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ROOT COLONIZATION STUDIES OF BIOHARDENED TISSUE CULTURE PLANTS OF *ALBIZIA AMARA*

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Abstract: The morphological, physiological and anatomical features of the plantlets developed under *in vitro* conditions, have a great impact on plant survival rate after transplantation to *ex vitro* conditions. The leguminous tree, *Albizia amara* has been selected for tissue culture studies in the present investigation, due to its immense medicinal properties. During acclimatization of tissue culture raised plants, the effect of microbial inoculants on biohardening and root colonization was determined in terms of serial dilution followed by plating on Kings B specific medium and trypan blue staining of the root samples. The two bioinoculants used in the present study were *Trichoderma viride* and *Pseudomonas fluorescens* . These microbes were used either individually (or) in combination. Maximum root colonization of both *P. fluorescens* (158×10^6 CFU/g FW) and *T.viride* (82.6 %) was observed in dual inoculation after 60 days of biohardening. Better root development was observed when shoots with root primordia were treated with a combination of bioinoculants and plant survival rate was increased to 82 %.

Key words - Albizia amara, Trichoderma viride, Pseudomonas fluorescens, root colonization, biohardening

I. Introduction

Albizia amara is a potential plant with medicinal value commonly found in dry forests of India. Its common name is Nallaregoo (or) Chigaraku in telugu and it belongs to the family leguminaceae. Phytochemical constituents extracted from *A. amara* are widely used in traditional medicine(Sastry et al, 1967, Rajkumar and Sinha, 2010; Sartaj et al, 2012; Gopinath et al, 2013; Rohith et al, 2014; Thippeswamy et al, 2014; Indravathi et al, 2016) . Minimum work was done on tissue culture studies of *Albizia amara* (Tomar and Gupta, 1988; Rammurthy and Savithramma, 2003). Earlier studies reported plantlet regeneration from cotyledonary bud explants derived from aseptically grown seedlings but during transplantation limitations were observed (Indravathi and Pullaiah, 2013). The present work uses an efficient protocol for increasing the survival rate of tissue cultured plants of *A.amara* by using auxin dip for *in vitro* root induction followed by biohardening and root development under *ex vitro* conditions

The use of microorganisms for seed treatment was an age-old science, but the inoculation of microbes for hardening of tissue culture plantlets is a new aspect. When *in vitro* grown plantlets were treated with microbial cultures, there is a change in plant metabolic response which is called as Biotization. Biotization leads to the development of plantlets morphologically and physiologically by providing biotic and abiotic stress resistance (Nylund et al. 1989; Indravathi et al, 2019a). In the present scenario, microorganisms like fungi and bacteria were used as biopriming agents for successful acclimatization. Biotization can be done during root formation either under *in vitro* (or) *ex vitro* conditions. Thus, application of beneficial plant growth-promoting microbes during biohardening envisages the overall development of micropropagated plantlets (Indravathi et al.2019b).

The present paper analyses the efficacy of bioinoculants in enhancing root colonization and promoting root development of the tissue culture plants after transplantation to *ex vitro* conditions.

II. MATERIALS AND METHODS

2.1. Establishment of Aseptic Cultures, Multiple Shoot Induction & Root Induction

Healthy seeds of *A.amara* were surface sterilized and inoculated in Murashige and Skoog (MS) half strength medium**.** All cultures were maintained at a temperature of 25 ± 2^0 C, 2000 lux light and with a photo period regime of 16 hrs light/8 hrs dark diurnal cycles. From two week old aseptic seedlings, cotyledonary node explants were separated and subjected to multiple shoot induction using MS medium containing sucrose (2.0 %), agar (0.8%) fortified with BAP (1mg/l). Healthy microshoots from the proliferating shoot cultures were excised and immediately dipped in different types of auxin solution and subjected to root induction under *in vitro* conditions.

