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The Acknowledgments of people, grants, funds, etc should be brief.

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Table of Content: Volume 10 Number 7, July 2016

ARTICLES

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Full Length Research Paper

Anatomical characteristics of Nigerian variants of *Caladium bicolor* (Aiton) Vent. (Araceae)

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Four *Caladium bicolor* variants collected from different parts of Nigeria were subjected to anatomical comparison to enhance the taxonomic status of the species. Fresh samples (leaf and petiole) of these variants were fixed in formalin, acetic acid and alcohol (FAA), dehydrated in alcohol series, peeled or sectioned. Peeled specimens were stained with safranin, while sectioned ones were stained with Alcian blue and counterstained with safranin. Good preparations were mounted on slides, viewed and photographed with Optika B-1000 FL LED microscope. Epidermal cells from the variants are mainly pentagonal-hexagonal but rarely heptagonal while the anticlinal cell walls are mainly straight and partly arced/curved. Variants A, B and D are amphistomatic while variant C is hypostomatic. Isotricytic, anisocytic, tetracytic, anomocytic and contiguous stomata were observed among the taxa. The stomata index (SI) varied from 4.35 to 11.76 on the adaxial surface, and from 6.25 to 47.62 on the abaxial surface. Calcium oxalate crystals (druses, 8.18 to 19.09 µm and raphides, 21.82 to 68.18 µm) occur in all variants. Raphides are predominantly found in the petiole while druses and raphides are found in the midrib and petiole. The shapes of the adaxial surface of the midrib are relatively different from each other, and include curved (convex) and flat surfaces. The leaf lamina comprised one layer of palisade and spongy mesophylls each. The number of vascular bundles varied from 11 to 23 in the midrib. These characters can be used to distinguish these taxa especially when combined with the existing data on the species. The similarities among these variants point towards the same evolutionary origin; however may suggest that intraspecific or interspecific hybridizations may have produced the variants.

Key words: Amphistomatic, *Caladium*, druses, hypostomatic, midrib, petiole, raphides.

INTRODUCTION

Caladium bicolor (Aiton) Vent. belongs to the family Araceae and is characterized by various leaf colourations (Hutchinson and Dalziel, 1954). There are over 2000 cultivar names for *Caladium,* and over a hundred cultivars are grown today (Wilfret, 1993). The genus *Caladium* comprises 12 species (Mayo et al., 1997), of which *C. bicolor* is the major source of cultivars. *C. picturatum* and *C. marmoratum* are now considered synonyms of *C. bicolor* (Madison, 1981). It is a monotypic genus that is found in most West African countries (Hutchinson and

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Table 1. Description of voucher specimens studied.

S/N	Species name	Herbarium number	Locality	Date of collection	Coordinates
1	*C. bicolor* (variant A)	UPH/V/1176	Ngor Okpala, Imo State	30/04/2015	N05° 25' 27.60"; E007° 11' 48.10"
2	*C. bicolor* (variant B)	UPH/V/1128	University of Port Harcourt, Abuja Park, Rivers State	19/09/2014	N04° 55' 29.80''; E006° 55' 40.20''
3	*C. bicolor* (variant C)	UPH/V/1126	Obiga-Asa, Ukwa-West L.G.A. Abia State	09/07/2014	N04° 55' 45.40", E007° 14' 28.70"
4	*C. bicolor* (variant D)	UPH/V/1242	Opposite Afam Power Plant, Rivers State	15/04/2015	N05° 21' 49.20", E006° 57' 30.00"

Dalziel, 1954).

In South and Central America other species of *Caladium* like *C. marmoratum* Mathieu, *C. picturatum* C. Koch, and *C. schomburgkii* Schott including *Caladium bicolor* (Aiton) Vent. have been reported (Birdsey, 1951; Hayward, 1950; Wilfret, 1993). Breeding of this species of plant has led to many variants (Wilfret, 1993). Also, intraspecific or interspecific hybridizations among these American species may have produced the cultivated species *C. hortulanum* and other variants of economic values (Hayward, 1950; Birdsey, 1951; Wilfret, 1993; Deng and Harbaugh, 2006a). Members of this genus are used as ornamentals and other economic purposes (Evans et al., 1992; Deng et al., 2005).

The anatomy of vascular and support tissues in the leaf and petiole in Araceaea showed and inferred an interesting relationship among them (Keating, 2000, 2002, 2004; Goncalves et al., 2004). These could suggest correlations between changes in vascular bundle and collenchyma characters and the appearance and further evolution of the unisexual-flowered, aperigoniate aroids (Natalie et al., 2011). Morpho-anatomical character patterns seem to imply a major adaptive shift in the evolution of aroids (Hesse, 2006a, b), and could be used to distinguish closely related plants (Kemka-Evans et al., 2014; Osuji and Nwala, 2015).

The naming and identification of *C. bicolor* cultivars is difficult. This is due in part to the lack of up-to-date reference material which illustrates all cultivars in colour, as well as the sale of cultivars without reliable names (Jin et al., 1999). Among the Araceae family, calcium oxalate deposits can be diagnostic, including their presence, type, diversity, occurrence and distribution have been noted to enhance the delimitation of members of this family (Osuji and Nwala, 2015; Osuji, 2013; Nurul et al., 2013; Mais and Amal, 2012; Gary, 2009) and raphide idioblasts are known as storage facilities for Aroids (Okoli, 1988; Okoli and Green, 1987; Okoli and McEuen, 1986).

Except the morphological description by Metcalfe and Chalk (1968), there is a dearth of information on the biology and taxonomic status of Nigerian species of *Caladium*. The ornamental and economic values of these species and their variants underscore the need for their taxonomic characterization using different systematic lines of study. This study bridges the gap by providing information on the biology and comparative anatomical features (including calcium oxalate crystal types) of four *C. bicolor* variants in Nigeria. The results of the study shall enhance the establishment of the taxonomic status of the species.

MATERIALS AND METHODS

Source of materials

The materials for this study were collected from different parts of the country (Table 1), properly identified using Flora of West Tropical Africa (Hutchinson and Dalziel, 1954), and deposited at the University of Port Harcourt Herbarium. Live specimens are being maintained at the Ecological Center, University of Port Harcourt.

Petiole and midrib studies

Subsamples from the original materials (Table 1) were fixed in formalin, acetic acid and alcohol (FAA) for 12 h. Thereafter, the specimens were dehydrated in ethanol series of different concentration (30 and 50%) and stored in 70% ethanol until when needed. The leaf portion of the petiole, and the central portion of the midrib were sectioned following the method of Agbagwa et al. (2007). The sections were stained in Alcian blue and counter-stained with 1% Safranin red for two minutes, mounted on a slide, viewed and photographed with an Optika B-1000 FL LED microscope.

Epidermal studies

Foliar materials for epidermal studies were collected fresh from plants in the Ecological Centre. 0.5 to 1 cm square leaf cuttings were obtained from identical regions of each fresh leaf, generally from mid-way between the leaf base and apex of lamina including

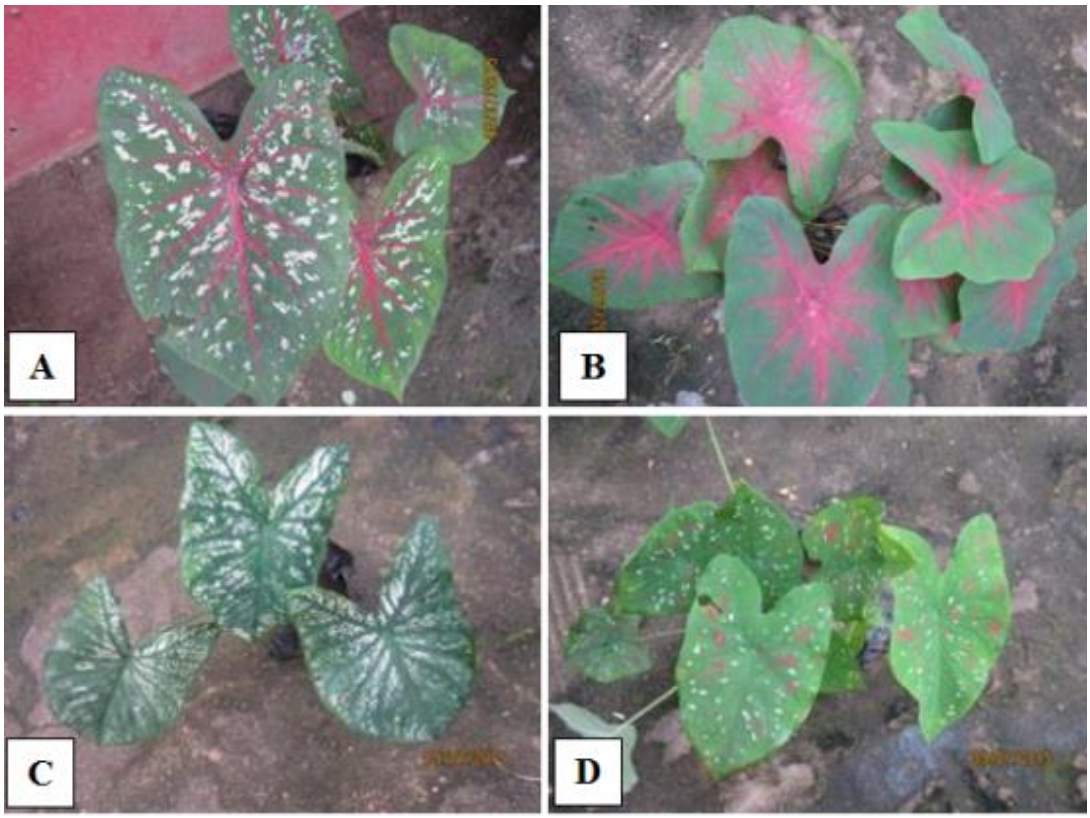

Figure 1. (A-D) Varaints of C.bicolor collected from different locations in Nigeria.

the mid-rib. The adaxial and abaxial epidermal peels were obtained using sharp pointed forceps. Peels were stained with 1% safranin or alcian blue, rinsed with distilled water to remove excess stain and mounted in a drop of pure glycerol on clean glass slides. A cover glass was placed over the drop and sealed with nail vanish to prevent dehydration (Okoli and Ndukwu, 1992). The epidermal features that were observed include: organization of the epidermis, arrangement of the epidermal cells, nature of trichomes, shape of epidermal cells and nature of the anticlinal cell wall of the leaf epidermis, stomatal types, density and index. The stomatal index (SI) was determined based on Metcalfe and Chalk (1979), while the terminology for the stomatal type is taken after Malvey (2004).

Statistical analysis

The average (mean), standard deviation (STD) and range were determined using IBM SPSS Statistics 20.

RESULTS AND DISCUSSION

Four variants of *C. bicolor* were identified. These specimens display a variety of colors including red, white, pink, yellow and green, and are tagged *C. bicolor* var. A, B, C and D (Figure 1). The coloration of the collections conformed to previous reports by Metcalfe and Chalk (1968). These authors reported that the genus *Caladium* in West Africa is monotypic with various colorations.

Epidermal characteristics

No variation was observed on the shapes of the leaf epidermal cell among the different variants of *Caladium* studied. For instance, the shape of the upper (adaxial) and the lower (abaxial) epidermal surfaces in variant A, C and D are mainly pentagonal-hexagonal but rarely heptagonal, while the anticlinal cell wall are mainly straight and partly arced/curved. However, in variant B the shape of the adaxial and the abaxial epidermal surfaces are pentagonal-hexagonal (Figures 2 and 3, Table 2).

