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# MORPHOLOGICAL DIVERSITY OF ARBUSCULAR MYCORRHIZA COLONIZING TWO AROMAIC GRASSES *VETEVIRIA ZIZANIOIDES* AND *CYMBOPOGON JWARANCUSA*

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**Abstract**

A survey of two aromatic grasses *Cymbopogon jwarancusa* and *Veteviria zizanioides* was conducted to determine the morphological diversity of mycorrhiza collected from various habitats of southern Punjab and northern areas of Pakistan. The mycorrhizal colonization has been categorized as extraradical and internal colonization along with Arum-type and Paris-type mycorrhiza. The extraradical phase consists of various types of hyphae while internal phase is comprised hyphae and a variety of endogenous structures (i.e. vesicles, arbuscules, hyphal coils and cuttings of hyphae in the cortical regions of the fine roots). Hyphal coiling was frequently observed in C. jwarancusa, coiled hypha were mostly aseptate, and coenocytic, while hyphal coiling was uncommon in *V. zizanioides*. No arbuscule had been observed in *C. jwarancusa* but feeder roots in *V. zizanioides* had extensive arbuscular colonization. Higher morphological diversity was observed in the roots of *C. jwarancusa* as compared to *V. zizanioides*. Both *Paris* and *Arum* type of Mycorrhiza were observed in the studied grasses. Many extraradical and intraradical non-mycorrhizal dark septate endophytic fungi along with unique type of hyphae with hyaline wall were also observed. The dark septate endophytic fungi had melanized hyphae and microsclerotia. The dimeter of microsclerotia ranged from 1.9 to 3.8µm.

## Introduction

Mycorrhizae establish a symbiotic association with plant root, arbuscular mycorrhizal fungi (AMF) are predominantly involved in nutrient transfer from soil, especially phosphorus and other poorly mobile nutrients (O'Keefe & Sylvia, 1991). They have very broad host range as they colonize members of more than 80% of all vascular plant families (Trappe, 1987). Several mycorrhizal types have been described but two types, the *Arum*-type and the *Paris*-type are mostly recognized based upon the morphological characteristics and the way they colonize the roots (Gallaud, 1905; Smith & Read 2008).

In the past, *Arum* type of AMF thought to be more common than the *Paris* type of AMF but Smith & Smith (1997) suggested that *Paris* type of AM is also found in wide range of genera such as *Parnassia, Colchicum, Gentiana, Erythronium, Trillium*, *Asarum* and *Acer* (Gallaud, 1905; Brundrett & Kendrick, 1990a).

The diagnostic features of arbuscular mycorrhiza (AM) are the highly branched arbuscules and vesicles of various shapes and sizes that develop in the root cortical cells. The fungus initially grows between cortical cells but soon penetrates the host cell wall and grows within the cell lumen. Neither the fungal cell wall nor the host cell membrane is branched (Bonfante & Perotto, 1995). As the fungus grows, the host cell membrane invaginates and envelops the fungus, creating a new compartment, where materials of high molecular complexity are deposited. This apoplastic space prevents a direct contact between the plant and the fungus cytoplasm and allows efficient transfer of nutrients between the symbionts. Other structures produced by VAM fungus are auxiliary cells, extrametrical hyphae, and spores (Jakobsen *et al*., 1992).

It is found that AMF morphology is highly influenced by host plant identity and genotype (Smith & Smith, 1997). Early studies revealed that a single glomaline isolate might produce *Arum* type in *Zea mays* and *Paris* type AMF in *Solanum tuberosum* (Barrett, 1958). Thus, the combinational studies with variation of either AMF species or Plants suggest that the identity of either partner might effect the morphological development of fungi. For instance by inoculating six different species of mycorrhiza on *Allium porrum*, Cavagnaro *et al*., (2001) found that out of these six AMF species, *Glomus intreadicus, G. mosseae* and *G. versiform* formed *Arum* type while *Gigaspora margarita*, *Glomus coronatum* and *Scuttellosporea calospora* formed Paris type mycorrhizae. Another important aspect is the type of plant root anatomy itself, as AMF morphology had been shown to be changed with the presence or absence of air spaces in roots (Brundrett & Kendrick, 1988).

A little work has been carried out on the mycorrhizal associations of essential oil bearing plants from Pakistan. Hussan & Ali (1992) studied on *Cymbopogan citrates* from the morphological point of view. However, Chattha *et al*., (1993), Hamed *et al*., (1994) and Iqbal *et al*., (1994) had reported the effect of root geometry on mycorrhizal associations in *Cymbopogan jwarancusa* from Cholistan desert.

In the present study, we are reporting the diversity of AMF morphology associated with two aromatic grasses i.e. *C. jwarancusa* and *V. zizanioides,* collected from various sites of Punjab differing in geography and vegetation. The main objective was to identify different morphological characteristics, which are unique to these plant species. The relationship between morphological variations of AMF and plant species identity has also been explored.

