Research Paper :

Evaluation of AM spores and mycorrhizal root fragment as fungal inoculum for establishing colonization on the Ri – T DNA transformed hairy roots

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SUMMARY

Arbuscular mycorrhizal fungi are ubiquitous and form symbiosis with roots of a majority of higher plants for establishing *in vitro* cultures. Hairy roots can serve as potential host under root organ culturing. Lab experiments were conducted to evaluate the potential of AM spores as well as AM roots to establish colonization in hairy roots. *Glomus mosseae, Glomus intraradices* and *Glomus caledonium* in spores as well as colonized roots pieces were used to develop colonization in Ri-TDNA transformed hairy roots of cowpea and tomato using Modified White's Medium (MWM) and Minimal medium (MM). Inoculated plants were kept under dark at 25^{0}C for about 3-4 weeks. When surface sterilized spores were used as inoculum for the establishment of AM colonization in Ri T-DNA transformed hairy roots, *G. intraradices* produced in M medium the maximum colonization in cowpea and tomato hairy roots, respectively. When mycorrhizal root fragments were used as inoculum, *Glomus mosseae* produced more colonization in M medium (30.3 %) than others in hairy roots of both cowpea and tomato. While comparing the efficiency of two sources two to three fold increase in root colonization was observed in cowpea and tomato hairy roots with the inoculation of AM spores rather than AM root pieces.

Key words : AM fungi, Mycorrhizal root fragment, AM spores, *Glomus,* Hairy roots

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Arbuscular Mycorrhizal (AM) fungi are a unique group of ubiquitous soil microorganisms known to form symbiotic association with roots of economically important crop plants. The symbiosis between the two biotropic organisms is mainly characterized by bi-directional transfer of nutrients which gives access for the plant to low mobile elements like phosphorus (Smith and Gianinazzi – Pearson, 1988). Compatibility with AM fungi enable plants to explore and conquer a novel ecosystem and continues to provide a selective advantage because of the nutritional benefit it provides to plants (Karandashov and Bucher, 2005).

AM fungi produce structures such as, vesicles and arbuscles in cortical roots (Bowen, 1987). AM propagules as isolated spores, vesicles and sheared mycorrhizal roots are virtually able to initiate AM symbiosis and establish the pre-symbiotic phase with the transformed root. Chlamydospores of *Glomus* sp. (Mosse and Hepper, 1975; Mugnier and Mosse, 1987) and non-sporocarpic azygospore of *Gigaspora margarita* (Becard and Fortin, 1988; Becard and Piche, 1989; Diop *et al.*, 1992) are also preferred as starter inoculum.

Spores, used as propagules to initiate monoxenic culture, harbor many saprophytic microorganisms that can influence both spore germination and AM formation and thus, require sterilization (Fracchia *et al.,* 1998). For all AM propagules, proper selection and efficiency of sterilization process are keys for the success of axenic or monoxenic AM fungal cultures.

Mycorrhizal roots used to initiate monoxenic cultures come from plants grown in pot cultures, with field collected soil or AM propagules. Leek (*Allium porrum* L.) plants are widely used because of their susceptibility to colonization. Two sources of AM inoculum *viz.*, spores and AM roots were tried for establishing the colonization in hairy roots of cowpea and tomato using three different species of AM fungi.

MATERIALS AND METHODS

AM spores as fungal inoculum for establishing colonization on the Ri – T DNA transformed hairy roots :

Hairy root sources :

Tomato and cowpea explants (tomato hairy roots produced with *A. rhizogenes* strain 2364 and cowpea hairy roots with *A. rhizogenes* strain 532 were used in this experiment). The hairy roots produced in

MWM and M medium were taken for the study. For establishing colonization in hairy roots, spores of different species of *Glomus* were used as given below:

Treatment details :
Tomato hairy roots :
- Hairy roots in MWM + *G. mosseae*
- Hairy roots in MWM + *G. intraradices*
- Hairy roots in MWM + *G. caledonium*

Cowpea hairy roots :
- Hairy roots in MM + *G. mosseae*
- Hairy roots in MM + *G. intraradices*
- Hairy roots in MM + *G. caledonium*

All the above six treatments were replicated thrice with appropriate controls.