Variants A, B and D are amphistomatic (with stomata on both surface of the leaf) while variant C is hypostomatic (stomata on only one surface). This character makes this variant different from the other variants studied. The stomata types observed among the different *Caladium* variants are isotricytic, anisocytic, tetracytic, anomocytic and contiguous (Figures 2 and 3, Table 2). Among these stomata types, tetracytic and anomocytic stomata were dominant (Figures 2 and 3) and were observed on both leaf surfaces of variants A, B and D while in variant C they were recorded only on the lower surface (Table 2).

Isotricytic and anisocytic occurred only on the lower leaf surface of variant D. The occurrence of the same stomata type among these variants of *Caladium*

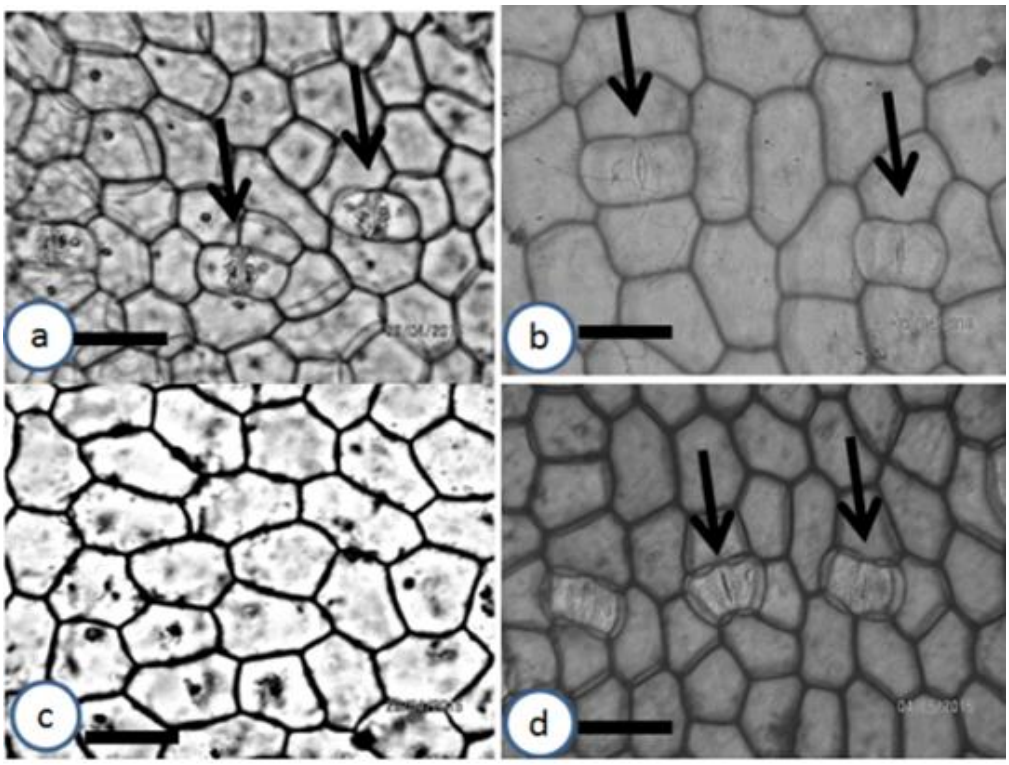

Figure 2. Upper epidermal surface of C. bicolor variants A to D. Bar = 36µm and arrows show stomata.

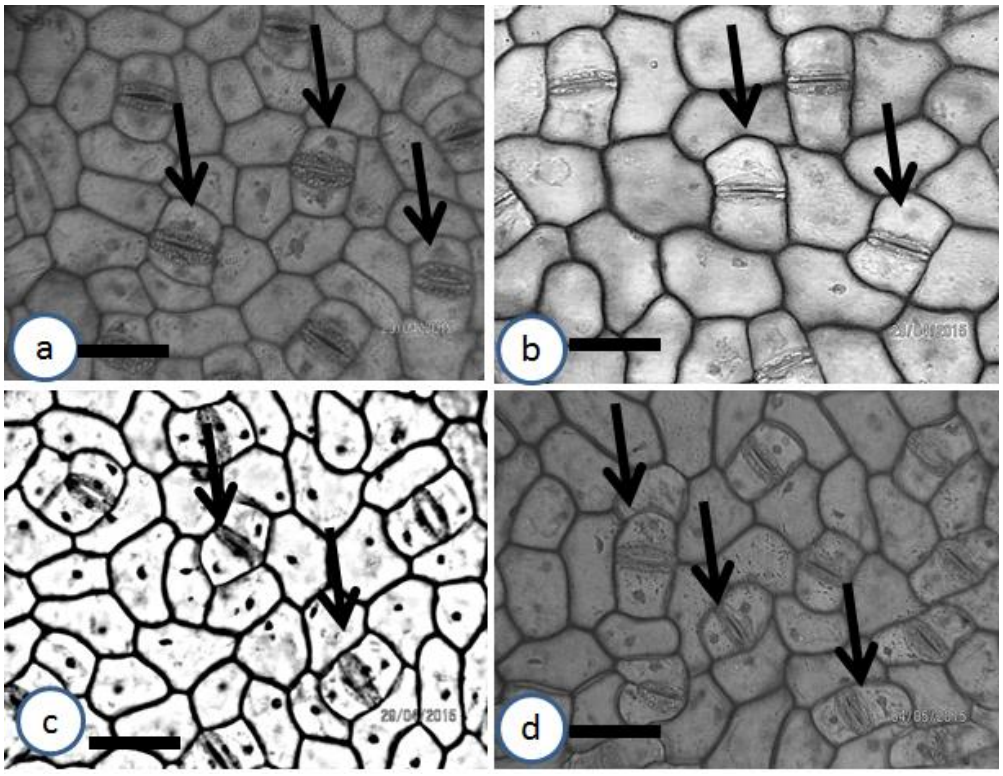

Figure 3. Lower epidermal surface of C. bicolor variants A to D. Bar = 36µm and arrows show stomata (a – d).

Table 2. Upper and lower epidermal characteristics of the *Caladium* variants studied.

S/N	Species name	Lower epidermis				Upper epidermis			
		Shape of epidermal cell	Nature of anticlinal cell wall	Stomata type	Stomatal index (SI)	Shape of epidermal cell	Nature of anticlinal cell wall	Stomata type	Stomatal index (SI)
1	*C. bicolor* (variant A)	Mainly pentagonal-hexagonal but rarely heptagonal	Mainly straight and partly arced/curved	Mainly tetracytic and rarely anomocytic	25.93-37.5 (31.26±4.05)	Mainly pentagonal-hexagonal but rarely heptagonal	Mainly straight and partly arced/curved	Mainly tetracytic and rarely anomocytic	6.25 - 8.11 (7.08±0.94)
2	*C. bicolor* (variant B)	Pentagonal-hexagonal	Mainly straight and partly arced/curved	Mainly tetracytic and rarely anomocytic	22.22 - 37.50 (30.92±5.93)	Pentagonal-hexagonal	Mainly straight and partly arced/curved	Mainly tetracytic and rarely anomocytic	4.35- 11.76 (6.94±3.01)
3	*C. bicolor* (variant C)	Mainly pentagonal-hexagonal but rarely heptagonal	Mainly straight and partly arced/curved	Mainly tetracytic and rarely anomocytic	6.25-36.36 (19.93±10.15)	Mainly pentagonal-hexagonal but rarely heptagonal	Mainly straight and partly arced/curved	Absent	0.00
4	*C. bicolor* (variant D)	Mainly pentagonal-hexagonal but rarely heptagonal	Mainly straight and partly arced/curved	Isotricytic, anisocytic, tetracytic, anomocytic and contiguous stomata	26.67- 47.62 (36.64±7.68)	Mainly pentagonal-hexagonal but rarely heptagonal	Mainly straight and partly arced/curved	Tetracytic, anomocytic and contiguous stomata	4.55 - 8.82 (6.31±2.24)

Table 3. Number of vascular bundles in the midrib and petiole of the *Caladium* variants.

S/N	Species name	Petiole	Midrib
1	*C. bicolor* (variant A)	19	19
2	*C. bicolor* (variant B)	27	11
3	*C. bicolor* (variant C)	22	13
4	*C. bicolor* (variant D)	22	23

indicates very close phylogeny as shown in *Sphenostylis stenocarpa* (Hochst *ex* A. Rich) Harms by (Nyananyo and Osuji, 2007). The presence of isotricytic, anisocytic and contiguous stomata on variant D distinguishes it from the other variants. In the same vein, the presence of brachyparacytic stomata in the abaxial leaf epidermis of an accession of *Xanthosoma* 'Ede Uhie' and its absence in other cultivars indicate divergent advancement in its evolution (Osuji and Nwala, 2015).

This suggests evolutionary divergence between *Caladium* variant D and the other variants. In a similar study among the Araceae, different stomata types namely anomocytic, actinocytic, paracytic, cyclocytic and transitionary types between paracytic and cyclocytic have been noted (Wang and Zhao, 2002). They observed that the epidermal cells are nearly isodiametric in outline with straight, arched and undulate anticlinal walls. Striate ornamentation occurs on periclinal walls of epidermal cells in some species. They noted that though the stomatal apparatus types in Araceae are of little taxonomical significance at intra-family level of Araceae, the combined characters of stomatal apparatuses, the shape of anticlinal wall and ornamentation of cuticules in guard cells may be useful for species identification (Wang and Zhao, 2002; Osuji and Nwala, 2015).

The stomata index (SI) varied from one variant to another. For instance, the stomatal index on the abaxial surface include- 25.93 to 37.5 (31.26±4.05), 22.22 to 37.50 (30.92±5.93), 6.25 to 36.36 (19.93±10.15) and 26.67 to 47.62 (36.64±7.68) for variants A, B, C and D respectively (Table 3). On the other hand, the stomatal index on the adaxial surface of the variants varied from 4.55 to 8.82 (6.31±2.24) in variant D to 6.25 to 8.11 (7.08±0.94) in variant A, and 4.35 to 11.76 (6.94±3.01) in B. The differences in stomata types, their distribution and indices on the leaf surfaces of these *Caladium* variants are diagnostic and could be used to distinguish among variants.

