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THE EFFECT OF BIOINOCULANTS IN INDUCING SYSTEMIC RESISTANCE IN TISSUE CULTURE PLANTS OF *ALBIZIA AMARA*

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ABSTRACT

Albizia amara is a plant with potential medicinal value. The entire plant possess pharmaceutical constituents of great significance, so it is exclusively used as traditional medicine for curing various diseases like diarrhea, gonorrhea, skin diseases, leprosy, cancer, inflammation and poisonous bites. 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 antioxidant 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. The phytochemical analysis of plant extracts revealed that methanol was the best solvent in giving maximum extract yield (17.3%). In the present study high antioxidant activity (99.48 AAE mg/g DW) and phenolic content (57.82 mg GAE mg/g DW) was observed in the methanolic extract of mixed inoculum treated plants. Further, high DPPH activity by low concentrations of the methanolic extract (IC_{50} value at 6 μg/ml) was also observed. The above strategy helped in overall improvement of morphological, physiological and phytochemical characteristics of tissue culture plantlets.

Keywords: *Albizia amara*, methanolic extract, secondary metabolites, systemic resistance, phenolic content, antioxidant activity

INTRODUCTION

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

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. A systemic approach for increasing the survival rate of tissue culture plantlets using bioinoculants was explored in the present study. Previous 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 morphologically and physiologically by providing biotic and abiotic stress resistance [5, 6, 7]. During acclimatization, the effect of bioinoculants in inducing systemic resistance through pharmacological studies was determined in terms of screening effective solvent extract, secondary metabolites, Total Antioxidant Activity (TAA), Total Phenolic Content (TPC) and DPPH Free Radical Scavenging Assay.

MATERIALS & METHODS

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.

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 1×10^6 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.

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

Effect of Bioinoculants in Inducing Systemic Resistance through Phytochemical Studies

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. It was determined in terms of screening effective solvent extract, secondary metabolites, Total Antioxidant Activity (TAA), Total Phenolic Content (TPC) and DPPH Free Radical Scavenging Assay.

Preparation of solvent extract

Plant samples from biohardened and control plantlets 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 (Fig.1). The extracts were filtered, concentrated under reduced pressure and stored at 4 ^{o}C for performing phytochemical studies.

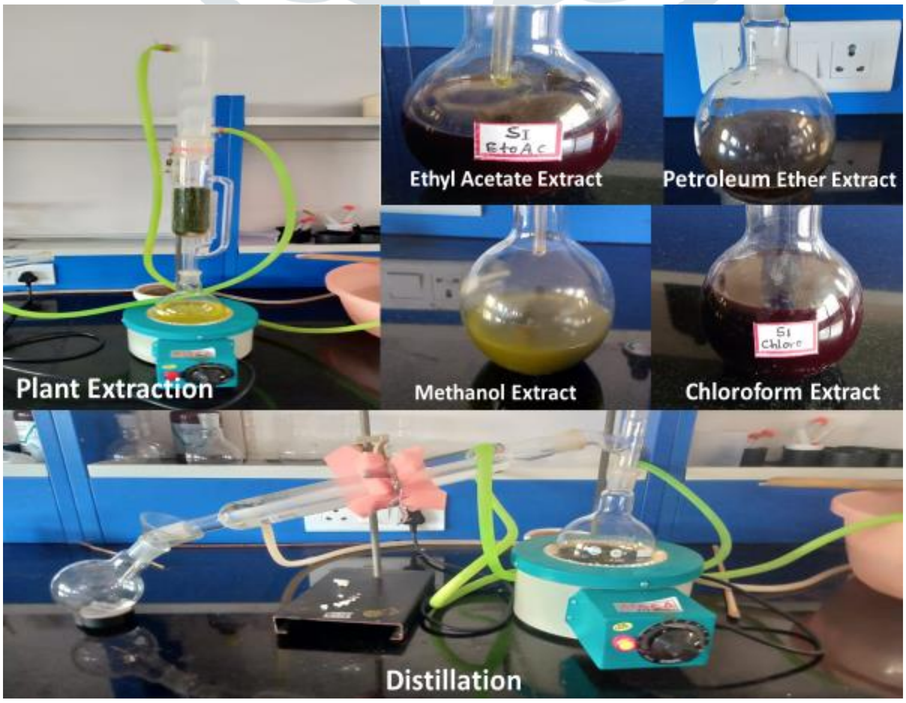

Figure 1: Soxhlet extraction & distillation using different solvents.

Qualitative Screening for Secondary Metabolites

By standard qualitative procedures, preliminary phytochemical analysis was done:

Alkaloids

The concentrated residue of the extract was digested with 2% HCl. The digest was tested by adding Mayer's reagent and Wagner's regent separately. The formation of cream-colored precipitate indicates the presence of alkaloids [8].

Glycosides

Treatment of the extracts with 2.5 % ammonium hydroxide gives red colour which indicates the presence of glycosides [9].

Anthraquinones

Fresh plant material should be taken to test the presence of anthraquinones. For extraction of anthraquinones, 0.5% KOH was used to soak the plant material overnight. One ml of each component (Hydrogen Peroxide, Acetic acid, Benzene) was added to the alkaline filtered extract. To the sample mixture an equal amount of dilute ammonia was added which gives red colour and it indicates the presence of anthraquinones [10].

Coumarins

The concentrated residue of each extract was taken in the test tube and dissolved in water. To this 1 N NaOH solution was added and closed with filter paper and kept in boiling water for few minutes. Later the samples were observed under the UV light for yellow fluorescence which indicates the presence of coumarins [11].

Flavonoids

The concentrated residue of the extract was dissolved in 50% methanol and heated on sand bath. Appearance of yellow/red colour, by the addition of HCl and magnesium powder to the sample mixture indicates the presence of flavonoids [12].

Tannins and Catecholamines

The extracts were diluted with water and few drops of ferric chloride was added. Appearance of green black and blue black colour indicates the presence of catecholamines and tannins respectively [13].

Phenols

The extracts were dissolved in water or alcohol and few drops of neutral ferric chloride solution was added. A change in colour indicates the presence of phenols [14].

Saponins

The extracts were tested for the presence of saponins [15]. The extracts were diluted with distilled water and agitated in a graduated cylinder for 15 minutes. The presence of saponins is indicated by the appearance of persistent foam.

Steroids and Terpenoids

The concentrated residue of the extract was dissolved in conc. HCl. Appearance of green and violet colour indicates the presence of steroids and terpenoids respectively [16].

Oils:

The extracts were tested for the presence of fatty acids. Few drops of extracts were placed on a filter paper and subjected to evaporation. The presence of oils is indicated by the formation of transparent spot [17].

