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Optimization of *in vitro* root organ culture and co-culture of AM fungi *Claroideoglomus claroideum*

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi, the obligate symbiont of plant roots require establishment of functional symbiosis with host to complete their life cycle. Among the various methods of mass production of AM inoculum, *in vitro* method in host root organ culture is a promising method to obtain contamination free high quality inoculum. Besides inoculum production, *in vitro* method provides opportunity for research in different aspects of host AM interaction. The present study was under taken to optimize the *in vitro* root organ culture of dicot plants *Solanum lycopersicum* and *Daucus carota,* monocot plants *Zea mays*, *Triticum aestivum* and *Sorghum bicolor* for co-culture of AM fungi *Claroideoglomus claroideum.* Optimization was carried out for root organ proliferation and AM fungi root colonization during co-culture under different cultural conditions such as media consistency and light.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are obligate symbiont of plant roots (Brundrett and Brundrett, 2009) belonging to the phylum Glomeromycota (Schuessler *et al*., 2001). AM fungi cannot complete their life cycle without establishing functional symbiosis with host root. The AM symbiotic association provides carbon in form of sugar and lipid to the fungal partner (Luginbuehl *et al*., 2017), in return there is improvement in growth, mineral nutrition and water uptake (Lekberg and Koids, 2005; Tekaya *et al*., 2017), disease resistance (Jung *et al*., 2012; Berdeni, *et al*., 2018), abiotic stress tolerance of host plant (Porcel *et al*., 2012; Panigrahy *et al*., 2019). Considering the benefits of AM symbiosis, large scale mass production of AM fungal inoculum is imperative for sustainable agriculture.

Various methods are available for mass production of AM fungi (Akhtar and Abdullah, 2014). Conventional substrate based *in vivo* pot culture method is most cost effective and highly adapted large scale production system (Gaur and Adholeya, 2002). However, *in vivo* pot culture is open method and it cannot guarantee production of contamination free inoculum (Kumar and Yadav, 2018).The substrate free systems like hydroponic and aeroponics have been developed, but their high maintenance cost limit their use to small scale. The *in vitro* culture system on root organ culture can guarantee production of high quality pure AM fungi (Ijdo *et al*., 2011).

The first report on the *in vitro* root organ culture system was from the work of White (1943). Mosse and Hepper (1975) were the pioneers in establishing *in vitro* culture of AM fungi in root organ culture system. Subsequently several workers developed many *in vitro* system for culture of AM fungi (Declerck *et al*., 1996; Fortin *et al*., 2002; Voets *et al*., 2005). Besides large scale inoculum production, AM propagation in *in vitro* root organ culture system provide opportunity of research in mycelium

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development studies (Bago *et al*., 1998), functional aspects mycorrhizal root symbiosis (Debiane *et al*., 2009), sporulation dynamics (Ijdo *et al*., 2011), reproduction cycles and nutritional requirements of AM fungi (Labidi *et al*., 2011), secondary metabolite production (Kumar and Yadav, 2018) etc. Reports on *in vitro* culture of some species of AM are available (Mosse and Hepper, 1975; Chabot *et al*., 1992; Declerck *et al*. 2000; de Souza and Declerck, 2003) however, reports on *C. clareoidum* is scanty. Therefore, the present study was undertaken to develop a simple *in vitro* excised root organ culture system and optimize AM root colonization for subsequent mass production of AM spores.

2. Materials and methods

2.1 Mass production of starter culture of AM fungi

The pure culture of AM fungi *Claroideoglomus claroideum* (CMCC/AM-2705) was procured from Centre for Mycorrhizal Culture Collection (CMCC), TERI, New Delhi, India. Mass propagation of the starter culture was carried out in pot culture with *Zea mays*, *Triticum aestivum* and *Sorghum bicolor* as host plant. Substrate for pot culture constituted of dried soil and sand in the ratio 3:1 (v/v) which was mixed with organic manure at 2:1 ratio (v/v). The Potting mix was cleaned thoroughly by sieving in 2mm sieve and sterilized by autoclaving three times on alternate days at 120^{o}C in 15psi for 30 mins to kill the other mycorrhizal spore in the substrate. In 2 kg a poly pot 5 seeds of host plants were sown and after 7 days of seedling growth *C. claroideum* spores were inoculated into the pots. The pots were irrigated on alternated days and host plants were allowed to grow for 4 months for AM root colonization and sporulation.

2.2 Assessment of AM root colonization and spore density in pot culture

After 4 months of host plant growth, roots were collected by careful uprooting. Cleaned roots were cut into 1 cm length, pre-treated and stained with trypan blue following Phillips and Hyman (1970). Root colonization of AM fungi was observed under microscope and expressed as percentage of colonization (Giovannetti and Mosse, 1980). The extraction of AM spore from pot culture substrate was done by wet sieving and decantation technique (Gerdemann and Nicolson, 1963) followed by sucrose density gradient centrifugation (Daniel and Skipper, 1982). The extracted spores were counted under stereozoom microscope and spore density was expressed as number of spores / 100 g soil.