The spores were surface sterilized with 96% ethyl alcohol for 60 seconds. Spores were transferred separately to the filter funnel with cellulose nitrate membrane (0.2 μm Sartorius, Germany) connected with vacuum pump for sterilization and washing with water. Spores present in the membrane filter were washed several times with sterilized distilled water to remove the traces of sterilizing agent. There after aseptically the spores were transferred to Petri dishes containing water agar and the plates were incubated for 2-3 days at 25^0C to check the contamination. The spores which did not develop contamination and appeared densely filled with cytoplasm and liquid droplets were taken and aseptically placed near (1 cm) to the transformed hairy roots in MWM and M media. A single spore was inoculated in each plate. Then the plates were kept inverted and incubated in dark at 25^0C for the AM spores to establish the colonization of hairy roots for about 3 to 4 weeks.

Mycorrhizal root fragment as fungal inoculum for establishing colonization on the Ri – T DNA transformed hairy roots :

Mycorrhizal root fragments containing mycelium of AM fungus were considered to be one of the potential sources for colonizing the hairy roots as well as for the development of mycelium. Hence, to assess the potential of AM roots to initiate the colonization in hairy roots, this experiment was conducted.

Treatment details :
AM root sources :
- *Glomus mosseae* colonized root fragments
- *Glomus caledonium* colonized root fragments
- *Glomus intraradices* colonized root fragments

Media used :
- M medium
- Modified White's medium

Hairy root sources :
- Cowpea
- Tomato

Host plant
- Maize

Production of AM roots :

Maize plants were raised under *ex vitro* condition (growth chamber) in the pots using sterilized substrates as 1:1 sand, soil mixture. The plants were grown for a period of 35-40 days.

After 35 days, young healthy AM colonized roots were selected, cut into small pieces and surface sterilized with ethanol for 2 min and used for inoculation. The roots were kept near the hairy roots in the plates containing MM as well as MWM and incubated for 3-4 weeks towards development of AM colonization in hairy roots.

Assessment of AM colonization in Ri – T DNA transformed hairy roots :

After 3 - 4 weeks of incubation AM inoculated (spores as well as roots) Ri- T DNA transformed hairy roots were collected from the plates and processed for the assessment of AM colonization using root clearing and staining technique as per Phillips and Hayman (1970) with slight modifications.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Colonization of hairy roots by AM spores :

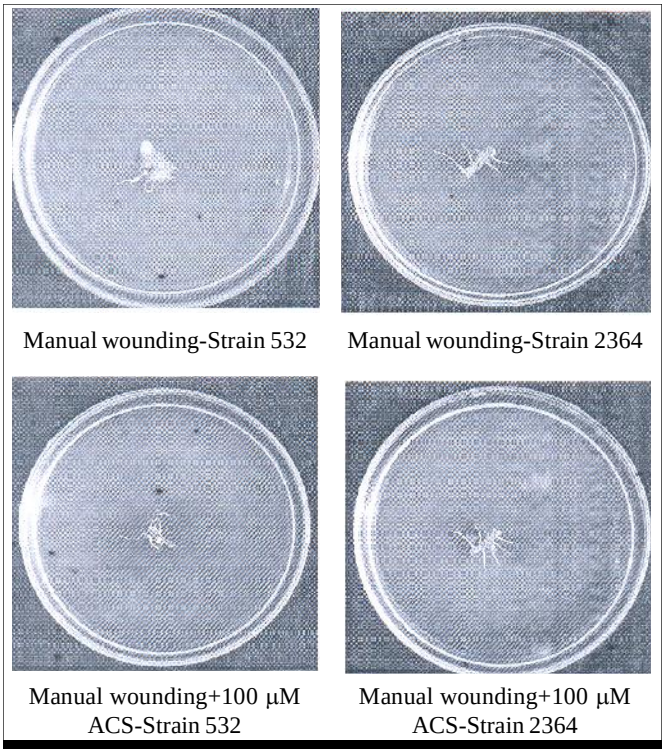
Establishment of AM fungal species, *G. mosseae, G. intraradices* and *G. caledonium* on the hairy roots were periodically observed under microscope. Presence of vesicles, hyphal network and sporulation were observed after 3 weeks of incubation by root clearing and staining technique (Plate 1)

The AM colonization in the transformed roots of cowpea is presented in Table 1. Maximum root colonization was recorded by *G. intraradices* (55.70 %) spores in MW medium against the least colonization by *G. caledonium* (18.0 %) in MW medium. Among the AM species inoculated, *G. intraradices* produced higher colonization in MW medium, whereas *G. mosseae* and *G. caledonium* produced higher colonization in minimal medium. Significant interaction was observed between AM spores as well as media used on root colonization.

