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The Effect of Biohardening on Plant Physiology –A Case Study in *Albizia amara*

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Abstract:- The physiological, anatomical and morphological features of the plantlets developed under *in vitro* conditions, have a strong impact on plant survival after transplantation to *ex vitro* conditions. The leguminous tree, *Albizia amara* has been selected for tissue culture studies in the present investigation, as it possesses immense medicinal properties. During acclimatization, the effect of bioinoculants on plant physiology was determined in terms of autotrophic activity by measuring the amount of fructose, chlorophyll and carotenoids of the leaf samples. The nutrient content in biotized plants was observed by estimating NPK % and micronutrient content. The water retention capacity of leaves was measured in terms of percentage of water loss. The present study reported that biohardening of tissue cultured plantlets with mixed inoculum of *Pseudomonas fluorescens* and *Trichoderma viride* enhanced plant survival rate due to its increased photosynthetic rate, efficient uptake of water, macro and micronutrients.

Keywords:- *Albizia Amara, Autotrophic Activity, Biohardening, Nutrient Content, Water Retention Capacity.*

I. INTRODUCTION

Albizia amara is a plant of potential 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 extensively used in traditional medicine(Sastry et al.1967) . Minimum work was done on tissue culture studies of *Albizia amara*. Earlier studies reported plantlet regeneration from cotyledonary bud explants derived from aseptically grown seedlings but during transplantation limitations were observed (Indravathi et al.2019a). 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 (Indravathi et al.2020).

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). 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).Earlier studies reported the improvement of morphological, anatomical, physiological and pharmacological features of the plantlets successfully survived under *ex vitro* conditions during acclimatization after application of bioinoculants (Indravathi et al.2019a).

The present paper analyses the efficacy of bioinoculants in enhancing certain physiological characteristics of the tissue culture plants after transplantation to *ex vitro* conditions. The effect of biohardening on plant physiology was determined in terms of autotrophic activity by measuring the amount of fructose, chlorophyll and carotenoids of the leaf samples. Efficient nutrient uptake in bioinoculant treated plants was observed by estimating NPK % and micronutrient content by using dried plant material. The water retention capacity of leaves was measured in terms of percentage of water loss.

II. MATERIALS AND METHODS

A. 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.

B. 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.

C. 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 (Fig.1). Various plant growth parameters of biohardened and control plantlets were recorded sixty days after transplantation. In the present paper the efficacy of bioinoculants on certain physiological characteristics (autotrophic activity, nutrient content and water retention capacity) of the tissue culture plants after transplantation to *ex-vitro* conditions were studied.

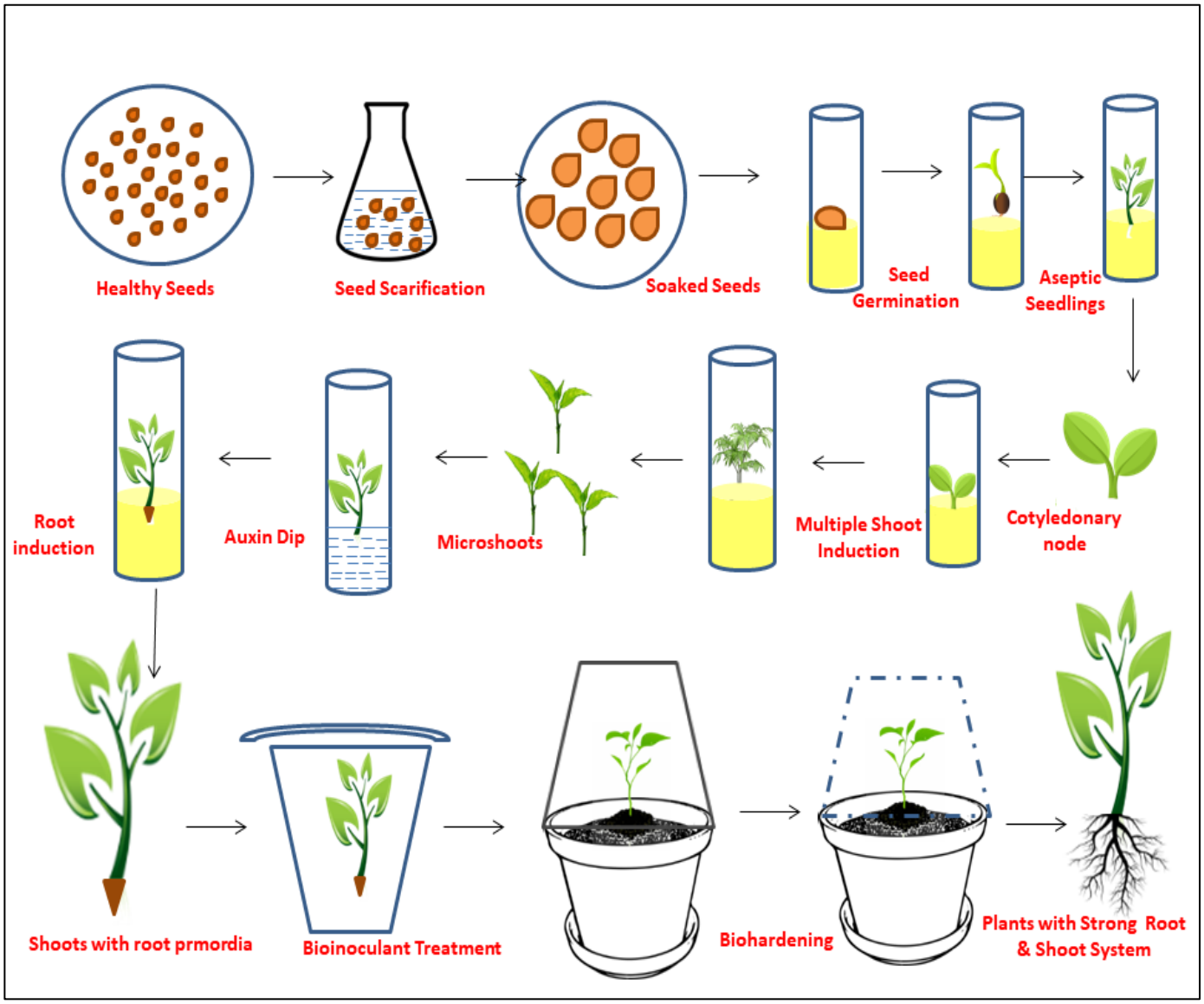

Fig 1: Illustration on Strategy to Improve Rooting & Acclimatization of Tissue Culture Plants

D. Effect of Biohardening on Plant Physiology- Autotrophic Activity

The autotrophic activity of biohardened and control plantlets were estimated by measuring the content of carotenoids, chlorophylls, free fructose and total fructose. For each treatment, plant material was taken from 60-day old plants and 3 replications were carried out. Shoots and Leaves were taken randomly from three types of biohardened plants [(Tv), (Pf), (Pf+Tv)] and control plants to study the effect of bioinoculants on plant physiology. Five plants were taken randomly from each type of treatment and each experiment was repeated thrice.