In this study, variants A and B have the same stomata types but the indices varied slightly from each other. Also, variant C has the same stomata types with A and B but it is hypostomatic while variant D has the same stomata types with these

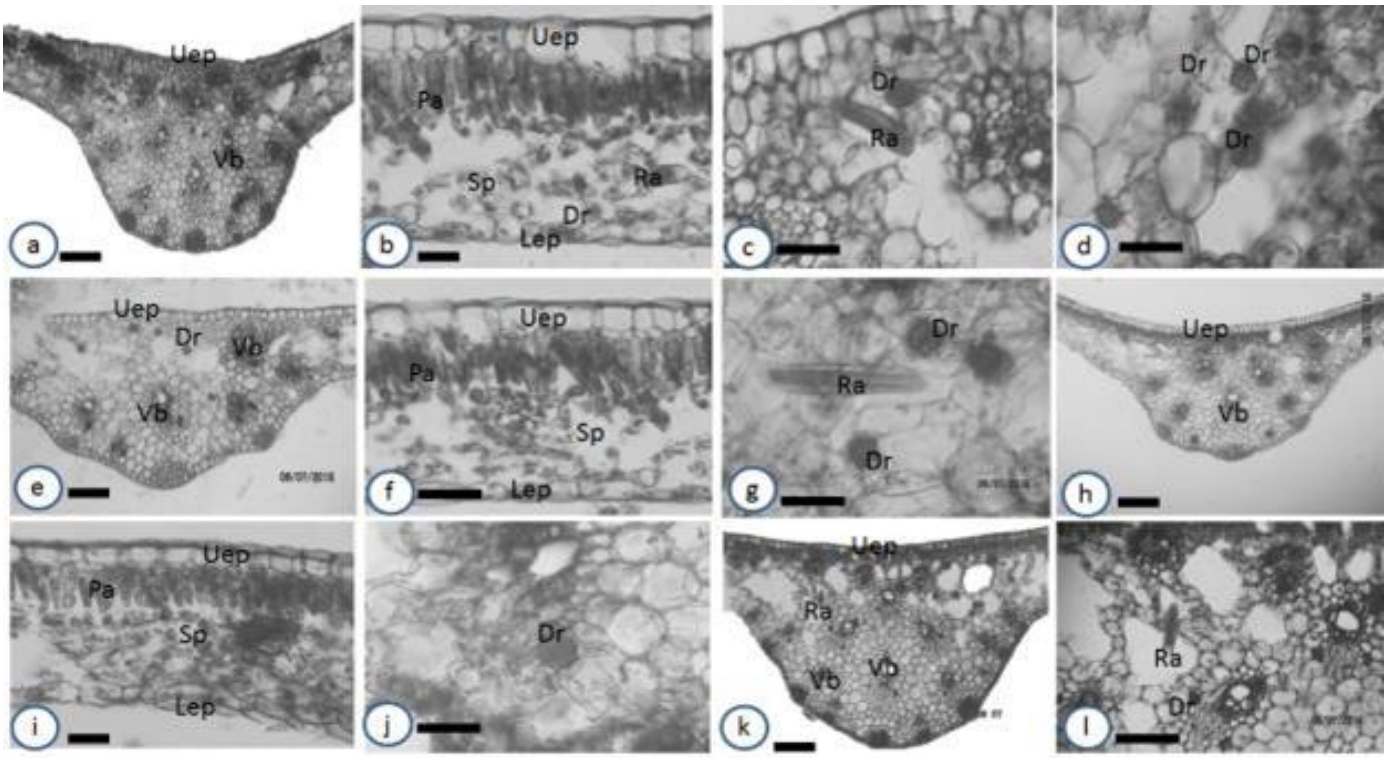

Figure 4. Midrib and leaf laminar of C. bicolor variants- (a-d) – variant A; (e-g) – variant B; (h-j) – variant C and (k-l) – variant D; Vb = vascular bundle; Uep = upper epidermis; Pa = palisade mesophyll; Sp = spongy mesophyll; Lep = lower epidermis; Dr = druse crystal; Ra = raphide bundle; bar = 40µm.

variants in addition to isotricytic, anisocytic and contiguous stomata. This finding however conforms with Osuji and Nwala (2015) who noted the presence of stomata on both upper and lower epidermis of both *Colocasia* and *Xanthosoma* spp. They further noted that stomata were more on the lower epidermis than the upper epidermis. Also, they observed epidermal variations and differences in stomatal indexes within the cultivars of *Xanthosoma* and *Colocasia* and suggested that this could account for their ecological adaptation to variation in the degree of wetness of the environment. Also, stomata types and nature of the epidermal cells have been of diagnostic importance in other members of angiosperm namely *Emilia* (Ndukwu and Agbagwa, 2006), *Abrus* (Agbagwa and Okoli, 2006), *Vernonia* (Kemka-Evans et al., 2014) and *Ixora* (Essiett and Umoh, 2014).

Shape of midrib and petiole

The shapes of the adaxial surface of the midrib are relatively different from each other. In variants A (Figure 4a) and C (Figure 4h), the shapes are curved (convex); in variant B (Figure 4e), it is flat while in variant D it is relatively flat (Figure 4k). This character is fairly diagnostic among the different variants. The leaf lamina comprised one layer of palisade and spongy mesophylls each (Figures 4b, f and i). The upper epidermal cells in the lamina of all the variants are isodiametric or oval. They elongate periclinally (Figures 4b, f and i). Also, the shapes of the petiole in all the variants studied are oval (Figures 5a, c, e and g). These similarities confirm that the variants have the same evolutionary origin and are of the same monotypic taxon (Metcalfe and Chalk, 1968); however intraspecific hybridizations may have produced the variants (Hayward, 1950; Birdsey, 1951; Wilfret, 1993; Deng and Harbaugh, 2006b).

Number of vascular bundle in the midrib and petiole

Variations in number and arrangement of vascular bundles in petiole and midrib have been used to differentiate species of the same genus or family (Ekeke and Mensah, 2015; Agbagwa and Ndukwu, 2004; Metcalfe and Chalk, 1979). For instance, in their studies, Ekeke and Mensah (2015) noted that the number of vascular traces in the midrib of members of Asteraceae varied from one species to another and is diagnostic. Also, Agbagwa and Ndukwu (2004) reported that the number of vascular bundles in the petioles of members of *Cucurbita* could be used to distinguish them (*C. moschata,* 10; *C. maxima,* 16 and *C. pepo,* 14).

In this study, the number of vascular bundles in the midribs and petioles varied from one variant to another

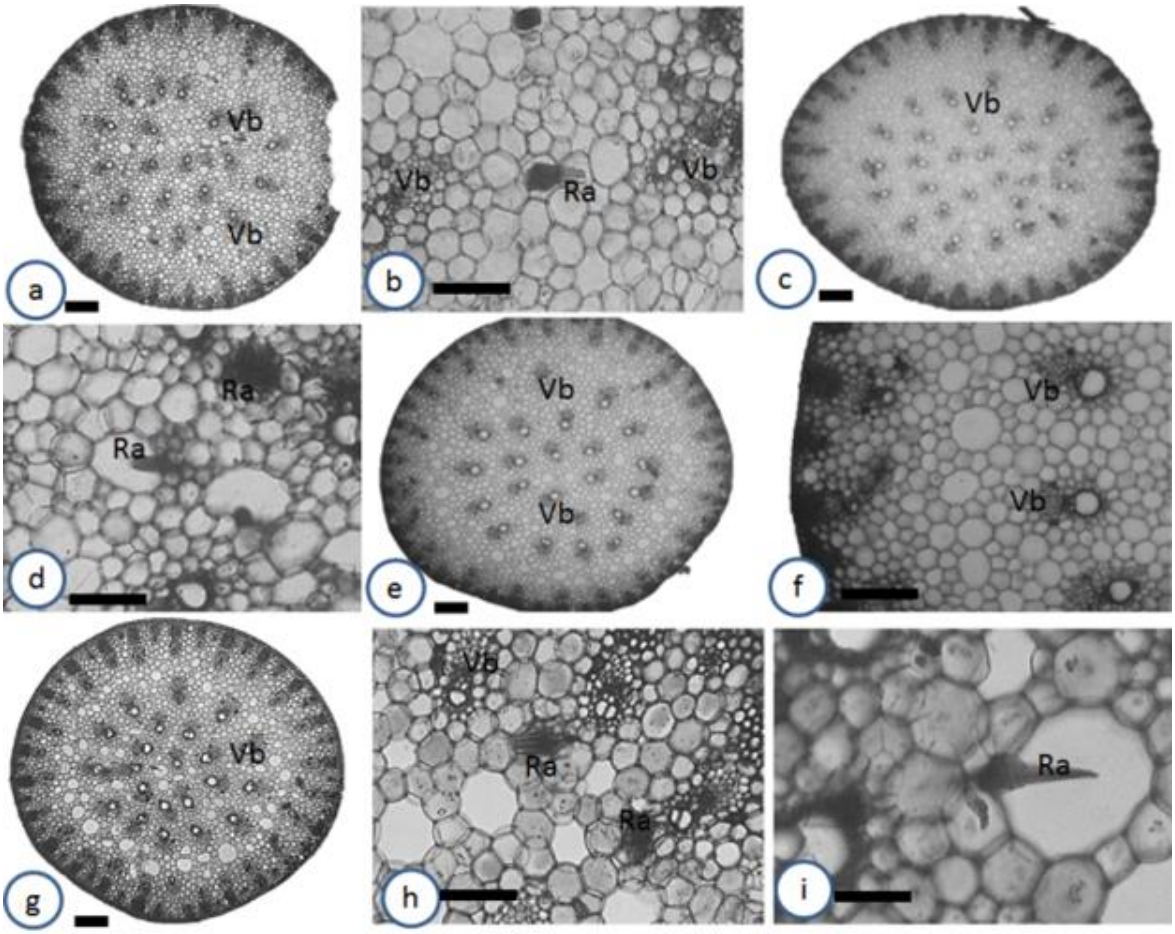

Figure 5. Petiole anatomy of C. bicolor (a-d) – variant A; (e-g) – variant B; (h-j) – variant C and (k-l) – variant D; Vb = vascular bundle; Ra = raphide bundle; bar = 42µm.

(Table 3). The number of vascular bundles in the petiole of the different variants are variant A (19), variant B (27), variant C (22) and variant D (22). In the midrib (Table 3), variant A has 19 vascular bundles, variant B (11), variant C (13) and variant D (23). This variation in the number of vascular bundles is diagnostic and could be used to distinguish the different *Caladium* variants. Though variants C and D had the same number of petiolar vascular bundle, the number of vascular bundle in their midrib differed and could be used to differentiate them. This finding therefore supports that the variation in number of vascular bundle in the petiole and midrib are diagnostic (Ekeke and Mensah, 2015; Agbagwa and Ndukwu, 2004; Metcalfe and Chalk, 1979) and however may suggest that intraspecific or interspecific hybridizations may have produced the variants.

Calcium oxalate types and sizes

Two main calcium oxalate crystal types were observed among the taxa studied. These are druses and raphides (Table 4, Figures 4 and 5). The druses are ubiquitous, raphides are predominantly found in the petiole while druses and raphides are found in the midrib. The occurrence of some of these crystals is specific or restricted to some tissues. For instance, among the taxa studied, the occurrence of the raphides is specific. They are found in the midrib and petiole but not in the lamina. This observation has been made in *Dieffenbachia seguine* (Araceae) (Gray, 2009). He noted that druses appear to be nearly ubiquitous throughout this plant occurring in most of plant parts except in the ovaries.

Furthermore, he observed that different portions of the same organ may have different crystal types such as the leaf margins which have a greater density of druses than the lamina, as well as overlapping raphide bundles not present in the lamina (Gray, 2009).

In the lamina, the calcium oxalates (druses) are embedded in the palisade and spongy mesophylls. They are identified by their dark and spiny nature. The accumulation of the calcium oxalate crystals in the leaf suggests that the taxa are poisonous. This explains why the leaves are not normally consumed by animals and

Table 4. Anatomical characteristics of the petiole and midrib.

