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Native AM fungal colonization in three *Hibiscus* species under NaCl induced Salinity

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Abstract : *The pot experiments were conducted to study influence of sodium chloride salinity on Native Arbuscular Mycorrhizal fungal consortium (NAMC) with respect to three Hibiscus species viz., H. cannabinus, H. sabdariffa and H. tiliaceus (Fam. Malvaceae). All three plant species exhibited appreciable colonization and showed excellent salt tolerance potential at 300 mM NaCl treatment. Results obtained revealed that Arbuscular Mycorrhizal fungal (AM) species spore recovery (SR) was in the range of 50-100% in treated plants. AM species studied in present paper were viz., Claroideoglomus etunicatum, Sclerocystis coremoides (H. cannabinus); Acaulospora myriocarpa, Acaulospora spp. (H. sabdariffa) and Acaulospora foveata, Acaulospora spp., C. etunicatum, Glomus botryoides, G. caledonicum & G. formosanum (H. tiliaceus).*
Keywords - *AM, Hibiscus, root colonization, NAMC, salinity.*

I. INTRODUCTION

Present paper deals with study of sodium chloride (NaCl) salinity effect on native Arbuscular Mycorrhizal fungi (AM) harboring in rhizosphere soil and roots of economically important three plant species *viz., Hibiscus cannabinus* L., *H. sabdariffa* L. and *H. tiliaceus* L. belonging to family Malvaceae. Of the first plant species *H. cannabinus* is an annual or perennial herb which is distributed throughout India and known as *Kenaf*. In India seeds, leaves and flowers of *H. cannabinus* are medicinally used. The seeds are used as appetizer, aphrodisiac, flattening and also for external application in pains and bruises. The leaves are purgative, and used in dysentery and diseases of blood, bile and throat while an infusion is administered in cough [1]. The Cooked young leaves are also used as a potherb or added to soups. In African folk medicine, *H. cannabinus* is used as an anthelmintic [2]. In Northern Cameroon, the plant is used as vegetable and in Southern Cameroon as a panacea in anaemic therapy [3]. Besides, it is used in the treatment of polluted water with low nitrogen and phosphorus concentrations [4]. The plant also has rich fibre content and hence serves as a good material in the paper industry [5].

H. sabdariffa or sour tea is another species of genus *Hibiscus*, which is typically called as *Roselle*. Originally from Angola, it is now cultivated throughout tropical and subtropical regions as hardy herbaceous shrub for its medicinal value. In Egypt and the Sudan, it is used as a beverage that helps to lower the body temperature, to treat cardiac conditions, and as a diuretic [6], likely Nigerian people drinks this beverage called as *Zobo* as remedy of hypertension [7]. In African folk medicine it has been used for its spasmolytic, antibacterial, cholagogic and anthelmintic properties. Whereas, in North Africa it is used to treat cough and sore throat, external wounds and abscesses [8].

Hibiscus tiliaceus commonly known as *Cottonwood Hibiscus*, is one of the most widespread species in the genus, with a pantropical distribution. It is a tree native to the shores of the Pacific and Indian oceans, today cultivated or naturalized throughout the tropical and subtropical regions of the world, particularly in coastal areas. The species grows in coastal thickets along brackish rivers [9-13], and in areas inland of mangrove forests [14]. In folk medicine, the leaves of this plant are used to treat fevers, soothe coughs, ulcer, wounds and various skin diseases [15]. In Indian system of medicine *H. tiliaceus* had been used as febrifuge, laxative, resolvent and emollient. The leaf infusion is used to wash ulcer and wounds whereas fruit juice is rubbed on skin to cure weakness [16-17]. Traditionally, the plant has been used for diabetes [18]. The flower extract has free radical scavenging activity [15].