Total Antioxidant Activity (TAA)

The Total Antioxidant Activity of extracts was tested with phosphomolybdenum reagent using ascorbic acid as the standard compound [18]. The reduction of transition metal ions denotes the antioxidant capacity of the samples and it can be estimated using PhosphoMolybdenum (PMo) assay. The samples were diluted in methanol and mixed with sodium phosphate (28 mM), sulfuric acid (600 mM) and ammonium molybdate (4 mM). The sample mixture was heated at 90°C and cooled which results in the production of green complex whose absorbance was read at 695 nm. The main concept of phosphomolybdenum assay was the reduction of Mo (VI) to Mo (V) by the antioxidant components of the plant extract. Samples with more absorbance indicates high antioxidant activity. The antioxidant activity of solvent extracts was compared with that of standard ascorbic acid. The total antioxidant activity of the plant extracts was represented as milligrams of Ascorbic Acid Equivalents per gram Dry Weight (mg AAE/ g DW) of the sample.

Total Phenolic Content (TPC)

Total phenolic content of the plant extracts was determined in terms of gallic acid content**.** The concentration of the total soluble phenolic compounds in chloroform, petroleum ether, methanol and ethyl acetate extracts were determined using Folin Ciocalteu reagent [19]. To 100 µl of each extract, 50 µl of the phenol reagent (1 M) was added and allowed to stand for 3 min. To this sample mixture, 300 µl Na_2CO_3 was added and vortexed. Final volume was made to 2ml by distilled water and the samples were incubated at room temperature in dark conditions for 1 hour. The absorbance was measured at 725 nm. The total phenolic content of the plant extracts was represented as milligrams of Gallic Acid Equivalents per gram Dry Weight (mg GAE/ g DW) of the sample.

DPPH activity

The free radical scavenging activity of the Petroleum Ether Extract (PEE), Ethyl Acetate Extract (EAE), Methanolic Extracts (ME) and Chloroform Extract (CE) was measured by 1,1-Di Phenyl-2-Picryl Hydrazyl (DPPH) assay. To each plant extract 1 ml of 0.004% DPPH solution was added, shaken well and incubated for 30 min at room temperature in dark conditions. The positive reaction was indicated by the disappearance of violet colour. At 517 nm the absorbance of the samples was measured using a spectrophotometer [20]. High free radical scavenging activity of the reaction mixture indicates lower absorbance. The concentration (µg/ml) of the extract at which 50% DPPH radicals were inhibited was referred as IC_{50} value. The plant methanol extract solution without DPPH was used as a blank.

The percentage of inhibition (I %) is calculated by: I % = 100 x (AC – AS)/AC

Where, AC is the absorbance of the control & AS is the absorbance of the sample.

RESULTS

Plant survival rate

The plant survival rate was recorded at different time intervals after acclimatization to *ex vitro* conditions. Mixed inoculum treated plants showed highest survival rate compared to single inoculations with either *P. fluorescens* (or) *T.viride* and also over control plantlets. Sixty days after transplantation, highest survival percentage (82%) was observed in dual inoculated plantlets compared to *T.viride* inoculated plantlets (63%), *P. fluorescens* inoculated plantlets (55%) and control plantlets (5 %).

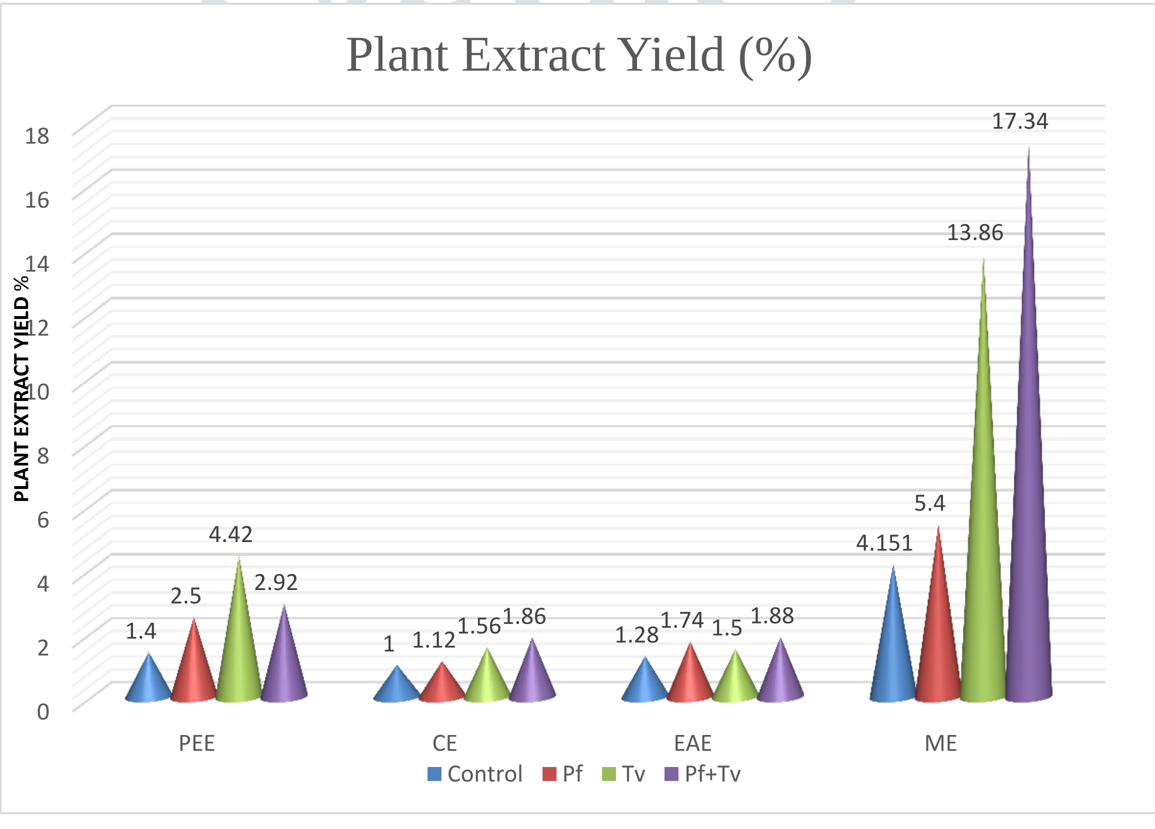

Figure 2: Plant extract yield (%) of biohardened and control plantlets

P.fluorescences treated plants **(Pf)**, *T.viride* treated plants **(Tv)**, *P.fluorescences* & *T.viride* treated plants **(Pf +Tv)**, Petroleum Ether Extract **(PEE)**, Chloroform Extract **(CE)**, Methanol Extract **(ME)**, and Ethyl Acetate Extract **(EAE)**.