S/N	Species name	Petiole		Midrib		Calcium oxalate sizes Range (Mean±standard deviation) µm	
		Shape of petiole	Calcium oxalate type bundle	Shape of upper epidermal surface	Calcium oxalate type bundle	Druses	Raphides
1	*C. bicolor* (variant A)	Oval	Raphide	Curved (Convex)	Raphide and druse	10.91-13.64 (12.43±1.27)	21.82- 40.91 (31.52±7.85)
2	*C. bicolor* (variant B)	Oval	Raphide	Flat	Raphide and druse	10.91-15.00 (13.03±1.54)	31.36-68.18 46.82±13.91)
3	*C. bicolor* (variant C)	Oval	Raphide	Curved (Convex)	Raphide and druse	9.55-19.09 (13.03±2.74)	27.27-43.64 (39.85±5.05)
4	*C. bicolor* (variant D)	Oval	Raphide	Relatively flat	Raphide and druse	8.18-15.00 (11.52±2.27)	24.55-49.09 (36.21±8.35)

humans except after being properly processed. These crystals could therefore be a source of defense to the plant against herbivores. This observation is in line with previous studies on *Xanthosoma* and *Colocasia* (Osuji, 2013; Uno et al., 2001).

The size of druses varied from 8.18 µm in variant D to 19.09 µm in variant C which corresponds to smallest and largest sizes of druse found in *Caladium* variants studied (Table 3). However, the sizes of the raphides ranged from 21.28 µm in variant A to 68.18µm in variant B (Table 4). In similar study in Araceae, Gray (2009) recorded different sizes of crystals in *Dieffenbachia seguine.* In this present study, the sizes of the calcium oxalate observed among the different variants include; druses (8.18 µm to 19.09 µm) and raphides (21.28 µm to 68.18 µm). These ranges fall within 1.0 µm to 250 µm recorded in *Amorphophallus mueleri* (Nurul et al., 2013).

Though there is difference between this value and that recorded in *Caladium* variants, this result supports the placement of both genera in the same family as having the same evolutionary origin. They are genetically controlled (Mais and Amal, 2012) and are useful in germplasm characterization and classification. Furthermore, the result conforms to the previous work in Araceae in the sense that druses and raphides were observed but in contrast because prismatic and styloid were not observed (Osuji, 2013; Nurul et al., 2013; Mais and Amal, 2012; Gray, 2009).

Conclusion

This study has revealed the different calcium oxalate crystals, their sizes and occurrence including the epidermal and anatomical characteristics of leaf, petiole and midrib among the different variants of *Caladium bicolor* in Nigeria. These are useful taxonomic characters for delimiting the variants especially when combined with the existing data on the species.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of plant stage dependency of QTLs to homologous and heterologous rust pathogen isolates of barley

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A disease test at different leaf layers (plant stages) of homologous rust and heterologous rust species were studied. The result from homologous rust species showed that those quantitative trait loci (QTLs) (Rphq2 and Rphq11) which were effective at seedling stage were also effective across all plant stages with gradually decreasing effect as plants grew older. Rphq3 which had consistent effect in all leaf layers confirmed the same result, that it is a plant stage independent QTL. For heterologous rusts, the effect of Rnhq-V was studied on three rust species; P. hordei-murini (Phm), P. hordei-secalini (Phs) and P. triticina isolate 'Flamingo' at three stages (leaf layers). Infection frequencies are higher at seedling stage and dramatically decrease as plants grow older in all three rust species tested on both SusPtrit and Su-Rnhq-V. The difference between lines tends to be reduced with higher leaf layer in all three tested inappropriate rust species. However, this would be not because of less effectiveness of the Rnhq.

Key words: Plant stage, homologous rust, heterologous rust.

INTRODUCTION

Developmental conditions of the host plant may determine the outcome of pathogen infection; on the other hand, pathogen infection can change the developmental program of the host (Haffner et al., 2015; Grant and Jones, 2009). The influence of plant development on disease resistance is very crucial in understanding of plant-pathogen relationship. Resistance to infectious pathogens appears at different stages of host development, varies with plant age or tissue maturity, may be specific or broad-spectrum and is driven by diverse mechanisms, depending on plant pathogen interactions (Develey-Riviere and Galiana, 2007). These responses of plants to infectious pathogens include basal response through transcription of genes in response to pathogen-associated molecular pattern recognition, hypersensitive response at the site of infection, systemic acquired resistance making the entire plant resistant to infection, jasmonic acid response and non-host immunity (Boyajyan et al., 2014).

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Plants are generally more susceptible to disease in early stage than in late period and this could be due to the fact that there is an increase in resistance through time, with plants already resistance to a pathogen increasing their ability to overcome infection and colonization at a later growth stage (Develey-Riviere and Galiana, 2007). Many studies have been published on this phenomenon and reported for large number of crop plants.

So far different barley (*Hordeum vulgaris* L.) genotypes to *Puccinia hordei* were assessed in greenhouse tests at seedling growth stages and in the field at adult plant growth stages (Marcel et al., 2008; Yeo, 2008). For this barley leaf rust *P. hordei* isolate 1.2.1 was used to evaluate the level of partial resistance. Also, a number of quantitative trait loci (QTLs) were found to confer resistance to *Puccinia triticina (Pt), Puccinia hordei-murini (Phm),* and *Puccinia hordei-secalini(Phs)* when inoculated on three barley populations (L94xVada, Steptoe x Morex, and Oregon Wolfe Barley populations) (Bettgenhaeuser et al., 2014). From these, diversity of QTLs govern resistance in host system and also indicated that qualitative R genes are not involved in near nonhost resistance, however, genes conferring partial resistance to adapted pathogens may play role in nonhost resistance (Jafary et al., 2008; Niks, 2014).The effects of QTLs developed from those three barley populations, namely: *Rphq2*, *Rphq11* and *Rphq16* are plant stage dependent (Marcel et al., 2007), that they are effective only at seedling stage. *Rphq3* on the other hand has a strong and consistent effect at both seedling and adult plant stages. *Rnhq* is a QTL for nonhost resistance, and was effective to *Phm* and *Phs* at seedling stage (Jafary et al., 2006).

Near-isogenic lines (NILs) differing with regard to disease resistance QTLs provide valuable material for a more detailed study into the genetic and molecular dissection of the mechanisms underlies the emergence of disease resistance during host development. Such NILs allows the evaluation of QTL in a nearly uniform genetic background, overcoming the difficulties of identifying QTL phenotypes (Marcel et al., 2007). QTL-NILs do not only provide a better estimate for the effect of single QTL alleles, but also provide a better insight into QTL x pathogen and QTL x environment interactions.

Therefore, this study used the NILs developed for partial resistance QTLs *Rphq2, Rphq3, Rphq11,* and *Rphq16*, and a nonhost resistance QTL, *Rnhq* with the objective to evaluate whether these QTLs show plant growth stage dependency on resistance to homologous and heterologous rust pathogen isolates.

MATERIALS AND METHODS

Description of QTL parental lines

The QTLs that were used in this study were mapped in different barley mapping populations namely L94xVada recombinant inbred lines (RIL) mapping population (Neervoort and Parlevliet, 1978), Steptoe x Morex mapping population (Rasmusson and Wilcoxson,1979) and the Oregon Wolfe Barley (OWB) population (Costa et al., 2001).

Table 1. Quantitative trait loci near isogenic lines (QTL-NILs) used in this study.

QTL-NILs	Donor line
Rphq2-BC$_5$S$_1$	Vada
Rphq3-BC$_6$S$_1$	Vada
Rphq11-s.F$_2$.BC$_5$S$_1$	Steptoe
Rphq16-BC$_6$S$_1$	Dom
Qnh.L-F$_2$.BC$_5$S$_1$	L94
Qnh.V.F$_2$.BC$_5$S$_1$	Vada

Development of a research line and NILs

Earlier screens of barley accessions for susceptibility to *P. triticina* and *P. hordei-murini (Phm)* allowed identification of several accessions that showed some degree of susceptibility to these rust fungi. Crosses were made between these barley accessions which exhibited relatively high number of pustules and/or high infection types when infected with *P. triticina* and *Phm*. The F_2 lines for susceptibility to *P. triticina* and *Phm* at the seedling stage grown to adult plant stage and crossed between the two crossing combinations to obtain double cross (DC) plants. Each DC plant was grown to develop DC-S_1 lines by selfing. The most susceptible plants within the most susceptible DC-S_1 lines were selected and selfed for several cycles without selection. Later, susceptible DC-S_5 lines were challenged with *P. triticina* and *Phm*. The DC-S_5 line with the highest number of pustules per leaf and the highest infection type (IT) was selected and named SusPtrit (Sus = Susceptible, P = *Puccinia*, trit = *triticina*). SusPtrit was used as a recurrent parent in near isogenic lines (NILs) development program for the QTLs of our interest. (that is, PR QTLs - *Rphq2*, *Rphq3*, *Rphq11* and *Rphq16* and Nonhost resistance QTL- *Rnhq*).

As stated above, near isogenic lines (NILs) with SusPtrit genetic background (Table 1) and having resistance QTLs, *Rphq2*, *Rphq3*, *Rphq11, Rphq16* and *Rnhq* (*Rnhq-V* and *Rnhq-L*), were used for this study.

The parental lines for each respective NILs were used as a reference. For *Rphq2-BC$_5$S$_1$* and *Rphq3-BC$_6$S$_1$* besides SusPtrit and Vada were used as reference, L94, L94-NILs (L94-*Rphq2* and -*Rphq3*) and Vada-NILs (Vada-*rphq2*, and -*rphq3*) were included as well. The QTLs-NILs seeds were sown together with their respective reference lines. The sowing was done two times a week to ensure sufficient plants of each QTLs-NIL and reference lines at the required stage and was extended for eight weeks to have different plant stages. For each QTL-NIL and reference lines, 2 and 3 seeds, respectively were sown in a pot of 14 cm diameter. At the 8th week, the seeds were sown in boxes (39 cm × 37 cm). The plants were raised in the greenhouse compartments in three replications.

Inoculum

Eight different stages of plants for each partial resistance QTL-NIL were inoculated with barley leaf rust *P. hordei,* isolate 1.2.1. For Su-*Rnhq*, only three different growth stages (first, second and third leaf

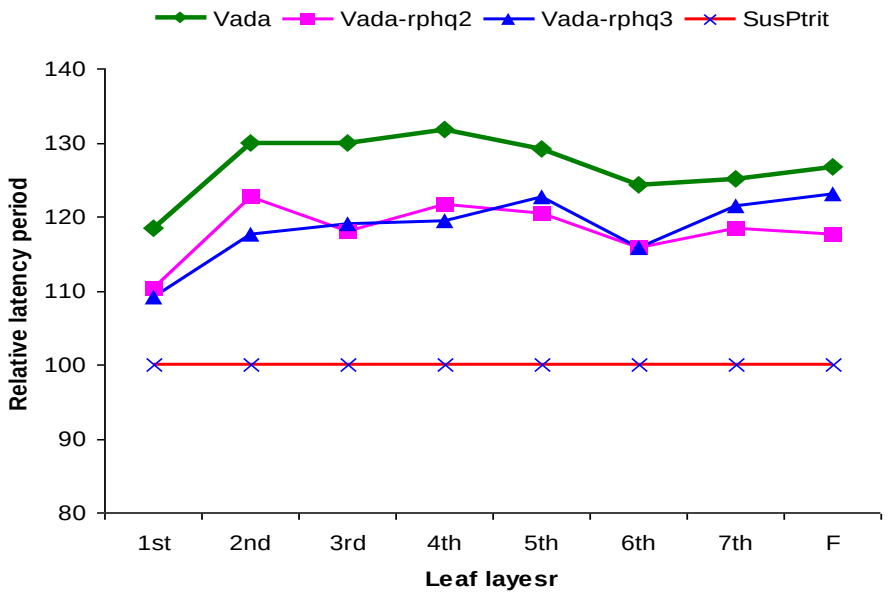

Figure 1. RLP50 of Vada and Vada-NILs relative to SusPtrit infected with *P. hordei*.

stages) were inoculated with *P. triticina (Pt)*, *P. hordei-murini(Phm)* and *P. hordei-secalini (Phs)* because the adult plants are resistant to these inappropriate rusts.

