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ORIGINAL ARTICLE

In vitro Establishment of Arbuscular Mycorrhizal Fungi (AMF) in Cucumber (*Cucumis sativus*) Roots

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ABSTRACT
The technique of in vitro cultivation of *Arbuscular mycorrhizal* fungi has been developed over the past few decades and opens up areas of studying plant-fungi interactions. It is a scientific breakthrough, especially for the study of the ubiquitous arbuscular mycorrhizal fungi, since these obligate symbionts depend on host plant. The present study is aimed to ascertain whether root colonization of AMF could be effected in vitro without undertaking complex and complicated culture conditions. Ultimate being to use the system either for sporal dissemination or hyphal inoculum as biofertilizer. Also this could form an economically viable technique for root organ cultivation of AM fungi.
Keywords: - In-vitro, AMF, Cucumber roots, Root culture.

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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are symbiotic obligate associations between a group of fungi and the roots of higher plants [1]. Nearly 80 to 90 percent plant species of all groups studied, more so angiospermic, studied show this symbiotic relationship [2; 3]. The principle beneficiary being the host plant due to higher nutrient uptake from the soil and in exchange AMF receive a rhizospheric niche and organic nutrients for its growth and development. It therefore, becomes imperative that the fungi will predominantly flourish only under strict host rhizosphere.

The strictness produced complexities therefore become detrimental in the *in vitro* culturing of these fungi. AMF have of late established themselves as a category of biofertilizers [4; 5; 6]. Wide use of these fungi with or without or in combinations with other manure and synthetic fertilizers is coming up to drastically reduce use and subsequent deleterious effects of industrial fertilizers in the agro and crop ecosystem. [7; 8]. There are some reports of successful root-organ culturing of AMF [9; 10; 11; 12). Some relevant results indicate failed efforts for successful growth of AM fungi independent of a plant host (13; 14]. The constraints that one experiences in having pure *in-vitro* cultures of AM fungi are due to their rigid symbiotic obligate association which is further complicated by the biotrophic and hypogenous nature of the micobionts involved. To overcome these debilitations several attempts have been made during the last few years to obtain symbiosis under *in-vitro* conditions. The use of root organ culture has proved particularly successful. A list of such *Glomalean* species which are either successfully or near successfully are cultivated on root organ cultures has been provided by Fortin *et al.,* (15 2002).There have been efforts by workers to have *in-vitro* cultures of AM fungi on specified culture media grown roots and then infected by AM with colorizations in the rhizospheres [16]. After drying such roots, these could be utilized as biofertilizers. However, culturing conditions could involve substantial economical inputs. The present preliminary study with cucumber (*Cucumis sativus*) seedlings is an effort to ascertain whether the roots of these seedlings could be inoculated with AMF spores after providing simple growing in conditions under petri plates and also with different media.

MATERIAL AND METHODS

Cucumber seeds (*Cucumis sativus*) *Var. long green* were floated on water and only those seeds were further used which sank to the bottom. These were then surface sterilized for 10 to 15 minutes with 0.01% cetrimide and thoroughly washed with sterile distilled water. Acid washed, dry, sterilized petri plates of 10 cm dia. were lined with Whatman filter paper discs and resterilized. The 10 or 20 seeds were placed in each petri dish under laminar air flow and 5ml of either sterile distilled water or sucrose

solution or nutrient solution added accordingly to wet the filter papers. These were then placed under constant illumination conditions of 1000 lux soft fluorescent white light on the culture rack at a constant temperature of 25 to 27^{0}C. Record for seed germination percentage and radical length was maintained. Radical emergence was taken as the first sign of germination. The petri plates were inoculated with 10 sterilized AMF spores each segregated from the fresh soil. Each medium was used in five replicates. 0.5 percent and 1.0 percent sucrose solution was prepared by dissolving 500 mg and 1gm of AR grade sucrose in 100 ml of double distilled water respectively. The solutions were sterilized by autoclaving before use for treatments. The nutrient medium was employed as suggested by Tahat *et al*., [17]. All reagents in the nutrient solution were of HIMEDIA AR grade. Per 1000 ml composition was constituted by adding appropriate volumes of specific nutrient solution previously made into a concentrated stock. The final composition was made as follows:

Nutrient	mg/lit
$Ca(NO_3)_2.4H_2O$	236
$FeSO_4.7H_2O$	2.0
KCl	65.0
KH_2PO_4	12.0
$MgSO_4.7H_2O$	36.0

The nutrient medium was used only after it was autoclaved.