Plant extract yield & preliminary analysis of phytochemical constituents:

Different extracts like petroleum ether, methanol, ethyl acetate and chloroform extracts of biohardened and control plantlets of *A. amara* were subjected to phytochemical analysis and the Table-1 depicts the result.

Methanol was the best solvent (Fig.2) in giving maximum extract yield (17.3%), followed by Petroleum ether (4.42%), ethyl acetate (1.88%) and chloroform (1.86%). The presence of flavonoids, phenols, coumarins, steroids, alkaloids, tannins and oils were observed from phytochemical analysis (Table 1).

Table 1: Distribution of secondary metabolites in biohardened & control plant extracts

S.No	Treatment	SOL	ALK	AQ	CU	CC	GLY	FLV	SAP	STE	TA	TER	PHE	OILS
1.	**Pf**	PEE	+	-	+	-	-	+	-	-	+	-	+	+
		CE	+	-	+	-	-	+	-	-	+	-	+	+
		EAE	+	-	+	-	-	+	-	+	+	-	+	+
		ME	++	-	+	-	-	+	-	+	+	-	+	+
2	**Tv**	PEE	+	-	+	-	-	+	-	-	+	-	+	+
		CE	+	-	+	-	-	+	-	-	+	-	+	+
		EAE	+	-	+	-	-	++	-	+	+	-	+	+
		ME	++	-	+	-	-	++	-	+	+	-	+	+
3	**Pf+Tv**	PEE	+	-	+	-	-	+	-	-	+	-	+	+
		CE	+	-	+	-	-	+	-	-	+	-	+	+
		EAE	+	-	+	-	-	++	-	+	+	-	+	+
		ME	++	-	+	-	-	++	-	+	+	-	++	+
4	**Control**	PEE	+	-	+	-	-	+	-	-	+	-	+	+
		CE	+	-	+	-	-	+	-	-	+	-	+	+
		EAE	+	-	+	-	-	+	-	-	+	-	+	+
		ME	+	-	+	-	-	+	-	+	+	-	+	+

SOL: Solvent used for extraction; **ALK:** Alkaloids; **AQ:** Anthraquinones; **CU:** Coumarins; **CC:** Catecholamines; **GLY:** Glycosides; **FLV:** Flavonoids; **SAP:** Saponins; **STE:** Steroids; **TA:** Tannins; **TER:** Terpenoids; **PHE:** Phenolic compounds.

Total antioxidant Activity (TAA):

Phosphomolybdenum Assay was used to test the total antioxidant activity in all plant extracts and the result was noted as AAE/g DW (Ascorbic Acid Equivalents per gram dry weight). The methanol extract exhibited highest antioxidant activity followed by ethyl acetate and chloroform extract. The least antioxidant activity was shown by petroleum ether extract. In the methanol extract, TAA was highest in Pf+Tv treated plants (99.48$\pm$ 0.97 mg/g DW) and least in control plantlets (32.98 $\pm$0.97 mg/g DW). In methanol extracts of Tv treated plants the antioxidant activity was 90.32±2.72 mg/g DW and in Pf treated plants it was 81.15±2.49 mg/g DW (Table- 2). The dual inoculation treated plants had highest antioxidant activity in all the extracts followed by Tv treated plants. Different extent of antioxidant activity was observed from various extracts of biohardened plants. The TAA of different extracts of all treatments are represented in Fig.5

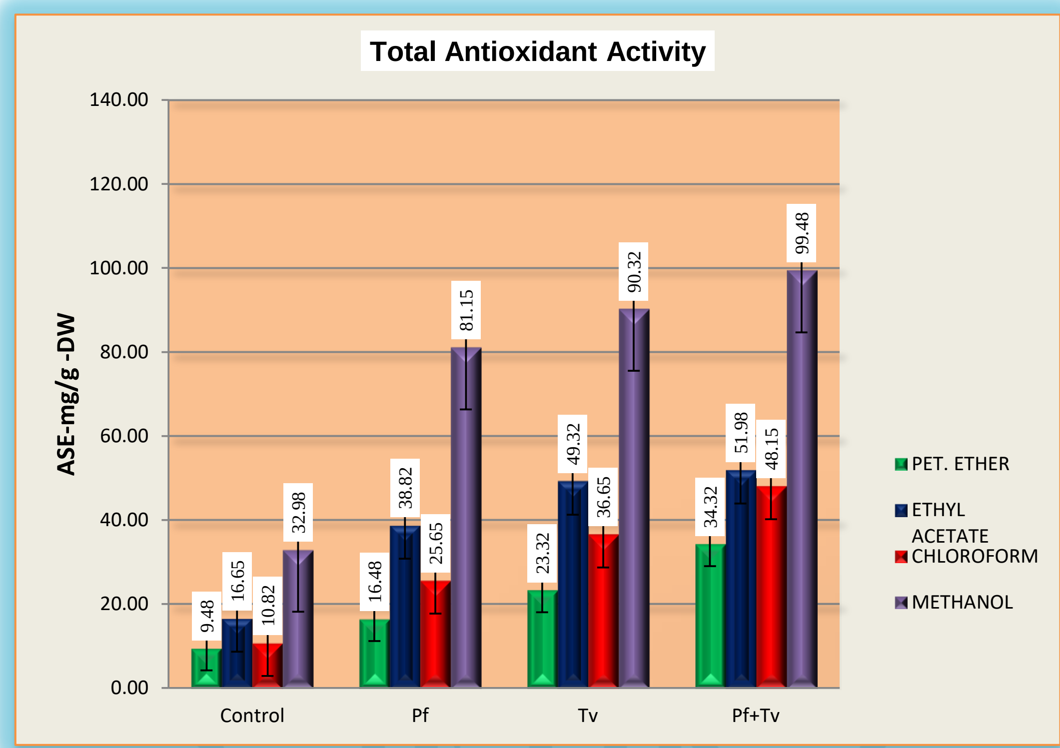

Figure 2: Total antioxidant activity of various solvent extracts of bioinoculant treated and control plantlets

Table 2: Total Antioxidant Activity (mg/g DW) of Various Solvent Extracts of Bioinoculant Treated and Control Plantlets

	Pet. Ether	Ethyl Acetate	Chloroform	Methanol
Control	9.48±0.41	16.65±1.03	10.82±0.62	32.98±0.97
Pf	16.48±0.41	38.82±1.25	25.65±0.62	81.15±2.49
Tv	23.32±1.70	49.32±2.87	36.65±1.03	90.32±2.72
Pf+Tv	34.32±2.39	51.98±1.02	48.15±2.09	99.48±0.97

Total Phenolic Content (TPC)

Folin–Ciocalteu assay was the method adapted to estimate the total amount of phenolic content in all plant extracts. The result was noted as GAE/gm DW (Gallic acid equivalents per gram dry weight). The extracts prepared from Pf+Tv treated plants exhibited highest phenolic content and it was 57.82±1.43 mg GAE/g DW in ME, 15.99 ±1.03 mg GAE/g DW in EAE, 13.49±0.85 mg GAE/g DW in CE, and 7.32±0.24 mg GAE/g DW in PEE (Table-3). The maximum phenolic compounds were extracted in methanol extract compared to other solvent extracts. The amount of phenolic compounds were significantly more in all biohardened plants compared to control (Fig.3).