2.2. Treatment of Bioinoculants

The microshoots of A.amara with root initials were taken out from root induction medium and hardened with microbial inoculants namely - *Pseudomonas fluorescens* (MTCC-8127) and *Trichoderma viride* (MTCC-4329). The microbial pure cultures were obtained from IMTech, Chandigarh. The inoculum density of the fungal spores used in the present study was 2 x 10^6 spores/ml whereas for bacterial inoculation diluted broth sample was used with an inoculum density of 1 x 10^6 CFU/ml. Four treatments were employed one is control, the other two include biohardening with *T.viride* (**Tv**), *P. fluorescens (***Pf***)* individually and the last treatment was done by using mixed inoculum i.e. *P. fluorescens + T.viride (***Pf + Tv***)*. The treated microshoots were transferred to sterile potting mix (vermiculite: peat: perlite: soil in 1:1:1:2) in plastic pots covered with plastic bags containing holes. All plants were maintained under standard greenhouse conditions.

2.3. Acclimatization Studies

The biohardened and control plantlets were later transferred to pots containing sand, farmyard manure and soil in 1:1:1 ratio and grown under shade conditions. The results on plant survival rate were recorded at distinct time intervals (15, 30 and 60 days) after transfer to *ex vitro* conditions. Various plant growth parameters of biohardened and control plantlets were recorded sixty days after transplantation. In the present paper the efficacy of bioinoculants in enhancing root colonization and promoting root development of the tissue culture plants after transplantation to *ex vitro* conditions was studied.

2.4. Effect of Bioinoculants on Root Colonization

To know the efficacy of bioinoculants on root colonization, root samples were tested for the presence of *P.fluorescens* on the root surface by serial dilution followed by plating on Kings B specific medium. The extent of root colonization of *T.viride* was observed by staining root samples with trypan blue. Effective colonization of bacteria and fungi in the root samples determines its symbiotic association in plant development.

For each treatment, plant material was taken from 30 day and 60-day old plants and 3 replications were carried out. Roots were taken randomly from three types of biohardened plants [(Tv), (Pf), (Pf+Tv)] and control plants to study the effect of bioinoculants on root colonization. Five plants were taken randomly from each type of treatment and each experiment was repeated thrice.

2.4.1. Fungal Colonization:

The observations were carried out by staining root samples of one- and two-month-old plants after transplantation under *ex vitro* conditions. Roots of biohardened and control plantlets were cleaned carefully with water, cut into small pieces and boiled in 10% potassium hydroxide solution for 5 minutes. Thereafter, the root pieces were rinsed 2–5 times with sterile water. Later the root tissue was softened using 1% HCl for 2–4 minutes. Finally, the root segments were stained with 0.05% trypan blue in lactophenol (Giovannetti and Mosse, 1980). The stained root segments were examined under microscopic camera (10X).

Assessment of Root Colonization

Fungal root colonization was assessed by adapting the method used by Giovannetti and Mosse (1980). From the stained sample mixture, root segments were picked randomly and noticed under microscopic camera for fungal hyphae and spores (10X). In each observation, 25 root segments were randomly taken and examined with the microscope to calculate fungal root colonization [98].

$$\text{Fungal colonization (FC)} = \frac{\text{No. of root segments colonized} \times 100}{\text{Total no. of root segments examined}}$$

2.4.2. Bacterial Colonization:

Epiphytic colonization of *P. fluorescens* was examined by recovering bacteria present on root surfaces of one- and two-month-old plants after transplantation under *ex vitro* conditions. Epiphytic populations of bacteria were isolated by taking roots from biohardened and control plantlets. The roots were placed in a conical flask containing 10 ml of 0.1 M Magnesium sulphate and 0.02% Tween 20 solution. The sample mixture was subjected to agitation on a rotary shaker for 30 minutes. Serial dilution and standard agar plating technique was employed to estimate bacterial colonization. Serial dilution of the sample suspensions was prepared and 0.1 ml from the appropriate dilution were plated on Kings B medium according to Miles and Misra (1938). The agar plates were incubated for 48 hours at room temperature and the number of colonies were counted. The mean CFU per gram fresh weight of tissue was determined. Prior to bacterial isolation, fresh weights of roots were recorded. Based on colony morphology, gram staining and motility test the identity of *P.fluorescens* was established.