Table 1 : Colonization of AM fungi (spore inoculation) in Ri–T DNA (*A. rhizogenes* 532) transformed cowpea hairy roots under different media (after 3 weeks)

Cultures	AM colonization (%)		
	Media		
	MWM	MM	Mean
Glomus mosseae	28.00[d]	47.40[b]	37.70[b]
Glomus intraradices	55.70[a]	38.20[c]	46.95[a]
Glomus caledonium	18.00[c]	28.40[d]	23.20[c]
Mean	33.90[b]	38.00[a]	
	S.E. ±	C.D.(P=0.05)	
Media (M)	1.75	3.82	
Culture(C)	2.15	4.68	
M X T	3.04	6.62	

MWM - Modified white's medium
MM - Minimal medium



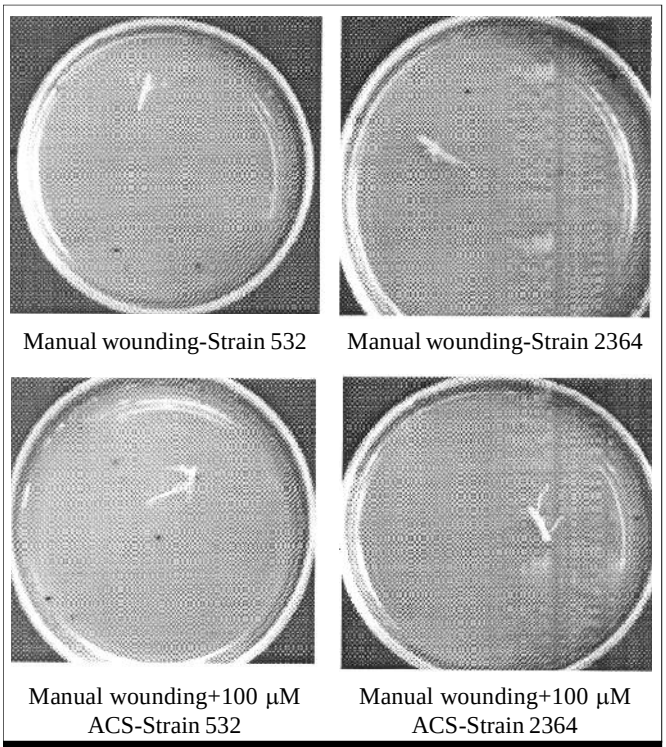
Manual wounding-Strain 532 | Manual wounding-Strain 2364

Manual wounding+100 µM ACS-Strain 532 | Manual wounding+100 µM ACS-Strain 2364

Plate 1 : Hairy root induction in cowpea (after 20 days)

By comparing the two media used, irrespective of the AM species, minimal media was observed superior in recording higher colonization (38.0 %) of cowpea hairy roots and irrespective of media used *Glomus intraradices* produced higher (46.95 %) colonization in cowpea hairy roots (Plate 2)

Similarly in tomato hairy roots also, *G. intraradices* recorded higher colonization (50.3 %) under MW medium, whereas *G. mosseae* and *G. caledonium* recorded higher colonization in M medium. Performance of AM species as well as media was similar to cowpea explants (Fig. 1).



Manual wounding-Strain 532 | Manual wounding-Strain 2364

Manual wounding+100 µM ACS-Strain 532 | Manual wounding+100 µM ACS-Strain 2364

Plate 2 : Hairy root induction in tomato (after 20 days)

By looking into the overall results, inoculation of *G. intraradices* resulted maximum colonization followed by *G. mosseae* and *G. caledonium* in both the explants used. It was reported that exudates from transformed carrot roots stimulated the hyphal growth of *Gi. margarita* (Poulin *et al.*, 1993). Presence of formononetin and biochain A in clover roots that stimulated the mycorrhizal colonization by *Glomus* sp. (Nair *et al.,* 1991). Also in the present study MWM was found suitable for the establishment of AM *in vitro* cultures on transformed roots of cowpea and tomato. However *G. mosseae* grown on these medium colonized the transformed roots but failed to sporulate, but the other species *viz.*, *G. intraradices* and *G. caledonium* developed intraradical spores in the same medium (Plate 3 and 4). Similarly the intraradical sporulation was observed earlier in transformed hairy roots of carrot with *Gi. margarita* (Gadkar and Adholeya, 2000).