Table 3: Total Phenolic Content (mg/g DW) of Various Solvent Extracts of Bioinoculant Treated and Control Plantlets

	Pet. Ether	Ethyl Acetate	Chloroform	Methanol
Control	1.82±0.25	3.15±0.41	2.65±0.22	16.65±0.41
Pf	2.99±0.62	9.32±0.62	5.15±0.41	33.65±2.86
Tv	3.99±0.24	15.15±0.82	7.49±0.62	43.82±0.47
Pf+Tv	7.32±0.24	15.99±1.03	13.49±0.85	57.82±1.43

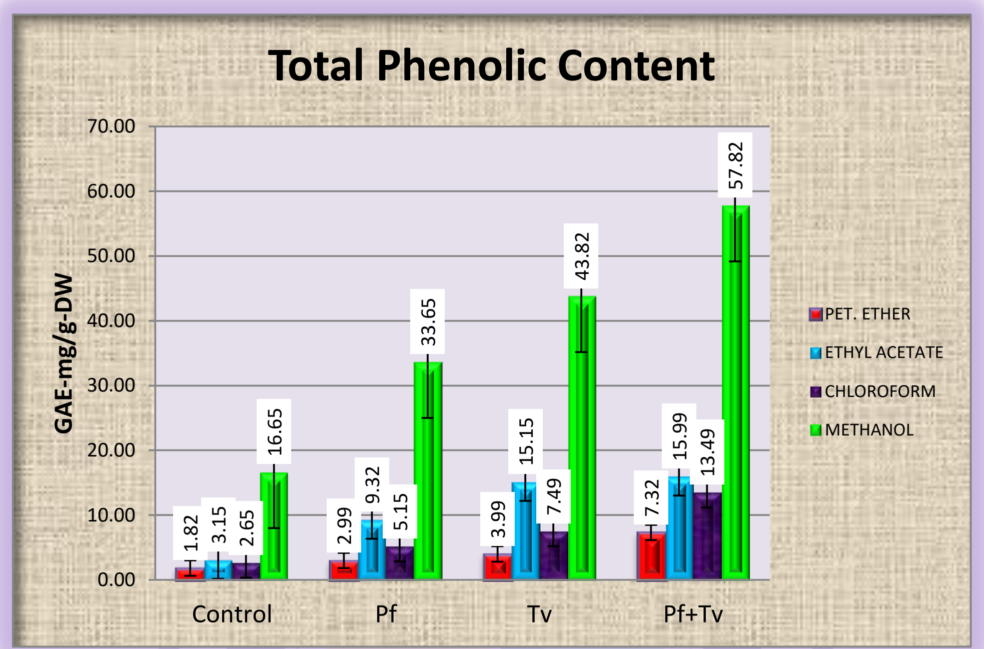

Figure 3: Total phenolic content of various solvent extracts of bioinoculant treated and control plantlets

DPPH activity:

The free radical scavenging activity was high in all extracts prepared from biohardened plants and also the activity increased with increase in its concentration (Fig. 4, 5, 6, 7). Best free radical scavenging activity was observed in methanolic extract of all treatments. In methanolic extract of Pf+Tv treated plants, the IC $_{50}$ value was found to be 6 µg/ml and it was 10 µg/ml for single inoculant treated plants (Fig.7).

IC $_{50}$ value of Pf+Tv treated plants was found to be 30 µg/ml for ethyl acetate and chloroform extracts (Fig.5 & 6), but for petroleum ether extract it was 50µg/ml (Fig.4). In terms of solvent extract, the DPPH radical scavenging activity was highest in the methanol extract (IC_{50}–6 µg/mL) followed by ethyl acetate and chloroform extracts (IC_{50} – 30 µg/mL). The lowest scavenging activity was noted in petroleum ether extract (IC_{50}–50 µg/mL). The DPPH activity was significantly more in all biohardened plants compared to control (Table 4, 5, 6 & 7).

Table 4: DPPH Activity of PEE of Bioinoculant Treated and Control Plantlets

PEE-Conc-ug/ml	10.00	20.00	30.00	40.00	50.00
Control	3.56±0.61	11.42±1.20	17.58±1.63	21.39±1.60	27.42±0.18
Pf	4.90 ±0.98	22.88±1.50	31.34±0.98	37.25±1.96	49.35±1.40
Tv	12.09±2.04	20.88±1.54	31.05±1.53	40.33±1.69	47.78±1.08
Pf+Tv	12.42±1.50	27.52±1.80	36.11±0.75	42.91±0.57	55.59±0.72