2.3 Seed collection and surface sterilization

Seeds of *Solanum lycopersicum* L., *Daucus carota* L., *Zea mays* L., *Triticum aestivum* L., and *Sorghum bicolor* (L.) Moench were collected from local market. The seeds were washed thoroughly under running tap water for 30 min, then immersed in an aqueous solution of 5% teepol and 7% sodium hypochlorite for 8-10 min, followed by washing with distilled water for 5-6 times. The explants were finally treated with 0.1% aqueous solution of mercuric chloride for 8-10 min for surface sterilization and washed 5-6 times with autoclaved distilled water.

2.4 Seed germination and development of explants

Surface sterilized seeds of dicot plant *S. lycopersicum* and *D. carota* were germinated *in vitro* in half strength MS (Murashige & Skoog, 1962) agar media (0.8% w/v). After two weeks of seedling growth, roots were excised to be used as explants for root organ culture. The seeds of monocot plants *Z. mays*, *T. aestivum* and *S. bicolor* were germinated *in vivo* in sterilized sand. After 10 days of seedling growth healthy roots were excised with sterilized scalpel and used as explants for *in vitro* root organ culture.

2.5 Optimization of in vitro root organ culture

The nutrient medium used for *in vitro* root organ culture is full strength MS with sucrose (3%). For optimization of root organ culture, the culture media was fortified with a range of concentrations (0.5-1.0 mgL^{-1}) of auxins like Indole Acetic Acid (IAA) or Indole Butyric Acid (IBA) separately. The pH of the culture medium was adjusted to 5.6-5.8 with either 0.1N HCl or 0.1N NaOH as per the requirement. The media was gelled with 0.8% (w/v) agar agar (Hi-media). Molten media (35ml) were dispensed into 150ml Erlenmeyer flasks. The flaks were capped with aluminum foil and autoclaved at 15psi at 121^{o}C for 20 min. for sterilization.

Excised root explants of *in vivo* germinated monocot plants *Z. mays*, *T. aestivum* and *S. bicolor* were cleaned by washing with distilled water several times. Surface sterilization of root explants were done by treatment with 0.1% (w/v) aqueous solution of $HgCl_2$ for 5 min. followed by washing with distilled water 5-6 times. Root explants of *in vitro* germinated *S. lycopersicum* and *D. carota* were washed with autoclaved distilled water to remove traces of the germinating media. The roots were the cut into 1cm length and inoculated to flaks containing sterilized media. All the operations including surface disinfection, inoculation and transfer of disinfected explants were conducted aseptically in laminar air flow chamber (Klenzaids, India). The inoculated flasks were maintained in the culture room at 25±1°C and 16 h photoperiod of 35-40 µmol m^{-2} s^{-1} irradiance level provided by cool white fluorescent tubes (Phillips, India) and 60% relative humidity.

2.6 In vitro co-culture of AM fungi and root organ

Arbuscular mycorrhizal spores were extracted from the soil substrate of mass propagated pot by Wet Sieving and Decantation technique (Gerdemann and Nicloson, 1963) followed by sucrose gradient centrifugation (Daniel and Skipper, 1982). The AM spores were subsequently surface sterilized in a solution of 0.05% Tween 20 (v/v) for 1 min., a solution of Chloramine T 2% (w/v) for 10 min., then in a solution of streptomycin (0.02% w/v) and gentamicin (0.01% w/v) for 10 min. (Bécard and Piché, 1992). Surface sterilization of the AM spores was carried out asceptically in laminar airflow cabinet. Viability of the surface sterilized AM spores were tested through spore germination in water agar petri plates.

The co-culture of root organ culture and AM fungi was carried out in AM fungi MS basal media (MS_0) containing sucrose (3% w/v) fortified with optimized concentration auxin (IAA / IBA) for different plant species such as *S. lycopersicum*s, *D. carota*, *Z. mays*, *T. aestivum* and *S. bicolor*. On the basis of their consistency 3 types media were prepared such as liquid, semisolid and porous. For the preparation of semi- solid media 0.8% agar was added as the gelling agent. For the porous media 40g of vermiculite was added per 100ml of MS_0 media. The *in vitro* proliferated roots which were used for the co culture experiment were cut in to 1cm (approx.) and inoculated into the flask containing sterilized culture media. The surface sterilized AM spores (approx. 100 no.) were inoculated ascetically into flask containing the root organ culture using a sterilized brush. The inoculated flasks were maintained in the culture room in same conditioned maintained for root organ culture. With respect to light condition 3 levels of irradiance were maintained such as dark, diffused light (10-15 µmol m^{-2} s^{-1}) and bright light (35-40 µmol m^{-2} s^{-1}) provided with cool white fluorescent tubes. For each condition 3 flasks with 5 root explants were taken and one set of flasks without AM inoculation was taken as control. Further, each set of experiment was repeated thrice.

2.7 Assessment of root morphology during co-culture

The changes in root morphology such as number of lateral roots induced and average length of lateral root proliferated were recorded after 30 and 60 days of co-culture in different cultural condition. The number of lateral root formed per explants were counted and expressed as number. Root length at stipulated day was measured and expressed in centimeter (cm).