Besides the medicinal potential of these three plants (*Kenaf*, *Roselle* and *Cottonwood Hibiscus*) a very common application is their consumption as leafy vegetables. Since, leafy vegetables are mostly taken for their valuable richness in nutrients. Amongst the three plants species first two are most frequently used as source of leafy vegetable, but third one is used by aboriginal Australians as leafy vegetable. The inventories on dependence of genus *Hibiscus* on AM fungi are very recent, which were carried with reference to *H. canbnabinus* [19], *H. meraukensi*s and *H. leptocladus* [20], *H. rosasinensis* [21] *H. sabdariffa* [22] and *H. tiliaceus* [23]. However, AM fungal status in *Hibiscus* spp. under salinity condition is remained unfocused with the exceptions of very few contributors like Maas [24] and Ashraf et al. [25].

It has been suggested that root hairs and mycorrhizal fungi were two alternative mechanisms for plant nutrient uptake [26-27]. The symbiosis between the roots-AM fungi improves water use and nutrient uptake, especially elements with low soil mobility, such as phosphorus, zinc, and copper. It also increases plant tolerance to various biotic and abiotic factors [28]. AM fungi are associated with the roots of over 80% of terrestrial plant species [29]. It is now well proved that, plants colonized by AM fungi have optional nutritive requirements and maintain physiological statues in stress situations and disturbed ecosystems [30-31]. Salinity is a harsh environmental factor that has the major effect on plant quantity and quality [32]. In order to survive in salt stress condition, plants develop the network responses of physiological and biochemical defence mechanisms to protect themselves against stress [33].

The presence of the AM fungi in Malvaceae species is a common feature. Although, in last decade, Malvaceae crops *viz.*, *Gossypium hirsutum* L, *H. cannabinus*, *H. sabdariffa* [24] and *Hibiscus esculentus* L. [25] etc. were tested for salinity tolerance. However, such attempts were not made in Maharashtra neither the attention has been paid to the study of mechanism of salt tolerance in this species. Since *Hibiscus* spp selected for present work were growing in salinity influenced soil along the coast and tidal mangrove swamps, that is why their potential as crop plants is underestimated in Maharashtra. As proved earlier [34-37] AM fungal association may increase plant tolerance to salinity, and it improves physiological process like photosynthetic activity or water use efficiency as well as production of osmoregulators etc. thereby enhances mineral nutrition [38-39]. Hence, in present paper it was thought worthwhile to investigate the extent of AM fungal association in three *Hibiscus* species subjected to sodium chloride salinity.

II. Material And Methods

2.1 Sample collection:

Roots and rhizosphere soil samples of three plants under study were collected from saline habitats at West coast in Maharashtra. In the laboratory, roots of each plant sample were pulverized and mixed with their own rhizospheric soil samples, which was further used as native AM fungal inoculum as consortium (NAMC) during pot experiment.

2.2 Salinity induction:

The physio-chemical properties of pot filling soil were analyzed following the Wiled et al.[40] prior to set experiment and after treatment study. The pot experiments were carried out in wide earthenware pots containing about 15 kg of air dried garden soil and compost amended with vermi-compost [3:1]. The soil was autoclaved (110˚C/1 h, twice at 48 h intervals) to remove indigenous AM fungal propagules prior to use. 200g of consortia *viz.*, *NAMC^{HC}: [*Ce+Sc*], $^{\#}$NAMC^{HS}: [*Am+Asp*], $^{@}$NAMC^{HT}: [*Af+Asp+Ce+Gb+Gc+Gf*] (Table 2) were applied beneath the 10 cm of potting mixture surface layer prior to raise *H. cannabinus*, *H. sabdariffa* and *H. tiliaceus* respectively**.** The plants were allowed to grow under natural condition. After eight weeks the plants were treated once a week with 300mM concentration of NaCl. Salinity induction treatments were continued for next eight weeks, each treatment was followed by a dose of fresh water to avoid excessive salt accumulation. The plants treated with fresh tap water served as control.