For inoculation of those plants at each stage, 1 mg (*P. hordei* 1.2.1) and 2 mg (heterologous rusts) of spores diluted 10 times with lycopodium spores were used as inoculum for each pot. Then, the inoculum was sprayed over the plants as uniformly as possible. The plants were then placed in a humidity chamber overnight (8 h) at 100% relative humidity in the dark at 18ºC to allow the spores to germinate. After incubation, the plants were transferred to a greenhouse compartment where the temperature was set at 14 ± 3°C with 30 to 70% relative humidity.

Data collection and analysis

Five to eight days after inoculation, when the infection flecks appear, observation zones containing proper density of flecks were delimited by marker. The observations started when the susceptible line showed the first mature pustules. The latency period (LP50) of three to five plants per QTL-NILs and two to three plants per parental line per stage in three replications and averages were considered to reflect the level of partial resistance for each QTL-NILs and donor lines. For all lines two leaves per pot per plant stages were scored.

For heterologous rusts, the frequency of visible infection sites (VIS; the number of both flecks and pustules per cm^2) and infection frequency (IF; the number of pustules per cm^2) following Jafary et al. (2006) were evaluated. Also, the latency period (LP) of the fungi on each plant was calculated. The data collected were analyzed using GenStat 12th edition statistical software.

RESULTS

Plant stage dependency of partial resistant QTLs

All lines tested show an increase in LP from the primary leaf up to the flag leaf. However, the LP of susceptible check, SusPtrit, was lower than the QTL-NILs and other parental lines which carry resistance genes.

LPs for SusPtrit were the shortest, averaging from 192 to 200 h from first leaf to forth leaf. Compared with SusPtrit, LP differences were slightly larger for QTL-NILs (201 to 240 h), Vada NILs (218 to 258 h) and L94-NILs (205 to 248 h) across all leaf layers (growth stages).

Rphq2

From first leaf to third leaf layers, *Rphq2* has longer RLP than *Rphq3* and up to second leaf layers compared to *Rphq11* on NILs with SusPtrit genetic background. Its effect starts to gradually decrease from second and fourth leaf layer onwards on L94-*Rphq2* and Sus-*Rphq2*, respectively. In general, the effect of *Rphq2* in Sus-*Rphq2* and L94-*Rphq2* is not significant above forth leaf layers (Figures 1 to 3). On the other hand, in Vada background NIL with *rphq3*, longer LP was observed after six leaf layer due to the presence of *Rphq2*. Its effect was lower than *Rphq3* on first, second and forth leaf layers.

Rphq3

The effect of this QTL is gradually increased after third leaf stage showing consistent effect in all developmental stages (Figures 1 to 3), except in Vada-*rphq2*. The effect of *Rphq3* was lower than *Rphq2* on L94-*Rphq2* from first to second leaf layers (Figure 2). It is also, lower than *Rphq2* and *Rphq11* from first leaf to third leaf layers on Su-*Rphq2* and Su-*Rphq11*, respectively (Figure 3). On the sixth leaf layer, its effect becomes equal to *Rphq2*

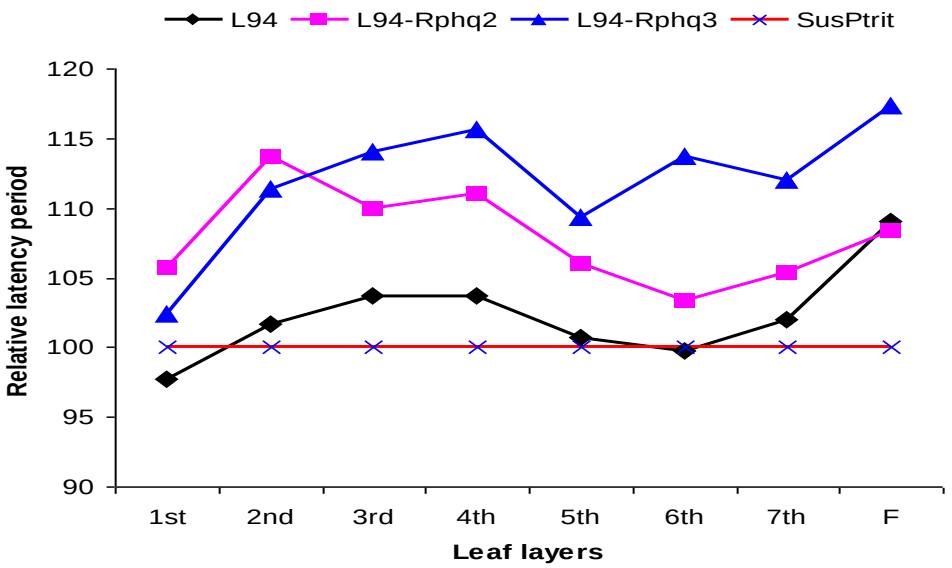

Figure 2. RLP50 of L94 and L94-NILs relative to SusPtrit infected with *P. hordei*.

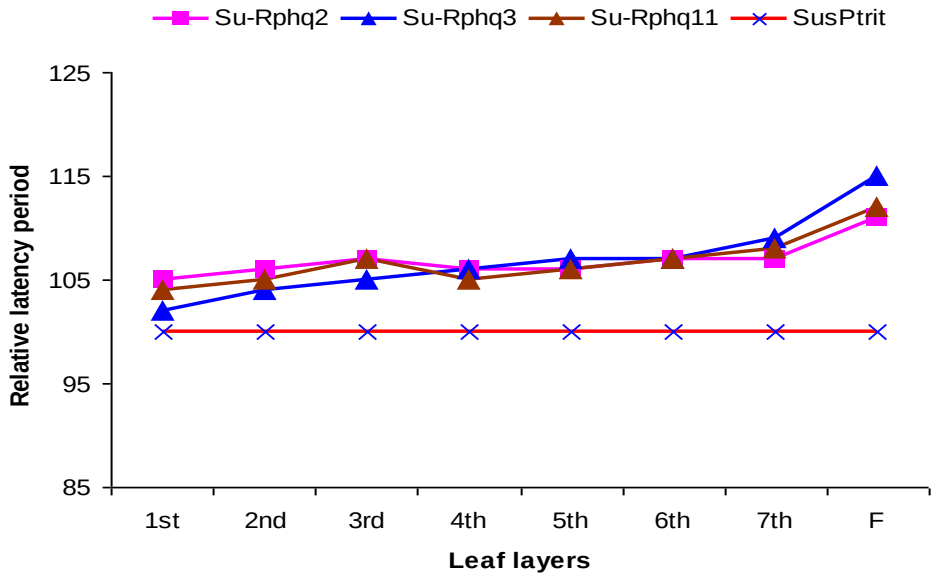

Figure 3. RLP50 of Su-*Rphq2,* Su-*Rphq3* and Su-*Rphq11* relative to SusPtrit infected with *P. hordei*.

and *Rphq11* on Su-*Rphq2,* and Su-*Rphq11,* respectively. However, its effect increases afterwards (Figure 3). In Vada-NILs with *rphq2*, the effect of *Rphq3* was higher than that with *rphq3* up to forth leaf layers though it has lower effect at third and sixth leaf layer afterwards.

Rphq11

The effect of *Rphq11* on Su-*Rphq11* was higher than Su-*Rphq3* from first leaf to third leaf layers (Figure 3). As plants grow higher, the RLP of Su-*Rphq11* increased as in other QTLs-NILs.

Plant stage dependency of nonhost resistance QTLs (*Rnhq-V*)

Effects of Su-*Rnhq-V* to the three inappropriate rust fungi were assessed by determining the relative latency period (RLP) and number of macroscopically visible infection sites. The level of infection established by inappropriate rusts rage from immune (no pustules and less than three flecks per cm^2) to susceptible. Infection frequencies are

Table 2. RIF of Su-*Rnhq*-V, SusPtrit, and L94 infected with *Pt, Phm* and *Phs*.

Lines	*P. Triticina* (Flamingo)			*P. hordei-murini*			*P. hordei-secalini*		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Su-Rnhq-V	66.3	38.0	33.0	37.5	22.0	18.0	39.7	33.7	25.0
Vada	2.1	-	-	-	-	-	6.0	2.0	-
L-94	73.1	47.0	39.5	63.0	57.0	53.0	77.3	69.0	63.0
SusPtrit	100.	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 3. RLP50 of Su-*Rnhq*-V, SusPtrit, and L94 infected with *Pt, Phm* and *Phs*.

Lines	*P. Triticina* (Flamingo)			*P. hordei-murini*			*P. hordei-secalini*		
	1	2	3	1	2	3	1	2	3
Su-Rnhq-V	101	105	103	102	104	103	104	103	103
Vada	-	-	-	-	-	-	117	122	-
L-94	103	105	108	103	105	109	101	103	107
SusPtrit	100	100	100	100	100	100	100	100	100

higher at seedling stage and dramatically decrease as plants grow higher in all three rust species tested (Table 2). The uredia size of infected leaves was small and had chlorosis on Su-*Rnhq-V* NILs at seedling stage as compared to that of SusPtrit.

As shown in Table 2, for infection with *P. triticina,* effect of *Rnhq-V* showed significant effect on Su-Rnhq-V only at second leaf stage. In case of *Phm* at first leaf stage, the effect is not significant; however there was positive effect at second leaf stage. It had positive effect on RLP at first and second leaf stage for *Phs*. However, the effect seems to decrease at second and third leaf stages. In general, the effect of *Rnhq-V* on Su-*Rnhq-V* on RLP tends to decrease with increment in leaf layer in all three tested inappropriate rust species. Parental line 'Vada' showed no sporulating uredia on both *P. triticina* and *Phm* except very few on *Phs*, as a result RLP was not scored for this line on those two rust species (Table 3).

DISCUSSION

Adequate standardization of plant age, inoculum density and quality, and environmental conditions are required to recognize true differences in susceptibility. In this research, the environmental conditions during plant growth prior to inoculation, during exposure of inoculated plants to dew, and during post-dew development were sufficiently defined and controlled to provide an acceptable level of variation in disease development attributable solely to environmental factors.

Near-isogenic lines (NILs) differing with regard to disease resistance QTLs provide valuable material for a more detailed study into the genetic and molecular dissection of the mechanisms underlying the emergence of disease resistance during host development. Such NILs allows the evaluation of QTL in a nearly uniform genetic background, overcoming the difficulties of identifying QTL phenotypes (Marcel et al., 2007). QTL-NILs do not only provide a better estimate for the effect of single QTL alleles, but also provide a better insight into QTL x pathogen and QTL x environment interactions.

In this study, three most effective QTL-NILs with SusPtrit background, *Rphq2, Rphq3,* and *Rphq11* contributed in resistance to homologous rust were used to evaluate the effect of each QTL at different plant development stages.