## Material and Methods

**Collection of samples:** The details for the collection of root and soil samples are given in table 1(a, b, and c). Soil and fine root samples for *Cymbopogon jwarancusa* were collected from twenty different locations in the Thal and Cholistan deserts, while samples for *Veteviria zizanioides* were collected from seven different locations in Islamabad and its outskirts. The number of plants excavated at each site was randomly selected and depends upon the size of population. Large populations allowed us to collect more number of root and soil samples as compared to that from small populations. All the sampling were commenced during April 2007 and 2008 (Tables (1a, 1b, 1c)) when the plants were at their flowering stage and maximum photosynthetic activity favored the development of mycorrhiza. All the samples of respective plants were brought to the laboratory in polythene bags and placed at 4°C until further selection for root examination (Quilliam & Jones, 2010).

**Table 1(a). Collection sites for *C. jwarancusa* in Thal desert with their coordinates and date of collection.**

| Collection site | Number of plants sampled | Number of root samples examined | Latitude (N) | Longitude (E) | Date of collection |
|---|---|---|---|---|---|
| Belkana | 10 | 8 | 30º 31' | 71º 33' | 03-04-2008 |
| Garh Maharaja | 10 | 7 | 30º 49' | 71º 54' | 03-04-2008 |
| Islam Wala | 13 | 8 | 30º 58' | 72º 02' | 03-04-2008 |
| Kotli Baqar Shah | 12 | 8 | 31º 16' | 72º 04' | 04-04-2008 |
| Garhi Fateh Ullah | 12 | 7 | 30º 43' | 71º 49' | 04-04-2008 |
| Karluwala | 12 | 8 | 31º 34' | 71º 39' | 05-04-2008 |
| Littan | 12 | 10 | 31º 32' | 71º 29' | 05-04-2008 |
| Mankera | 10 | 8 | 31º 23' | 71º 25' | 05-04-2008 |
| Nawan Kot | 12 | 10 | 31º 09' | 71º 36' | 06-04-2008 |
| Chaubara | 10 | 8 | 30º 52' | 71º 33' | 06-04-2008 |

**Table 1(b). Collection sites for *C. jwarancusa* in Cholistan desert with their coordinates and date of collection.**

| Collection site | Number of plants sampled | Number of root samples examined | Latitude (N) | Longitude (E) | Date of collection |
|---|---|---|---|---|---|
| Haiderwali Dahir | 10 | 8 | 29º 03' | 72º 08' | 23-04-2008 |
| Nagra | 13 | 8 | 29º 01' | 71º 52' | 24-04-2008 |
| Dingarh | 10 | 6 | 28º 56' | 71º 50' | 24-04-2008 |
| Badalwala | 10 | 8 | 28º 45' | 71º 38' | 25-04-2008 |
| Derawar Fort | 10 | 7 | 28º 45' | 71º 21' | 25-04-2008 |
| Kot Murad | 13 | 10 | 28º 23' | 70º 44' | 26-04-2008 |
| Varni | 15 | 8 | 28º 25' | 70º 26' | 26-04-2008 |
| Tarechri | 13 | 10 | 28º 16' | 70º 20' | 27-04-2008 |
| Fort Marot | 10 | 8 | 29º 13' | 72º 24' | 27-04-2008 |
| Januwala | 10 | 8 | 29º 07' | 72º 04' | 27-04-2008 |

**Table 1(c). Collection sites for *V. zizanoiodes* in Islamabad and its outskirts with their coordinates and date of collection.**

| Collection site | Number of plants sampled | Number of root samples examined | Latitude (N) | Longitude (E) | Date of collection |
|---|---|---|---|---|---|
| Islamabad | 10 | 8 | 33º 43' | 73º 04' | 12-04-2007 |
| Bar Tamma | 10 | 7 | 33º 44' | 73º 05' | 12-04-2007 |
| Nurpur Halan | 12 | 10 | 33º 45' | 73º 06' | 25-04-2007 |
| Katarian | 12 | 10 | 33º 42' | 73º 06' | 25-04-2007 |
| Islamabad Airport | 10 | 8 | 33º 42' | 73º 06' | 26-04-2007 |
| Lara | 10 | 8 | 33º 52' | 73º 17' | 26-04-2007 |
| Marglla Hills | 12 | 10 | 33º 43' | 73º 08' | 27-04-2007 |

At the time of root processing for mycorrhization only the healthy looking plants with best fine feeder roots were selected. A total of 82 samples for *C. jwarancusa* and 61 samples for *V. zizanoiodes* were processed to observe the variations in the morphology of mycorrhiza. At least 5g roots were removed from each plant, gently washed to remove soil particles and preserved in FAA (Formaline–Acetic acid-Alcohol, 5:5:90 ml). The roots were cleared and stained according to a modification of the procedure used by Phillips and Hayman (1970). The modification included clearing the roots in 10% KOH in autoclave for 10 minutes at 121°C and 1.05 kg/cm$^2$ pressure, bleaching them with 20% hydrogen peroxide, neutralization with 10% HCl and staining with 0.05% trypan blue in lactophenol by simmering for about 15 minutes at 90°C.