2.3 AM fungal colonization status and species identification:

After stable visible morphological differences in the plant species, the microscopic observations were recorded for evaluation of AM fungal status in roots. The roots and rhizosphere soil samples were collected from each pot, made free from soil debris by washing after following clearing and staining method of Phillips and Hayman [41]. Processed roots were observed under a binocular microscope to evaluate mycorrhizal colonization following the technique of Giovannetti and Mosse [42]. Percentage colonization of AM fungi (C_z) was calculated using Nicolson formula [43].

After root removal, the soil samples from each replicate pot were combined to obtain a single composite sample per species. AM fungal spores from control and salinity stress responding soil samples were extracted directly using the wet sieving and decanting method of Gerdemann and Nicolson [44]. Traditional taxonomic methods, based on root screening, and spore morphology were used to identify the AM fungal species associated with *Hibiscus spp*. Original descriptions of Schenk and Perez [45] were referred for the same. The AM fungal species spore recovery (*SR*) was calculated for each plant as $SR = (N_O/T_N) \times 100$, where: N_O is number of AM fungal species observed and T_N is total number of AM fungal species present in consortium.

III. Result And Discussion

Dry gravel red Soil with Electrical conductivity (Ec) 0.20 $dS.m^{-1}$ and 8.45pH was used for pot filling. After salinity induction experiment in presence of NAMCs, physio-chemical properties of dried potting soil were again analyzed. This soil was contained 0.25, 0.34, 0.37 % carbon; total nitrogen was 0.04, 0.66, and

0.98%; available phosphorous: 1.84, 1.38 and 1.43%; available potassium: 5.75, 5.50 & 3.25%; Ec: 0.39, 0.12 & 0.18 $dS.m^{-1}$; 7.15, 7.72 & 7.18 pH in controlled pots of *H. cannabinus*, *H. sabdariffa* and *H. tiliaceus* respectively. Whereas, soils of all three plants treated with 300 mM NaCl to induce salinity (Ec 0.85, 2.95 & 2.36 $dS.m^{-1}$ respectively) were notably influencing C%, N%, P%, K% and pH (Table 1). The colonization of AM fungal components such as vesicles, arbuscules and hyphae were significantly influenced at saline condition and thereby showed enhancement in percentage colonization in all three species of *Hibiscus* in comparison with control plants (Table 1).

3.1AM fungal colonization

The frequency of mycorrhizal colonization in root tissue and the rate of arbuscular formation in root segments was increased with induction of saline condition and were measured at level of 300 mM NaCl. The highest stimulation rate of colonization was measured in *H. cannabinus* (90%), followed by *H. sabdariffa* (70%), whereas in *H. tiliaceus* it was 50%. In all the three plant species, AM fungal colonization was found to be improved as compared with controlled plants (Table 1). The AM fungal components occurrence intensity can be evident from photo-micrographic illustrations which are presented in Fig. 1. Formation of extraradical hyphal colonization (*em*) in *H. cannabinus* (Fig 1*c*), intense intra-traradical hypahal (*h*) colonization in *H. sabdariffa* (Fig 1*g*), and stimulated arbuscular colonization in *H. tiliaceus* (Fig 1*c*) were consistently recorded which are strongly supporting that AM fungal colonization under salinity stress improves formation of their structural components. It is clearly reported that, "Besides the intraradical phase, AM fungi have an extraradical phase comprising a network of hyphae which ramify within the soil, exploring and exploiting it for mineral nutrients"[46] and "these hyphae actively take up P, N, and several micronutrients[47-50] which are afterwards transferred to the host plant, thus improving its mineral nutrition"[46]. In present paper consistent formation of extraradical hyphae are reported in *H. cannabinus* under salinity influence which is ultimately beneficial to the plant.

Table 1: AM fungal colonization in Hibiscus roots in salinity induced soil.