As shown in Figure 3, *Rphq2* on Su-*Rphq2* had higher effect from first to third leaf layers as compared to *Rphq3* on Su-*Rphq3*. Its effect starts to relatively decrease after fourth leaf layers in SusPtrit background NILs. *Rphq2* is effective at seedling stage and gradually lose its effect as plant grows higher. The effect of *Rphq3* on Su-*Rphq3* consistently increases from first leaf layer to adult plant stage. This consistency in effect indicates that this QTL is stage independent. On the other hand, *Rphq11* on Su-*Rphq11* had no as such statistically significant difference from *Rphq2* and *Rphq3 o*n Su-*Rphq2* and *Su-Rphq3,* respectively. It tended to have an effect intermediate between *Rphq2* and *Rphq3* (Figure 3).

In previous studies (Niks et al., 2000; Marcel et al., 2008) it was reported that *Rphq2* had a strong effect in the seedling stage but almost no effect in adult plant stage, while *Rphq3* was effective in seedling and adult plant stages indicating that *Rphq3* is plant stage independent. Also it was reported (Yeo, 2008) that, *Rphq11* was effective in seedling stage. However, in present study, it was observed that those QTLs which were effective at seedling stage were also effective across all plant stages with gradually decreasing effects

as plants grew older. Previously, these QTLs were reported to be plant stage dependent because they were mapped at seedlings stage but not at adult stage. Here the QTL-NILs used for this study were only those in which QTLs of interest is in the plant material. So, that the QTLs which were reported to be plant stage dependent may not be as reported due to the fact that QTLs do function throughout the plant stage but its effect is smaller than other QTLs detected in a mapping population. However, the effect of *Rphq3* is consistent in all leaf layers observed, indicating that this QTL is plant stage independent as reported in previous studies. As far as *Rnhq* is concerned, Su-*Rnhq-V* had positive effect in resistance at first leaf layer; however, its effect seems to decrease as plants grew older.

Generally, as reviewed in detail in Develey-Rivière and Galiana (2007), resistance acquisition during development has been reported for a large number of crops from both monocots and dicots (wheat, rice, maize, soybean, common bean, tomato, grapevine, tobacco). This resistance to diseases associated with major transitions happening during plant life cycle (Kus et al., 2002; Poethig, 2003; Rusterucci et al., 2005; Baurle and Dean, 2006), function of the maturity of tissue or organ (Zeier, 2005), acquired resistance with development (Panter et al., 2002; Rusterucci et al., 2005), the functional regulation of plant resistance (R) genes (Panter et al., 2002; McDowell et al., 2005), induction of defense mechanisms (Cameron and Zaton, 2004; Dong, 2004; Hugot et al., 2004; Xu et al., 2006). In this study, we observed the same phenomenon of resistance at different developmental stage of nearly isogenic barley lines.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Response of Sesbania (*Sesbania sesban* L. Merr.) to inoculation with indigenous isolates of Rhizobium strains

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Nitrogen fixation through legume-rhizobium symbiosis serves as a cost effective, sustainable and eco-friendly source of N to fodder and grain legume crops. However, there is a need to identify effective rhizobial inoculants compatible with a particular legume. An experiment was conducted to evaluate the effectiveness of forty indigenous isolates of Rhizobium strains on *Sesbania sesban* L. Merr. Each strain was cultured in yeast manitol broth for 3-5 days and inoculated to sesbania seedlings. Unfertilized (-N) and N fertilized (+N) treatments were also included as control treatments. Results revealed that Rhizobium strains have significantly affected nodulation, growth and N content (NC) of sesbania. Based on their relative effectiveness on seedling growth of sesbania, the test strains were grouped into six clusters. Eight strains (20%) in clusters VI, V and VI produced significantly higher nodulation, growth and NC on seedlings of sesbania than those produced by all other strains and +N treatment. On average, these strains increased shoot dry matter and NC by 50 and 50.8 % over +N treatments, respectively. Their mean symbiotic effectiveness (SE) values were > 85 % and hence are classified as highly efficient strains. In conclusion, there is a significant possibility of being able to isolate effective strains, which can be used as inoculants for sesbania, from rhizobial biodiversity resources in Ethiopian soils.

Keywords: Nitrogen, Rhizobia, green manure, Sesbania.

INTRODUCTION

Almost all Ethiopian soils are by far most deficient in Nitrogen (N), severely limiting crop production and productivity (Bekere et al., 2014). Inadequate uses of organic and inorganic fertilizers, continuous cropping, soil erosion and decreasing or abandoning of traditional soil fertility restoration practices are some of the causes that account for its deficiency (Haile and Abay, 2013).

In an effort to overcome the problem, application of fertilizers containing N has long been practiced in Ethiopia. Dramatic increases in the yield of several crops

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have been obtained due to this practice. There were several occasions where the yields of crops were increased by over 100% (Gebre, 2007). Due to this fact, the adoption of N fertilizers by farmers in Ethiopia was far higher than any other agricultural technology. Consequently, the importation and consumption of the fertilizer increased year on year (Haile and Tekalign, 2013). However, the per capita fertilizer consumption is still very low and farmers are applying sub-optimal levels of fertilizers (IFPRI, 2010). The unprecedented increase in the price of fertilizers is the major challenge for low levels, or a lack of, fertilizer use by farmers. Moreover, continuous use of N fertilizers on the same piece of land acidifies soil and aggravate cation losses and pollution (Rowell, 1994; Haile and Abay, 2013).

These problems necessitate the development and use of alternative sources of N. One such alternative is the use of biological nitrogen fixation (BNF), a process in which atmospheric N is converted into a plant available form of N by free living and symbiotic N_2 fixing microorganism. BNF is a cost effective, sustainable and eco-friendly means of supplying N to plants (Hungria and Vargas, 2000). The scientific exploitation of BNF can greatly decrease our dependence on artificially produced commercial N fertilizer and improve the quality and quantity of internal resources (Arujo et al., 2012).

The use of leguminous green manure crops and trees is one of the mechanisms of taking advantage of BNF. Legumes belonging to the family of Fabaceae form symbiotic association in their roots with a group of microorganisms which belong to the genus Rhizobium. In the process of association the roots of legumes and Rhizobia form a structure known as a nodule in which atmospheric N_2 fixed and converted into plant useable form them and for none N_2 fixing crop (Peoples and Craswell, 1992). Sesbania (*Sesbania sesban* (L) Merr.) is one of the most important and widely used green manure and improved fallow species to replenish N depleted soils by farmers in eastern and southern Africa (Makatiani and Odee, 2007). It is a fast growing, leguminous N_2-fixing, multi-purpose tree adapted to subtropical and tropical environments (Makatiani and Odee, 2007). When sesbania biomass is incorporated into the soil as green manure, it decomposes fast and release substantial amounts of crop available N and organic carbon into the soil (Nigussie and Alemayehu, 2013). For instance, Weerakoon (1989) reported that green biomass of *Sesbania sesban* applied at 4.4 tha^{-1} with N equivalence value 83 kg N ha^{-1} increased the grain yield of maize from 1.9 t ha^{-1} in the unfertilized control plot to 3.9 t ha^{-1} in Sri Lanka.

Sesbania was introduced to Ethiopia in the 1970s and thought as the most promising species in the highlands due to its N_2-fixing ability, deep rooting tree with good quality foliage to be used as feed for animals and protein supplement (Mekoya et al., 2009) and for erosion control and soil fertility restoration (Degefu et al., 2011). It can be integrated into the farming system for soil fertility improvement as alley cropping, where its biomass can be pruned and incorporated into the soil, or for the improvement of the fallow system.

The high value of sesbania as indicated above as an organic N fertilizer source for soil fertility improvement and/or as forage is due to its N-fixing ability in symbiotic association with bacteria, commonly known as rhizobia (Wolde-meskel et al., 2004a). Through this association sesbania can fix up to 542 kg N ha^{-1} $year^{-1}$ (Shaheen et al., 2004). Similarly, Degefu et al. (2011) reported that N-fixation by sesbania is in the range of 500 to 600 kg N ha^{-1} $year^{-1}$.

However, the amounts of N fixed by tree legumes, including sesbania among others, depends on the symbiotic effectiveness of rhizobia nodulating the particular legume. This is due to the fact that there are wide variations among legumes in their specificity for a particular rhizobia strain. Soils may contain several types of rhizobia and yet a strain specific and effectively nodulating a particular legume species may be absent or the population of the appropriate strain could be too small for nodulation to occur (Dart et al., 1991). Thus, in such soils there is a need to inoculate leguminous crops and trees with the effective rhizobia strain which is specific to a particular legume species. There are also promiscuous types of rhizobium strains which are able to effectively nodulate different species of leguminous trees and crops. If such strains are obtained, it will be more advantageous than specific strain nodulating and inducing N-fixation in only one species. In any case, developing a specific or/and promiscuous rhizobia strain for enhanced N-fixation by legumes requires isolation and screening such bacteria from diverse agro ecologies and soil types in the laboratory, greenhouse and fields.

Once such effective rhizobium strains are obtained they can be commercially produced and supplied to farmers or any user so that they can be used as inoculants of sesbania. In this regard, Wolde-meskel et al. (2004a) isolated 241 different rhizobium strains from roots of 15 woody species most of them being indigenous to Ethiopia and three leguminous crops. They further reported that they were belonging to genera of *Agrobacterium*, *Bradyrhizobium*, *Mesorthizobium*, *Rhizobium* and *Sinorhizobium* based on bio-chemical and molecular methodologies. However, information on nodulating ability of these strains and their effects on the growth and N content of legumes is lacking. Such information is important to identify effective rhizobium strains that can be used as inoculants of a particular legume which ultimately helps to produce high quality sesbania green manures that will be used to fertilize the soil. Thus, an experiment was conducted to investigate the ability of selected indigenous strains of rhizobia in inducing nodulation in sesbania and to evaluate the effectiveness of each strain on the growth and N content of sesbania in the greenhouse.

Table 1. Indigenous rhizobium strains, their geographical origin, host plant and their genera.