III. RESULTS

3.3.1. Bacterial Colonization:

Maximum colonization of both *T.viride* and *P. fluorescens* increased in mixed inoculum treated plants after 60 Days After Hardening (DAH). Dual inoculated plants had the highest populations of *P.fluorescens* i.e 108 x 10^6 CFU . g-1 FW (30 DAH) and 158x10^6 CFU. g-1 FW (60 DAH). Pf treated plants had the second highest population of *P.fluorescens* i.e 27x 10^6 CFU . g-1 FW (30 DAH) and 42x10^6 CFU. g-1 FW (60 DAH). The bacterium isolated from epiphytic root regions of *A.amara* is a motile, gram-negative rod. After 2 days growth on King's B Medium colonies were entire, smooth, creamy and 2 to 3 mm in diameter (Fig.1).

3.3.2. Fungal Colonization:

At the end of the acclimatization phase of 30 and 60 days, microscopic analysis of roots highlighted the absence of *T.viride* fungal structures in control plants. A great difference in the level of root colonization was detected among the three different inoculum treatments. The highest fungal colonization levels were observed in mix inoculum treated plantlets (FC%= 63.3%) followed by the plants treated with *T.viride* (FC%= 31.3%) after 30 DAH (Fig.2). After 60 days the fungal colonization levels were further increased in Pf + Tv treated plants (FC%= 82.6%) followed by Tv treated plants (FC%= 52%). The results were presented in Table 1.

Table 1: Extent of Microbial Colonization after 30 & 60 Days After Hardening (DAH)

Treatments	Colonization of Tv (%)		Colonization of Pf (x10 6 / g soil)	
	30 DAH	60 DAH	30 DAH	60 DAH
Control	0	0	0	2
Pf	0	0	27	42
Tv	31.3	52	0	4
Pf+ Tv	63.3	82.6	108	158

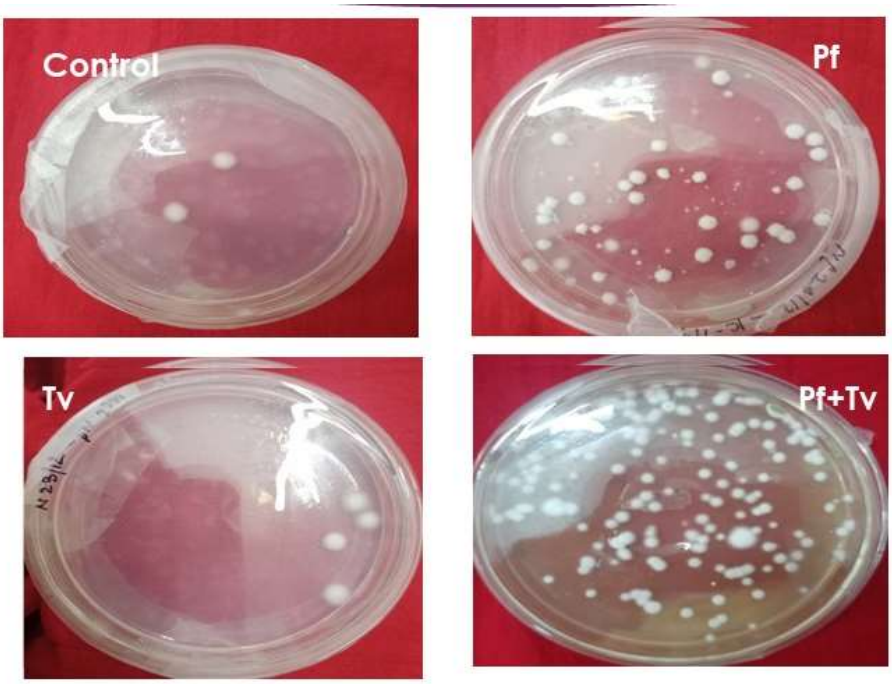

Figure 1: Effect of *P.fluorescens* on root colonization after 60 days.