2.8 Assessment of AM root colonization during co-culture

AM root colonization during co-culture was assessed at 30 and 60 days interval. For each culture condition, roots were cleaned with distilled water then cut into 1cm length. Root segments of replicate flaks were mixed together to get a composite sample. The root pre-treatment and staining (0.05% Trypan blue) was done as per Phillips and Hayman (1970). From the composite sample 50 root segments were observed and AM root colonization is express as percentage According to Giovannetti and Mosse (1980) by the formula:

$$\text{AM root colonization (\%)} = \frac{\text{No. of root segment with AM colonization}}{\text{Toral no. of root segments observed}} \times 100$$

3. Results

3.1 Mass production AM and root colonization in pot culture

After 4 months of pot culture, host plant roots were examined for AM colonization. It was observed that AM fungi root association was 100% in *Z. mays*, *T. aestivum* and *S. bicolor*. However, the spore density was 425, 562 and 700 per 100g soil in *Z. mays*, *T. aestivum* and *S. bicolor* respectively.

3.2 Optimization of in vitro *root organ culture*

Optimization of *in vitro* root organ proliferation of dicot plants *S. lycopersicum* and *D. carota*, monocot plants *Z. mays*, *T. aestivum* and *S. bicolor* were tested in a range of concentrations (0.25 to 1.5 mgL^{-1}) of IAA and IBA. *In vitro* root organ proliferation of in terms of highest number of lateral root per explants and highest root length was recorded for *S. lycopersicum* with IAA ($1mgL^{-1}$) and for *D. carota* IBA ($1mgL^{-1}$) (Table 1). For monocot explants optimum concentration of auxin for *in vitro* root proliferation was IAA ($1mgL^{-1}$) for *Z. mays*, IBA ($1mgL^{-1}$) for *T. aestivum* and *S. bicolor* within 30 days of culture (Table 2).

3.3 Effect of media consistency on root morphology during co-culture

The effect of different culture media consistency on the induction of lateral root and increase in the root length was assessed during co-culture under bright light condition. It was observed that highest number of lateral root was induced in semi solid media, followed by porous media and least number in liquid media. After 30 days of co-culture *D. carota* developed highest number (7.67) of lateral root in semi solid media, followed *S. lycopersicum* (6.2) in porous media under AM inoculated condition. After 60 days of co-culture *D. carota* among the dicot and *S. bicolor* could induce highest number of lateral roots in semi solid and porous media (Figure 1). With respect to root length, after 30 days of co-culture *S. lycoperiscum* showed maximum root length followed by *D. carota* in semi solid and porous

Table. 1

Effect of IAA and IBA concentration of on root organ proliferation of *Solanum lycopersicum and Daucus carota*

Explant Source	Auxin	Conc. of Auxin (mgL^{-1})	No of root lateral roots	Root length (cm)
Solanum lycopersicum	IAA	0.25	6.8 ± 2.75	2.28 ± 1.60
		0.5	7.13 ± 3.73	2.94 ± 1.79
		1.0	8.53 ± 5.31	3.26 ± 1.69
		1.5	5.86 ± 3.33	2.76 ± 0.24
	IBA	0.25	5.46 ± 3.33	2.16 ± 1.64
		0.5	6.06 ± 3.17	2.45 ± 1.76
		1.0	6.5 ± 2.18	2.82 ± 1.49
		1.5	5.62 ± 2.30	1.99 ± 1.70
Daucus carota	IAA	0.25	5.53 ± 2.58	1.62 ± 0.08
		0.5	7.66 ± 1.49	2.78 ± 1.81
		1.0	8.86 ± 3.44	2.90 ± 1.74
		1.5	7.25 ± 2.84	2.58 ± 1.28
	IBA	0.25	6.26 ± 3.30	2.67 ± 1.94
		0.5	7.2 ± 3.52	2.95 ± 1.99
		1.0	8.46 ± 2.89	3.64 ± 1.52
		1.5	6.4 ± 3.71	3.07 ± 1.86

Data(Mean± SE) pooled from 3 independent experiment each with 5 replicates.