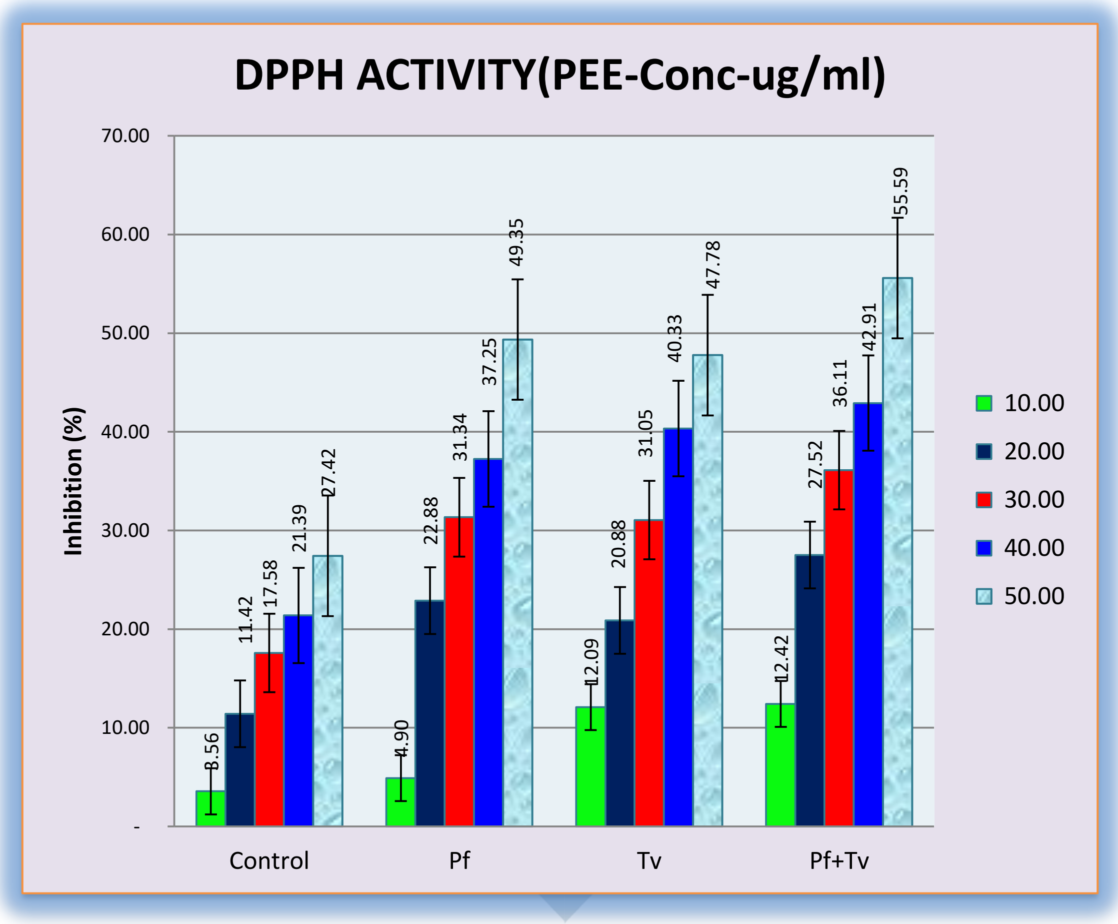

Figure 4: DPPH Activity of PEE of Bioinoculant Treated and Control Plantlets

Table 5: DPPH Activity of EAE of Bioinoculant Treated and Control Plantlets

EAE-Conc-ug/ml	10.00	20.00	30.00	40.00	50.00
Control	9.71±2.28	12.76±0.69	17.75±1.64	20.76±1.31	26.67±1.16
Pf	17.88±2.41	30.65±0.46	47.48±1.08	62.65±1.64	82.94±2.50
Tv	28.37±0.98	40.75±1.59	48.56±1.55	61.60±1.16	89.48±1.16
Pf+Tv	31.60±1.59	44.15±2.68	52.55±1.64	66.57±1.31	99.01±0.56

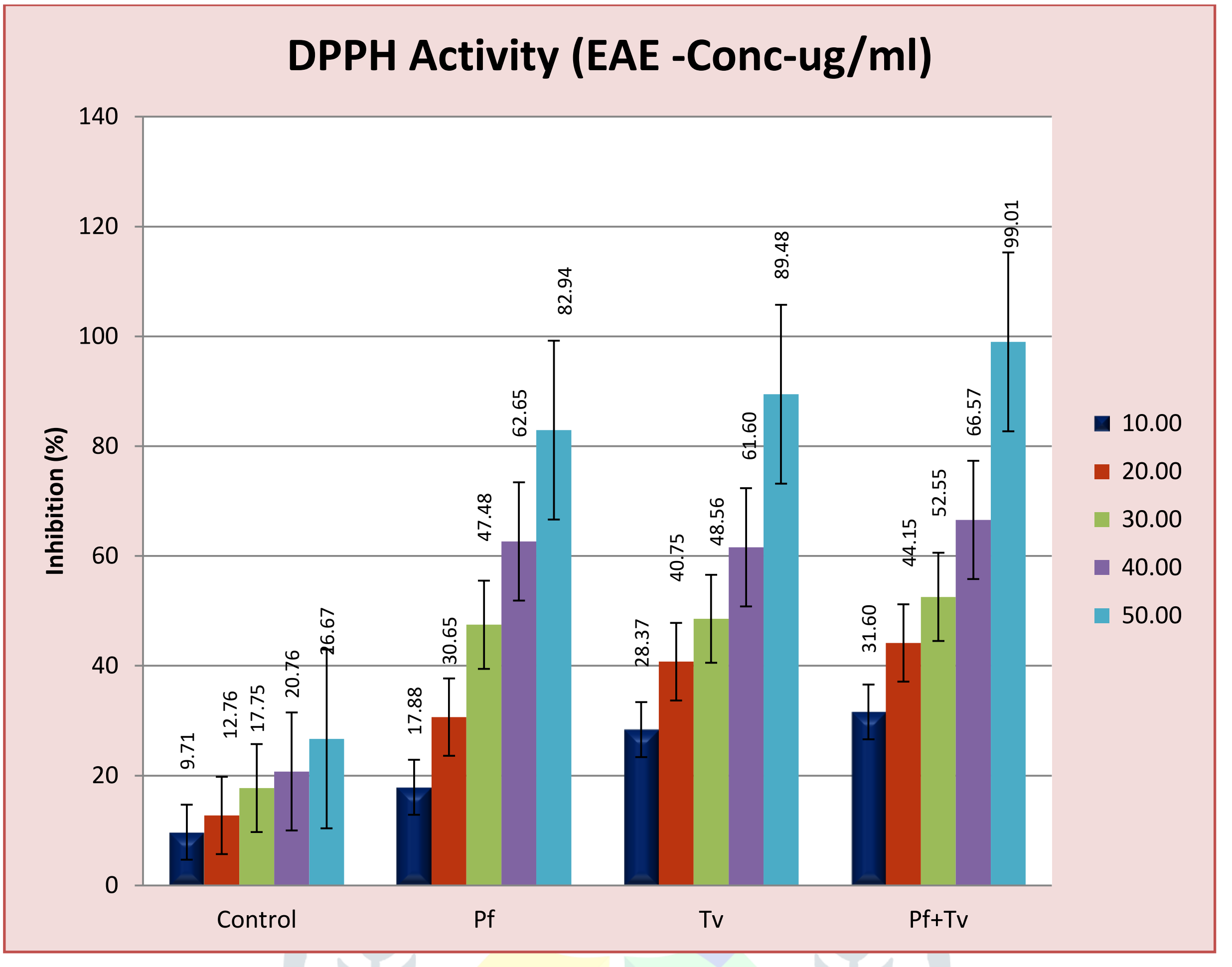

Figure 5: DPPH Activity of EAE of Bioinoculant Treated and Control Plantlets

Table 6: DPPH Activity of CE of Bioinoculant Treated and Control Plantlets

CE-Conc-ug/ml:	10.00	20.00	30.00	40.00	50.00
Control	4.02±1.62	10.28±1.19	14.71±1.48	20.85±1.21	25.89±1.56
Pf	5.23±1.50	14.31±1.22	26.80±1.19	38.59±1.61	57.06±1.73
Tv	12.12±1.49	23.46±1.11	37.88±1.04	58.33±1.70	69.12±0.60
Pf+Tv	16.83±1.98	28.86±1.74	52.42±1.45	64.97±1.58	88.76±1.26