➢ *Autotrophic Activity- Total Chlorophyll & Carotenoid Content*

The concentrations of Chlorophyll a (C_a), Chlorophyll b (C_b), and Carotenoids (C_{x+c}) pigments were tested by adapting Lichtenthaler method (Lichtenthaler et al.2001). Fresh leaves were collected from biohardened and control plantlets. The leaf samples were weighed (100mg) and placed in glass tubes. To the leaf sample, 10 ml of 100% acetone was added and blended by a homogenizer. The sample was subjected to centrifugation (2500 rpm for 5 minutes) and the supernatant was transferred to glass vials and used for chlorophyll and carotenoid estimation. Since carotenoids and chlorophylls are extremely light sensitive and easily become photobleached, the extraction and concentration procedures were performed in dim light. For correct pigment values the quantitative determination of chlorophylls and total carotenoids in the whole leaf extract were carried out immediately after preparation of the extract. The carotenoid, chlorophyll a and chlorophyll b content of the samples were recorded by UV-visible spectrophotometer (Model No. Systronics -118) at 470 nm, 662 nm and 644 nm wavelengths respectively (Fig.2). 100% acetone solution was taken as blank. The results were calculated and represented as amount of pigment in ug / g Fresh Weight of the sample.

Chlorophyll a: Ca = 12.25 A663.2 – 2.79 A646.8
Chlorophyll b: Cb = 21.50 A646.8 – 5.10 A663.2
Total carotenoids: Cx+c = (1000 A470 – 1.82 Ca – 85.02 Cb) / 198

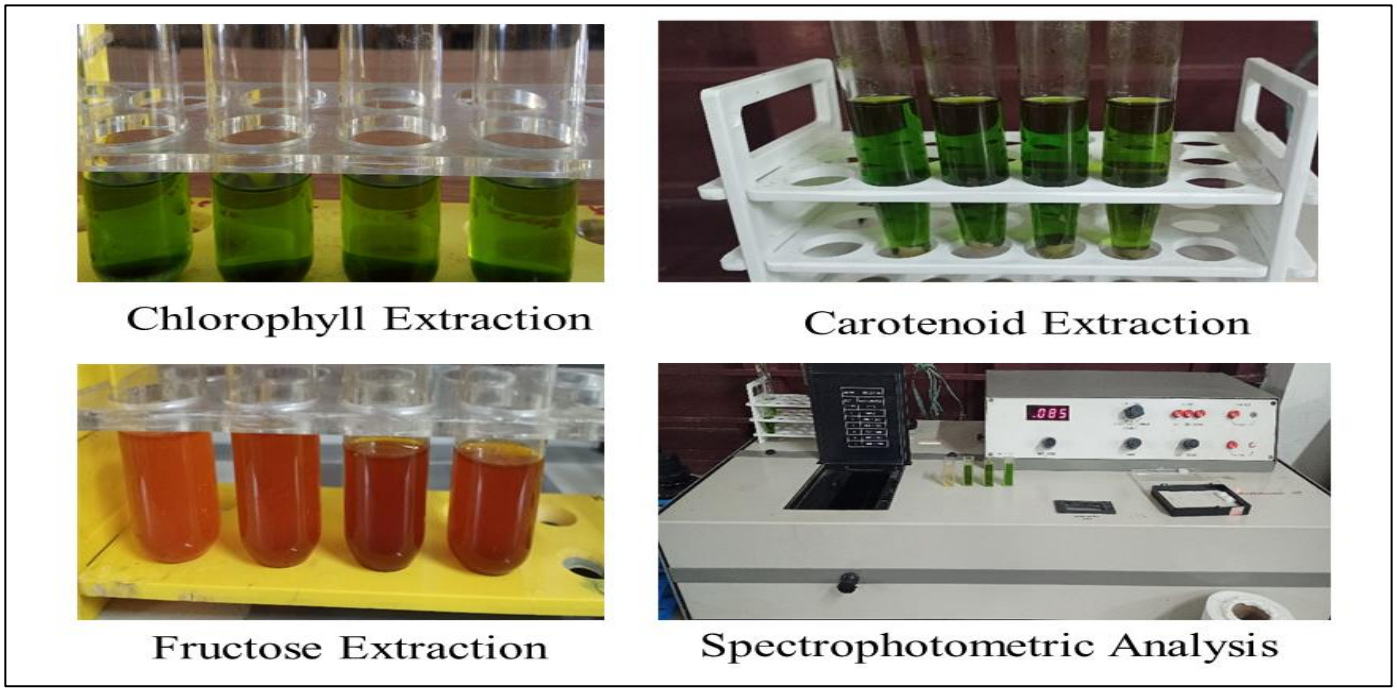

Fig 2: Extraction & Estimation of Chlorophyll, Carotenoid and Fructose

➢ *Autotrophic Activity- Fructose Content*

The fructose content (total and as sucrose) of biohardened and control plantlets was estimated using modified Roe's method (Davis et al.1967). The chromophoric substance obtained in this method was evaluated by spectrophotometer at 420 nm, which was proportional to the amount of fructose present in the unknown sample. Fructose standard was prepared by dissolving 10 mg of fructose in 100 ml of distilled water.

Shoots were collected and shade dried from inoculated and control plants. The dried sample was weighed and to this 0.05% Resorcinol and HCl (12 M) was added. The sample mixture was heated at 77°C for 8 minutes and cooled immediately. This determines the total fructose present in the sample. This test does not distinguish between fructose fraction present in sucrose and free fructose of the tested sample. The fructose fraction in sucrose was estimated by destroying the free fructose in the sample using hot potassium hydroxide (2N KOH). The difference between total fructose and the fructose fraction contained in the sucrose represents the content of free fructose. The experiment was repeated thrice and the absorbance was measured at 420 nm . The experiment results were represented as amount of fructose in mg / g distilled water of the sample using an equation that was obtained from the fructose standard graph. Free fructose (%) was calculated as (Free fructose/Total fructose) x 100

E. Effect of Biohardening on Plant Physiology- Nutrient Content:

Sixty day old biohardened and control plants were collected, dried and evaluated for nutrient content using standard procedures. The plants were examined for nitrogen, phosphorus and potassium (NPK) content and micronutrients such as Zinc, Copper, Manganese and Iron. NPK content was evaluated by micro Kjeldahl method, Vanadomolybdate method, and Flame photometry respectively. The micronutrients were estimated using atomic absorption spectroscopy.