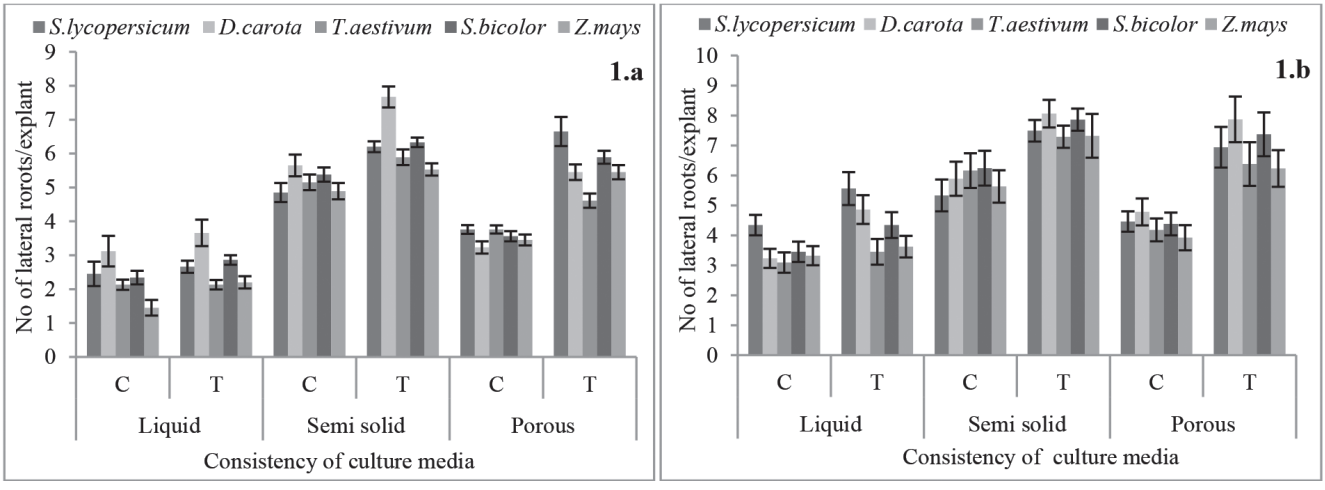


Figure 1. Number of lateral root per explant under different media consistency after 30 (a) and 60 (b) days of co-culture in bright light condition. (C: Uninoculated Control, T: Inoculated Treatment)

media. After 60 days of co-culture *D. carota* (7.68cm) had highest root length followed by *S. lycopersicum* (7.31cm) in semi solid media, however in porous media *S. lycopersicum* (6.87cm) showed highest root length followed by *D. carota* (6.54cm) which are statistically not significant. Among the monocots *Z. mays* had highest root growth in both semi solid and porous media after 30 and 60 days of co-culture. In general AM inoculated explants showed higher number of lateral roots and longer root length compared to the un-inoculated control (Figure 2).

3.4 Effect of light on root morphology during co-culture

Under different light conditions, highest number of lateral root induction was observed in *D. carota* under bright light condition followed diffused light condition after 30 and 60 days of co-culture. Among the monocot *Z. mays*

Table 2

Effect of IAA and IBA concentration of on root organ proliferation of *Zea mays*, *Triticum aestivum* and *Sorghum bicolor.*

Explant Source	Auxin	Conc. of Auxin (mgL^{-1})	No of root lateral roots	Root length (cm)
SZea mays	IAA	0.25	3.33 ± 1.87	1.69 ± 0.96
		0.5	5.66 ± 2.69	1.71± 0.65
		1.0	10.93 ± 4.07	3.53 ± 1.88
		1.5	4.93 ± 1.98	1.56 ± 0.98
	IBA	0.25	3.46±1.95	1.92±1.07
		0.5	5.73±2.18	2.06±1.42
		1.0	10.26±4.66	3.11±0.98
		1.5	4.66±1.79	1.79±0.90
Triticum aestivum	IAA	0.25	3.86 ± 1.45	1.58± 0.67
		0.5	6.06 ± 1.94	2.2 ± 0.72
		1.0	4.33 ± 2.12	1.56 ± 0.94
		1.5	3.73 ± 2.15	1.42±0.93
	IBA	0.25	3.73±2.15	1.64±0.89
		0.5	5.8±1.93	3.03±1.15
		1.0	4.46±2.09	2.38±1.39
		1.5	3.53±1.88	2.04±1.66
Sorghum bicolor	IAA	0.25	3.26 ± 2.18	1.42 ± 0.86
		0.5	5.33 ± 1.95	1.80 ± 0.84
		1.0	6.46 ± 3.39	2.32 ± 0.85
		1.5	3.55 ± 1.50	1.4 ± 0.74
	IBA	0.25	2.93±1.70	2.30±1.68
		0.5	4.66±1.63	2.49±1.462
		1.0	5.93±1.86	2.68±1.33
		1.5	3.4±1.54	2.24±1.74

Data (Mean± SE) pooled from 3 independent experiment each with 5 replicates.

