 6°C.

Storage period (days)	T1 (Log cfu/mL)	T2 (Log cfu/mL)	T3 (Log cfu/mL)	T4 (Log cfu/mL)	T5 (Log cfu/mL)	T6 (Log cfu/mL)	Test F
0	10.340 ± 0.0400^{c}	10.507 ± 0.0586^{b}	10.160 ± 0.0458^{d}	10.650 ± 0.0458^{a}	10.627 ± 0.0252^{a}	10.700 ± 0.0173^{a}	P<0.0001
7	10.287 ± 0.0473^{c}	10.453 ± 0.0208^{b}	10.083 ± 0.0513^{d}	10.637 ± 0.0416^{a}	10.587 ± 0.0153^{a}	10.623 ± 0.0252^{a}	P<0.0001
14	9.357 ± 0.0981^{b}	10.010 ± 0.6165^{ab}	9.833 ± 0.2082^{ab}	10.353 ± 0.1680^{a}	9.767 ± 0.0751^{ab}	9.713 ± 0.0981^{ab}	P=0.0204
21	8.900 ± 0.0700^{b}	8.877 ± 0.0850^{b}	9.543 ± 0.0513^{a}	8.947 ± 0.0153^{b}	8.763 ± 0.1026^{bc}	8.600 ± 0.0361^{c}	P<0.0001
28	7.360 ± 0.1473^{b}	7.477 ± 0.0503^{b}	8.493 ± 0.1102^{a}	7.330 ± 0.0985^{b}	8.267 ± 0.2779^{a}	7.367 ± 0.1069^{b}	P<0.0001

Data represent the means ± S.D of three replicates. Values followed by same superscript are not significantly different (p> 0.05).

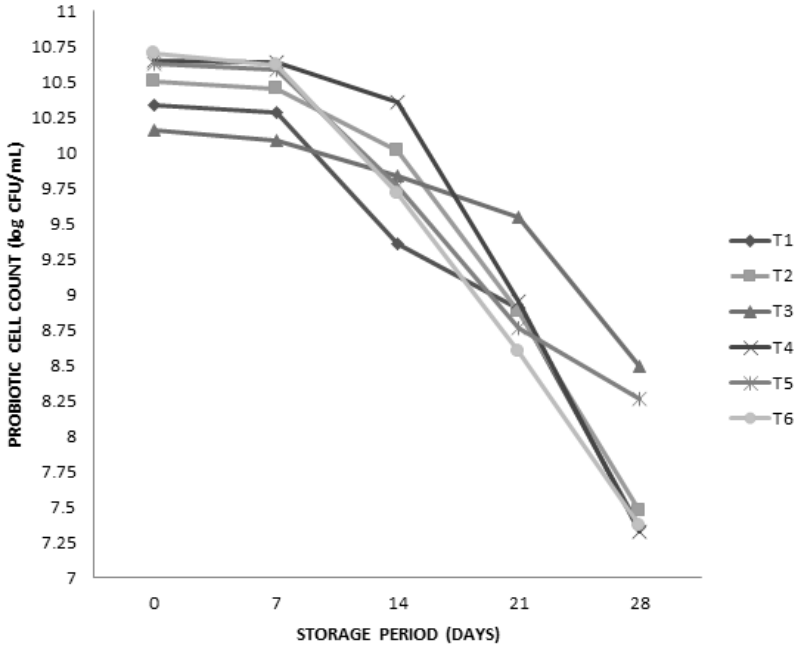
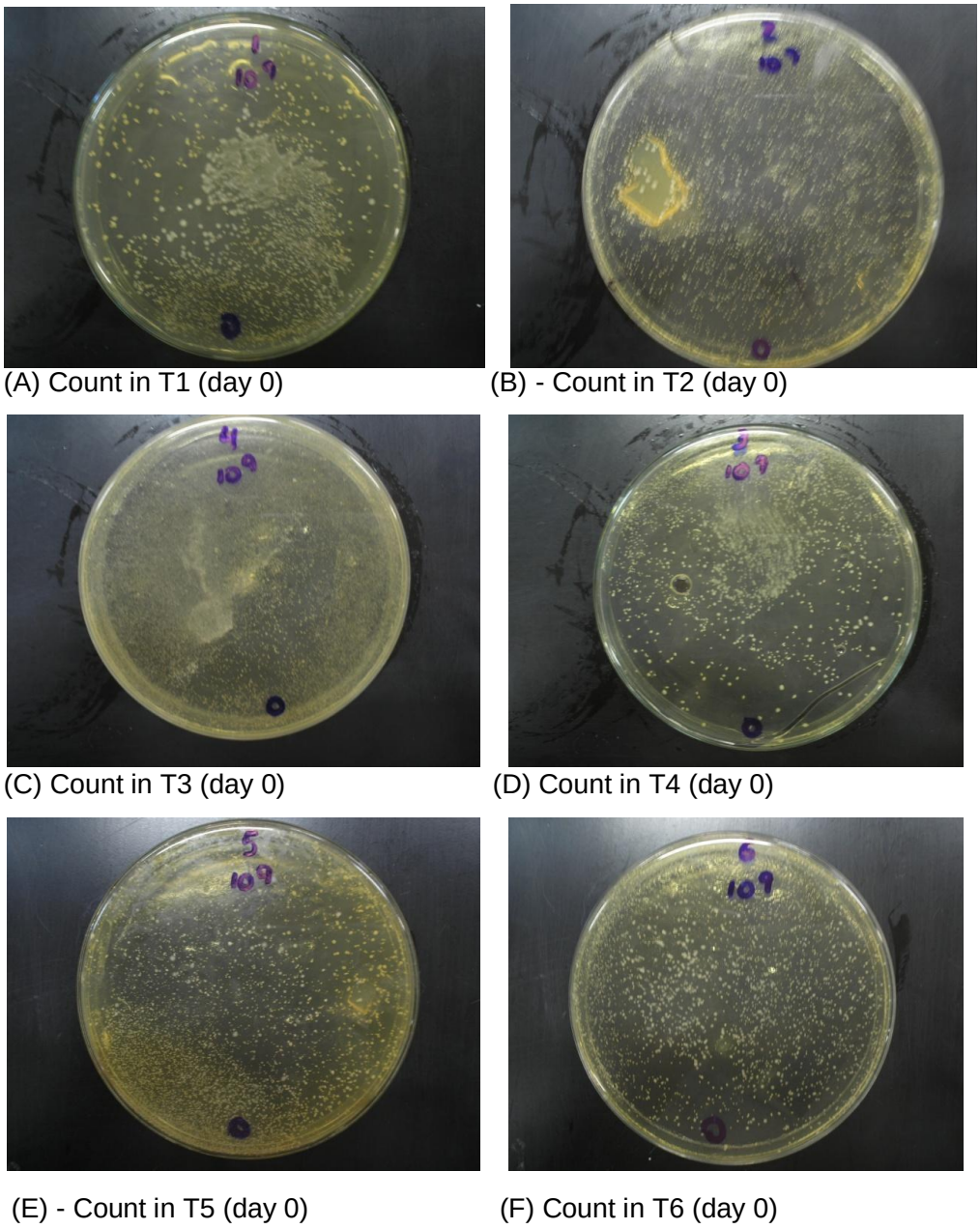