- Nutrient Content - Determination of Total Nitrogen in Plant Samples

Total nitrogen in the plant samples was tested by adapting Micro-Kjeldahl method and calculations (AOAC,1995). The procedure consists of 3 steps:

- Digestion: Plant sample was dried, weighed (0.5g) and transferred to digestion tube. To the plant sample, 10ml of conc. H_2SO_4 and catalyst mixture (K_2SO_4, $CuSo_4$2 H_2O, Selenium in 50:10:1) was added and loaded to the digestion tubes. The sample mixture was heated at 100 ^{0}C until foam disappears. At the end of digestion process the sample turns to light green colour or colourless.
- Distillation: The digestion tubes were cooled, and the samples were transferred to distillation unit. 40 % NaOH was added automatically to distillation unit. The digested sample liberates ammonia which was absorbed by boric acid and causes a colour change from pink to green. Blank sample was also maintained.
- Titration: The sample obtained from distillation unit was titrated with H_2SO_4 (0.02N). This changes the distillate colour from green to pink. The titer reading of both sample and blank (ml) was noted. Finally, the nitrogen content of the samples was calculated as follows:

$$\text{Nitrogen Content (N\%)} = \frac{R \times \text{Normality of Acid} \times \text{Atomic Wt. of } N_2 \times 100}{\text{Sample Weight (g)} \times 1000}$$

$$= \frac{R \times 0.1 \times 14 \times 100}{0.5 \times 1000}$$

Where R = (Sample titre- Blank titre)

- Nutrient Content - Determination of Phosphorous in Plant Sample

The phosphorus content of the plant samples was evaluated by Vanadomolybdate method using colorimeter. The phosphorus present in the plant sample reacts with Vanadate molybdate to give a yellow colour complex compound in acidic medium. The colour intensity form the basis of quantitative estimation of total phosphorus present in the plant samples(Koenig et al. 1942).

Plant sample was dried, weighed (1g) and transferred to digestion flask. Diacid (3:1 - Nitric acid: Perchloric acid) mixture was added to the sample, mixed well and heated .The solution was filtered and the volume was made to 100ml using distilled water and stored in air tight container. To 10 ml dilute, 10 ml ammonium molybdate vanadate solution was added and the contents were mixed well. The volume was made to 50 ml and the reading was recorded using spectrophotometer at 420 mm and the P content was measured using a standard curve.

$$\text{Phosphorus content (P \%)} = \frac{F \times R \times 100 \times 100}{10000 \times 1000 \times 10 \times 1}$$

Absorbance of plant sample : R
Absorbance of standard : A
Concentration of standard : B ppm
F (Factor) : B / A

- Nutrient Content -Determination of Potassium in Plant Samples

The estimation of potassium was done using flame photometry (Black 1965). Plant sample was dried, weighed (1g) and transferred to digestion flask. 20-25 ml of acid mixture (750 ml conc. HNO_3 + 150 ml conc. H_2SO_4 + 300 ml of $HClO_4$) was added, mixed well and heated on a hot plate. Digestion process gets completed when sample becomes colourless. The sample was cooled and 20 ml H_2O was added and filtered into a 100 ml volume flask. By using flame photometer, the potassium content of the aliquots was measured using red filter at selected wave length (767 mm).

Potassium content (P %) = Reading obtained x 4x 10^{-3}

➢ *Determination of Micronutrients in Plant Samples*

For micronutrient analysis, the plant samples were digested in a diacid mixture which was prepared by mixing per-chloric acid and nitric acid in the ratio of 4:9 (Lindsay 1978). Plant sample was dried, weighed (1g) and transferred to conical flask. Di-acid mixture was added to the plant sample, mixed well and heated in a digestion chamber at high temperature until the red fumes of nitrogen dioxide disappears. Digestion process gets completed when sample becomes colourless. The digest volume was made up to 25ml with double distilled water and filtered. Finally the filtrates were used for the estimation of Fe, Mn, Zn and Cu with Atomic Absorption Spectrophotometer (Model No. ELICO-SL243). Calculation was done as follows:

- Micronutrient Content (ppm) = AAS Reading x 25 (Dilution Factor)

F. Effect of Biohardening on Plant Physiology- Water Retention Capacity (WRC)

The Water Retention Capacity (WRC) of plants was evaluated by measuring the percentage of water loss using Fresh Weight (FW) and Dry Weight (DW) of excised leaves. The leaves were detached from biohardened and control plantlets and the loss of water was measured simply by weighing the samples at different time intervals (Debergh 1991). Leaves used in this experiment were excised from sixty-day-old biohardened and control plantlets. Water loss was measured by weighing the leaf

samples at 5-minute time interval for a total time period of 50 minutes at 50% RH and 18°C temperature. Each experiment was repeated three times. The Water Retention Capacity (WRC) was calculated according to the method proposed by Debergh (1991). After that, the leaves were kept overnight in an oven at 55^0C to determine Dry Weight (DW)

$WRC = (FW_0 - FW_t / FW_0 - DW) \times 100$

Where FW_0 is the fresh leaf weight at time 0 minutes

FW_t is the leaf weight at time t minutes
DW is the leaf dry weight.

III. RESULTS

A. *Effect of Biohardening on Plant Physiology- Autotrophic Activity*

Variations in chlorophyll and carotenoid contents between inoculated and control plantlets were recorded during hardening (Table 1). The highest chlorophyll contents were observed in inoculated plantlets compared to uninoculated ones. Of the biohardened plants highest chlorophyll contents were present in Pf+ Tv inoculated plantlets (1125.59±1.34 ug/g FW) followed by Pf (1055.70±1.50 ug/g FW), Tv (977.15±1.34 ug/g FW) treated plants and control plantlets (583.09±2.24). About 335.35±1.50 ug/g FW carotenoids were present in Pf+Tv inoculated plantlets in comparison to 314.07±2.42 ug/g FW in Tv inoculated plants, 286.82±1.50 ug/g FW in Pf inoculated plants and 100.79±1.29 ug/g FW uninoculated control (Fig.3).

