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Antimicrobial Activity of Biohardened Plant Extracts of Albizia Amara

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Abstract

Albizia amara is an important medicinal plant commonly found in dry forests of India. It belongs to leguminaceae and its common name in telugu is Nallaregoo (or) Chigaraku. All of the plant parts i.e., roots, shoots, leaves, flowers, seeds and bark, were exclusively used in traditional medicine for curing different diseases like diarrhea, skin diseases gonorrhea, poisonous bites and leprosy. Due to its wide pharmacological activities and presence of several therapeutic constituents, Albizia amara has been selected for tissue culture studies in the present investigation. A systemic approach for increasing the survival rate of tissue culture raised A.amara plantlets using bioinoculants was explored . The studies reported that biohardening of tissue cultured plantlets with mixed inoculum of Pseudomonas fluorescens and Trichoderma viride enhanced plant survival rate (82 %). This microbial association helped in inducing systemic resistance in plants to overcome abiotic and biotic stress caused during acclimatization. The phytochemical studies of biohardened plants exhibited high antimicrobial activity due to the presence of more secondary metabolites. These secondary metabolites probably due to their antagonistic properties gave protection against pathogenic attack during transplantation. Phytochemical analysis of different plant extracts like, ethyl acetate, chloroform, methanolic, and petroleum ether extracts were studied. The ethyl acetate extract of dual inoculant treated plants showed maximum antimicrobial activity. In bacteria, it showed maximum zone of inhibition on B.cereus (11.5±1.73), followed by E.coli (10±0.82 mm) . In fungi, it showed maximum inhibition on F.oxysporum (13.25±0.28mm) followed by C.albicans (8.5±0.17mm). The above strategy helped in overall improvement of morphological, physiological and phytochemical characteristics of tissue culture plantlets.

Keywords: Albizia amara, ethanolic extract, secondary metabolites, systemic resistance, antimicrobial activity.

1. Introduction

Albizia amara is an important medicinal plant commonly found in dry forests of India. It belongs to leguminaceae and its common name in telugu is Nallaregoo (or) Chigaraku. All of the plant parts i.e., roots, shoots, leaves, flowers, seeds and bark, were exclusively used in traditional medicine for curing different diseases like diarrhea, skin diseases gonorrhea, poisonous bites and leprosy [1,2]. The crude extract and the bioactive compounds isolated from various solvent extracts of Albizia amara were used in curing different diseases. In addition, the plant extract possesses various pharmacological properties like antihyperlipidemic, anticancer, antimicrobial, anti-inflammatory analgesic and antioxidant activities [3,4]. These pharmaceutical compounds were also used as an alternative to chemical preservatives for

the management of pre-harvest and post-harvest fungal infestations and mycotoxin contamination in food grains [5,6].

Due to its wide pharmacological activities and presence of several therapeutic constituents, Albizia amara has been selected for tissue culture studies in the present investigation. The morphological, physiological, and anatomical features of the plantlets developed under in vitro conditions, have a strong impact on plant survival after transplantation to ex vitro conditions. In the present study a systemic approach for increasing the survival rate of tissue culture plantlets using bioinoculants was explored. Earlier studies reported that when in vitro grown plantlets were treated with microbial cultures, there is a change in plant metabolic response which leads to the development of plantlets physiologically and morphologically by providing abiotic and biotic stress resistance [7, 8, 9, 10, 11]. In the present paper the effect of bioinoculants in inducing systemic resistance in tissue culture plants of Albizia amara after 60 days of transplantation to ex-vitro conditions were explored through pharmacological studies and it was determined in terms of antimicrobial activity

2. Materials & 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 & Acclimatization studies

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.

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 and various parameters of biohardened and control plantlets were recorded sixty days after transplantation.

2.3. Preparation of solvent extract

Plant samples from biohardened and control plantlets of 60 days old were shade dried, weighed and transferred to the thimble and successively extracted using a Soxhlet extractor with 250 ml of petroleum ether, chloroform, methanol and ethyl acetate. The extracts were filtered, concentrated under reduced

pressure and stored at 4 ^{o}C for performing phytochemical studies. By standard qualitative procedures, preliminary phytochemical analysis was done for qualitative screening for secondary metabolites [12, 13].