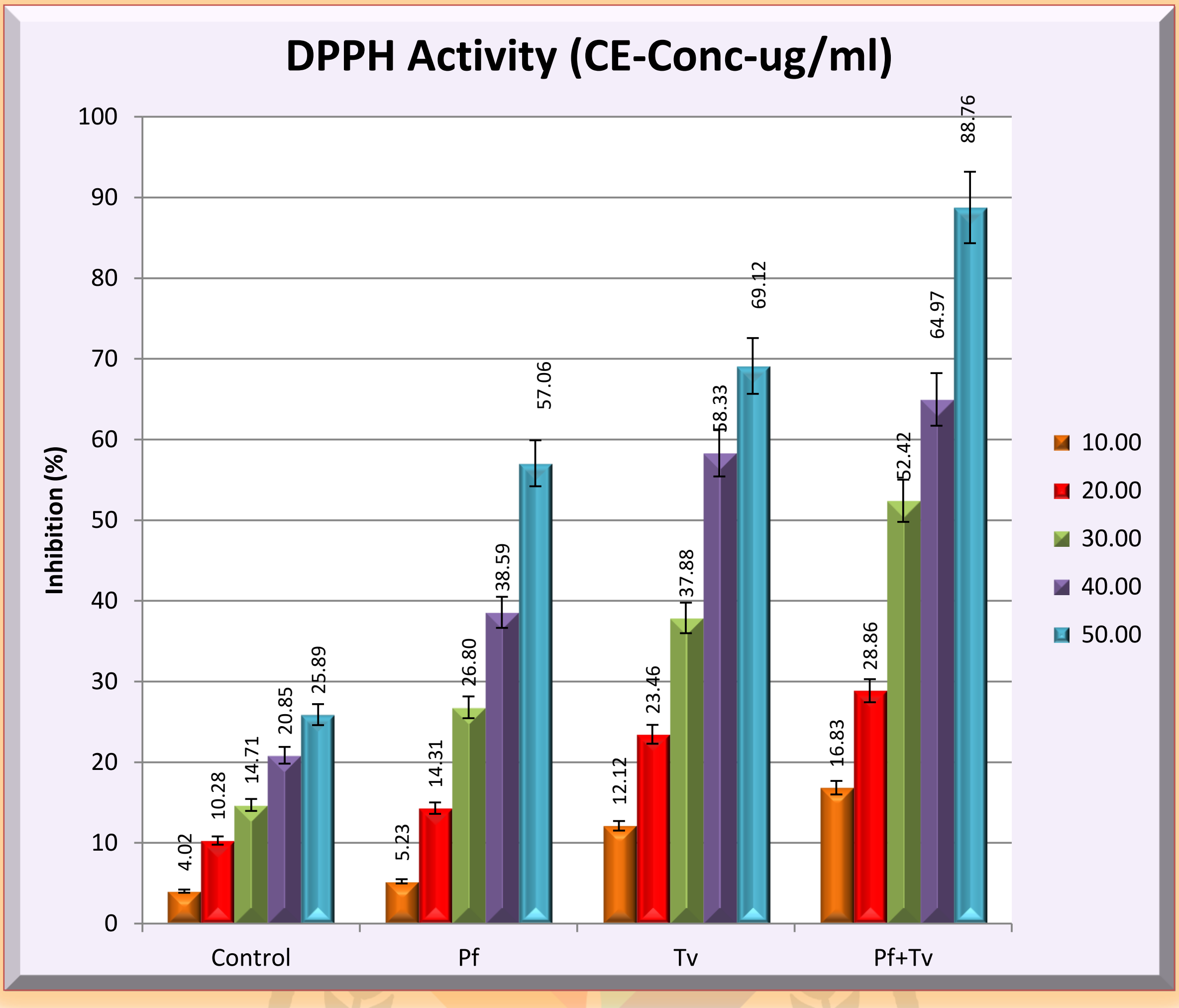

Figure 6: DPPH Activity of CE of Bioinoculant Treated and Control Plantlets

Table 7: DPPH Activity of ME of Bioinoculant Treated and Control Plantlets

ME- Conc-	**2.00**	**4.00**	**6.00**	**8.00**	**10.00**	**20.00**
Control	11.69±0.93	18.71±1.68	26.11±0.57	33.14±1.21	39.71±0.98	45.94±1.44
Pf	14.51±0.74	25.10±2.18	37.12±1.55	46.57±1.41	68.92±0.45	83.69±0.65
Tv	17.71±2.45	28.89±0.82	38.01±0.98	46.99±1.96	71.54±0.67	87.12±0.98
Pf+Tv	23.46±0.93	34.22±1.51	55.95±0.54	66.21±1.17	84.12±1.62	97.71±1.50