Mycorrhizal colonization of hairy roots :

Considering the inability of some AM fungi to produce spores (Johnson, 1977; McGree, 1989), the use of excised AM roots as inocula offers an opportunity for taxonomic and genetic studies of these obligate biotrophic microorganisms. However, proper selection of sheared-root inocula is the key of the success of this cultivation system. Use of mycorrhizal roots as the source of

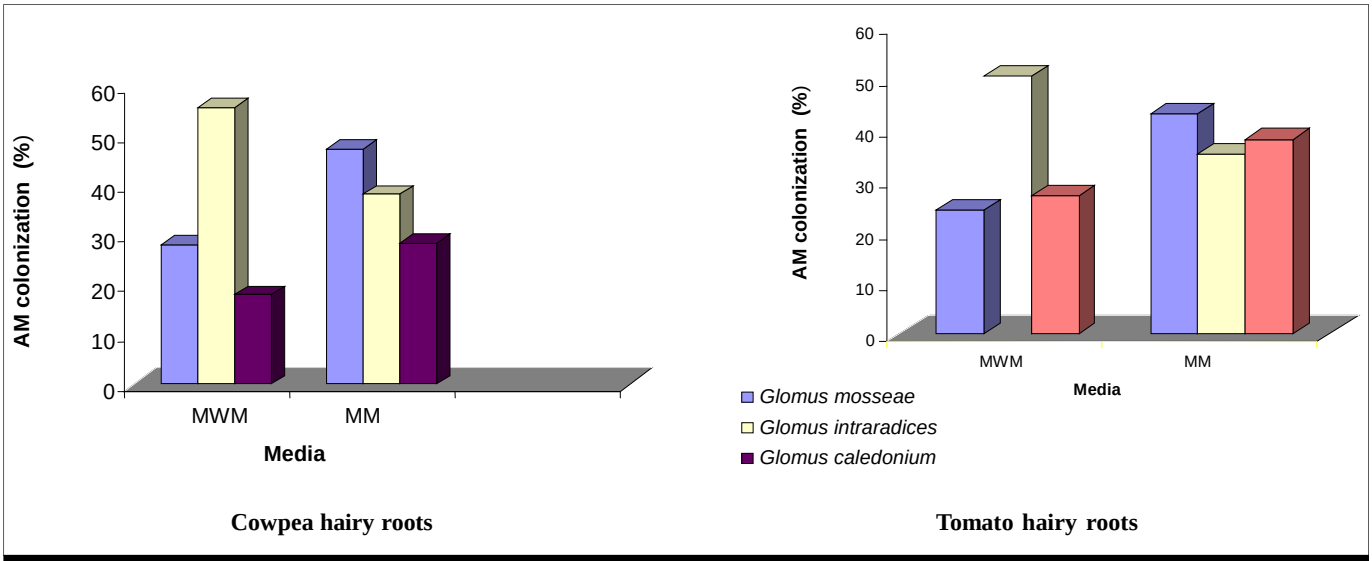
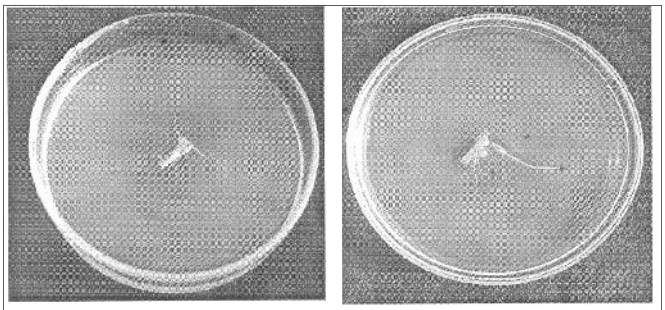