2.4. Antimicrobial studies

Twenty milligrams of leaf extracts were dissolved in one mL of Dimethyl Sulphoxide (DMSO) and 10 µL (250 µg/disc) portions were impregnated on sterilized Whatmann No.1 filter paper discs (6 mm diameter). The solvent was allowed to dryness at room temperature in a laminar air flow bench. The antimicrobial activity of the extracts was evaluated by disc diffusion method [14]. Filter paper discs containing 200 µg/disc were placed on surface of the solidified nutrient agar medium, pre seeded with microbial strains (10^{5}CFU/ mL). Standard antibiotics, viz., Gentamycin(10 µg/disc), Vancomycin (30 µg/disc) and Flucanozole (10 µg/disc) obtained from Hi-media, Mumbai, were used as positive controls. The discs containing petroleum ether, ethyl acetate, ethanol or DMSO served as negative controls. The solution of DMSO (100 µL/mL) served as the negative control. All the samples were tested in triplicates to confirm the activity. The plates were incubated for 24- 72 h at 30- 37 ^{o}C and the diameter of the inhibition zones was recorded in millimetre (mm). The antimicrobial activity of the extracts was measured by observing bacterial free zones formed around the discs. Three independent trials were conducted for each concentration to confirm the activity.

2.5. Antibacterial activity assay

Antibacterial activity of different extracts of A. amara were determined by disc diffusion method on the Nutrient Agar Medium. In this method, 6 mm sterilized filter paper discs (Whatmann no. 1) were saturated with sterilized plant extracts at 1mg/ml concentration. The impregnated discs containing plant extracts were placed on to the surface of NAM medium. The NAM media was pre-inoculated with test bacteria E.coli (MTCC1687) and B.cereus (MTCC1133) (inoculum size 1 X 108 CFU/ml). For each treatment three replicates were maintained. The disc devoid of extract consisting of only dimethyl sulfoxide (DMSO) served as control. The plates were kept at 4 ^{0}C for 1 hour for diffusion of extract, thereafter the plates were incubated at 37 ^{0}C for 24 hours. After incubation, zone of inhibition if any around the disc was measured in mm (millimetre). Vancomycin (10ug/disc) and Gentamycin (10ug/disc) were used as positive reference to determine the sensitivity of each bacterial species tested.

2.6. Antifungal activity:

The disc diffusion method was employed for the determination of zone of inhibition (ZOI) according to the method described by Ebrahimabadi et al. (2010) with slight modifications [15]. In this method, 6 mm sterilized filter paper discs (Whatmann no. 1) were saturated with sterilized plant extracts. The impregnated discs are then placed on to the surface of PDA medium pre-inoculated plates (inoculum size: 100 µl of 10^{4} spores/ml) and incubated at 30°C for 72 hrs. .The two fungal cultures – Candida albicans (MTCC854) and Fusarium oxysporum (MTCC284) were maintained on PDA, and 7-days-old cultures were used for further investigations. For each treatment three replicates were maintained. The disc devoid of extract consisting of only dimethyl sulfoxide (DMSO) served as control. Vancomycin (30ug/disc) and Fluconazole (10ug/disc) were used as positive reference to determine the sensitivity of each fungal species tested

3. Results

3.1. Plant extract yield & Preliminary analysis of phytochemical constituents:

Different solvents like petroleum ether, methanol, ethyl acetate and chloroform were used to extract the phytochemical of biohardened and control plantlets of A. amara. The presence of flavonoids, phenols, coumarins, steroids, alkaloids, tannins and oils were observed from phytochemical analysis.

3.2. Antibacterial Activity:

The inhibitory activity of different solvent extracts of A. amara against two bacteria – E.coli and B.cereus are presented in Table 1. Among the different extracts tested, ethyl acetate extract recorded highest antibacterial activity followed by chloroform and methanol, whereas no significant antibacterial activity was observed in petroleum ether extract. The control DMSO did not inhibit any of the bacteria tested. In E.coli , the ethyl acetate extract recorded highest antibacterial activity with zone of inhibition ranging from 9.25±0.29 to 10±0.82 mm. The chloroform exhibited antibacterial activity with zone of inhibition ranging from 6.25±0.50 to 8.5± 0.40 and for methanol extract it was 6.25±0.50 mm (Figure.1) . In B.cereus , the ethyl acetate extract recorded highest antibacterial activity with zone of inhibition ranging from 8± 0.82 to 11.5±1.73 mm (Figure.2).The chloroform exhibited antibacterial activity with zone of inhibition ranging from 7±0.50 to 7.25±0.50 and for methanol extract it was 6.5±0.50 mm. The most susceptible organism in the present investigation was B.cereus followed by E.coli .The present study clearly indicates Gram positive bacteria were more susceptible than Gram negative bacteria.