Figure 1. Total viable count of *Lactobacillus acidophilus* during the storage period. T1 = fermented drink with 1% starter and fruit salad flavor; T2 = fermented drink with 1% starter, fruit salad flavor, and supplementation with 2% inulin; T3 = fermented drink with 1% starter, fruit salad flavor and the addition of 4% inulin; T4 = fermented drink with 2% starter and fruit salad flavor; T5 = fermented drink with 2% starter, fruit salad flavor and supplementation with 2% inulin; T6 = fermented drink with 2% starter, fruit salad flavor and the addition of 4% inulin.

microorganisms (Gomes and Malcata, 1999) have generally agreed that products with high acidity (e.g., yoghurt) generate an increased loss of viability compared to probiotic products with low acidity (° Dornic). This concept also explains the high counts of probiotic microorganisms observed in this study throughout the 28 days of storage.

Of the two variables studied (inoculum and prebiotic fiber), a larger amount of inoculum resulted in significantly increased growth of the probiotic microorganism (p-value <0.05). Considering that autolysis reduces the number of probiotic bacteria (Kang et al., 1998; Koch et al., 2008) and that there is a reduction in autolysis in the presence of prebiotics (Saran et al., 2012), the efficient growth of the probiotic bacteria in this study can also be explained by the addition of inulin.

According to Collado and Sanz (2006), Mattila-Sandholm (2002), and Ouwehand et al. (1999), survival of the probiotic bacteria in a food product is fundamental, and sufficiently populations (typically greater than 7 log CFU/mL or g) are of physiological importance to the consumer. This value was achieved in all six treatments in this study. Figure 1 shows that the total lactic bacteria count required for fermented milk (7 log CFU/g),



(A) Count in T1 (day 0) (B) - Count in T2 (day 0)

(C) Count in T3 (day 0) (D) Count in T4 (day 0)

(E) - Count in T5 (day 0) (F) Count in T6 (day 0)

Figure 2. Photos showing the *L. acidophilus* count for the six treatments under study on the first day of storage.

according to the Brazilian Legislation (Brasil, 2000), was reached in all six treatments.