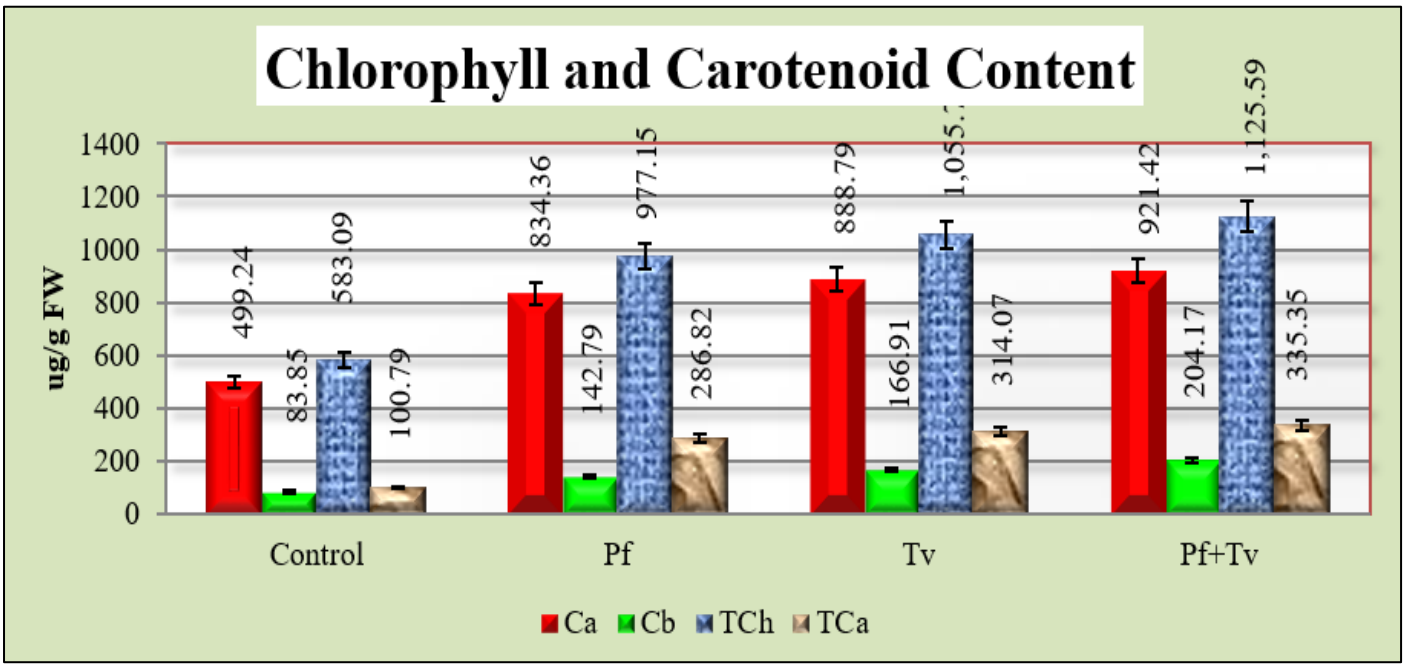

Fig 3: Estimation of Chlorophyll and Carotenoids in Bioinoculant treated and Control Plantlets

Table 1: Estimation of Chlorophyll & Carotenoids in Biohardened and Control plantlets

Chlorophyll Conc-ug/g FW	C_a	C_b	C_{a+b}	C_{x+c}
Control	499.24±2.41	83.85±0.65	583.09±2.24	100.79±1.29
Pf	834.36±1.13	142.79±2.00	977.15±1.34	286.82±1.50
Tv	888.79±1.62	166.91±2.05	1055.70±1.50	314.07±2.42
Pf+Tv	921.42±1.13	204.17±2.00	1125.59±1.34	335.35±1.50

The fructose fraction (36.42 mg/g DW) in sucrose, free fructose (21.6 mg/g DW) increased in the dual inoculant treated cultures causing overall increase of total fructose content (58.02 mg/g DW) (Table 2). The total fructose content for single inoculations is 43.88 mg/g DW (Tv) and 36.62mg/g DW (Pf) respectively. In control plantlets, the free fructose (8.00±0.09), fructose fraction (7.68±0.24) and total fructose content was 15.68 mg/g DW was recorded. The free fructose % was lowest in mixed inoculum treated plants - 37.22±0.68 mg/g DW (Fig.4).

Table 2: Estimation of Fructose in Bioinoculant Treated and Control Plantlets

Fructose Conc-mg/g DW	Total Fructose	Fructose Fraction	Free Fructose	Free Fructose (%)
Control	15.68 ±0.24	7.68±0.24	8.00±0.09	50.98±1.00
Pf	36.62±0.16	21.48±0.33	15.13±0.41	41.28±0.59
Tv	43.88±0.67	27.88±0.33	16.44±0.43	37.48±0.5
Pf+Tv	58.02±0.09	36.42±0.33	21.6±0.43	37.22±0.68

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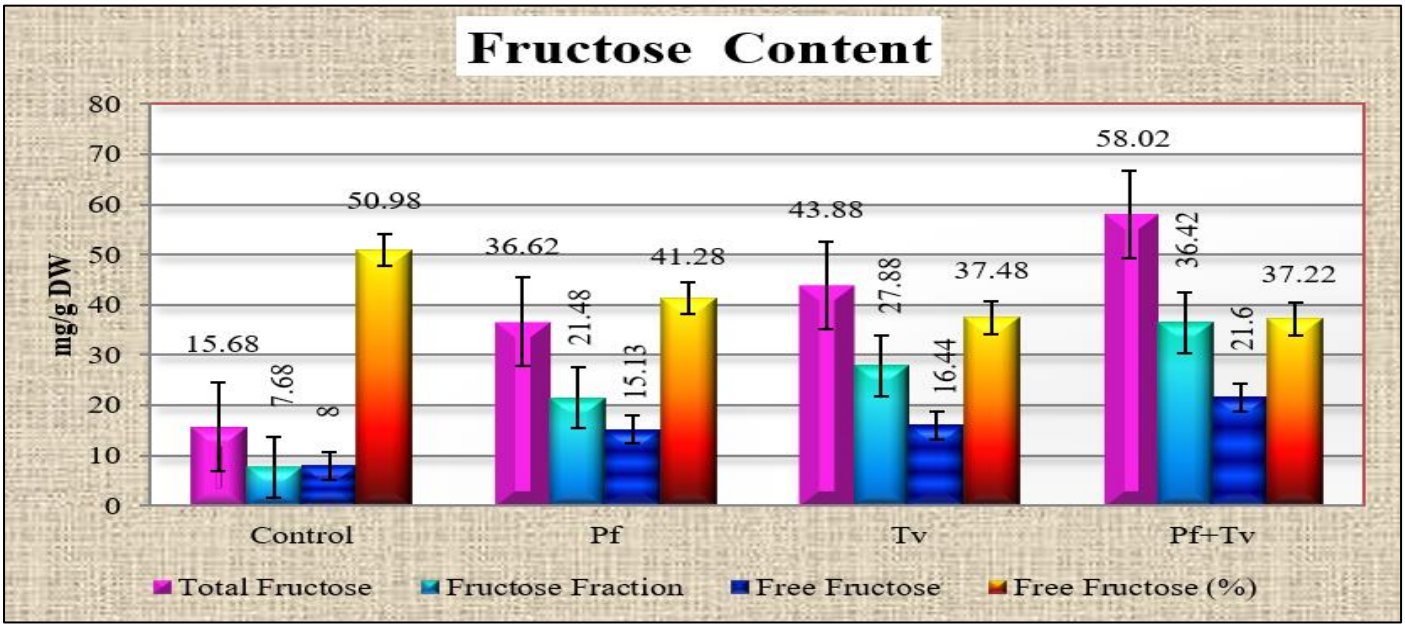