Figure 1. Antibacterial Activity of Various Solvent Extracts of Biohardened and Control Plantlets against Escherichia coli

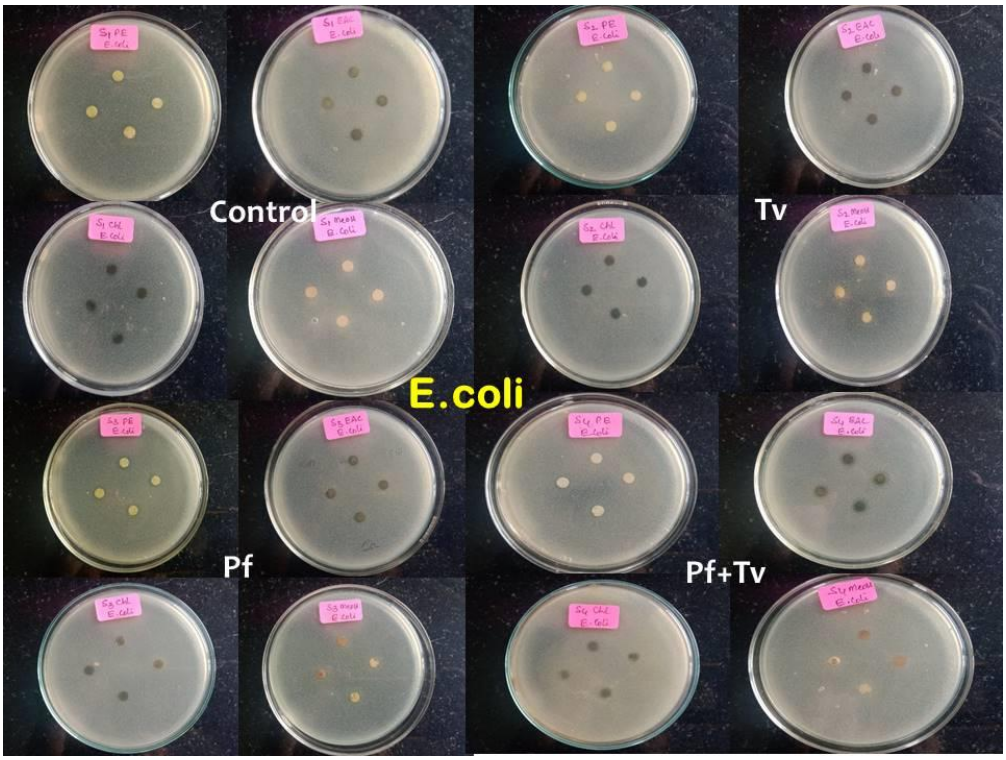
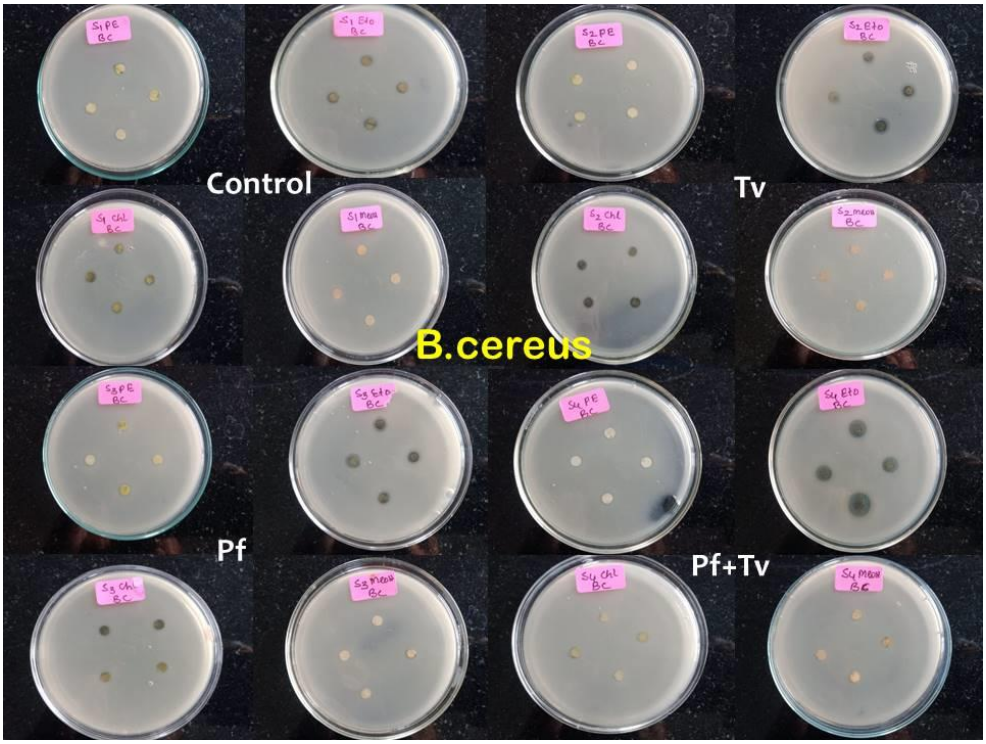