Roots from 5 seedlings from each media were separated, washed, and then serially cut into 1 cm long pieces. These were subsequently treated by the method of Phillips and Hayman [18] and as modified by Kormanik *et al*., [19] for root clearing and their staining with trypan blue. The root length colonization was assessed using frequency distribution by Biermann and Lindermann [20] in which the colonization was assessed as proportion of root length colonized by the fungi and Percent seedling root colonization was calculated using following relation:

$$\text{Percent total seedling root colonization} = \frac{\text{Total number of colonized seedling root piece}}{\text{Total number of root pieces examined}} \times 100$$

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RESULTS

Depending on the medium, the seeds of cucumber (*Cucumis sativus*) var. long green, showed germination ranging from 80 to 100 percent after 96 hours. A 100 percent seed germination seed was recorded with sucrose. Nutrient media showed lower 80 percent germination as against 95 percent with distilled water only (Table: 1).

Observations showed that the seedlings growing on distilled water were healthy upto about 25 to 30 days after germination, which was extended to 40-45 days by providing sucrose and to 45 to 50 days by the nutrient media. Irrespective of the media, presence of AMF spores do not seem to effect germination or radical length significantly under petri plate conditions (Table 1). After 30 days of germination of cucumber seeds nutrient media seems to score better over sucrose and sucrose better over distilled water as far as the observable health of the seedlings are concerned.

In the presence of AMF spores cucumber seedlings in the distilled water showed only 10.87 percent of roots being infected after 30 days. The increase in the availability of roots by increasing the number of seedlings per plate does not seem to change the percentage seedling roots infected appreciably. Sucrose 0.5 percent with AMF spores however, showed a two fold increase in root infection irrespective of the root density in the petri plates. In the nutrient medium root infection of cucumber seedlings seems having a relation with the root density. Higher root density showed higher endomycorrhizal infection; the increase being fourfold higher than the distilled water (Table 2). Maximum total seedling root colonization and total percent number of pieces showing colonization was observed in nutrient medium with 88.39 and 92.86 percent respectively in the nutrient medium (Table 3).

DISCUSSION

The results of these experiments are discussed in the light of the pioneering work of Moose and Hepper [21] and Quilambo [22] used root cultures from *Lycopersicum esculentum* and *Trifolium pretense* to establish *in vitro* mycorrhiza and demonstrated first time that the spores of AMF could be successfully used to colonize excised roots growing on mineral based medium. In the present study too the minimal

conditions of nutrients were employed to effect the root colonization of a cucurbit *Cucumis sativus* and to assess any success thereof.

From the present study it is obvious that the sucrose and mineral nutrients do add to the longevity of the seedlings. Thereby providing more time for the roots for getting inoculated by hyphae from germinated spores and also develops AM vesicles and arbuscules inside. Earlier *in vitro* experiments of Fortin *et al.,* [15] where a better AM endomycorrhizic establishment was reported to vary between 15- 45 days from the day of inoculation with spores in the medium agrees with the present observation also. This observation was the basis for extension of the observational part to 50 days after germination at 25-27 ^{0}C.The compensatory role of mineral uptake by roots in the health of the seedling was much too obvious in the present experiments also. Fortin *et al.,* [15] further said that two types of fungal inoculums can be used to initiate monoxenic cultures; either extraradical spores or propagules from the intra radical phase i.e. *mycorrhizal* root fragments and isolated vesicles. In the present case the latter inoculum method may not be helpful in achieving the objective than the former. The reason being that AMF identifications are predominantly based upon spore characteristics and less on hyphal endo-root conlonizations. Further monoxenicity study of the inoculum was not the aim; the aim being to see whether cucumber roots can be a medium of infection under *in vitro* conditions by AM fungi.