Ten 1cm segments from each plant sample were mounted in lactophenol slide. A slight pressure on the cover slip flattened the KOH–treated segments. Two hundred root segments of each plant per sample were examined under the compound research microscope Olympus CH2 at the magnification of 200x and 400x. The data for types of AMF structures such as arbuscules, coils, vesicles, intra and extraradical hyphae and non-

mycorrhizal colonization were collected from each slide and presented in respective figures. At the same time, data for the size of vesicles, hyphal diameter and staining intensity were also recorded. To calculate the size of each kind of vesicle and hyphae, fifty readings were taken and mean values were calculated.

**Microphotography:** Either all the microphotographs were taken with Olympus BH2 polarizing compound research microscope fitted with PM-10AK automatic exposure photo-micrographic system or digital Lebomed USA microscope fitted with digital camera digi-1500 coupled with P-IV computer system at the magnification of 200x or 400x. The slides and microphotographs of the specimens have been deposited to the Mycological Laboratory of Cholistan Institute of Desert Studies.

## Results

**Extraradical colonization:** The extraradical mycorrhizal association comprised hyphae showing various types of structural differences. Two distinctive types of hyphae were observed i.e., runner and absorbing hyphae. The runner hyphae were running parallel along the axis of root and were mostly darkly stained with thick or double walls, with or without septa and of variable diameters (Fig. 1B and 4C). The absorbing hyphae were mostly aseptate, much branched, less stained and thin walled (Fig. 1D).