Figure 2. Antibacterial Activity of Various Solvent Extracts of Biohardened and Control Plantlets against Bacillus cereus



3.3. Antifungal activity:

The antifungal activities of the plant extracts were evaluated against two fungi i.e- Candida albicans and Fusarium oxysporum. The negative control, DMSO, did not inhibit any of the fungus tested. Various plant extracts exhibited varying degrees of growth inhibition against the mould – C.albicans with ZOI ranging from 6.25±0.50 to 8.5±0.17 mm. Among the fungi tested, the fungi C.albicans, was inhibited more strongly by ethyl acetate extract of Pf+Tv treated plants (8.5±0.17 mm)than the other plant extracts(Table 1). The chloroform extract exhibited antifungal activity with zone of inhibition ranging from 7.5±0.58 to 8.25±0.96 mm and for methanol extract it was 6.25±0.50 mm (Figure.3). In F.oxysporum , the ethyl acetate extract recorded highest antifungal activity with zone of inhibition ranging from 6.57±0.43 to 13.25±0.28 mm .The chloroform exhibited antibacterial activity with zone of inhibition ranging from 6.5±0.46 to 10±0.57mm and for methanol extract it ranged from 6.2±0.43 to 8.35±0.40 mm (Figure.4). The most susceptible fungal organism in the present investigation was F.oxysporum followed by C.albicans .The present study clearly indicates plant pathogenic fungus F.oxysporum was more susceptible than human pathogenic fungi C.albicans.

Figure 3. Antifungal Activity of Various Solvent Extracts of Biohardened and Control Plantlets against Candida albicans