Salinity treatment	**Soil physio-chemical properties**							**AM fungal status**	
	Texture	**pH**	**Ec $dS.m^{-1}$**	**C %**	**N %**	**P %**	**K %**	**C_z%**	**N_O**
***Hibiscus cannabinus* with $^{*}NAMC^{HC}$**									
control	GS	7.15	0.39	0.25	0.04	1.84	5.75	60	-
300 mM NaCl	GS	6.93	0.85	0.23	0.84	1.72	1.75	90^{t}	02
***Hibiscus sabdariffa* with $^{\#}NAMC^{HS}$**									
control	GS	7.72	0.12	0.34	0.66	1.38	5.50	45	-
300 mM NaCl	GS	6.82	2.95	0.40	1.06	2.15	4.15	70^{t}	02
***Hibiscus tiliaceus* with $^{@}NAMC^{HT}$**									
control	GS	7.18	0.18	0.37	0.98	1.43	3.25	30	02
300 mM NaCl	GS	6.60	2.36	0.54	0.90	1.93	4.75	50^{t}	04

[GS- Gravel red soil, Ec-Electrical conductivity, C-Carbon, N- Nitrogen, P- Phosphorus, K- Potassium; ^{t}Values for root colonization represent treatment means obtained by pooling five replications of NaCl treatments; C_z: AM fungal colonization percentage, N_O: Number of AM fungal species observed, ($^{*}NAMC^{HC}$, $^{\#}NAMC^{HS}$, $^{@}NAMC^{HT}$): see foot note of Table 2]

Salinity treated and control plants root segments stained in Trypan blue dye for comparative discussion of AM colonization are showed in Fig. 1. In present work, salinity emphasized roots of *H. cannabinus* were possessed of numerous vesicles, and chlamydospores; arbuscules, an internal hyphal network ramifying along the length of the root tissue and cluster of young chlamydospores of *Glomus botryoides* in *H. sabdariffa* was consistently observed. Whereas, young or mature spores were characteristically observed in the roots of *H. tiliaceus*. It is routinely noticed that, besides the development of arbuscules vesicles or mycorrhizal hyphae, occurrence of other fungal endophytes (*ofe*) is very a common observation in most of psammophytes. In present study root segments of *H. tiliaceus* such *ofe* were observed along with mycorrhizal chlamydospores (Fig. 1*l*).

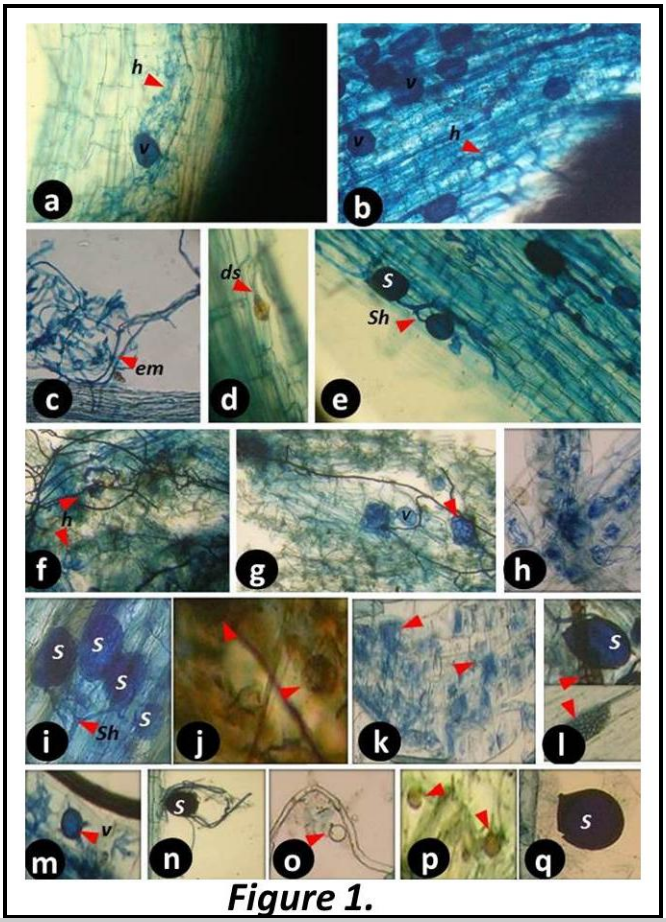

Figure 1.