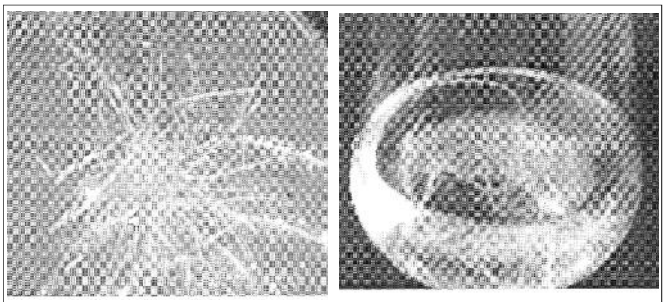
Fig. 1 : Colonization of AM fungi (spore inoculation) in Ri – T DNA (*A. rhizogenes* 532) transformed hairy root under different media (after 3 weeks)



5 min of infection with *A. rhizogenes*

15 min of infection with *A. rhizogenes*

Plate 3 : Hairy root induction in cowpea with different infection period



In solid medium

In liquid medium

Plate 4 : Cowpea hairy roots

inoculum to initiate colonization in hairy roots was evaluated in the present study.

Mycorrhizal root as fungal inocula :

Establishment of AM fungal species *G. mosseae*, *G. intraradices* and *G. caledonium* on the hairy roots were periodically observed under microscope. Presence of vesicles, hyphal network and sporulation was observed after 3 weeks of incubation by root clearing and staining technique.

The result showed that, in cowpea hairy roots, colonization was observed significantly higher with *Glomus mosseae* in MM (30.30 %), followed by *Glomus intraradices* in MWM. Not much difference in root colonization was recorded with *Glomus caledonium* inoculation in MWM and MM (Table 2)

In case of tomato also, *Glomus mosseae* registered higher colonization (22.9 %) in MM, followed by *Glomus intraradices* (16.0%) in MWM.

These results suggest that inoculation of root fragments colonized with *Glomus mosseae* recorded significantly higher colonization in hairy roots of cowpea (25.15 %) and tomato (16.35 %) than other species evaluated. By comparing the sources of inoculation, with spore inoculation AM colonization in hairy roots was observed two fold higher with cowpea and three fold higher colonization with tomato explants than inoculation by mycorrhizal root fragments.

As that of spores, there is no evidence that mycorrhizal root pieces need specific exogenous condition or a host plant for hyphal regrowth (Diop *et al.*, 1994). However root exudates might stimulate vesicle germination and hyphal growth. The growth promoting

Table 2 : Colonization of AM fungi in transformed hairy roots by maize mycorrhizal root fragments (after 3 weeks)

AM Cultures	AM root colonization (%)					
	Cowpea hairy roots			Tomato hairy roots		
	MM	MWM	Mean	MM	MWM	Mean
Glomus mosseae	30.30[a]	20.50[c]	25.15[a]	22.90[a]	9.80[c]	16.35[a]
Glomus intraradices	15.00[c]	27.40[b]	21.20[b]	11.00[b]	16.00[a]	13.50[b]
Glomus caledonium	13.20[d]	12.50[d]	12.85[c]	5.00[c]	5.50[c]	5.25[c]
Mean	19.41[b]	19.96[a]		12.96	10.44	
	S.E. ±		C.D.(P=0.05)	S.E. ±		C.D.(P=0.05)
Culture(C)	1.17		2.09	0.06		1.33
Media(M)	0.96		2.56	0.74		1.62
C*M	1.66		3.63	1.05		2.29

MWM - Modified white's medium
MM - Minimal medium

substance derived from host cells might accumulate in the intraradical structures of the mycorrhizal root pieces (Strullu and Plenchette, 1992), which helps in the initial proliferation of AM hyphae.

When comparing with the two sources of inocula, present results showed the higher colonization of hairy roots when spores were used for inoculation rather than using AM roots. These results are in conformity with the comparison made between the use of *in vitro* produced spores and vesicles from leek plants grown in pot culture (Nantais, 1997) and it was reported that for a given number of propagules, root colonization was more efficient when using spores rather than isolated vesicles. The results suggested that the use of AM spores was observed better to establish the monoaxenic cultures of AM fungi using Ri- T DNA transformed hairy roots.

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