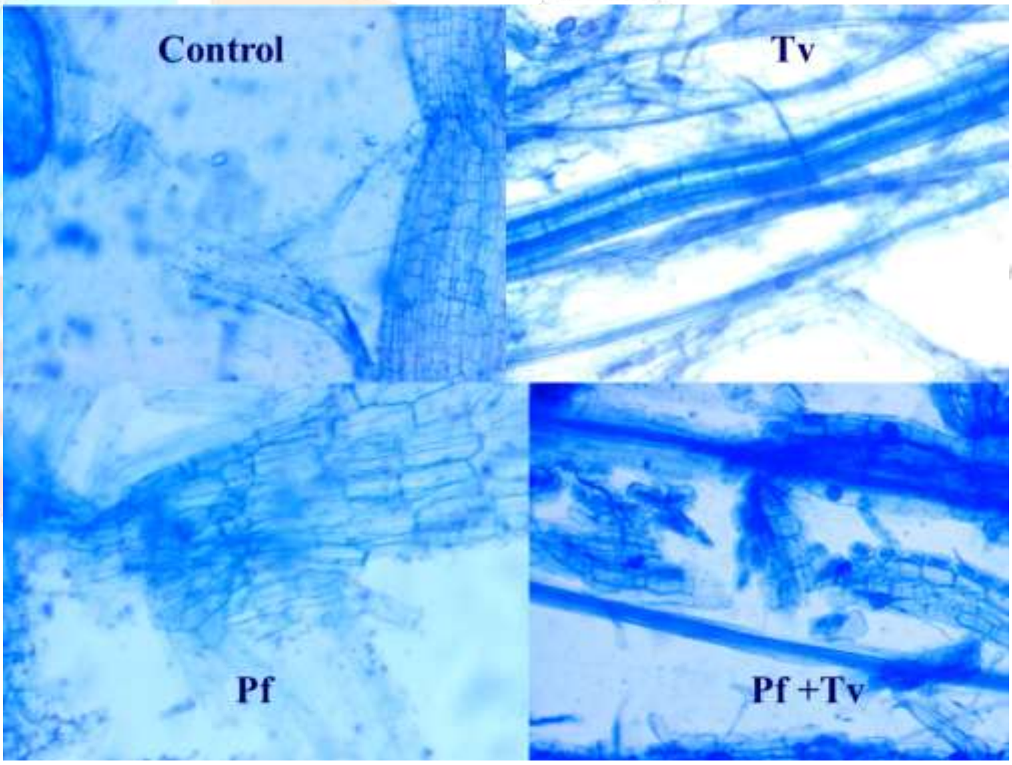

Figure 2: Effect of *T.viride* on root colonization after 60 days.

IV. DISCUSSION

Examination of biohardened stained roots of *A.amara* plantlets under microscope showed the presence of fungal mycelium , hyphae and spores of *T.viride*. Colonization of *T.viride* was observed maximum in dual inoculated plants followed by *T.viride* inoculated plants after 30 and 60 days of biotization. The fungal hyphae penetrated the root system both intra and intercellularly. This led to a significant increment in overall growth and devolpment of biohardened plantlets. Similar reports of high fungal colonization on overall plant growth and development were reported in *Ranunculus asiaticus* L. and *T. bellerica* (Chittora et al, 2010; Borriello et al, 2017).

Maximum establishment of *P. fluorescens* was observed after 60 days of transplantation on the surface of dual inoculum treated plant roots followed by *P. fluorescens* inoculated plant roots. Similar reports on epiphytic root colonization of *Pseudomonas* sp. PsJN strain with tomato plants was observed (Pillay and

Nowak, 1997). This observation suggests a good symbiotic success of both *P.fluorescens* and *T.viride* inoculum in deeply colonizing the root apparatus and rhizosphere region of the plants rather than single inoculation of plantlets. The degree of colonization and the extent of plant growth promotion depended upon plant genotype, temperature, and inoculum size. There was a strong correlation between plant growth promotion and microbial colonization.

V. CONCLUSION

The present strategy employed during the acclimatization step of tissue culture raised plantlets of *A.amara* by using microbial inoculants has given a positive result. Biohardening of tissue cultured *A.amara* plantlets with a combination of *T.viride* and *P. fluorescens* enhanced plant survival rate (82%) compared to single inoculum treatment either with *P. fluorescens* or *T.viride*. Effective root colonization occurred in Pf+ Tv treated plants which had helped in increased nutrient uptake and plant biomass.

1. Better root development was observed when shoots with root primordia were treated with a combination of bioinoculants (Pf+Tv).
2. Maximum colonization of both *P. fluorescens* (158×10^6 CFU/g FW) and *T.viride* (82.6 %) increased in dual inoculation after 60 days after biohardening.
3. After sixty days of biohardening, an improvement in plant growth characteristics and survival rate (82%) was observed in dual inoculum (Pf+Tv) treated plants.

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