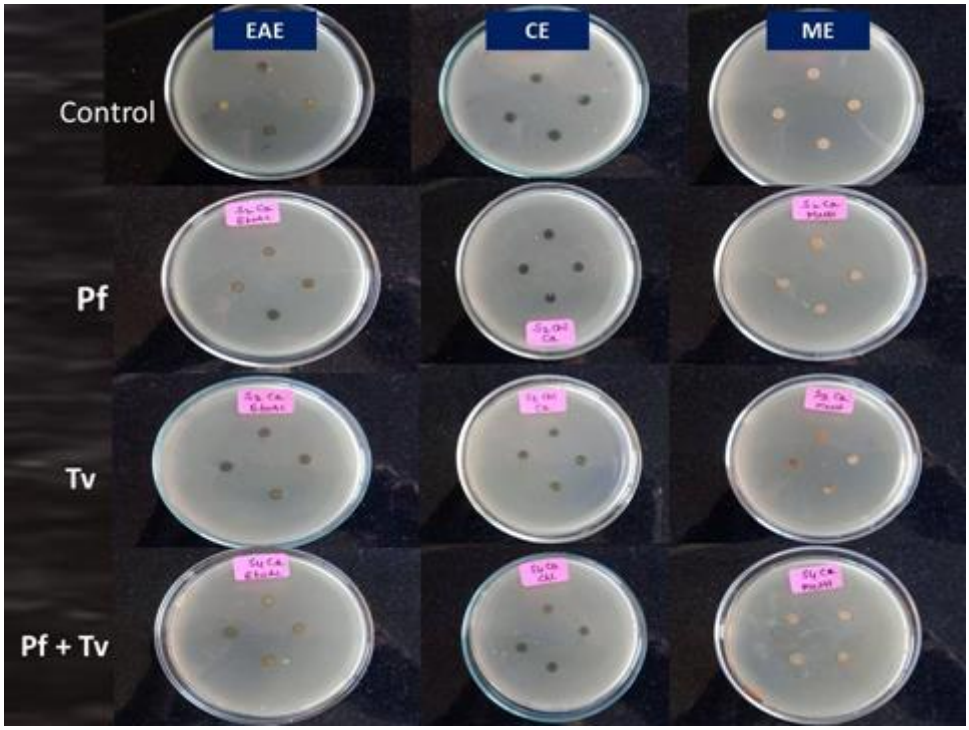


Figure 4. Antifungal Activity of Various Solvent Extracts of Biohardened and Control Plantlets against Fusarium oxysporum

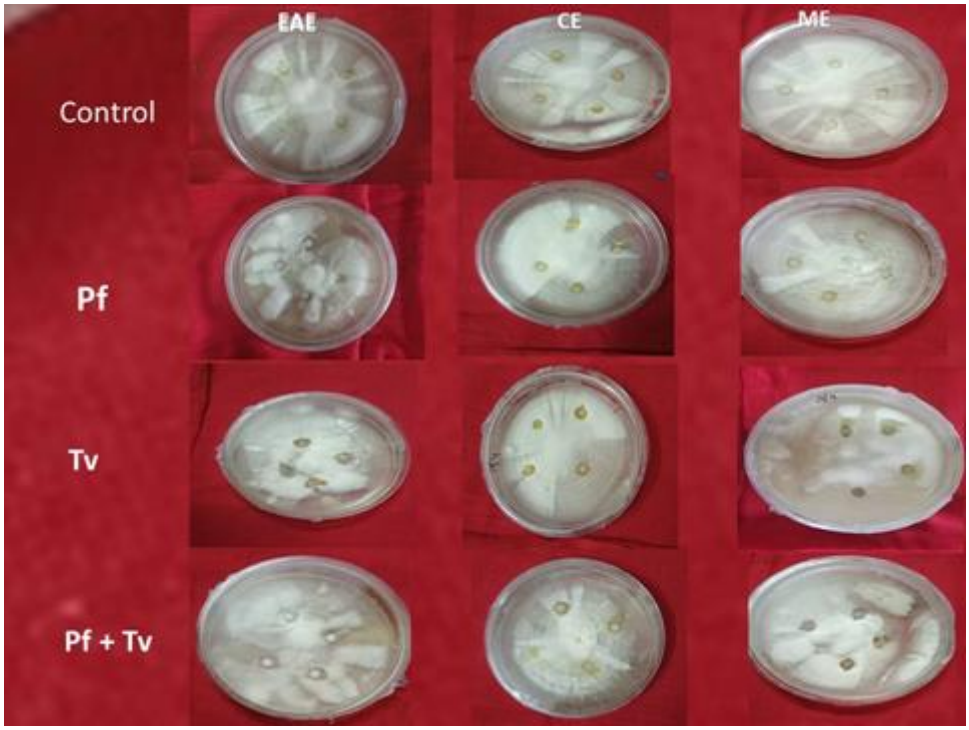


Table 1. Antimicrobial Activity of Various Solvent Extracts of Biohardened and Control Plantlets of Albizia amara (Zone Of Inhibition -mm)