In the present effort it seems that under the petri plate culture conditions AMF spores are induced to first germinate and then infect and subsequently establish in itself the roots of cucumber. Generally, AM fungi spores do not initially need specific conditions or the presence of a host root to germinate [12], however, root exudates can stimulate germination and / or post germination hyphal growth (19; 20). In the experiments here, since spores do germinate and do infect the roots, it seems therefore, that the model as presented by Poulin *et al*. [23] and Buee *et al.,* [24] may be operational here too. It also means that the cucumber seedling roots provide stimulus for the AMF spore germination and subsequently induce a hyphal growth stimulus by putting exudates in the medium wherein they are growing together. The increase in the infected root percentage by increasing the number of roots available for infection under nutrient medium conditions also leads to that inference. Whatever be the case, it is conclusively encouraging that this simple technique can be exploited by further refinement for high grade infection under controlled conditions and then use the cucumber roots as inoculum for propagation and biofertilization of other commercially important plants. The technology once established can constitute a convenient mode of culturing and distribution of AMF biofertilizers. This can also become a handy employment for landless rural population as a cheap, home based vocation. Production of AMF root biomass under *in vitro* conditions on various solid media by exploiting their symbiosis with the specific root species is already a technique towards above mentioned aim [25]. However, the production costs, it seems can considerably be economized by the refinement of the system described here.

The potential of the *in vitro* system for the study of inter -specific AM fungal competition is yet to be investigated on a scale that it should have been and compatibility studies between AM fungi using root organ cultures are possible. Results thus might provide improvement that could be made use of to industrially produce the inocula [15]. The root organ culture of mycorrhiza has proved useful for taxanomists and physiologists and also geneticists. It is also a good medium for the study of interaction with root borne pathogens and other soil organisms. The system of these and other interactions could perhaps be achieved using an improved version of root hypocotyl system [26]. This statement could fully agrees with the present work, wherein the cucumber seedling is complete root hypocotyl system.

In any case it is evident from this study that the *in vitro* colonization of cucumber seedling roots is possible under simple petri dish conditions. The diversity of inoculate AMF species encourages one to assure that this present technique can be employed in both multiple as well as monoxenic inoculi. The observations are depictive of the diversity of various genera and species which can be made to infect the root individually or collectively and *Cucumis sativus* seeds can be employed as a good bioassay. Both arbuscules and vesicles with hyphae can be seen within the infected roots grown under *in vitro* root culture with AM fungi. The spore identifications showed infections were possible with various forms of AMF under petri plate root culture conditions from cucumber seedlings.

Table 1:- Percentage germination and root length with and without AMF to *Cucumber* seeds and seedlings respectively after 96 hrs. lying on petri plates containing either water or sucrose or nutrient medium.

Medium		Germination Percentage		Radical length.	
		Without Spore	With Spore	Without Spore	With Spore
Distilled Water		95.21±5.6	95.84±6.4	06.76±0.26	06.33±0.29
Sucrose	0.5 %	85.64±3.4	90.54±4.3	07.33±0.18	07.66±0.26
	1.0 %	100.00±00	100.00±00	08.22±0.38	08.84±0.42
Nutrient medium		80.48±4.2	80.78±2.6	06.92±0.16	07.13±0.27

Table 2:- Percentage infection of roots of *Cucumber seedlings* by AMF after 30 days of treatment with AMF spores on various growth media.

Medium		Percentage seedlings roots infected	
		Low root density	High root Density
Control Dist. Water	- AMF spore	0.00±00	0.00±00
	+ AMF spore	10.87±1.21	12.34±1.13
Sucrose	0.05%	23.17±1.23	25.32±1.87
	0.1%	08.62±0.27	11.87±.13
Nutrient Medium		12.53±0.89	47.52±2.76

Table 3:- Percent total seedling root colonization and Percent total root colonization after 30 days of treatment with AMF spores on various growth media.

Medium		Percent total seedling root colonization	Percent number of pieces showing colonization from each seedling.
Distilled Water		36.25±2.02	40.21±2.89
Sucrose	0.5 %	48.52±3.20	52.68±3.18
	1.0 %	72.31±4.89	72.89±5.26
Nutrient medium		88.39±4.56	92.86±6.12

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