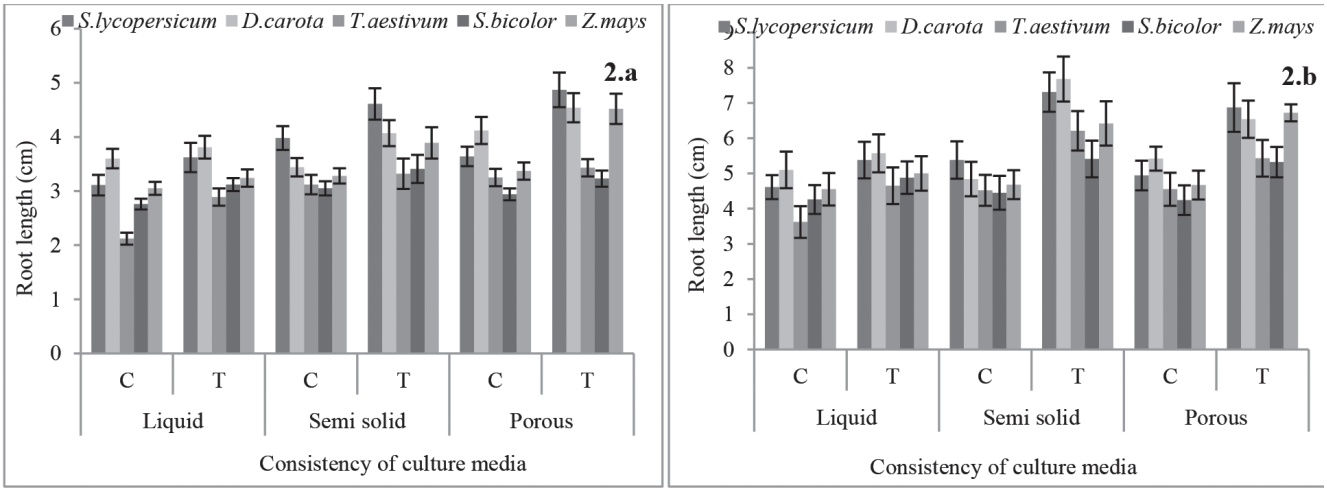


Figure 2. Root length (cm) of explants under different media consistency after 30 (a) and 60 (b) days of co-culture in bright light condition. (C: Uninoculated Control, T: Inoculated Treatment)

showed maximum number of lateral roots in all the light condition. Under dark conditions all the explants could induced least number of lateral roots (Figure 3).With reference to root length, all the explants showed higher root growth in bright light conditions followed by diffused light condition and least in dark condition in semi solid media. Among the dicots *S. lycopersicum* and among monocots *Z. mays* exhibited highest root growth after 30 and 60 days of co-culture.. The AM inoculated explants showed more number of lateral roots and more root length compared to their uninoculated control in all the light conditions (Figure 4).

3.5 AM root colonization in co-culture

The AM root colonization of the explants in different media consistency and different light conditions were analyzed. For different media consistency, highest percentage of AM colonization was recorded in semi solid media followed by porous media and least colonization was observed in liquid media after 30 and 60 days of co-culture. Among the dicots *D. carota* in semi soil and porous media, but *S. lycopersicum* in liquid media showed highest percentage of AM colonization. Among the monocots highest percentage of AM colonization was recorded for *S. bicolor* in all the media consistency (Figure 5). Under different light conditions, highest percentage of AM colonization was found in dark condition followed by diffused light condition and least in bright light condition for all the explants. Among the dicots *D. carota* and among the monocots *S. bicolor* showed highest percentage of AM colonization in all the light condition after 30 and 60 days of co-culture (Figure 6).

6. Discussion

From the present study it was revealed that the AM fungi *C. claroideum* can form symbiotic association with roots of *Z. mays*, *T. aestivum* and *S. bicolor*. Many grass species have been used for mass production of AM fungi *in vivo* (Fracchia *et al*., 2001; Chaurasia and Khare 2005; Sun and Tang, 2012; Kadian *et al*., 2018). The fibrous root system of grasses provides larger surface area for AM fungi and root interactions. As AM fungal spore production has been reported to be influenced by plant species (Struble and Skipper, 1988), the present findings of highest spore AM production with *S. bicolor* is justified. Hence *S. bicolor* is most suitable among the three tested host for mass production of *C. claroideum* spore in pot culture.

The optimal auxin (IAA/ IBA) concentration for root organ proliferation of *S. lycopersicum* and *D. carota*, *Z. mays*, *T. aestivum* and *S. bicolor* was determined. For lateral root development, auxin is reported to be the dominant regulator (Benkova and Hejatko 2009; Fukaki and Tasaka 2009). Auxin in form of IAA and IBA has been used for successful *in vitro* root induction and proliferation in variety of plants (Shukla *et al*., 2009; Victório *et al*., 2012; Hanur and Krishnareddy, 2016). Optimum concentration of IAA /IBA for root proliferation varies from species to species under different *in vitro* cultural conditions (Tavares, *et al*., 2010). From the present study, optimum concentration of IAA and IBA for the *S. lycopersicum* and *D. carota*, *Z. mays*, *T. aestivum* and *S. bicolor* was found to be 1 mgL^{-1} in MS0, which was used in further experiment.

In the present findings higher number of lateral root and longer length of lateral root were found in AM inoculated root culture than non inoculated one. Root system architecture (RSA) gets frequent modification following AM interactions (Scannerini *et al*., 2001; Hodge *et al*., 2009). Despite fundamental differences in root system architecture of monocotyledons and dicotyledons (Osmont *et al*., 2007), in both lateral roots are preferentially colonized by AM fungi and their formation is induced upon symbiosis establishment (Gutjahr & Paszkowski, 2013). Lu & Wu (2017) reported AM inoculation increases number of lateral roots and root length in white clover *in vivo*. Formation of more number of lateral roots after AM colonization is reported in trifoliate orange (Chen, 2017). There are evidences of local elongation of root laterals following establishment of mycorrhiza (Yano *et al*., 1996, Torrisi *et al*, 1999), perhaps in order to increase the suitable sites for colonization (Harrison, 2005; Mukherjee and Ané, 2011). Thus, the present findings of higher number of lateral root and higher length of lateral root in AM inoculated root organ cultures than non inoculated explants is supported by the reports of above mentioned workers.