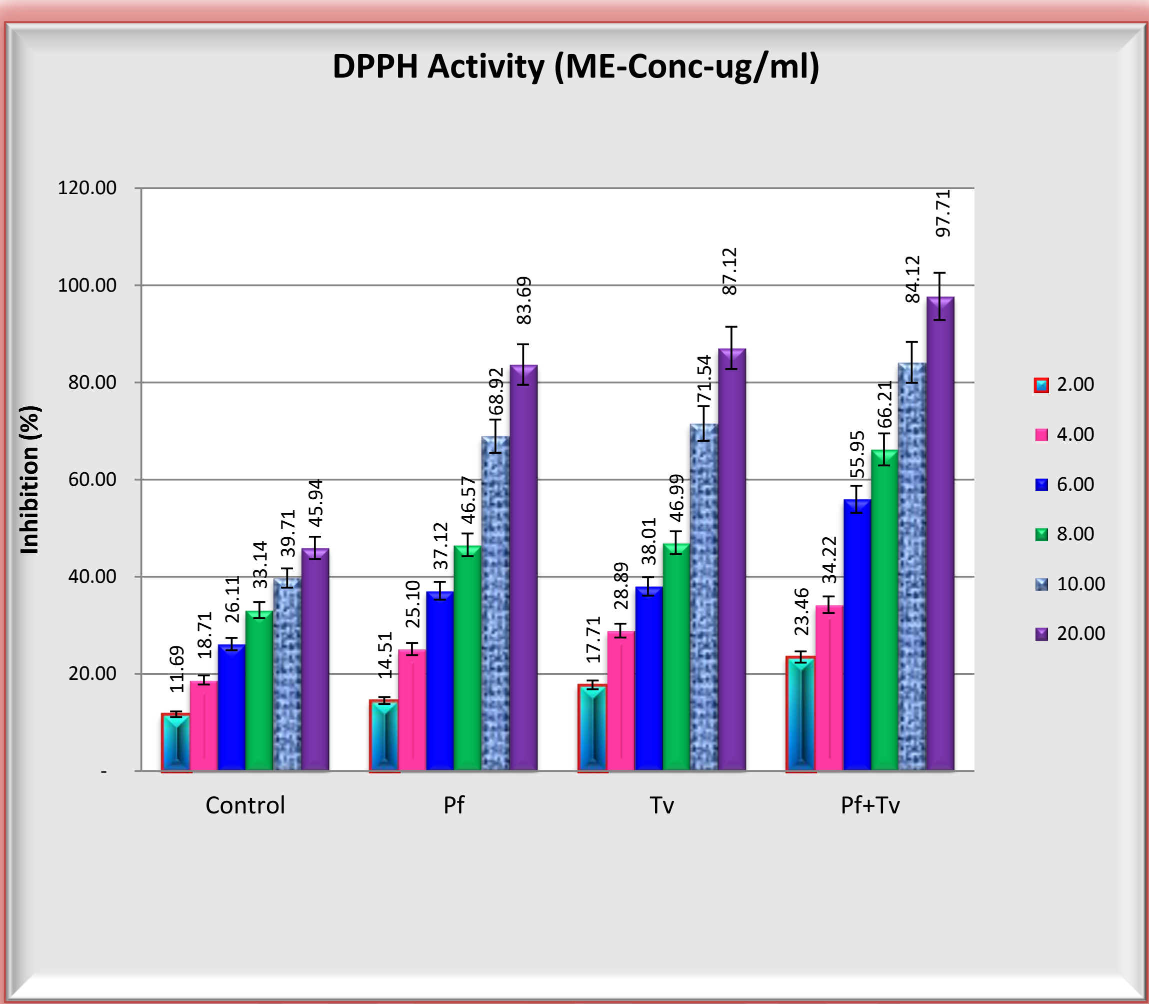

Figure 7: DPPH Activity of ME of Bioinoculant Treated and Control Plantlets

DISCUSSION

Effect of bioinoculants on plant survival rate

The inclusion of biological hardening step using beneficial microbes during transplantation of *in vitro* grown plantlets was the unique features of this study. Microbial inoculants such as *Trichoderma viride* and *Pseudomonas fluorescens* were found very efficient in increasing the plant establishment frequency. When bioinoculants were used, tissue culture plants were protected from shock caused due to transplantation The plant survival rate was observed maximum in mixed inoculum treated plants, which was due to the symbiotic interaction between *P. fluorescens* and *T.viride.* Plants treated with bioinoculants produced more secondary metabolites and organic acids which enhanced resistance towards abiotic and biotic stress factors. Similar reports of *P. fluorescence* and *T. viride* when used individually and in combination on plant survival and growth rate was studied by other researchers [21].

Plant extract yield & preliminary phytochemical analysis

Phytochemical analysis of ethyl acetate, petroleum ether, chloroform, and methanol extracts of the present work showed the presence of different secondary metabolites. The results were in coincidence with the extracts of wild plants of *A. amara* [22]. Preliminary phytochemical analysis of various extracts indicated the presence of tannins, flavonoids, saponins, alkaloids, steroids, coumarins and phenolic compounds. The methanolic extract of dual inoculum treated plants exhibited the presence of more flavonoids and phenols.

Total Antioxidant Activity

The antioxidant activity of medicinal plants was due to its alkaloids and flavonoid content. The methanolic extract of wild plants of *A.amara* [23] contains high antioxidant activity due to its alkaloid and flavonoid content. Similar reports of high antioxidant activity was observed in the present study with methanolic extract of biohardened tissue culture plants of *A.amara* .It was also observed that TAC was more in tissue culture plants (99.48± 0.97 mg/g) compared to wild plants (72.6 ± 2 mg g-1) of *A.amara*. Thus it can be concluded that methanol extract of biohardened *A.amara* plantlets have high antioxidant activity which was enhanced due to treatment with bioinoculants.

Total Phenolic Content

The antioxidant activity was directly related to the amount of phenolic compounds [24]. Due to the presence of hydroxyl groups, phenols exhibit high free radical scavenging activity and therefore were considered as very important plant constituents [25]. A positive relationship between antioxidant activity and total phenols was found in wild plants of *Albizia amara*. The total phenolic content of ME and PEE of wild *A.amara* plants were 243.37 and 1161.86 µg /ml GAE respectively [22] . Similar reports of high TPC content was observed in the present study with methanolic extract of biohardened tissue culture plants of *A.amara* . Further it was noted that TPC was more in tissue culture plants (47.82±1.43 mg GAE/g in ME) compared to wild plants . There was a significant difference between the total phenolic contents of the ME and other extracts both in wild plants and tissue culture plants of *A.amara*. Thus it can be concluded that methanol extract of *A.amara* plantlets have high phenolic content which was augmented due to biohardening with mixed inoculum. The results of TPC and TAA of *A.amara* leaf extracts revealed that methanol extracts had the highest phenolic content and antioxidant activity. The present findings reveal that the methanol extracts of dual inoculated *A.amara* plantlets exhibit strong antioxidant activity due to the presence of more phenolic compounds.

Polyphenols are the major antioxidant components of plant kingdom. The antioxidant activity of medicinal plants was estimated by measuring the phenolic contents of plant extracts. The hydroxyl groups of phenols give up hydrogen atoms and form phenoxy radicals exhibiting strong antioxidant activity. In Rubus species, Deighton *et al.,* (2000) reported a positive correlation between phenolic content and antioxidant activity [26]. Several workers reported linear relationship between antioxidant activity and the concentration of phenolic compounds [27].

DPPH Activity

DPPH is the most commonly used free radical to estimate the antioxidant capacity of different extracts isolated from plants. Due to the presence of antioxidant compounds in various plant extracts of *A.amara*, there was a significant decrease in the concentration of DPPH radicals. The hydrogen donated by the antioxidant components of the plant extract scavenges the free radicals. Among the various extracts tested, DPPH radical was strongly inhibited by the lowest concentration of methanol extract (IC_{50} value 6 µg/mL). Concentration dependent antiradical activity against DPPH radical was shown by all the plant extracts. The lower IC_{50} value is associated with a high DPPH radical scavenging activity. The DPPH scavenging activity of methanol extract was strong, due to its high phenolic content. A linear relationship existed between the concentration of the extract and free radical scavenging activity [28].