Treatments	**Control**		**Pf**		**Tv**		**Pf+Tv**		P - Value
BACTERIA	**E.coli**	**B.cereus**	**E.coli**	**B.cereus**	**E.coli**	**B.cereus**	**E.coli**	**B.cereus**	
PE	0±0.0	0±0.0	7.75±0.96	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	
EA	0±0.0	8±0.82	9.25±0.29	8.3±0.11	9.5±0.58	9.5±0.66	10±0.82	11.5±1.73	
CHL	6.25±0.50	7±0.50	7.5±0.58	7±0.40	8±0.58	7.25±0.50	8.5±0.40	7±0.50	
ME	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	6.25±0.50	6.5±0.50	
FUNGI	**F.oxysorum**	**C.albicans**	**F.oxysorum**	**C.albicans**	**F.oxysorum**	**C.albicans**	**F.oxysorum**	**C.albicans**	0.83
PE	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	7.5±0.58	0±0.0	0±0.0	
EA	6.57±0.43	6.25±0.50	9.62±0.47	7±0.40	11±0.40	8±0.58	13.25±0.28	8.5±0.17	
CHL	6.5±0.46	7.5±0.58	8.4±0.12	8.12±0.74	9.25±0.50	8.25±0.96	10±0.57	8.25±0.96	
ME	6.2±0.43	0±0.0	7±0.24	0±0.0	8.35±0.40	6.25±0.50	8±0.54	0±0.0	

4. Discussion

The plant , Albizia amara belong to the Leguminosae family, are rich in alkaloids, and their extracts have been reported to possess various bioactivities [16]. Previous reports indicate the antimicrobial and antiaflatoxigenic activities of crude extracts of wild plants of A. amara in which chloroform extract was more effective [17,18]. Other earlier reports on A. amara revealed the presence of a group of budmunchiamines in A. amara , the main alkaloid constituents having antimicrobial activity [19,20]. The results of present study are in coincidence with previous reports on antimicrobial activity of the wild plant extracts of A.amara. The ethyl acetate extract showed maximum inhibition on B.cereus, followed by E.coli in bacteria where as in fungus maximum inhibition was observed on F.oxysporum followed by C.albicans. The ethyl acetate extract showed maximum antimicrobial activity followed by CE and ME of dual and single inoculant treated plants . The PE extract exhibited faint inhibition against all test microbes . The Phytochemical analysis of ethyl acetate extract which recorded significant antimicrobial activity revealed the presence of flavonoids, phenols, coumarins, steroids and alkaloids. These secondary metabolites probably due to their antagonistic properties gave protection to biohardened plants against pathogenic attack.

5. 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. This microbial association helped in inducing systemic resistance in plants to overcome abiotic and biotic stress caused during acclimatization. The phytochemical studies of biohardened plants exhibited the presence of more secondary metabolites. These secondary metabolites probably due to their antagonistic properties gave protection against pathogenic attack during transplantation. Phytochemical analysis of different plant extracts like, ethyl acetate, chloroform, methanolic, and petroleum ether extracts were studied. The ethyl acetate extract of dual inoculant treated plants showed maximum antimicrobial activity. In bacteria, it showed maximum zone of inhibition on B.cereus (11.5±1.73), followed by E.coli (10±0.82 mm) . In fungi, it showed maximum inhibition on F.oxysporum (13.25±0.28mm) followed by C.albicans (8.5±0.17mm). The secondary metabolites were rich in mixed inoculum treated plants which had induced resistance to fight against biotic and abiotic stress during its transfer from in vitro to ex vitro conditions thereby improving plant survival rate .

The establishment of tissue culture raised plants successfully from lab to land require development of novel culture methods. Basic research on biotic and abiotic factors governing plant growth, development and survival,during transplantation of tissue culture plants from in vitro to ex vitro conditions is the absolute need. Special focus on plant microbial interaction at molecular level during biohardening needs to be envisaged. The molecular mechanisms of signal recognition and transduction in plants when transferred from in vitro to ex vitro conditions are the important areas of research to be explored.