Under different light conditions highest number of lateral root and highest length of lateral root was observed in bright light and followed by diffused light and least in dark condition. Positive effect of white light on root elongation is reported by Salisbury *et al*., (2007) and Costigan *et al*., (2011). Auxin and sucrose are considered as a major player in light regulated root elongation (Sassi *et al*., 2012). Increase in the rate of *in vitro* root elongation under light condition than in darkness in some plant species has been observed (Vinterhalter, 1990; Monteuuis, 2000). Thus, bright light induced root proliferation in *in vitro* root organ culture as found in the present study is similar to the mentioned reports.

The highest rate of root proliferation and highest percentage of AM root colonization was observed in semi solid media followed by porous media and least in liquid media. Abd Ellatif *et al.*, (2019) reported that mycorrhizal

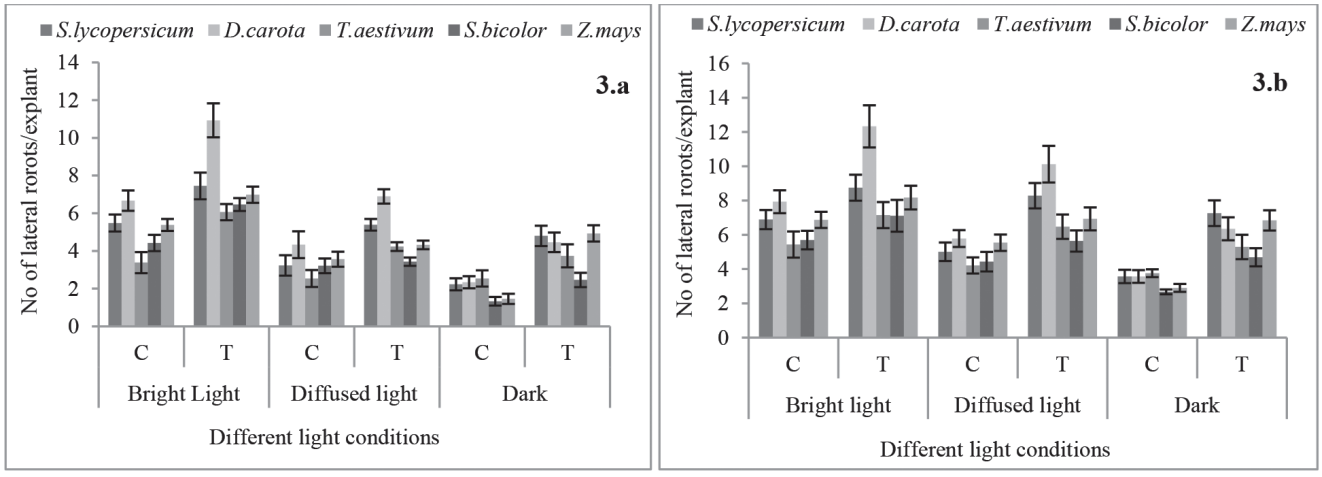


Figure 3. Number of lateral root per explants under different light conditions after 30 (a) and 60 (b) days of co-culture in semi solid media. (C: Uninoculated Control, T: Inoculated Treatment)

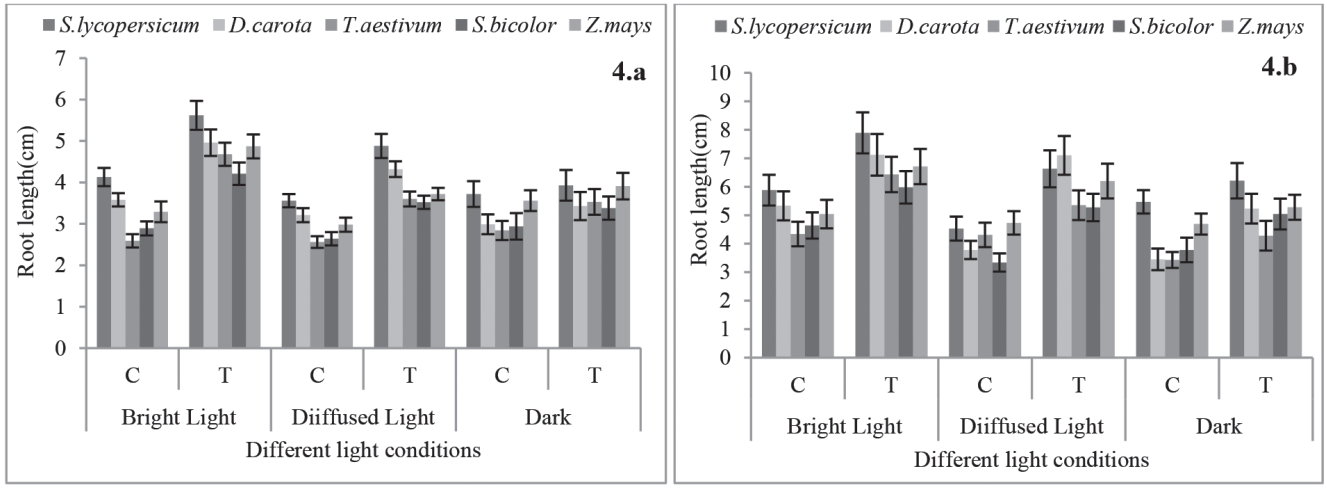


Figure 4. Root length (cm) of explants under different light conditions after 30 (a) and 60 (b) days of co-culture in semi solid media. (C: Uninoculated Control, T: Inoculated Treatment)