The acidophilus milk produced by Zacarchenco and Massaguer-Roig (2004) showed an initial L. acidophilus count of 8.869 log CFU / mL, a value which exceeded that in all six treatments of the present study on the first day of storage. On day 21 of storage, the probiotic count in the acidophilus milk was 8.322 log CFU/mL, which exceeded that observed in all six treatments in this study.

As expected, after 28 days of storage, the *L. acidophilus* count in the six treatments had decreased but remained above the minimum count of 7 log CFU/ mL. Thus, according to Jelen and Lutz (1998), this beverage could be classified as a probiotic food.

Illustration of probiotic microorganism growth

Figure 2 (A, B, C, D, E, and F) shows the growth of *L. acidophilus* in the six treatments on the first day of storage (day 0). The total probiotic counts in the six formulations ranged from 10.160 to 10.700 log CFU/mL

on the first day and from 7.330 to 8.493 log CFU/mL after 28 days of storage. These levels met the requirements described in the literature as well as those in the Brazilian legislation for fermented milk (Brasil, 2000), which recommends that all microorganisms producing lactic fermentation must be present and viable in the product at a level of 7 log CFU/mL.

Conclusions

With reference to the effects on the lactic acid bacteria population due to the amount of inulin and the inoculum used in various experiments, only changes in the inoculum led to a significant increase (p-value < 0.05) in the *L. acidophilus* population.

All treatments evaluated in this study showed good acceptability for the attributes of color, flavor, aroma and consistency, with the exception of T_6 (fermented drink with 2% starter, fruit salad flavor and the addition of 4% inulin), which presented low acceptability for the attribute of color. Thus, improvements should be made for this formulation to enhance the color via the addition of a larger amount of fruit pulp or a natural color additive.

Although all six treatments presented optimal microbiological, sensory and physicochemical results, the fermented beverage obtained from treatment one presented the best requisites for potential production on an industrial scale, considering the acidity and pH standards proposed by the Brazilian legislation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Impact of pineapple waste silage on intake, digestibility and fermentation patterns of West African dwarf sheep

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Pineapple waste (PW) is a by-product from pineapple processing, mostly dumped to pollute the environment. This study was conducted to recycle PW by ensiling with maize cobs and brewer dried grains at different levels. Twelve (12) West African dwarf rams weighing between 15.00 to16.10 kg were randomly distributed into four diets. Diets included: 0, 20, 40 and 60% PW as diets 1, 2, 3 and 4, respectively. Parameters studied were feed intake, nutrient digestibility and fermentation patterns: total volatile fatty acids, lactic acid concentrations, rumen pH, ammonia nitrogen and nitrogen metabolism. Silage pH ranged from 3.60 to 6.40 in all treatments. Temperature of silages varied from 31.10 to 31.50°C. CP, NDF and ADF ranges were: 12.15 to 13.74, 40.61 to 47.20 and 20.62 to 28.58%, respectively. Body weight intake, faecal output, crude protein intake, crude fibre intake, ether extract intake and ash-intake at 20% inclusion level were: 525.69, 73.27, 72.76, 26.63 and 31.17g/day, respectively. Highest total volatile fatty acid of 56.18 mg/dl was observed in 20% inclusion level. CP digestibility decreased linearly as the protein contents of the diets increased. Therefore, PW can be preserved for a longer period and utilized by ruminants at 20% PW inclusion rate.

Key words: Pineapple waste, silage, intake, digestibility, sheep.

INTRODUCTION

The need for self-reliance in food production especially animal protein is critical not only at national but also at family level. The average African diet is lopsided, excessively packed with carbohydrate sources with negligible quantity of animal protein. FAO (2011) recommended a protein consumption of 65 g per caput per day of which 35 g should be from animal sources. In 2009, world production of pineapple stood at over 18 million tons, pineapple ranks 12th among fruits crop worldwide (FAO, 2011). About 70% of the pineapple produced in the world is consumed as a fresh fruit in the country of origin (Loeillet, 1997), while the remaining 30% is exported or transformed into canned slices, chunks, crush (solid pack) and juice. The post-harvest processing of pineapple fruits yields skins, crowns, bud ends, cores, waste from fresh trimmings and the pomace of the fruit from which the juice has been extracted. Recently, pineapple production for fruit manufacture has developed

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Abbreviations: PW, Pineapple waste; **ADF,** acid detergent fibre; **NDF,** neutral detergent fibre; **ADL,** acid detergent lignin; **CP,** cassava peels.

rapidly, from this large quantity of pineapple; there are considerable quantities of by-products (50 to 80%) that can be used as a feed resource for raising livestock (Devendra, 2001). These by-products account for approximately 30 to 35% of the fresh fruit weight. They can be used as soil amendment or as feedstuffs for all classes of livestock (Hepton et al., 2003). Like other fresh fruit by-products, fresh pineapple cannery waste are rich in water (about 90%) and soluble carbohydrates and decay very quickly.