Figure 1. Comparative mycorrhizal colonization in Hibiscus root segments:

[*a*] ***H. cannabinus* control:** Vesicular & hyphal colonization; [*b-e*] ***H. cannabinus* NaCl treated:** dense colonization of Vesicles & hyphae (b), extraradical hyphal colonization on root surface (*c*); spore of *Sclerocystis coremoides* (d), chlamydospores of *Claroideoglomus etunicatum* with subtending hypahe stained with trypan blue (*e*); [*f*] ***H. sabdariffa* control:** hyphal colonization; [*g*] ***H. sabdariffa* NaCl treated:** Vesicular-hyphal colonization and arrow headed young spores of *Acaulospora myriocarpa;* [*h-j*] ***H. tiliaceus* control:** Arbuscular colonization (*f*), cluster of chlamydospores of *Glomus botryoides* formed from swollen endings of peridial hyphae which are frequently bifurcate (here subtending hypahe) stained with trypan blue (*i*); sloughing spores of *Glomus caledonicum* (*j*); [*k-o*] ***H. tiliaceus* NaCl treated:** dense arbuscular colonization (*k*), other fungal endophytes and mycorrhizal chlamydospore (*l*), Vesicular & hyphal colonization (*m*); *Glomus formosanum* spores with three hyphal attachments found on root surface (*n*), young spore of *Acaulospora spp.* with hyaline hypahe on root surface (*o*), mature spore of *Acaulospora foveata* on root surface (*p*), chlamydospores of *Claroideoglomus etunicatum* stained with trypan blue on root surface (*q*). **[*ds*-** degenerating spore, ***em*-** extraradical hyphae, ***h*-** mycorrhizal hyphae, ***s*-** chlamydospore, ***sh*-** subtending hypha and ***v*-** vesicle**]**.

3.2 Status of AM fungal spores

Rhizosphere soil samples collected from each plant from different locations were combined to obtain a single composite sample and subjected to extraction of AM fungal spores. Results obtained indicates that, 2 AM fungal species *viz.*, *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, and *Sclerocystis coremoides* Berk & Broome were encountering to *H. cannabinus.* 2 AM fungal species *viz.*, *Acaulospora myriocarpa* Spain, Sieverd. & N.C. Schenck, *Acaulospora* spp. (Unidentified) were dominating the *H. sabdariffa* composite rhizosphere sample. Whereas, *H. tiliaceus* was prevailing with 6 fungal species *viz.*, *Acaulospora foveata* Trappe & Janos, *Acaulospora* spp. (Unidentified), *C. etunicatum, Glomus botryoides* Rothwell & Victor, *Glomus caledonicum* (Nicol. & Gerd.) Trappe & Gerdmann, *Glomus formosanum* Wu & Chen. Thus, each representative composite sample (henceforth designated as HC, HS and HT respectively for three plants) in spite of possessing differences in number and spore density of AM fungal species were treated as consortium ($NAMC^{HC}$, $NAMC^{HS}$ and $NAMC^{HT}$) for pot experiment to evaluate the NaCl induced salinity effect on *Hibiscus.* Thus, AM fungal consortia used during present work comprised with bi-species spores ($NAMC^{HC}$ & $NAMC^{HS}$) and multi-species spores ($NAMC^{HT}$) for *H. cannabinus*, *H. sabdariffa* and *H. tiliaceus* respectively. Since three different NAMCs were used during study, the soil samples from pot experiment each of the three *Hibiscus* species possessed different spore representatives out of total eight AM fungal species

belonging to four genera (Table 2). Morpho-taxonomical descriptions of these AM species is beyond the scope of paper and hence are not included.