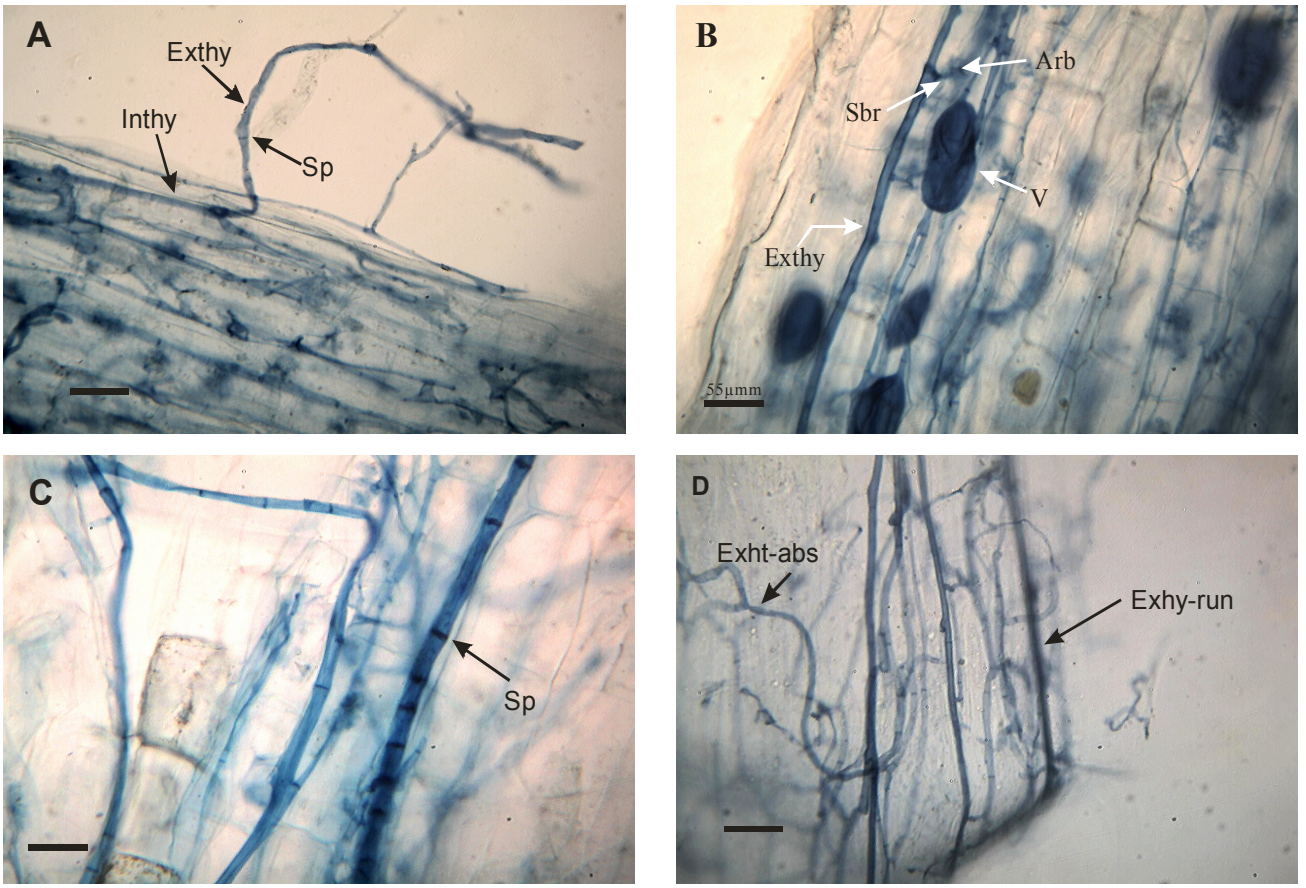


Fig. 1. Extraradical colonization in *Vetiveria zizanioides*. A) Ribbon like septate extraradical hyphe with the entry points and produce much branched, less stained network of aseptate hyphae inside the root. B) Extraradical septate darkly stained, thick walled hyphae running parallel to the axes of root. Such hyphae periodically produce very thin hardly stained side branches that produce either vesicles or arbuscules after entering into the cortical cells C) septate thin walled less stained hyphae that may enter into the cortical cells to produce arbuscules. These hyphae are much branched with variable diameters. D) both absorbing and runner hyphae which are septate and highly branched. Exthy, Extraradical hyphae; Inthy, Intraradical hyphae; Sp, Septata; Arb, Arbuscule; Sbr, Side branch; V, Vesicle; Ext-runhy, Extraradical runner hyphae; Ext-abshy, Extraradical absorbing hyphae. Bars, 55 µm.

**Intraradical colonization:** Other than various types of hyphae, internal colonization comprised of a variety of usual endogone structures (i.e. vesicles, arbuscules, hyphal coils and cuttings of hyphae in the cortical regions of fine roots). The diameters of internal hyphae fluctuate from 3.8 to 9.5 µm and no significant difference was found in this regard between the two grasses. The penetration of extraradical hyphae in the feeder root was initially intercellular but latter entered into the cortical cells to produce coils, vesicles, arbuscules and multifaceted hyphal cuttings.

Various types of vesicles were observed in *V. zizanioides* and C. *jwarancusa.* These vesicles were produced as terminal swelling of hyphae either intracellular or intercellular in the roots. They varied from globose (lightly stained, 32-38µm Fig. 5A and darkly stained 35-90 µm Fig. 5E) to sub globose (lightly stained 32 x 67 to 45.6 x 64.6 µm Fig. 1B, darkly stained 915 x 585 µm Fig. 2G) and cylindrical (darkly stained 21.6 x 54-27-132.3µm) in sizes (Fig. 4C, 5B and 5C).