Therefore, there is the need for rapid utilization of the waste, but, the canneries are often not located in areas of animal production and transportation of such bulky products is expensive and may require daily visits to the cannery (Nhan et al., 2009; O'Donovan, 1978). Consequently, fresh pineapple waste is often preserved by drying or ensiling. On the other hand, drying allows the soluble carbohydrates dispersed in water to be evaporated therefore, ensiling appears the best method for preserving fresh pineapple waste. Ensiling enhances nutritive value, eliminates anti-nutritional elements, prolongs storage life, improves intake and better digestion of the nutrients. Drop in pH during ensiling will eliminate unwanted microbes in the materials and accelerate the breakdown of any residual agro-chemical in the materials. A sustainable method for conserving pineapple waste is to ensile with other materials like maize cobs, cassava peels, which act as absorbent for excess water (Hung et al., 2009). Similarly, ruminants in the tropics encounter feed shortages during the dry season and ensiled pineapple waste can be fed to ruminants during this period of feed shortage. Therefore, this study was designed to determine the impact of pineapple waste silage on the voluntary intake, nutrient digestibility and rumen fermentation patterns of West African dwarf rams and to estimate the optimum level of the pineapple waste that can be effectively utilized for optimum performance.

MATERIALS AND METHODS

The experiment was carried out at the Teaching and Research Farm of the University of Ibadan, Nigeria. The farm is situated between the latitude 7.27°N and longitude 3.45°E at an altitude of 200 to 300 m above sea level. The average annual rainfall was about 1250 mm with mean temperature of 25 to 29°C and average humidity of about 84% during the rainy season and 76.6% during the dry season.

Twelve (12) WAD sheep aged between six to seven months weighing between 15.00 to 16.10 kg were used for the trial. Four diets comprising dried cassava peels (DCP), pineapple waste (PW), brewers dried grain (BDG), ground maize cobs (GMC) and urea (U) at various inclusion levels were formulated as shown in Table 1.

Preparation of pineapple waste (silage)

The silage weighing 50 kg for four different treatments were mixed and filled in plastic silos of 120 L capacity. The container was lined internally with polythene sheets. The silage was compacted manually to displace the air and final compaction was made after which the polythene was wrapped over the material. Sand bag of 45kg weight was later rolled over the filled materials and left for 21 days for fermentation.

Determination of silage quality

The silage was opened after 21 days to determine the silage quality. The assessed quality characteristics were colour, smell, texture, temperature and pH according to Babayemi and Igbekoyi (2008). Immediately the silage was opened, a digital thermometer was inserted to determine the temperature. Sub-samples from different points and depths were later taken and mixed together for dry matter determination by oven drying at 65°C until a constant weight was achieved. The samples were later milled and stored in an air-tight container until ready for chemical analysis. The pH of sub-sampled silage was done by macerating 100 g of the sub-sample in a beaker containing 100 ml of distilled water; pH meter was used with buffer liquid to determine the level of the pH. Colour assessment was ascertained using visual observations with the aids of colour charts. The odour or smell of the silage was relatively assessed by setting up a seven-man panel to determine whether fruity, pleasant or alcoholic, structures of the silage was also determined whether dry, wet, firm, separable.

Silage intake and dry matter digestibility

The sheep were allotted to different metabolic cages specially made for a separate collection of urine and faeces. The sheep were fed with the silage at 5% body weight. Feed intake was determined on the following day by weighing the remnants and subtracting it from the feed supplied. Representative sample (10%) aliquot was obtained from the orts for dry matter and nutrient analysis. 10% aliquot from urine was also taken to determine the nitrogen metabolism; this was well preserved by adding 0.5 ml of concentrated sulphuric acid to trap nitrogen available in the urine and later stored in the refrigerator prior to the chemical analysis. Fresh water was supplied daily *ad-libitum*. Sheep were adapted to the metabolic cages for 14 days and another seven days for the collection of data. In the morning, before animals were fed, faecal output was evacuated and weighed, 10% aliquot taken from each animal and kept inside oven for dry matter determination. These faeces were later bulked, milled and stored in an air-tight container pending chemical analysis.