Rhizobium strains codes	Geographical origin (locations in Ethiopia)	Host plant	Genera
AC51a$_1$	Nazret	*S. sesban*	*Rhizobium*
AC51a$_2$	Nazret	*S. sesban*	*Rhizobium*
AC51c	Nazret	*S. sesban*	*Rhizobium*
AC61a	Debrezeit	*V. unguiculata*	*Rhizobium*
AC 61d	Debrezeit	*V. unguiculata*	*Rhizobium*
AC73d	Debrezeit	*P. vulgaris*	*R.huautlense*
AC73b$_1$	Debrezeit	*P. vulgaris*	*Rhizobium*
AC73b$_2$	Debrezeit	*P. vulgaris*	*Rhizobium*
AC73c	Debrezeit	*P. vulgaris*	*Rhizobium*
AC47c	Arba-minch	*S. sesban*	*S.fredii*
AC73e$_2$	Debrezeit	*P. vulgaris*	*Rhizobium*
AC100e	Leku	*A. senegal*	*M. abyssinicae*
AC100c	Leku	*A. senegal*	*M. abyssinicae*
AC28c$_2$	RFC	*A. tortilis*(Meki)	*Mesorhizobium*
AC39a	Chofa	*A. abyssinica*	*M. shonense*
AC39e$_1$	Chofa	*A. abyssinica*	*M. shonense*
AC 39d	Chofa	*A. abyssinica*	*M. shonense*
AC38b$_2$	Akaki	*A. abyssinica*	*Sinorhizobiumi*
AC39e$_2$	Chofa	*A. abyssinica*	*M. shonense*
AC99a	Wondogenet	*S. sesban*	*M. hawassense*
AC99b	Wondogenet	*S. sesban*	*M. hawassense*
AC99c	Wondogenet	*S. sesban*	*M. hawassense*
AC99e	Wondogenet	*S. sesban*	*M. hawassense*
AC98a	Wondogenet	*A. abyssinica*	*M. abyssinicae*
AC98b	Wondogenet	*A. abyssinica*	*M. abyssinicae*
AC98c	Wondogenet	*A. abyssinica*	*M. abyssinicae*
AC98e	Wondogenet	*A. abyssinica*	*M. abyssinicae*
AC40a	Debrezeit	*A. abyssinica*	*Sinorhizobium*
AC21c$_2$	Nazret(A.minch)	*A. tortilis*	*M.plurifarium*
AC51e	Nazret	*S. sesban*	*Rhizobium*
AC50b	Debrezeit	*S. sesban*	*Rhizobium*
AC50c	Debrezeit	*S. sesban*	*Rhizobium*
AC50d	Debrezeit	*S. sesban*	*Rhizobium*
AC18a	Debrezeit	*A. tortilis*(Abergele)	*S.fredii*
AC20b	Akaki	*A. tortilis*(A. minch)	*S.fredii*
AC21c$_1$	Nazret	*A. tortilis*(A. minch)	*Sinorhizobium*
AC25a	RFC	*A. tortilis*(Mega)	*Sinorhizobium*
AC47a	Arba-minch	*S. sesban*	*Sinorhizobium*
AC47b	Arba-minch	*S. sesban*	*Sinorhizobium*
AC46d	Arba-minch	*S. sesban*	*Sinorhizobium*

MATERIALS AND METHODS

Brief description of the study site

The experiment was conducted in Hawassa, southern Ethiopia in the greenhouse of the College of Agriculture, Hawassa University, which is located at 7° 05" N and 38° 47" E in 2013. It has a mean altitude of 1750 m above sea level with mean minimum and maximum temperature of 12.06 and 26.68°C respectively. It receives a mean annual rainfall of 952 mm.

Treatments and experimental procedure

Treatments included 40 Rhizobia strains isolated from roots of different plant species and diverse locations of Ethiopia (Table 1). Moreover, un-inoculated negative (-N) and un-inoculated but N fertilized (+N) control treatments were also included in the experiment for comparison. The rhizobium strains were originally isolated, authenticated and their phylogenetic identities were established by Wolde-meskel et al. (2004 a, b). They were maintained in soil microbiology laboratory, College of Agriculture,

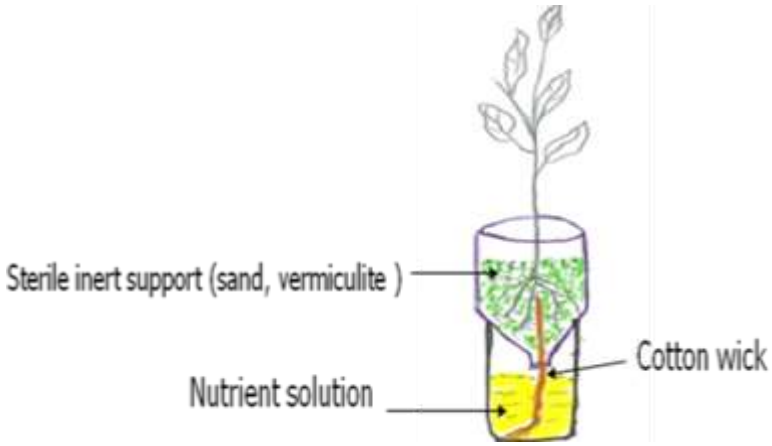

Figure 1. Model of modified Leonard jar containing cotton wick, nutrient solution and sterile inert support.

Hawassa University, Ethiopia and were generously made available by the college for this study. The list of strains along their geographic origin and host plants from which they were first isolated are indicated in Table 1.

The experiment was conducted in the greenhouse using modified Leonard jars, assembled as described in Vincent (1970). The jars were made of two plastic jugs of different sizes. The upper part of the jar had a diameter of 6 cm wide at the mouth and was filled with sterilized sand. The lower part of the jar was used to fill the N free nutrient solution (Figure 1).The cotton wick in the middle makes the nutrient available to the growing plant.

Parallel to preparing the Leonard jars, the laboratory sterilization and pre-germination of sesbania seeds were conducted. Visually healthy looking seeds of sesbania were selected and the surface sterilized with 96% alcohol followed by surface disinfection with 3% sodium hypochlorite for one minute (Somasegaran and Hoben, 1994). Then, the seeds were rinsed with distilled water five times. The disinfected seeds were aseptically transferred into petri-dishes containing 1% water agar medium and allowed to germinate for 12 days at room temperature.

The germinated seeds of sesbania were transplanted to the Leonard jar using sterilized forceps. Two seedlings were planted per jar and later thinned to one seedling per jar. The seedlings in the Leonard jar were fertilized with quarter strength of Modified Jensen N- free solution (Broughton and Dilworth, 1970) twice a week. In the meantime, each strain indicated in Table 1 was grown on yeast extract manitol broth (YMB) for 3 to 5 days, depending on the genera specific of strain. After the emergence of the first leaf (5 days after transplanting), the seedlings of were inoculated with each YMB cultured strains containing approximately 10^9 cell ml^{-1} as per the treatment. Un-inoculated and un-fertilized (-N) control treatment was also supplied with N fertilizer free medium. The un-inoculated but N fertilized (+N) treatment plants were supplied with 1 g of KNO_3 L^{-1} of the nutrient solution (Broughton and Dilworth, 1970). The experiment consisted of a total of 42 treatments and was laid out in completely randomized design (CRD) with three replications. The plants were grown for 9 weeks.

Two weeks after seedlings were inoculated with rhizobium strains, data on plant height (PLHT) and number of leaves (LN) were collected every week until the end of the experiment. The plants were harvested at the end of 9^{th} week. Tops were severed at sand level dried at 70°C in an oven for 48 h and weighed. The roots were carefully separated from the sand from each jar and immediately brought to the nearby soil laboratory where they were washed over 1 mm sieve by gently flowing tap water in order to remove adhering sand and other dust particles. Then nodules were counted. Both the nodules and roots were then weighed after oven drying at 70°C as per the treatment, and the replications were taken.

The dried shoots of each seedling were further processed to demine the total tissue N content. The dried tissues of each seedling were pass through a 0.5mm sized sieve. Then the processed samples were analysed for total N content (NC) by using Kjeldhal digestion procedure as described in Rowell (1994).

Determination of symbiotic efficiency of Rhizobia isolates

Symbiotic efficiency (SE) of each isolate was calculated using the following formula:

$$\text{SE (\%)} \frac{N \text{ in the shoot dry matter of inoculated plant } X\ 100}{N \text{ in the shoot dry matter of N fertilizered plant}}$$

Symbiotic effectiveness (SE) values were rated as ineffective (< 35%), effective (35-85%) and highly effective (>85%) as described in Beck et al. (1993).

Statistical analysis

Data on the number of nodules per plant (NN), nodule size (NS), nodule dry weigh (NDW), shoot dry weight (SDW), root dry weight (RDW), PLHT, NL and tissue N contents (NC) were subjected ANOVA using SAS software version 8.1 (SAS, 2000). Further, mean separations were done using least significant difference method at 0.05 probability level. Cluster analysis of the rhizobium strains studied in experiment was performed using the same software based on nodulation, growth parameters and the NC of sesbania (functional diversity).

RESULTS AND DISCUSSION

Functional diversity of test strains

All strains induced nodulation in sesbania seedlings except AC100e and the results of cluster analysis

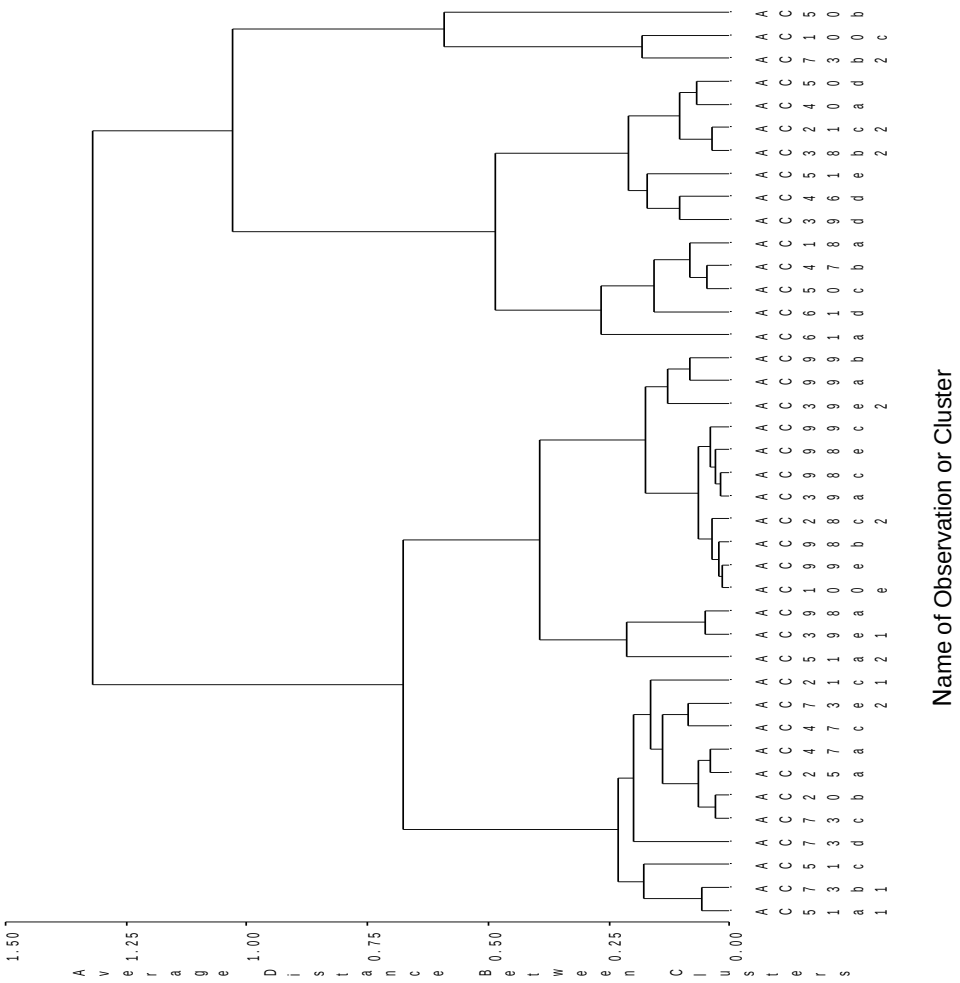

Figure 2. Dendrogam showing the functional relationship or relationships among rhizobium strains nodulating sesbania.

revealed that all the forty rhizobium strains were classified into six clusters. The dendrogram of the six cluster developed based on the functional relationship of the strains in each of the six cluster is shown in Figure 2 and the list of rhizobium strains in each cluster and their proportion are presented in Table 2. The strains in cluster-I account for the largest proportion (35%) compared with all other clusters, followed by cluster-II (27.5%), and the least proportion (2.5%) of strains were found in cluster-VI.