Fig 4: Estimation of Fructose in Bioinoculant Treated and Control Plantlets

B. Effect of Biohardening on Plant Physiology- Nutrient Content

NPK content was maximum in mixed inoculum treated plants (0.91 %) compared to *T.viride* (0.54 %) and *P.fluorescens* (0.48 %) treated plants. The Nitrogen, Phosphorus and Potassium content of mixed inoculum plants treated plants were 0.7%, 0.107% and 1.91% respectively. Least NPK content was recorded in control plants i.e., 0.102%, 0.04 and 0.387%. Micronutrients like Zn, Fe, Cu and Mn acquisition was highly improved by microbial inoculum treated plants. Dual inoculation resulted in maximum (673 PPM) uptake of all micronutrients followed by *T.viride* (610.75 PPM) and *P. fluorescens* (597.25 PPM) inoculated plants. All bioinoculant treated plantlets showed more nutrient content when compared to control (277 PPM). The acquisition of micronutrients was significantly higher in mixed inoculum treated plants plants and least in control plants (Fig.5 & 6). Present work indicated the differential uptake of macro and micronutrients by the bioinoculant treated and control plantlets (Table 3 & 4)

Table 3: NPK Content (%) in Bioinoculant Treated and Control Plantlets

Treatments	N	P	K	NPK %
Control	0.102	0.04	0.387	0.18
Pf	0.392	0.071	0.989	0.48
Tv	0.532	0.092	1.01	0.54
Pf+Tv	0.7	0.107	1.91	0.91

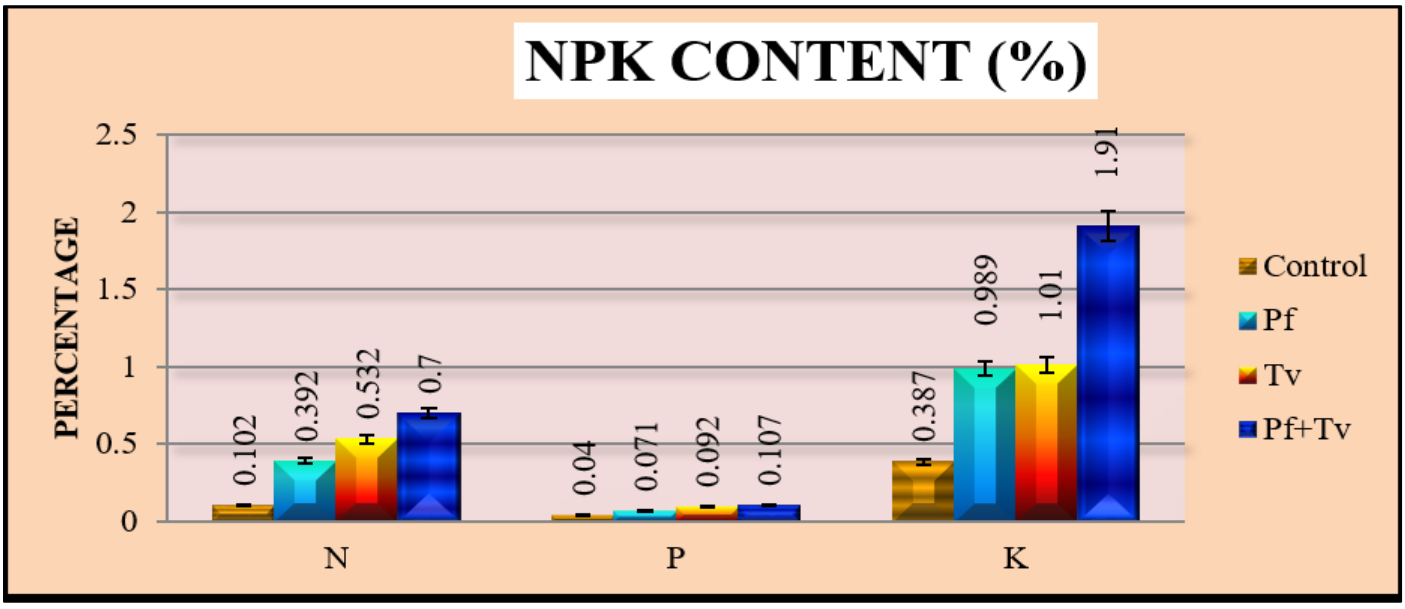

Fig 5: NPK Content (%) in Bioinoculant Treated and Control Plantlets

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Table 4: Micronutrient Content (PPM) in Bioinoculant Treated and Control Plantlets

Treatments	Zn	Fe	Cu	Mn	Micronutrient
Control	14	741	10	343	277
Pf	25	1574	16	774	597.25
Tv	26	1591	22	804	610.75
Pf+Tv	46	1723	38	885	673