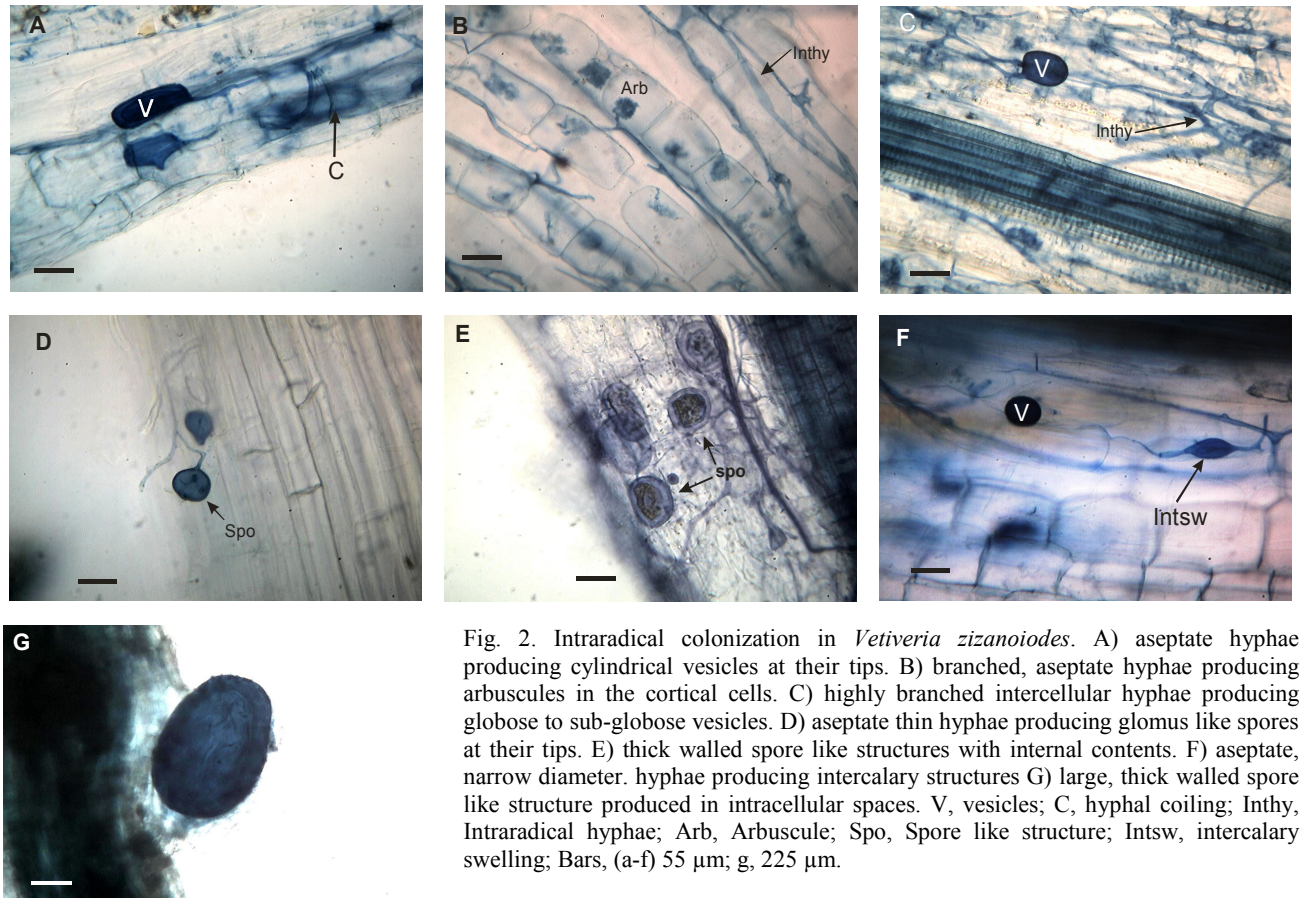


Fig. 2. Intraradical colonization in *Vetiveria zizanoiodes*. A) aseptate hyphae producing cylindrical vesicles at their tips. B) branched, aseptate hyphae producing arbuscules in the cortical cells. C) highly branched intercellular hyphae producing globose to sub-globose vesicles. D) aseptate thin hyphae producing glomus like spores at their tips. E) thick walled spore like structures with internal contents. F) aseptate, narrow diameter. hyphae producing intercalary structures G) large, thick walled spore like structure produced in intracellular spaces. V, vesicles; C, hyphal coiling; Inthy, Intraradical hyphae; Arb, Arbuscule; Spo, Spore like structure; Intsw, intercalary swelling; Bars, (a-f) 55 µm; g, 225 µm.

Some thin walled glomus spore like structures produced at the tips of very thin almost hyaline aseptate hyphae were recorded in *V. zizanioides* (Fig. 2D). Apart from that very thick walled chlamydospores from small globose (60.8 µm) to subglobose (45.6 x 87.4 µm, Fig. 2E) and very large chlamydospores (585 x 915 µm) were also observed in it. These large chlamydospores were unique in *V. zizanioids* and had wall of uneven thickness (Fig. 2G). Intercalary hyphal swellings were only observed in the *V. zizanioides* samples collected from Islamabad Airport (Fig. 2F). Hyphal cuttings and extensive coiling in the cortical cells of roots were only found in *C. jwarancusa* (Fig. 4A, 4B and 5F). However, no arbuscular colonization was observed in *C. jwarancusa*.

The runner hyphae were more numerous and pronounced in *C. jwarancusa* than in *V. zizanioides*. Their diameter varied from 5.7-10.8µm in *C. jwarancusa* (Fig. 3A, 3B and 4A) and 10.45 - 17µm in *V. zizanioides* (Fig. 1b and 1c). The morphology of absorbing hyphae was almost similar in roots of both grasses and their diameter ranged from 3.8 to 17.1µm (Fig. 1D, and 4B). Another kind of hyphae with diameter ranged from 3.7 to 5.7µm having similar morphology to that of absorbing hyphae were observed in *V. zizanioides*. These hyphae were with septa at either regular or irregular intervals, entering directly into the root producing aseptate interacellular hyphae (Fig. 1A). On the other hand, some darkly stained, thick walled hyphae were observed in *C. jwarancusa*. These septate hyphae were rare and dichotomously branched with the diameter of 10.8µm. (Fig. 3A).