Determination of rumen fermentation patterns

Rumen fluid samples (80 ml) were taken from 12 rams (three replicates per treatment). The collection of rumen liquor was by the rubber tube suction method as described by Preston (1995). The ruminal fluid was strained through a clean cheese cloth into a thermos flask for different replicates. Rumen fluid pH was determined immediately after sampling with pH meter and the rumen fluid was fixed by adding 10% sulphuric acid solution (0.5 ml sulphuric acid to 9 ml of rumen fluid), for later analysis of ammonia nitrogen (NH_3-N) concentration using the methods of Anderson and Ingram (1979). Lactic acid concentration, volatile fatty acids - acetic acid, propionic acid and butyric acid concentrations as described by Gilchrist and Douglas (1969).

Chemical and statistical analyses

All samples of feed and faeces were oven dried at 105°C for 48 h to determine the dry matter content. When the final weight was

Table 1. Gross composition of experimental diets (g/100 g).

Parameter	Treatment			
	1	2	3	4
Pineapple waste (PW)	0.00	20.00	40.00	60.00
Dried cassava peel (DCP)	68.00	48.00	28.00	8.00
Brewers dried grain (BDG)	20.00	20.00	20.00	20.00
Ground maize cobs (GMC)	10.00	10.00	10.00	10.00
Urea (U)	2.00	2.00	2.00	2.00
Total	100.00	100.00	100.00	100.00

Treatment 1 = 0% PW, Treatment 2 = 20% PW, Treatment 3 = 40% PW, Treatment 4 = 60% PW.

Table 2. Proximate composition of experimental diet (g/100 g) on dry matter basis.

Parameter	Treatment			
	1	2	3	4
Dry matter	84.57	78.48	68.88	62.59
Crude protein	12.15	12.74	13.42	13.74
Crude fibre	12.44	12.65	13.03	13.68
Ether extract	5.51	4.63	3.75	2.85
Ash	6.06	5.42	4.64	3.58
Nitrogen free extract	63.84	64.56	65.17	66.15
Acid detergent fibre	24.44	26.67	28.58	20.62
Neutral detergent fibre	40.61	43.63	47.20	41.57
Acid detergent lignin	5.46	3.54	3.29	2.45
Hemicellulose	16.17	16.96	18.62	20.95
Cellulose	18.98	23.13	25.29	18.17

Treatment 1 = 0% PW, Treatment 2 = 20% PW, Treatment 3 = 40% PW, Treatment 4 = 60% PW.

reached, the samples were milled to analyze for crude protein, crude fibre, ether extract, ash and nitrogen free extract as described by (AOAC, 2000). The fibre fractions: acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) were determined according to Soest et al. (1991).

Data were analyzed using analysis of variance (ANOVA) by following the procedure of SAS (SAS, 2000). Differences between the treatment means were separated using the least significant difference of the same package.

RESULTS AND DISCUSSION

Proximate composition of experimental diet of WAD sheep fed pineapple waste silage are presented in Table 2. The dry matter values obtained were relatively low ranging from 62.59 to 84.57%; this might be due to high moisture of the PW before silage making which affected the digestibility of nutrients. Church (1993) reported that feed with lower moisture content has higher retention in the rumen for microbial degradation. The crude protein values obtained were higher than the 7% critical crude protein required for ruminal function (NRC, 1980) and within the range of the 10 to 13% recommended for growth and maintenance for ruminants (Ranjah, 1981) and 12 to 14% CP for finishing sheep (NRC, 1985). The higher level of crude protein might be due to the fortification of each diet with 2% urea which increased the nitrogen content and subsequently helped in silage fermentation.

The NDF and ADF recorded in this study were slightly higher than ADF and NDF reported by Bardiya et al. (1996). This difference might be due to the differences in the processing method employed in extracting pineapple juice and the level of water soluble carbohydrate available in the pineapple waste. The quality of silages containing PW as replacement for DCP in terms of colour, taste, texture, pH, odour and temperature are shown in Table 3. The colour of silages ranged from dark-brown to yellowish-brown. Differences were observed in the smell of the silages as all silages were characterized by pleasant smell. The only exception for the smell was the treatment 4 that exhibited pleasant-fruity smell. Kung and Shaver (2002) reported that pleasant smell is accepted for good or well prepared

Table 3. Silage characteristics (colour, smell, texture and temperature) of ensiled pineapple waste.