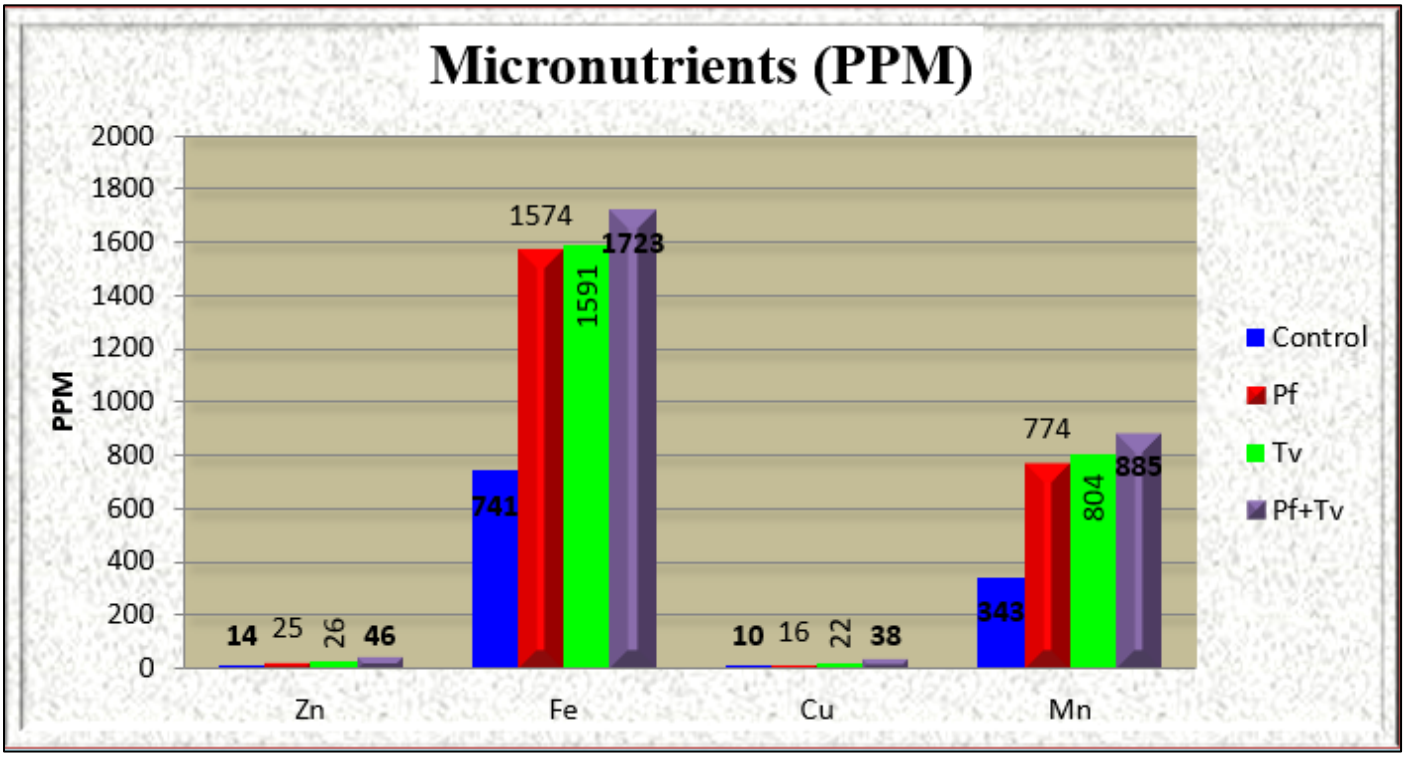

Fig 6 :Micronutrient Content (PPM) in Bioinoculant Treated and Control Plantlets

C. Effect of Biohardening on Plant Physiology- Water Retention Capacity (WRC)

The data on rate of water loss from leaves of biohardened and control plantlets during hardening were presented in Figure 7. The percentage loss of water is highest in control plants ranging from 20.35 to 78.56 % where as it is lowest in dual inoculant treated plants ranging from 5.49 to 29.81 %. When excised leaves of control plants were subjected to 18°C and RH of 50% for 1hour, water loss ranged 20.35 to 78.56 %. The leaves from bioinoculant treated plants displayed about 5.49% to 46.59 % reduction in water content after the same period of time. The percentage of water loss in Tv treated plants ranged from 10.16 to 32.7 % where as in Pf treated plants it ranged from 11.32 to 46.59 % (Table 5). The leaves of control plants showed highest reduction in water content (78.56 %).

Table 5: Water Retention Capacity in Bioinoculant Treated & Control Plantlets

Time Interval	Control	Pf	Tv	Pf+Tv
5	20.35±0.81	11.32±0.67	10.16±0.30	5.49±0.55
10	40.00±0.82	17.90±0.38	14.17±0.47	13.76±0.76
15	45.96±0.81	22.11±0.42	15.53±0.73	15.41±0.13
20	56.88±0.47	27.57±0.41	19.39±0.42	18.03±0.78
25	58.53±0.78	31.59±0.20	22.24±0.20	21.67±0.71
30	65.88±0.64	35.37±0.87	23.16±0.10	21.34±0.76
35	73.24±0.57	36.89±0.52	25.80±0.53	24.91±0.46
40	74.62±0.17	41.60±0.16	29.79±0.77	26.90±0.78
45	78.56±0.21	46.59±0.76	32.70±0.77	29.81±0.21

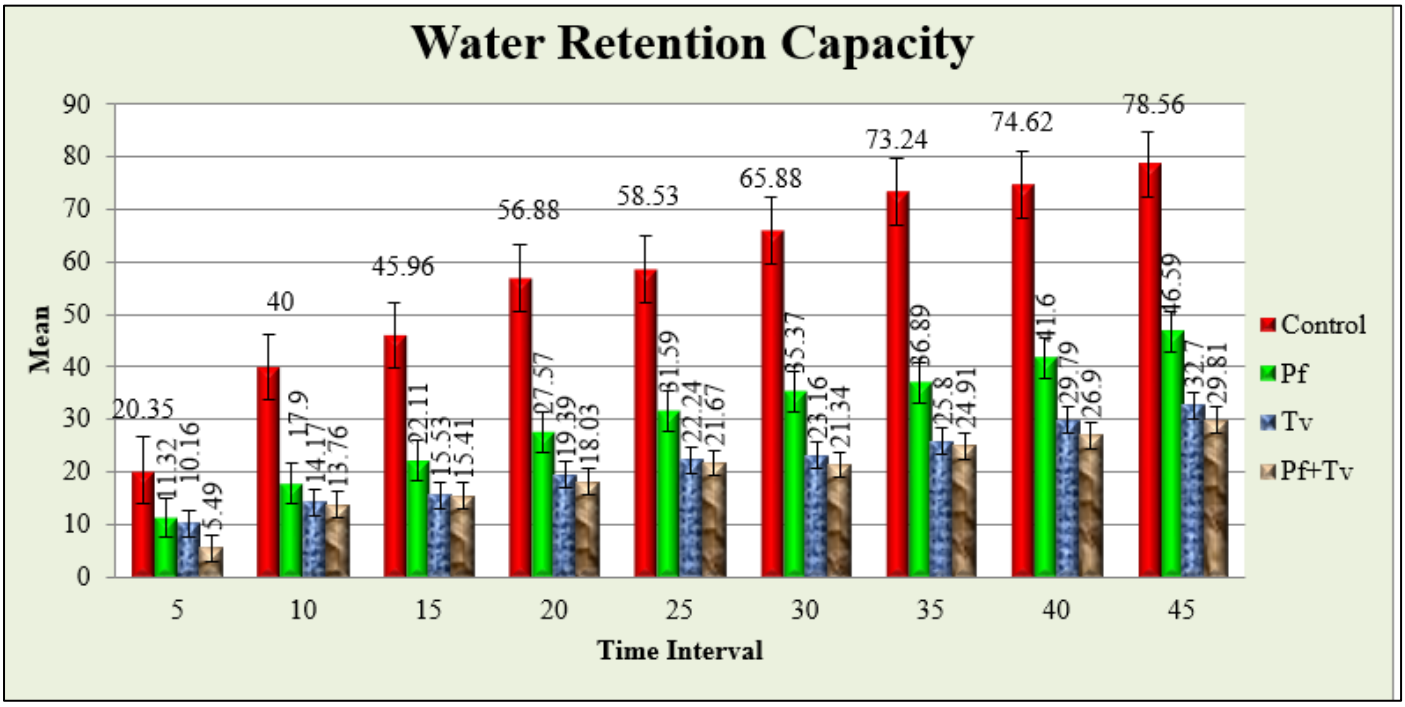

Fig 7: Water Retention Capacity in Bioinoculant Treated and Control Plantlets

IV. DISCUSSION

A. Effect of Biohardening on Plant Physiology- Autotrophic Activity:

The photosynthetic pigment quantitative analysis showed that the photosynthetic potential and primary productivity is highest in dual inoculum treated plants and least in control plantlets. The lower level of carotenoids and chlorophyll pigments in control indicated poor photosynthetic ability which was due to the inefficiency of photosynthetic apparatus for achieving a net positive carbon balance (Grout et al. 1986) Total chlorophyll contents significantly increased in inoculated plantlets, and this had contributed to high photosynthetic activity causing overall plant growth and survival of plantlets under *ex vitro* conditions. Inoculation of tissue culture plants with bioinoculants has been suggested for increasing stress resistance during acclimatization process, providing better establishment under *ex vitro* conditions and faster growth. The results were in coincidence with biohardened plants of *Boswellia* (Suthar 2012). The photosynthetic capacity per unit area of the leaf was indicated by its chlorophyll content ()Kozlowski 1991). It also determines the rate of photosynthesis in the plant (Bojovic 2005). Thus, plant productivity and its photosynthetic activity was indirectly estimated by its chlorophyll content (Dickmann et al. 1968).