Table 2: Status of AM fungal spores in salinity induced soil of Hibiscus

$Consortium	Salinity treatment	AM fungal spores recovered								¥SR %
		[1]Af	[2]Am	[3]Asp	[4]Ce	[5]Gb	[6]Gc	[7]Gf	[8]Sc	
*NAMC^{HC}	***Hibiscus cannabinus***									
	control	-	-	-	x	-	-	-	x	00
	300 mM NaCl	-	-	-	√	-	-	-	√	100
#NAMC^{HS}	***#Hibiscus sabdariffa***									
	control	-	x	√	-	-	-	-	-	50
	300 mM NaCl	-	√	x	-	-	-	-	-	50
@NAMC^{HT}	***@Hibiscus tiliaceus***									
	control	x	-	x	x	√	√	x	-	33
	300 mM NaCl	√	-	√	√	x	x	√	-	67

[*[1]A. foveata, [2]A. myriocarpa, [3]Acaulospora* spp., *[4]C. etunicatum, [5]G. botryoides, [6]G. caledonicum, [7]G. formosanum, [8]S. coremoides*; $Consortium- *NAMC^{HC}: [*Ce+Sc*], #NAMC^{HS}: [*Am+Asp*], @NAMC^{HT}: [*Af+Asp+Ce+Gb+Gc+Gf*]; (-) AM fungal species was not a counterpart of consortium; (√) AM fungal species recovered & (x) AM fungal species not recovered; ¥*SR*: AM fungal species recovery].

After stable visible morphological differences in treated and controlled *Hibiscus* spp, AM fungal spores from each potting soil per plant species were recovered and identified. The AM fungal species spore recovery (*SR*) was calculated for each plant species. The results obtained revealed that 100% , 50% and 67% AM fungal species spore recovery was possible at 300 mM NaCl treatment for *H. cannabinus*, *H. sabdariffa* and *H. tiliaceus* respectively over control plants (Table 2). Thus, it is very much clear that, AM fungal species spore recovery was greater than 50% over the controlled *H. cannabinus* and and *H. tiliaceus* plants, which confirms that salinity stress is influencing the tolerance of AM fungi and subsequently spores development. Most probable justification for failure in obtaining 100% *SR* for all the three plant species may be because of application of non-quantified NAMCs dosage at the rate of 200g/15kg potting soil mixture. Non-quantified NAMCs which consists of unequal or unknown amount of spores of individual AM fungal species. Hence, to evaluate salinity tolerance effect of individual AM species it is essential to design the experiments by isolating individual native spore and thereafter producing consortia with various combinations of AM species with equal proportion i.e. quantified NAMCs. However, results obtained in present paper strongly admire that AM-fungal association may increase plant tolerance to salinity [34-37].

Mycorrhizal symbiosis is a key component in helping plants to cope with adverse environmental conditions therefore, beneficial effects of mycorrhiza on growth under saline conditions have been studied recently in various plant species and families [51-53]. It is now confirmed that, the endomycorrhizal associations often result in greater yields of crop plants such as rice [54], tomato & onion [55] and bell pepper [56] even under saline conditions. Recently Aulia et al.[57] checked productivity of *H. sabdariffa*. affected by treatment of commercial product of mycorrhiza {MYCOgold$^{(TM)}$ (root enhancer) containing selected spores of *Glomus, Gigaspora*, *Acaulospora* and *Scutellospora* isolated from native Malaysian soils. The application of mycorrhiza on the productivity of Roselle, showed its positive effects on fruit number per plant, fruit weight per plant, fruit weight and calyx weight per fruit. However, such efforts have not yet been made in India. Hence, present work based on application of NAMCs provides significant information and suggests positive responses to NaCl salinity induced *Hibiscus* spp.

IV. CONCLUSION

All the three plants belonging to genus *Hibiscus* under present study *(H. cannabinus*, *H. sabdariffa* and *H. tiliaceus*) possess excellent salt tolerance potential at 300 mM NaCl concentration in presence of AM fungal consortium and exhibited appreciable colonization. These three species of *Hibiscus* till date are being ignored for cultivation practices in Maharashtra and hence their economic potential is yet remained untapped. Thus, to set mycorrhiza based technology for these plants there is urgent need for isolation of monosporic cultures of AM fungal species and thereby testing the efficacy of these cultures alone as well as in various consortia in salinity affected regions in near future.

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