Most plants exhibited strong antioxidant activity at a concentration ranging from 10 - 50 µg/ml [29]. Similar results were observed in PE, EAE and CE of *A.amara*. Thus it can be concluded that methanol extract of biohardened *A.amara* plantlets have high antioxidant activity which was enhanced due to treatment with bioinoculants..The wild plant extracts of *Albizia amara* were reported to have significant DPPH activity. IC_{50} value of wild plant extracts was found to be 164 µg/ml for methanolic extract and 213 µg/ml for petroleum ether extract [22]. Similar observations were reported in *C. esculenta, E.agallocha* where tissue culture grown plants showed high phenolic content and strong scavenging activity compared to wild plants [30, 31].

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 high antioxidant 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. Methanol was the best solvent in giving maximum extract yield (17.3%). In the present study high antioxidant activity (99.48 AAE mg/g DW) and phenolic content (57.82 mg GAE mg/g DW) was observed in the methanolic extract of Pf+Tv biohardened tissue culture plants of *A.amara* .It was also observed that total antioxidant activity is correlated to the phenolic content. Further studies reported that low concentrations of the methanolic extract (IC_{50} value at 6 µg/ml) of Pf+Tv biohardened tissue culture plants of *A.amara* exhibited high DPPH activity. The total phenolic content, antioxidants and other 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 .

Successful establishment of tissue culture raised plants from lab to land require development of innovative culture methods. Basic research on physical, chemical and biological factors governing plant survival, growth and development during acclimatization phase of tissue culture is the need of hour. 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]. 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.

[2]. 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.

[3]. Praveen P, Thippeswamy S, Mohana DC and Manjunath K. 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, 4,2011, 832-835.

[4]. Thippeswamy S, Mohana DC, Abhishek RU and Manjunath K. 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, 2013, 297-305.

[5]. Lazarovits G and Nowak J. Rhizobacteria for improvement of plant growth and establishment. Hort Science. 32,1997, 188.

[6]. Nowak J. Review benefits of *in vitro* "biotization" of plant tissue cultures with microbial inoculants. In vitro Cell Dev Biol Plant. 34,1998, 122.

[7]. 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.

[8]. Somolenski SJ, Silinis H and Fransworth NR. Alkaloid screening. I-VII Lloydia. 35 ,1972, 1-34 ; 36: 359-389; 37: 30-61; 506-536; 38: 225-255, 411-441, 497-528.

[9]. Peyer W. Detection of emodins in admixture with phenolphthalein in nostrums. Deutsche Apotheker Zeitung. 4,1931, 574.

[10]. Das AK and Bhattacharjee AK. A systematic approach to phytochemical screening. Tropical Science. 12,1970.

[11]. Casparis P and Manella E. Detection of Coumarins in drugs III. Pharmaceutica Acta Helvetica. 19,1944, 158 – 160.

[12] . Geissman TA. Anthocycnins, Chalcones, flavones and related water-soluble plant pigments. In: Paech, K. and Tracy, M.V. (Eds.) Modern Methods of Plant analysis, Springer- Verlang, Berlin. 3 ,1955, 450-498.

[13] . Gibbs RD. Chemotaxonomy of flowering plants I-IV. ,Montreal and London, 1974.

[14] . Kepm W. Qualitative organic analysis,Spectrochemical Techniques, Mc Grew-Hill, London. (ed.) ,1986.

[15] . Cambie RC, Cain BF and La Roche. A New Zealand phytochemical survey (III) ferns and fern allies. New Zealand J. Sci. 4, 1961, 707 -714.

[16] . Harborne JB. Phytochemical methods, Chapman and Hall, London, 1991.

[17] . Eckey EW. Vegetable Fats and Oils. Soil Science,78 (1), 1954, 83.

[18] . Priesto P, Pineda M, Aguilar M. Spectro photometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of Vit E. Anal Biochem. 269, 1999, 337-341.

[19] . Singleton VL and Rossi JA. Colorimetry of total phenolics with phosphomolybdic phosphor tungstic acid reagents. Am J Enol Viticul. 16, 1965, 144.

[20] . Blios MS. Antioxidant determinations by the use of a stable free radical, Nature, 26, 1958, 199–200.

[21] . Mishra DS, Gupta AK, Prajapati CR and Singh US, Combination of fungal and bacterial antagonists for management of root and stem rot disease of soybean. Pakistan Journal Botany, 43 ,2011, 2569.

[22] . Rajkumar T, Satheesh Kumar E, Sinha BN. Evaluation of antioxidant properties of *Albizia amara* leaves.International Journal of Advance Pharmaceutical and Biological Sciences, 2,2012, 99-106.

[23] . Suresh Kumar P, Sucheta1 S, Sudarshana Deepa V, Selvamani P and Latha S. Antioxidant activity in some selected Indian medicinal plants. African Journal of Biotechnology, 7, 2008, 1826-1828.

[24] . Sharma OP and Bhat TK. DPPH: antioxidant assay revisited. Food Chem., 113, 2009, 1202-1205.

[25] . Mani RS, Alam AM, Akter R and Jahangir R. In-vitro free radical scavenging activity of *Ixora coccinea* L. Bangladesh J Pharmacol. 3, 2008, 90-96.

[26] . Deighton N, Brennan R, Finn C and Davies HV. Antioxidant properties of domesticated and wild Rubus species. Journal of the Science of Food and Agriculture, 80, 2000, 1307-1313.

[27] . Cuvelier ME, Richard H and Berset C. Comparison of the antioxidative activity of some acid-phenols: structure activity relationship. Bioscience Biotechnology and Biochemistry, 56, 1992, 324-327.

[28] . Basu S, Sen A, Das M, Nath P and Datta G. Phytochemical evaluation and *in vitro* study of antioxidant potency of *Amorphophallus campanulatus, Alocasia indica* and *Colocasia esculenta*: a comparative analysis. Int. J. Pharm. Bio. Sci., 3(3), 2012, 170 -180.

[29] . Phongpaichit S, Nikom J, Rungjindamai, Sakayaroj N, Hutadiolk, Towatana, Rukachaisirikul V and Kirtikara K. Biological activities of extracts from endophytic fungi isolated from Garcinia plants. FEMS Immunol. Med. Microbiol., 51(3), 2007, 517-525.

[30] . Akshatha, MD, Smitha Kavadikeri and Nagashree N. Rao, *In vitro* Micropropagation and Antioxidant Assay in *Colocasia esculenta*, Plant Tissue Cult. & Biotech, 28(2), 2018, 183-190.

[31] . Arumugam M, Pawar UR and Gomathinayagama M, Antibacterial and antioxidant activity between micropropagated and field grown plants of *Excocaria agallocha* L. IRJP 3(3) 2012.