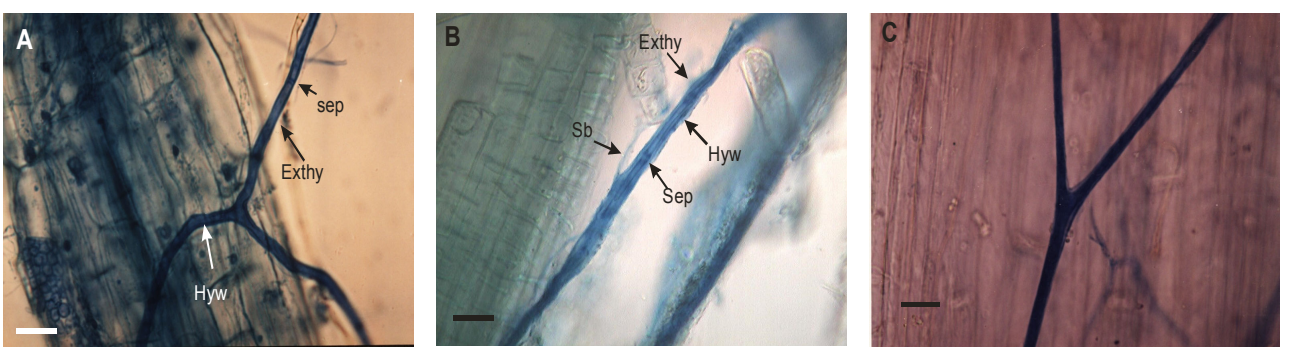


Fig. 3. Extraradical AMF colonization in *Cymbopogon jwarancusa*. A) darkly stained, septate, thick walled extraradical hyphae B) unique hyphae with distinctive external less stained wall and internal tube like cells. C) aseptate, darkly stained dichotomously branched hyphae with hyaline walls. Sep, Septa; Exthy, Extraradical hyphae; Hyw, Hyphal wall; Sb, Side branch; Cle, External clear zone; Int, Internal stained zone. Bars, 55µm.

In *C. jwarancusa* some darkly stained aseptate extraradical hyphae with the diameter from 2.6 to 3.0µm were observed to entering directly in to the cortical cells producing either packed cuttings (Fig. 4A) or producing extensive coiling in the cortical cells like *arum* type mycorrhiza (Fig. 4B). Another types of darkly stained, unbranded, aseptate hyphae with relatively wider diameter of 14.5 to 15µm were also observed in *C. jwarancusa*. These hyphae gave of short side branches, which after entering into the cortical cells produced cylindrical transparent vesicles and identified as *arum* type of AMF (Fig. 4C). In the case of *V. zizanioides*, normally stained septate hyphae with the diameter ranged from 11.4 to 15.2µm emerging small tapering branches that formed arbuscules in the cortical cells and identified as *paris* type of AMF (Fig. 1C).

A unique type of extraradical hyphae was observed in *C. jwarancusa*. Morphologically these hyphae looked very different from all the other extraradical or intracranial hyphae observed so far, due to their structural distinction and wider diameter (i.e. up to 18.6µm). These hyphae were less branched and less stained to hyaline, making enterence into the cortical cells producing almost hyaline vesicles (Fig. 3B).

**Non-mycorrhizal infection:** The non-mycorrhizal infection was represented by the brown fungus and very unique type of septate hyphae which have non-stained outer region and, darkly stained cytoplasmic, inner region. These hyphae were 17 to 19 µm in diameter (Fig. 5H). Both of these fungi could not be identified. The extraradical brown hyphae were regularly septate, highly branched, translucent, thin walled and their diameter ranged from 1.9 to 3.8µm. These hyphae were seen frequently entering into the cortical cells and forming either intercellular microsclerotia or hyphal coils and spore like structures inside the cortical cells (Fig. 5G).

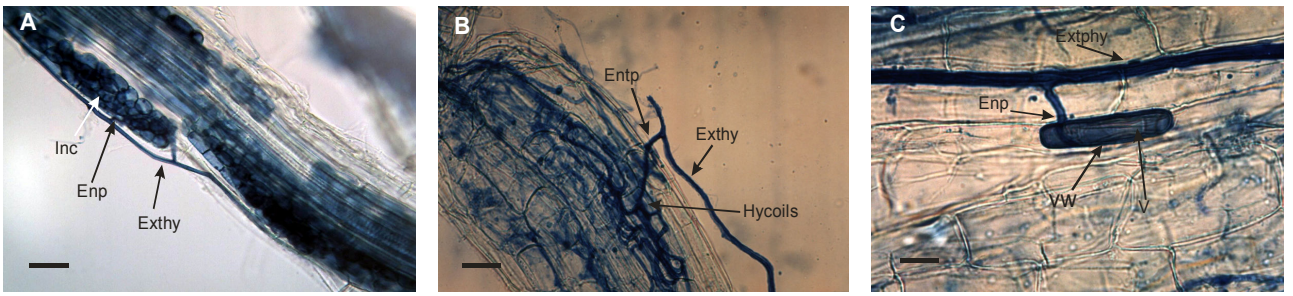


Fig. 4. Extraradical hyphae and variety of internal structures produced by AMF in *Cymbopogon jwarancusa*. A) darkly stained, aseptate extraradical hyphae produced elliptical to barrel shaped structures in the cortical cells B) darkly stained, aseptate hyphae with entry point in to the root and produced extensive inter and intracellular coiling C) Extraredical aseptate hyphae running parallel to the axis of root and produced cylindrical vesicles in the cortical cells. Inc, internal hyphal cuttings; Enp, entry point; Exthy, extraradical hyphae; Hycoils, hyphal coilings; VW, vesicle wall; V, vesicle. Bars, a-b 55µm, C) 40.5 µm.