Silage	pH	Smell	Texture	Temperature (°C)	Colour
Treatment 1	6.40	Pleasant	Not firm	31.50	Dark brown
Treatment 2	5.30	Pleasant	Fairly firm	31.60	Brown
Treatment 3	4.10	Pleasant	Firm	31.40	Yellow brown
Treatment 4	4.00	Pleasant and fruity	Strongly firm	31.20	Yellow brown
Pineapple waste	3.60	Pleasant and fruity	Strongly firm	31.10	Yellow

Treatment 1 = 0% PW, Treatment 2 = 20% PW, Treatment 3 = 40% PW, Treatment 4 = 60% PW.

Table 4. Nutrient digestibility of WAD sheep fed with silages of pineapple waste.

Parameter	Treatment				
	1	2	3	4	SEM
Dry matter digestibility (%)	78.16^{a}	71.62^{a}	70.67^{a}	62.83^{b}	1.81
Crude protein (%)	89.28^{a}	84.73^{ab}	83.71^{ab}	80.19^{b}	1.32
Ash (%)	87.79^{a}	86.29^{a}	84.78^{a}	73.61^{b}	1.33
Ether extract (%)	81.22^{a}	76.28^{ab}	75.81^{ab}	69.98^{b}	1.50
Crude fibre (%)	87.49^{a}	84.80^{ab}	82.88^{ab}	79.21^{b}	1.23
Nfe (%)	73.25^{a}	67.46^{ab}	65.33^{ab}	55.02^{b}	2.42
Neutral detergent fibre (%)	83.32^{a}	79.96^{ab}	79.08^{ab}	72.88^{b}	1.43
Acid detergent fibre (%)	82.06^{a}	80.11^{a}	77.23^{a}	63.01^{b}	1.87
Acid detergent lignin (%)	86.77^{a}	71.81^{a}	69.97^{a}	58.47^{ab}	2.13
Hemicellulose (%)	84.39^{a}	84.02^{a}	81.92^{a}	86.40^{a}	1.94
Cellulose (%)	80.76^{a}	81.39^{a}	78.18^{a}	59.24^{b}	2.54

^{abc}Means in the same row with the different superscripts are significantly different ($p<0.05$). Treatment 1 = 0% PW, Treatment 2 = 20% PW, Treatment 3 = 40% PW, Treatment 4 = 60% PW. SEM, Standard error of mean; NFE, nitrogen free extract.

silage. Well preserved silage should retain the original colour (Menesis *et al* (2007) and Oduguwa et al. (2007).

The temperature 31.10 - 31.6°C recorded in this study agreed with Menesis *et al*. (2007) who gave the temperature of best preserved forage as 28 to 32°C. The pH of these experimental silages fell below range of 4.5 - 5.5 reported by Babayemi (2009) for guinea grass harvested at 4 and 12 weeks re-growth. The reason for the differences might be the high content of water soluble carbohydrates in the PW which reduced the pH. This level of acidity in the ensiled PW as replacement for DCP was confirmed by Choopeng et al. (2005) that PW and corn bran are difficult to ensile due to their acidity and high moisture content.

The DM digestibility values obtained in this study as presented in Table 4 ranged between 62.81 to 78.16%. These values were different from 65.10 to 67.0% obtained for sheep fed forage supplemented with concentrates (Adeneye and Sunmonu, 1994). However, Hadjipanayiotu (1990) reported a similar dry matter digestibility for lambs (78 to 79%) and kids (78 to 80%) when concentrate was fed as supplement to hay.

Generally, DM digestibility recorded in this study followed a particular trend. It decreased progressively from T2 to T4. This trend agreed with the findings of Devendra (1977) and Prado et al. (2003) that digestibility decreased when citrus pulp and pineapple waste were included at levels in excess of 30% of sheep ration.

Crude protein digestibility were significantly different ($P<0.05$) between T1, T2, T3 and T4. It was observed that the higher the inclusion levels of PW in the diets, the lower the CP digestibility. Consequently, this observation agreed with the speculation of Quelle et al. (1997) and Giri et al. (2000) that digestibility of nutrients varies with nutrient composition. The higher CP digestibility reported in this study might due to the fortification of silages with 2% urea which improved the protein status of the silages and subsequently enhanced rumen micro-organism proliferation thereby encouraging a more rapid digestion of ingesta.

The value obtained for T2 to T4 were consistent with the range of 63.3 to 78.4% obtained by Olorunnisomo and Ososanya (2002) who fed maize offal and sorghum brewers grain as supplement to WAD goats. The highest

Table 5. Dry matter intake and percentage body weight intake of rams fed pineapple silages.