The present study demonstrated that consortium (Pf+Tv) and single inoculation treated plants increased autotrophic activity by increasing photosynthetic pigments and total fructose content. Bioinoculants caused progressive increase in chlorophyll and carotenoids which was due to decreased stomatal resistances and increased gas exchange capacity. The plant roots colonized by *Trichoderma* strains alters the gene expression related to plant physiology there by establishing chemical signal communication which results in the development of resistance to pathogens and increasing photosynthetic efficiency (Harman 2012, Hermosa 2012)

In mycorrhizal plants, the photosynthates were converted to intermediate sugars like trehalose and mannitol by the fungal mantle. These carbon metabolites produce new fungal biomass, strengthen plant root system and promote efficient nutrient uptake and increased photosynthetic rate (Bougher et al. 1990).

Thus plantlets of *A.amara*, acclimatized using bioinoculants were well adapted to *ex vitro* conditions as identified by their high levels of photosynthetic pigments, net photosynthetic rate and low transpiration rate leading to enhanced growth. Plant stress and nutritional deficiencies can be indirectly estimated by measuring its chlorophyll content. Dual inoculant treated plantlets scored highest chlorophyll content indicating improved photosynthetic efficiency during hardening progresses. Thus, the restoration of the autotrophic activity in biohardened plants was due to increase in chlorophyll, carotenoid pigments and total fructose content (Damiano et al. 2007).

B. Effect of Biohardening on Plant Physiology- Nutrient Content

The result of the present study showed that combination of the bioinoculants together, improved plant growth, thereby showing the symbiotic association of *P. fluorescens* with *T. viride*. Mixed inoculum treated plants produce more organic acids and improves phosphorus accessibility (Avis et al. 2008). Apart from the provision of macro and micronutrients to plantlets the bioinoculants like *T. viride* and *P. fluorescens* can efficiently produce secondary metabolites and plant growth promoting substances which enhance plant growth and promote nutrient uptake besides having biocontrol activity (Burr et al.1978, Harman et.al 2012).

Increased levels of P-content and micronutrients in dual inoculum treated plants explain their crucial role in phosphorus absorption from soil by extending their mycelium in to the root system of *A.amara*. The extramatricular mycelia deeply penetrate into the soil and helps in efficient uptake of phosphorus and micronutrients. Similar studies on phosphorus uptake by the plants inoculated with *P.indica* were reported (Gosal et al. 2010, Verma et al. 1998). In the present investigation, the plant biomass was observed to be positively affected by dual as well as single inoculations. Similar studies on increase in biomass and yield was observed during root colonization of a wide range of host plants by endophytic fungus, *P. indica* (Serfling et al. 2007). The enhanced vegetative growth of dual inoculant treated *A.amara* plantlets was due to the ability of the fungus to colonise plant roots efficiently. This in turn increased the solubility of phosphates, better nutrient absorption which enhanced plant biomass (John et al. 2010). The present work is in accordance with the other studies that a combination of *Pseudomonas* and arbuscular mycorrhiza fungi resulted in acquisition of more nitrogen and phosphorus [1]. Prominent increase in the NPK content indicates that bioinoculants played a vital role in the solubilisation of soil nutrients.

By increasing access to the growth-limiting nutrients, fungal hyphae can significantly enhance carbon fixation and increased photosynthetic rates (Smith et al. 1997). Similar observations has been reported for both endo- and ectomycorrhizas (Reid et al.1983). Thus higher photosynthetic rates in terms of chlorophyll content and biomass was recorded in biohardened plants. These studies are in coincidence with previous reports that the higher net photosynthetic rates is correlated with the effective microbial colonization of plant roots 9Nylund et al. 1989, Reid et al.1983). The main reason behind the increased photosynthetic rates was due to increased micro and macro nutrient absorption. Similar studies on improved nutrient status of biohardened plants in increasing the net photosynthetic rate has been reported for different species (Bougher et al. 1990, Longstrength et al. 1980 , Natr 1972, Osman et al. 1977).

Thus sustainable utilization of soil nutrients by plants helps to increase root length and root number which would have contributed to the increase in biomass. Similar studies on higher nutrient content was reported in the tea plants treated with mixed inoculum (Jibu et al. 2010)

C. Effect of Biohardening on Plant Physiology- Water Retention Capacity (WRC)

The high percentage of water loss in tissue culture plants was due to its morphological and physiological abnormalities. The reason behind rapid water loss in the initial stages of hardening could be due to less wax on leaves. Poor stomatal anatomy, high transpiration rate are the other factors which contribute to high water loss during initial stages of hardening (Damiano et al. 2007). High efficiency in controlling water loss by biohardened plants under dehydrating conditions was due to the active functioning of stomata (Frommel et al. 1991). Low ratio of potassium to other elements was related to impaired functioning of the stomata in control plants of *A.amara*. Similar reports on K-content in relation to stomatal functioning of tissue culture plants was reported in *Solanum lacinium* (Elkund 1968). Another main concern is that microplants often have a poor water retention capacity. According to Santamaria and Herstiens (1994) poor WRC is caused both by a poorly developed cuticle and by malfunctioning stomata.

The bioinoculants used in the present study helped in efficient nutrient and water uptake and promoted resistance against soil borne diseases. Effective plant microbial interaction facilitates the easy accessibility of mineral nutrients. Microbial association promotes hydraulic conductivity of the roots and improves plant -water relations thereby preventing wilting and desiccation of tissue culture plantlets.

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*. The autotrophic activity, water retention capacity and nutrient uptake of biohardened plants was significantly more compared to untreated plants. An improvement in plant physiological characteristics like chlorophyll content (1125.59 ug/g FW), carotenoids (335.35 ug/g FW), fructose content (58.02 mg/g DW), NPK Content (0.91%), micronutrient content (673 PPM) and water retention capacity was good in mixed inoculum (Pf+Tv) treated plants.

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REFERENCES

[1]. Akhtar MS and Siddique ZA (2008). *Glomus intraradices*, *Pseodomonas alcaligenes* and *Bacillus pumilus*: Effective agents for the control of root -rot disease complex of chick pea (*Cicer arietinum* L.). Journal of General Plant Pathology. 74: 53–60.

[2]. AOAC (1995). Official Methods of Analysis,16th Edn. Association of Official Analytical Chemistry, Washington, DC.

[3]. Avis TJ, Gravel V, Antoun H and Tweddell RS (2008). Multifaceted beneficial effects of rhizosphere microorganisms on plant health and productivity. Soil Biology and Biochemistry. 40:17-33.