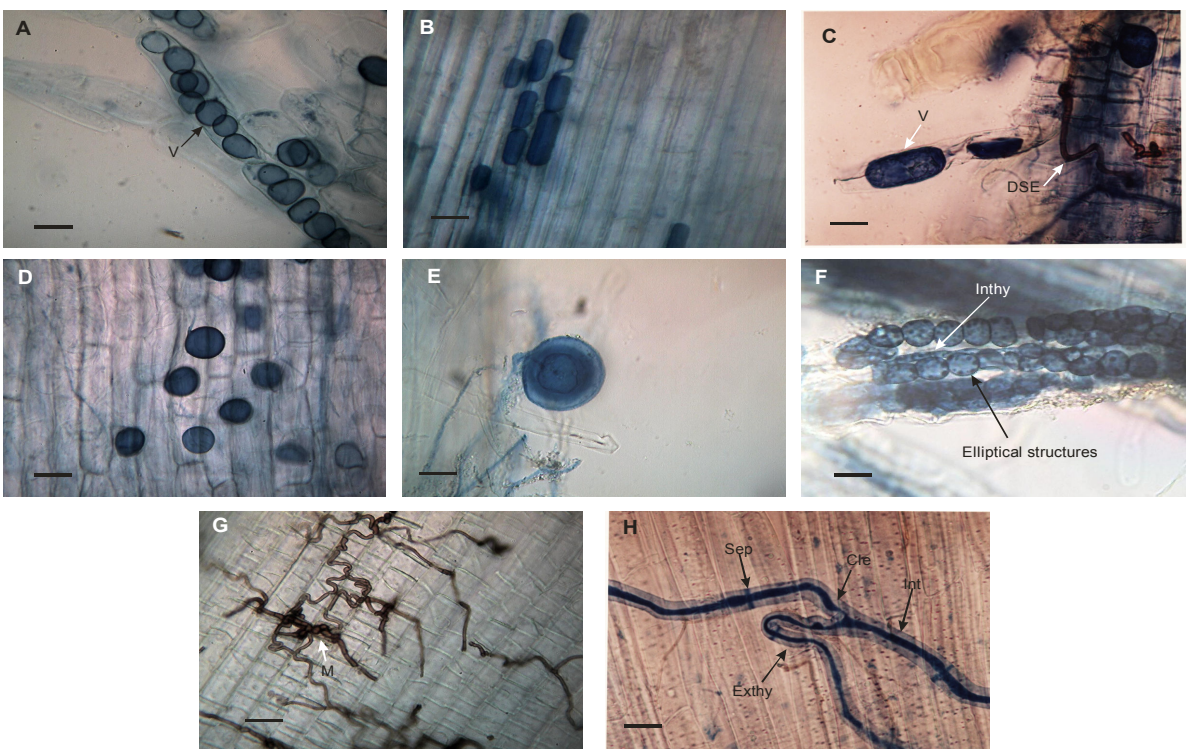


Fig. 5. Internal AMF colonization of *Cymbopogon jwarancusa*: A) globose to subglobose hyaline vesicles, B) darkly stained longitudinal to cylindrical vesicles in the cortical cells, C) elliptical vesicle with the internal contents, D) intracellular globose transparent vesicles, E) thick walled spore like vesicle, F) coenocytic intercellular barrel shaped structures produced by coenocytic aseptate hyphae. G-H) non mycorrhizal infection. V, Vesicle; DSE, dark septate endophytic fungus; Inthy, intraradical hyphae; M, microsclerotia. Bars, 40.5µm.

## Discussion

The aromatic grasses, *C. jwarancusa* and *V. zizanioides* had strong associations with mycorrhizae but various morphological differences in fungal colonization were observed in two grasses. In extraradical colonization, hyphae of AMF were observed to enter in to the roots and extending into the soil. Similar finding were reported by St. John *et al*., (1983). Extraradical soil hyphae are one of the main features of these fungi that are responsible for nutrient acquisitions, propagation of the association and spore formation. The AMF produce different types of soil hyphae including thick "runners" or distributive hyphae (Friese & Allen, 1991). The finer hyphae can produce "branched absorptive structures" (BAS) where fine hyphae proliferate and absorption of nutrition from soil is facilitated (Bago *et al*., 1998b).