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References

1. Gopinath P, Sundara Vadivel D, Kamatchiammal S,Saroja V. Anti-Cancerous Activity of Albizia Amara (Roxb.) Boivin using Human Breast Cancer Cells (MCF-7) by In Vitro Methods. International Journal of Pharma Research & Review. 2(8) (2013) 23-32.
2. Sastry CV, Rukmini C and Ramachandra Rao L, Chemistry of Saponins: part III – Isolation of new flavonol Glycoside, 4'-0- Methylquercetin-3-rutinoside, from Albizia amara Benth. Indian J.Chem. 5, 1967, 613.
3. Rohith Gundamaraju, Kim Kah Hwi, Rajeev K Singla, Ravi Chandra Vemuri, Sartaj Banu Mullapalli, Antihyperlipidemic potential of Albizia amara (Roxb) Boiv.bark against Triton X-100 induced condition in rats, Phcog Res. 6 (2014) 267-273.
4. Indravathi G, Sreekanth Reddy R and Suresh Babu P. Albizia amara - A Potential Medicinal Plant: A Review. International Journal of Science and Research. 5(3), 2016, 621-627.

5. Rajkumar T, Sinha BN. Chromatographic finger print analysis of budmunchiamines in Albizia amara by HPTLC technique. Int J Res Pharm Sci. 1(2010) 313–316.
6. Thippeswamy S, Mohana DC, Abhishek RU and Manjunath K. Inhibitory effect of alkaloids of Albizia amara and Albizia saman on growth and fumonisin B_1 production by Fusarium verticillioides. International Food Research Journal 21(3) (2014) 947-952.
7. Lazarovits G and Nowak J. Rhizobacteria for improvement of plant growth and establishment. Hort Science. 32,1997, 188.
8. Nowak J. Review benefits of in vitro "biotization" of plant tissue cultures with microbial inoculants. In vitro Cell Dev Biol Plant. 34,1998, 122.
9. Rammoorthy V, Viswanathan R, Raguchandar J, Prakasham T and Samiyappan R. Induction of systemic resistance by plant growth promoting rhizobacteriain crop plants against pest and diseases. Crop protection. 20, 2001, 1.
10. Indravathi G and Suresh Babu P **"**Enhancing acclimatization of tissue cultured plants by Biotization – a review" International Journal of Scientific Research and Reviews, 2019b, 8 (3), 564-575**.**
11. Indravathi G and Suresh Babu P "Enhancing acclimatization of tissue cultured plants of Albizia Amara by Biotization" Int. J. Sci. Res. in Biological Sciences, 2019a, 6 (4), 43-50.
12. Das AK and Bhattacharjee AK. A systematic approach to phytochemical screening. Tropical Science. 12,1970.
13. Harborne JB. Phytochemical methods, Chapman and Hall, London, 1991.
14. Cruickshnak R. 11thed. Medicinal microbiology: a guide to diagnosis and control of infection. Edinburgh and London: E and S, Livingston Ltd; 1968. p. 888.
15. Ebrahimabadi AH, Ebrahimabadi EH, Djafari-Bidgoli Z, Kashi FJ, Mazoochi A, Batooli H. Composition and antioxidant and antimicrobial activity of the essential oil and extracts of Stachys inflata Benth from Iran. Food Chemistry. 2010 Mar 15;119(2):452-8.
16. Karmegam, N., Jayakumar, M. and Karuppuswamy, S. 2012. Synergistic antibacterial activity of four medicinal plants collected from Dharapuram Taluk of Tiruppur District, south India. Journal of Plant Sciences 7: 32-38.
17. Praveen, P., Thippeswamy, S., Mohana, D.C. and Manjunath, K. 2011. Antimicrobial efficacy and phytochemical analysis of Albizia amara (Roxb.) Boiv. an indigenous medicinal plant against some human and plant pathogenic bacteria and fungi. Journal of Pharmacy Research 4: 832-835.
18. Thippeswamy, S., Mohana, D.C., Abhishek, R.U. and Manjunath, K. 2013. Efficacy of bioactive compounds isolated from Albizia amara and Albizia saman as source of antifungal and antiaflatoxigenic agents. Journal of Consumer Protection and Food Safety, 297-305
19. Rajkumar, T. and Sinha, B.N. 2010. Chromatographic finger print analysis of Budmunchiamines in Albizia amara by HPTLC technique. International Journal of Research in Pharmaceutical Sciences 1: 313-316.
20. Thippeswamy, S., * Mohana, D. C., Abhishek, R. U. and Manjunath, K. ,Inhibitory effect of alkaloids of Albizia amara and Albizia saman on growth and fumonisin B_1 production by Fusarium verticillioide, International Food Research Journal 21(3): 947-952 (2014)