According to Wolde-meskel et al. (2004b, c), AC61a AC61d and AC73b2 were found to be genetically highly related based on RFLP fingerprinting. Similarly, the same authors found that AC50, AC50c and AC50d were genetically and metabolically in the same group. However, the dendrogram developed based on functional diversity in this study has grouped test strains in different clusters (Figure 2). This shows that strains of similar genetic and physiological origin do not necessarily have similar symbiotic effectiveness.

Cluster mean effects of strains on nodulation and growth of sesbania

The mean nodulation and plant growth parameters, tissue N content in sesbania produced by rhizobium strains in each cluster and their mean symbiotic effectiveness values (SE) are presented in Table 3. Strains in cluster-I and II accounted for 62.5% of all the test strains and produced lower growth parameters and tissue N content in sesbania than that produced in N fertilized (+N) treatment. Furthermore, their mean SE values were less than 35% and thus, they were classified as symbiotically ineffective (Beck et al., 1993). Therefore, strains in these clusters were inferior to N fertilizer treatment based on all indices of symbiotic efficiency. On the other hand, the strains in cluster III produced growth parameters and N content in sesbania similar to that produced in N fertilized treatment. Their mean SE value was 101% which is greater than 85% indicating that they are symbiotically effective and have a potential to be

Table 2. List of rhizobium strains in each cluster and their proportions.

Clusters	List/codes of rhizobium strains	Number of strains in each cluster	Proportion (%)
I	AC100e, AC28c$_2$, AC39a, AC39e$_1$, AC39e$_2$, AC99a, AC99b, AC99c, AC99e, AC98a, AC98b, AC98c, AC98e, AC51a2	14	35
II	AC73c, AC51a1, AC20b, AC25a, AC51a$_1$, AC73b1, AC47c, AC73e2, C21c, Ac51c,AC73d	11	27.5
III	AC40a, AC21c$_2$, AC38b2, AC51e,AC50d, AC39d, AC46d	7	17.5
IV	AC50c, AC47b, AC18a, AC61d, AC61a	5	12.5
V	AC100c, AC73b2	2	5
VI	AC50b	1	2.5
Total	**-**	**40**	**100**

Table 3. Cluster mean effects of rhizobium strains on nodulation, growth and N content of sesbania and their symbiotic efficiency (SE) of Rhizobia isolates.

Rhizobia clusters	*NN	NS (cm)	NDW (mg plant^{-1})	SDW g plant $^{-1}$	RDW g plant $^{-1}$	PLHT (cm)	NL	N (mg plant^{-1})	SE (%)
Cluster-I	12	0.7	25	0.20	0.1	18	7.0	2.5	25.8
Cluster-II	43	1.9	62	0.7	0.3	32	11.2	3.3	34
Cluster-III	44	1.9	66	0.8	0.5	36	11.6	9.8	101
Cluster-IV	62	2.2	82	1.0	0.6	38	11.5	12.0	124
Cluster-V	68	2.7	115	1.2	0.7	41	13.8	14.3	147
Cluster-VI	76	2.8	130	1.4	1.0	51	12.7	19.6	202
-N control	0	0	0	0.11	0.08	12	4.7	1.2	-
+N control	0	0	0	0.8	0.46	31	10.3	9.7	-

*NN = number of nodulest, NS = nodule size, NDW = nodule, dry weight, SDW = shoot dry weight, RDW = root dry weight, PLHT = plant height, LN = leaf number, N = Nitrogen and SE = Symbiotic Efficiency.

exploited as inoculants of sesbania.

However, those strains in clusters IV, V and VI produced a superior mean growth and N contents than that produced in +N treatment. This finding has also been substantiated by their very high SE values (Table 3). The strains in these clusters accounted for only 20% of all strains screened.

Effects of individual strains in clusters from III-VI on sesbania seedlings

The effect of individual rhizobium strains in cluster III, IV, V and VI on the nodulation, growth and N content of sesbania are summarized in Table 4 and 5. Accordingly, there is a significant variation in the strains in relation to the nodulation and growth of sesbania. The highest NN, NS, NDW and SE were recorded in sesbania seedlings inoculated with AC50b followed by AC100c, AC73b2 and AC50c in that order, while AC39d and AC50d were the lowest (Table 4).

There is a significant variation in the Rhizobium strains in relation to the nodulation and growth of sesbania. Eight (20%) of all tested strains produced significantly higher shoot and root dry matter yields and higher plant height and tissue N content in sesbania seedlings than that produced in the N fertilized treatment. However, the highest growth and N content in sesbania seedlings were still obtained from inoculants of AC50b, AC100c and AC73b2. These inoculants for example increased SDW by 64, 39 and 40% over that obtained with N fertilizer treatment respectively. The corresponding increases in PLHT were 64, 36 and 29 % respectively. On the other hand, these inoculants increased the N contents of sesbania seedlings by 102, 41 and 53.6% over +N treatment respectively. This is in line with the findings of Makatiani and Odee (2007) who reported that inoculation of sesbania with indigenous rhizobia isolate GSS1 significantly increased SDW and NC by 23 and 20% respectively over the control in the pot experiment. The apparent superior growth and N contents observed in sesbania seedlings which were inoculated with these three strains could be due to their higher N input to the plant through N_2 fixation. Figure 3 shows the dramatic (left) effect of AC50b on sesbania seedling relative to the negative control (middle) and positive control (right)

Table 4. The effects of rhizobium strains on nodulation parameters of sesbania and their SE.

Treatment	NN	NS (cm)	NDW (mg plant^{-1})	SE (%)
-N control	0h†	0^{f}	0g	-
AC61a	74.0^{a}	2.6^{a}	76.7^{de}	127
AC61d	71.0^{a}	2.2^{bc}	95.0^{cd}	133
AC73b2	70.0^{a}	2.6^{ab}	107.5^{bc}	154
AC100c	73.3^{a}	2.7^{a}	116.2^{ab}	141
AC39d	39.3^{f}g	2.3^{bc}	55.1^{f}	109
AC38b2	49.3^{cde}	2.0^{de}	79.5^{de}	106
AC40a	34.0g	0.23^{f}	57.3^{f}	98
AC21c2	46.3^{def}	2.4^{bc}	72.0^{f}	98
AC51e	61.3^{b}	2.2^{cd}	73.4^{f}	106
AC50b	76.0^{a}	2.8^{a}	125.8^{a}	202
AC50c	56.7^{bc}	1.8^{e}	71.0^{ef}	120
AC50d	39.3^{f}g	2.2^{cd}	62.2^{f}	95
AC18a	57.0^{bc}	2.8^{a}	85.4^{de}	121
AC47b	52.0^{cd}	2.7^{a}	79.1^{de}	119
AC46d	43.7^{ef}	2.1^{ef}	57.8^{f}	97
+N control	0h	0^{f}	0g	-
LSD 0.05)	**7.9**	**0.33**	**18.3**	**-**
CV (%)	**9.5**	**10.2**	**15.4**	**-**

†Means within column followed by the same letter (s) are not statistically different from each other at 0.05 probability level.

Table 5. Effects of rhizobium strains on growth and N content of Sesbania 9 weeks after planting.

Treatments	SDW (g plant^{-1})	RDW (g plant^{-1})	PLHT (cm)	NL	NC (mg plant^{-1})
-N control	0.08^{f}	0.034^{g}			
AC61a	0.91^{de}	0.18^{cde}	35.0^{d}	10.7^{d}	12.3^{cde}
AC61d	1.02^{bc}	0.18^{cde}	39^{bcd}	11.0^{bc}	12.9^{cd}
AC73b2	1.15^{b}	0.22^{bc}	40.0^{bc}	13.0^{ab}	14.9^{b}
AC100c	1.15^{b}	0.25^{ab}	42.3^{b}	14.3^{a}	13.7.^{bc}
AC39d	0.9^{cde}	0.14^{ef}	39.3 ^{cd}	12.0^{bcd}	10.6^{efg}
AC38b2	0.86^{cde}	0.12^{ef}	37.3^{cd}	11.3^{cd}	10.3^{fg}
AC40a	0.81^{e}	0.18^{cde}	32.0^{f}	12.0^{bcd}	9.5^{g}
AC21c2	0.81^{e}	0.17^{de}	35.0^{ed}	12.3^{bcd}	9.5^{g}
AC51e	0.88^{cde}	0.16^{ef}	38.5^{cd}	13.7^{ab}	10.3^{fg}
AC50b	1.35^{a}	0.3^{a}	51.0^{a}	13.0^{ab}	19.6^{a}
AC50c	1.0^{bcd}	0.22^{bcd}	40.3^{bc}	12.0^{bcd}	11.6^{def}
AC50d	0.77^{e}	0.14^{ef}	36.7^{cd}	10.7^{d}	9.2^{g}
AC18a	0.95^{cde}	0.14^{ef}	38.7^{bcd}	12.0^{bcd}	11.7^{def}
AC47b	1.0^{bcd}	0.14^{ef}	37.0^{cd}	12.0^{bcd}	11.5^{def}
AC46d	0.84^{de}	0.13^{ef}	35.0^{de}	11.3^{cd}	9.4^{g}
+N control	0.83^{ed}	0.14^{ef}	31.^{f}	10.3^{d}	9.7^{g}
LSD 0.05)	**0.18**	**0.05**	**3.7**	**1.85**	**1.74**
CV (%)	**12.5**	**18**	**6.1**	**9.7**	**9.5**

Means within column followed by the same letter (s) are not statistically different from each other at 0.05 probability level.

treatments.

The majority of strains in Table 4 or 5 which were found to be effective inoculants of sesbania were found to belong to the genera of *Rhizobium* and *Sinorhizobia*. The

Figure 3. Sesbania seedlings as they were grown in Leonard Jars in the greenhouse. Seedling inoculated with AC50b (Left), seedling without N fertilizer and without inoculants (Middle), and seedling received N fertilizer (Right).

test strains in the two genera accounted for 53.3 and 33.3% of all the test strains. This is in line with Wolde-meskel et al., (2004a) who reported that most strains that produced higher plant height and dry matter in sesbania belonged to the genera of *Rhizobia* and *Sinorhizobia*. The apparent large number of N_2 fixing bacteria which we found to be effective in nodulating and enhancing the growth and N content of sesbania suggests that the tree is promiscuous both for nodulation and effectiveness. Similarly, Boivin et al. (1997) reported that sesbania species can enter into symbiosis with many other species of rhizobia.

Conclusion

It is concluded that out of the forty rhizobium strains screened for their symbiotic effectiveness in the greenhouse, 37.5% of them produced dry matter yield, plant height and tissue N content in sesbania similar or higher than that produced in +N fertilizer treatment. Of this number only eight (20%) of them produced significantly higher dry matter, plant height and tissue N content in sesbania than that produced in +N control treatment. This shows that there is a high potential to identify effective rhizobium strains from indigenous sources from the diverse agro ecologies of Ethiopia. This, further implies that some of the efficient strains identified in this study can be used as inoculants of sesbania for enhanced production and productivity of the tree which in turn can be exploited as green manure or/and forage. Most of the rhizobium strains identified as effective symbionts of sesbania were originally isolated from trees and low land food leguminous crops other than sesbania. This implies that there is possibility to use one or more of the rhizobium strains identified as efficient in this study as inoculants of many legumes.

Conflict of Interest

The authors have not declared conflict of interest.

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