Parameter	Treatment				
	1	2	3	4	SEM
Dry matter in silage	84.57[a]	78.48[a]	68.88[a]	62.59[b]	3.40
Dry matter Intake (g/day)	592.79[a]	525.69[a]	575.17[a]	574.92[a]	35.43
Faecal output (g/day)	129.48[a]	163.21[ab]	154.20[ab]	213.73[b]	3.57
Urinary output (mls/day)	185.62[a]	217.80[b]	216.42[b]	215.24[b]	4.64
Body wt intake (%)	3.73[a]	3.23[b]	3.47[ab]	3.45[ab]	0.12
Crude protein intake (g/day)	72.05[a]	73.27[a]	70.54[a]	78.99[a]	4.49
Crude fibre intake (g/day)	73.77[a]	72.76[a]	68.49[a]	78.65[a]	4.52
Ether extract intake (g/day)	32.67[a]	26.63[b]	19.66[c]	16.39[cd]	2.53
Ash intake (g/day)	35.91[a]	31.17[ab]	24.39[ab]	20.58[b]	2.11
Nitrogen free extract intake (g/day)	378.57[a]	371.33[a]	342.59[a]	380.33[a]	22.77

[abc]Means in the same row with the different superscripts are significantly different.

ADF digestibility reported in T1 was an indication of ADF content in the silage. Badamana, (1992) reported that diets with higher fibre fractions might have longer retention in the digestive system and hence higher digestibility.

The high digestibility of fibre fractions (NDF and ADF) recorded in T1 to T3 might be due to the ecology of the rumen of animals fed the diet which will favour fibre digestion. The rumen will be colonized mainly by fibre digester probably accounting for the higher ADF digestibility.

This was better explained by Preston and Leng, (1987) that rumen microbes' breaks fibrous feed constituents like soluble and structural carbohydrates providing energy for their own utilization and host animal.

The decreased ether extract digestibility (EED) from T1 to T4 could be best explained by Suksathit and Yanin (2011) who observed that increase in hay content resulted in linearly decreased apparent digestibility across the treatments for diets fed to cattle.

The crude protein intake of 78.99 g/day was highest in T4 followed by T2. The values obtained ranged from 70.54 to 78.99 g/day. However, no significant difference was observed among all treatments. This result does not agree with Correia et al. (2007) who reported a range of 51.83 to 61.51% crude protein intake for sheep fed with ensiled pineapple waste and maize cobs. Higher crude protein intake might be due to the fortification of the silage with urea at 2% which might have improved the intake of the animals.

Normally, fat content of ruminant diet is low (<50 g/kg) and if increased above 100 g/kg, the activities of rumen microbes are reduced (McDonald et al., 2002). Looper et al. (2001) suggested a limit in total fat of 6 to 7% of the ration dry matter. The ether extract intakes in this study were relatively low. The EE intake values ranged from 16.39 to 32.67%. There were significant differences across all treatments. This observation was in agreement with the report of Rogerio et al. (2007) with the range of 15.44 to 31.98% when pineapple waste was fed at different levels to sheep.

Presented in Table 5 are results of feed, dry matter intake, faecal and urinary output of WAD ram fed silages containing pineapple waste. Dry matter in silages ranged from 62.59 to 84.57%. The lowest dry matter intake (DMI) was exhibited by rams on T2 (20% PW) with mean value of 525.69 g/day. The percentage body weight intake on dry matter basis ranged between 3.23% in T2 (20% PW) and 3.73 in T1 (0% PW). The highest faecal output value 213.73 g/day was obtained in T4 (60% PW), followed by rams on T2 (20% PW) with mean value 163.21 g/day. The urinary output ranged from 185.62 to 217.80 ml/day.

The crude protein intake (CPI) values obtained were 72.05, 73.27, 70.54 and 78.99 g/day for rams on diets T1, T2, T3 and T4, respectively. The crude fibre intake (CFI) values ranged from 68.49 to 78.65 g/day. Ether extract intake (EEI) values obtained varied from 16.39 g/day for rams fed T4 (60%PW) to 32.67 g/day for rams on T1 (0%PW). Similarly, the ash intake (ASHI) values followed the same trend with T1 (0% PW) having the highest mean value 35.91 g/day while the lowest value 20.58 g/day was recorded in T4 (60% PW). The nitrogen free extract values ranged between 342.59 g/day in T3 (40% PW) and 380.33 g/day in T4 (60%PW).

The rumen pH ranged from 3.83 to 4.57 which is similar to the findings of Mom serg et al. (2001), Nguyen Van Thu (2001) and Promkot and Wanapat (2003). Vongsamphanh and Wanapat (2004) reported that pH was not significantly different (P<0.050) when ruminants were supplemented with different cassava hays. Optimum pH for maximum microbial growth is between 6.5 and 7.0 (Hungate, 1966).