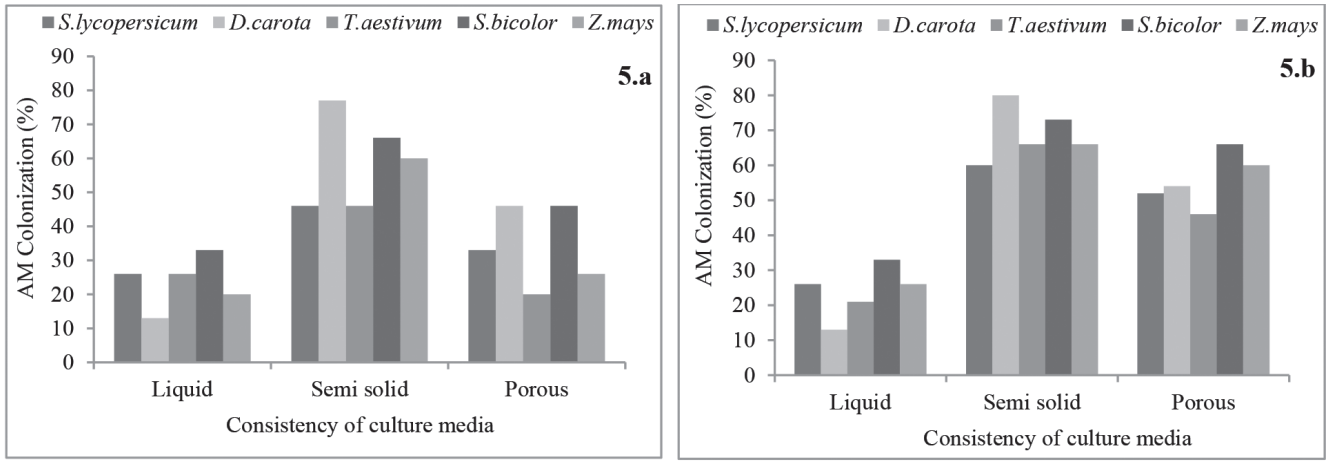


Figure 5. AM root colonization (%) in different media consistency after 30 (a) and 60 (b) days of co-culture in bright light condition.

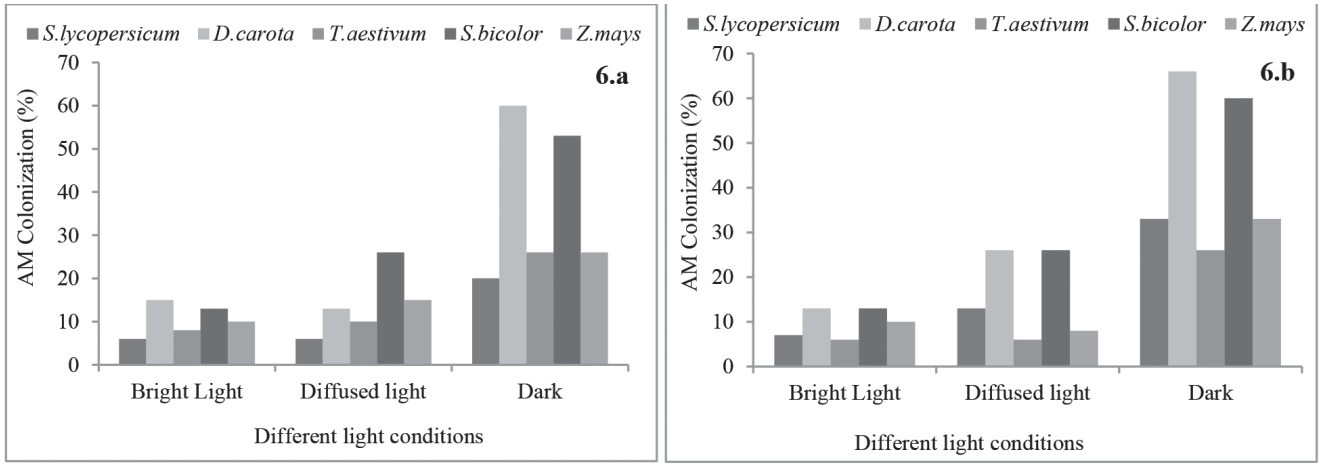


Figure 6. AM root colonization (%) in different light conditions after 30 (a) and 60 (b) days of co-culture in semi solid media.