The greater variety and quantity of extraradical hyphae in the rhizosphere of *C. jwarancusa* may be credited to enhance diversity of AMF species, however, hyphal production, branching, extent and distribution into soil also depends upon various biotic and abiotic factors such as plant fungal interaction, fungal identity, temperature and pH (Cavagnaro *et al*., 2001; Matsuda & Ymada, 2003). Another aspect is the ability of fungus to produce the external hyphae as some AMF species produce more extraradical hyphae than the other does. For instance Abbott & Robson (1985) found that *Glomus fasiculatum* produced less extraradical hyphae from Trifolium subterraneum colonized roots than did *Glomus tenne*, *Gigaspora alospora* and *Acaulospora laevis*. Similar results had been reported in *Citrus* by Graham *et al*., (1982).

The dimorphic nature of extraradical hyphae of AM fungi is not new. Mosse (1959) and Nicolson (1959) described coarse, thick walled hyphae having diameters of more than 20 µm and fine, thin walled hyphae with diameters ranging from 2 to 10 µm. In the present study, the 19µm diameter hyphae in *C. jwarancusa* appeared to be analogous to the hyphae observed by Mosse (1959). The physiology and capacity to absorb and translocate nutrients to the host is known to be affected by the diameter of the hyphae. Barber (1995) reported that hyphae with larger radius showed greater uptake per unit length than an absorbing structure of smaller radius. However, the narrower absorbing hyphae should be more efficient as they posses more surface area to that of broader hyphae. This smaller diameter of hyphae allows them to explore narrow pore spaces in soil to that of hyphae with larger diameter (O'Keefe & Sylvia, 1991; David, 1998).

The internal phase consists of mixed colonization of various kinds of hyphal, arbuscular and vesicular structures. We observed higher structural diversity of AM fungui in the cortical cells of *C. jawarancusa* where as in *V. zizanioides* less diverse vesicular structures were found. These observations also suggest that few mycorrhizal species are associated with *V. zizanioides* to that of *C. jwarancusa* which may be due to some kind of host specificity or host preference by the AM fungui. This idea is also supported by the presence of large thick walled chlamydospores in *V. zizanioides* and absent in *C. jwarancusa*. Such a capability of plants to selectively influence AM fungus colonization had also been reported in agricultural ecosystem by (Dhillion, 1992; Giovannetti & Hepper, 1985 & Remy *et al*., 1994).

Arum-type mycorrhizal colonization was more prominent in *V. zizanioides* roots than Paris-type. Intracellular hyphal colonization that produced arbuscules from cell to cell was absent in the *C. jwarancusa* Fig. 2B). It has been suggested that arbuscules and vesicles are the major organs through which nutrients are exchanged between the host and the endophyte. This assumption is based on the large surface area of these organs, but yet to be known correctly. The arbuscules are short lived and degenerate quickly depending upon the soil and climatic conditions. This may be the reason that we do not find arbuscules in *C. jawarancusa*. All the root samples of *C. jawarancusa* were collected from various localities of Thal and Cholistan deserts Table 1. Under the stress conditions, especially the scarcity of water, the life span of arbuscules seemed to be much lessened and quickly passed on to the formation of coiling and vesicles. In older plants, vesicles may develop thick walls and may function as propagules (John & Michael 1990). Hyphal coiling was observed in the cortical cells of both grasses; however, morphologically they differ from each other in the sense that in *V. zizanioides* coiling was coarse, much convoluted and less stained (Fig. 1B). While in *C. jawarancusa* coiling was refined, usually two to three coils per cell and the hyphae were thick and coenocytic (Fig. 4B), these morphological variations may be ascribed to the physiological difference in associated AMF species.

Entering points of extraradical hyphae were frequently observed in both the grasses during study but development of appressoria was not observed in either grass at the point of contact between extraradical hyphae and roots. However, extraradical hyphae may produce cuttings, coiling or vesicles just after entering into the cortical cell (Fig. 4A and 4C).

In the present study, we observed the morphological diversity of AMF in the roots of two aromatic grasses collected from various diverse habitats and geographical conditions. One way was to classify the AMF under different climate based up on morphological variations and other way was to ascertain the morphological diversity as a whole. We adopted the second approach because a mixed morphological diversity in AMF was observed in the samples collected from any site and no clear pattern of differentiation was emerged in the morphology of fungi under different geographic conditions. There are many studies showing that environmental conditions such as soil chemistry, temperature and light can influence the relative abundance of structures, level of colonization, and extent of colonization (Bret Hart *et al*., 2008; Sikes *et al*., 2010; Murray *et al*., 2010) but there is no evidence which shows that they can change the morphological types of AMF (Cavagnaro *et al*., 2001). We concluded that the morphological diversity of AMF in *V. zizanioides* and *C. jwarancusa* is largely controlled by the kinds of AMF species associated and plant genome. In the present study, we adopted the conventional protocols for recording the details of colonization and for microphotography,

however, in future planning has been made to use root sections, confocal and scanning electron microscopy in order to observe the AMF characteristics of morphological diversity more condusively.

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