Lactic acid concentration in the digester of WAD sheep was not significantly affected by increase on inclusion

Table 6. Volatile fatty acids, ammonia-nitrogen and rumen pH of rams fed silages containing pineapple waste.

Parameter	Treatment				
	1	2	3	4	SEM
pH	4.57[a]	3.93[b]	3.83[b]	4.00[b]	0.11
Lactic acid (mg/dl)	29.10[a]	31.41[a]	24.47[a]	23.10[a]	1.58
Propanoic acid (mg/dl)	12.93[ab]	13.96[a]	10.96[bc]	10.27[c]	0.70
Acetic acid (mg/dl)	19.40[ab]	20.94[a]	16.32[ab]	15.40[b]	1.06
Butyric acid (mg/dl)	19.72[a]	21.28[a]	16.59[a]	15.66[a]	1.07
NH_3 - N (mg/dl)	9.03[a]	9.07[a]	8.30[a]	7.96[a]	0.25

[abc]Means in the same row with the different superscripts are significantly different. Treatment 1 = 0% PW, treatment 2 = 20% PW, treatment 3 = 40% PW, treatment 4 = 60% PW. NH_3-N, ammonia nitrogen; SEM, standard error of mean.

levels of dietary PW. The lactic acid concentration values obtained as shown in Table 6 ranged from 23.40 to 29.10 mg/dl. These values were higher than the values reported by Nguyen Thi Hong et al. (2009) who fed sheep with ensiled pineapple waste with maize cobs. The difference might due to the higher content of water soluble carbohydrates in forms of fructose and sucrose that are converted to lactic acid by the lactic acid bacteria in the rumen as a result of extraction process.

The mean values of ammonia-nitrogen (NH_3-N) concentration in the rumen fluid of WAD sheep was not affected by supplementation of urea of 2% for all treatments and were 7.9 to 9.00 mg/dl. The results were similar to the findings Wanapat et al. (2005) who found that the ammonia nitrogen (NH_3-N) concentration in the rumen fluid was not significantly affected but tended to increase when supplemented with a high level of urea. This result agrees with Chathai et al. (1989) who reported a range of 4.5 to 12.4 mg/dl in swamp buffaloes fed untreated rice straw, ammonia nitrogen (NH_3-N) was less than 2 mg/dl and increased to 9 mg/dl when the straw was treated with urea. These observations agreed with range given by Hong et al. (2009) when sheep were fed pineapple waste ensiled with poultry litter (1.6 to 10.4 mg/dl). The ammonia nitrogen concentration reported in this study does not exceed the concentration that can lead to urea toxicity as reported by Church (1993) that 100 mg/dl could lead to urea toxicity in ruminants. The most suitable rumen ammonia-nitrogen levels for microbial activities were 5 to 20 mg/100 ml in ruminants fed on low quality roughages. Wanapat and Pimpa (1999) found that optimum range of NH_3-N was 13.6 to 34.4 mg/100 ml for microbial protein synthesis and digestibility in buffaloes.

A similar result was obtained by Thu and Preston (1999), who demonstrated that a concentration of rumen NH_3-N of 15 to 30 mg/100 ml was optimum for maximum feed intake and digestibility. Preston and Leng (1987) also reported that the optimum level of NH_3-N in rumen fluid for microbial growth ranged from 5 to 25 mg/dl and a range of 8.5 to over 30 mg/dl was considered optimum by McDonald et al. (1996). Increase in rumen NH_3-N in this study is an indication of some breakdown of the protein which could be facilitated by relatively higher pH in the pineapple waste ensiled with urea as non-protein nitrogen source.

Conclusion

Based on the findings of this study, it can be concluded that pineapple waste can be successfully ensiled with other feedstuffs, recycled into animal feed without any adverse effect on the health of the animals. Pineapple waste is a good source of energy and it appears to interact with other feedstuffs which reduces its moisture content and makes it more digestible. Though, it is not a good source of protein but with fortification, it does complement protein utilization of other feedstuffs.

This study showed that inclusion of pineapple waste up to 20% can be used as replacement for cassava peel to promote effective feed intake, improve digestibility, the total volatile acids increased at 20% PW and 48% DCP and this could be attributed to the fact that pineapple waste contains water soluble carbohydrates which can be easily digested. The use of PW as replacement for cassava peel at 20% PW and 48% DCP is therefore recommended to improve livestock production especially in the dry season when grasses are not readily available.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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