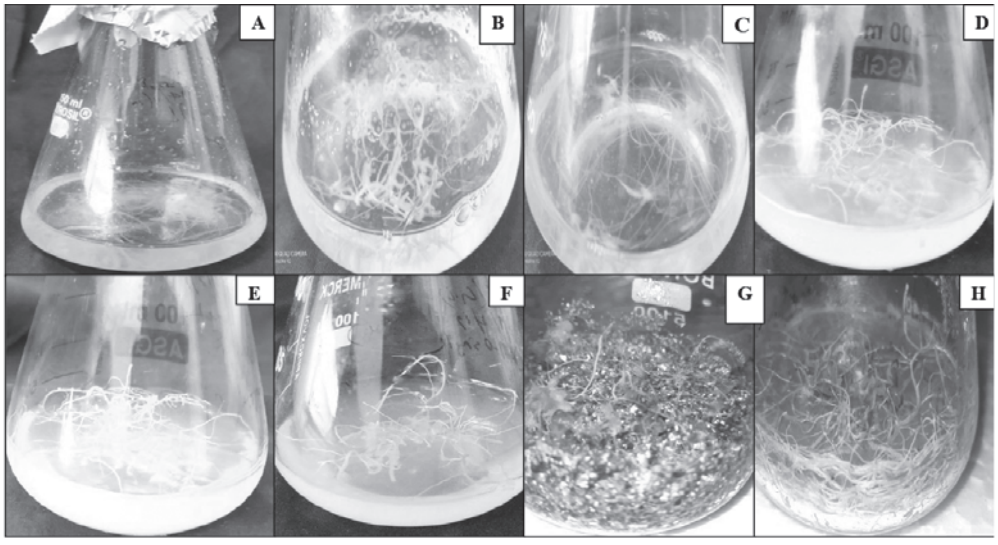
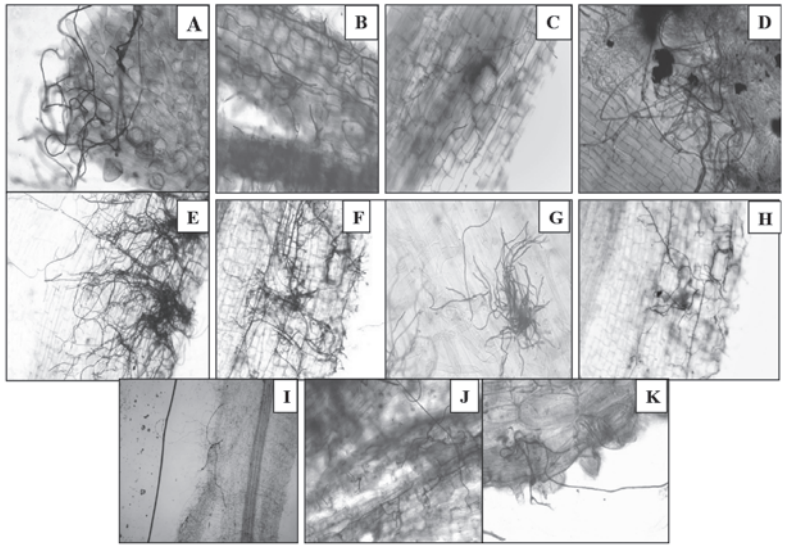


Photo plate 1: Co culture of AM spore with root organ culture in different culture conditions; Liquid media: *D. carota* (A), *S. lycopericum* (B) &*T. aestivum* (C), Semi solid media: *D. carota* (D), *S. bicolor* (E) & *Z. mays* (F), Porous media: *Z. mays* (G) & *D. carota* (H)



Photoplate 2: AM root colonization of *C. claroideum* in co-culture with *D. carota* (A &B), *S. lycopersicum* (C & D), *Z. mays* (E &F), *T. aestivum* (G & H) and *S. bicolor* (I, J & K)

colonization rate and arbuscular abundance of *S. lycopersicum* roots grown *in vitro* were recorded to be higher on solid media than in liquid media. According to the workers such finding might be because of the immobilization of spore inoculum on solid media resulting enhanced growth of hyphae. Besides, poor aeration in liquid media might hinder AM fungi development. *Glomus etunicatum* was reported to colonize better in culture media containing vermiculite than agar, which was most likely due to the vermiculite improved air diffusion through the media (Bressan, 2002). Since there is differential rate of AM root colonization in different media consistency, the present findings of higher *in vitro* root colonization of *C. claroideum* in media containing agar cannot be generalized for all AM species. As there is highest AM root colonization in semi solid media, which might have caused higher root proliferation in terms of higher number of lateral root and higher length of the lateral root in semi solid media than liquid media.

From the present study it was recorded that *C. claroideum in vitro* root colonization was better under dark condition than light condition. Higher level of photosynthetic photon stimulates root colonization by AM *Glomus intraradices* in *Solanum tuberosum* plantlets cultured in an *in vitro* tripartite system (Louche-Tessandier, 1999). The rate and maximum percentage of AM infection were found lower at high light intensities (15-2 0klux) than low ones (5-10klux) in *Allium cepa* by *Gigaspora calospora* (Furlan and Fortin, 1977). Hayman (1974) reported larger AM arbuscules at high light intensities than low one. Since there are reports on differential effect of light on AM root colonization, the present observation of higher *in vitro* root colonization of *C. claroideum* in dark than light condition cannot be generalized for the all the species of AM fungi.

7. Conclusion

From the present study it was concluded that semi solid and porous media are more suitable for root organ culture proliferation than liquid media during co culture with AM fungi *C. claroideum*. Among different light conditions bright light condition is most suitable for the root organ culture proliferation with the AM fungi. The AM root colonization occurred maximum in semi solid and porous media under dark condition. Hence, for root organ proliferation, initial co culture can be done in semi solid or porous media under bright light condition. Later, the co-culture should be kept in dark condition for better AM root colonization and subsequent sporulation.

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