[4]. Black CA. (1965). Methods of soil analysis. Part I. Am. Soc. Argon. Inc. Publi. Madison Wisconsin USA.
[5]. Bojovic B and Stojanovic J. (2005). Chlorophyll and carotenoid content in wheat cultivars as a function of mineral nutrition. Archives of Biological Sciences. 57(4): 283−290.
[6]. Bougher NL, Grove TS and Malajczuk N. (1990). Growth and phosphorus acquisition of karri (Eucalyptus diversicolor F. Muell.) seedlings inoculated with ectomycorrhizal fungi in relation to phosphorus supply. New Phytol. 114: 77.
[7]. Brix H. (1971). Effects of nitrogen fertilization on photosynthesis and respiration in Douglas -fir. For Sci. 17: 407-414.
[8]. Burr TJ, Schroth MN and Suslow TV (1978). Increased potato yields by treatment of seed pieces scientific strains of *Pseudomonas fluorescens* and *P. putida*. Phytopathology. 68: 137.
[9]. Damiano C, MD Arias P, La Starza SR and Frattarelli A (2007) . Temporary Immersion System for Temperate Fruit Trees. Acta Hort. 748: 87-90.
[10]. Davis JS and Gander JE (1967). A re-evaluation of the Roe procedure for determination of fructose. Anal. Biochem. 19: 72–79.
[11]. Debergh PC (1991). Acclimatization techniques of plants from in vitro. ActaHortic. 289: 291–300.
[12]. Dickmann DI and Kozlowski TT. Mobilization by *Pinus resinosa* cones and shoots of C-14 photosynthate from needles of different ages. American Journal of Botany. 55: 900−906.
[13]. Elkund E (1968). Secondary effects of some Pseudomonads in the rhizoplane of peat grown cucumber plants. Acta Agric Sc and Suppl. 17 (1970) 1-57 .
[14]. Frommel MI, Nowak J and Lazarovits G (1991). Growth Enhancement and Developmental Modifications of in Vitro Grown Potato (*Solanum tuberosum* ssp. *tuberosum*) as Affected by a Nonfluorescent *Pseudomonas* sp. Plant Physiol. 96: 928-936.
[15]. Gosal SK, Karlupia A, Gosal SS, Khhibba IM and Varma A (2010). Biotization with *P. indica* and *P. fluorescens* improves survival rate, nutrient acquisition,field performance and saponin content of micropropagated *Chlorophytum* species. Ind J Biotechnol. 9: 289-297.
[16]. Grout B WW, Taffs J and Donkin ME (1986). Sucrose independent strawberry cultures and indication of further development in micropropagation, Ann Botany (London) 55(1):129-131.
[17]. Harman GE, Herrera-Estrella AH, Horwitz BA and Lorito M (2012). *Trichoderma* – from basic biology to biotechnology. Microbiology. 158 (Special Issue) 1–2.
[18]. Hermosa R, Viterbo A, Chet I and Monte E (2012). Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology. 158: 17–25.
[19]. Indravathi G and Pullaiah T. (2013). In vitro propagation studies of *Albizia amara* (Roxb.) Boiv.African Journal of Plant Science.7: 1-8.
[20]. Indravathi G and Suresh Babu P (2019). Enhancing acclimatization of tissue cultured plants by Biotization – a review. Int. J. of Scientific Research and Reviews. 8 (3): 564-575.
[21]. Indravathi G and Suresh Babu P (2019). Enhancing acclimatization of tissue cultured plants of *Albizia amara* by biotization. Int. J. Sci. Res. in Biological Sciences, 6 (4): 43-50.
[22]. Indravathi G and Suresh Babu P (2020). Strategies to improve rooting and acclimatization of *Albizia amara* (Roxb.) Boiv. Journal of Advanced Scientific Research. 11(6): 93-100.
[23]. Jibu Thomas, Ajay D and Raj Kumar R (2010). Influence of beneficial microorganisms during in vivo acclimatization of in vitro-derived tea (*Camellia sinensis*) plants. Plant Cell Tiss Organ Cult, 101:365.
[24]. John RP, Tyagi RD, Prevost D, Brar SK, Pouleur S and Surampalli RY (2010). Mycoparasitic *Trichoderma viride* as a biocontrol agent against *Fusarium oxysporum*. sp. adzuki and *Pythium arrhenomanes* and as a growth promoter of soybean. Crop Protection. 29: 1452.
[25]. Koenig RA and Johnson CR (1942). Colorimetric determination of biological materials. Ind. Eng. Chem. Analyt. Edn.14: 155-156.
[26]. Kozlowski TT, Kramer JP and Pallardy GS (1991) .The physiological ecology of woody plants. Academic Press, Inc. San Diego. New York. 37−44.
[27]. Lichtenthaler, HK and Buschmann C (2001) . Chlorophylls and carotenoids – Measurement and characterisation by UV-VIS. Current Protocols in Food Analytical Chemistry (CPFA), John Wiley, New York. (Supplement 1) F 4.3.1 - 4.3.8.
[28]. Lindsay WL and Norvell WA (1978). Proc. Soil Sci. Soc.Am.42: 421-428.
[29]. Longstrength DJ and Nobel PS (1980). Nutrient influences on leaf photosynthesis. Effects of nitrogen, phosphorus and potassium for *Gossypium hirsutum* L. Plant Physiol. 65: 541-543.
[30]. Natr L (1972). Influence of mineral nutrients on photosynthesis of higher plants. Photosynthetica. 6: 80-99.
[31]. Nowak J. (1998) Review benefits of *in vitro* "biotization" of plant tissue cultures with microbial inoculants. In vitro Cell Dev Biol Plant. 34: 122.
[32]. Nylund JE and Wallander H (1989) . Effects of ectomycorrhiza on host growth and carbon balance in a semi-hydroponic cultivation system. New Phytol. 112: 389-398.
[33]. Osman AM, Goodman PJ and Cooper JP (1977). The effects of nitrogen, phosphorus and potassium on rates of growth and photosynthesis of wheat. Photosynthetica. 11: 66-75.
[34]. Reid CPP, Kidd FA and Ekwebelam SA (1983). Nitrogen nutrition, photosynthesis and carbon allocation in ectomycorrhizal pine. Plant Soil. 71:415-431.
[35]. Santamaria JM and Herstiens G (1994). The lack of control of water loss in micropropagated plants is not related to poor cuticle development. Physiologia Plantarum. 91: 191-195.

[36]. Sastry CV, Rukmini C and Ramachandra Rao L (1967), Chemistry of Saponins: part III – Isolation of new flavonol Glycoside, 4'-0- Methylquercetin-3-rutinoside, from *Albizia amara* Benth. Indian J.Chem. 5: 613.
[37]. Serfling A, Wirsel SGR, Lind V and Deising HB (2007). Performance of the Biocontrol Fungus *Piriformospora indica* on : 531.
[38]. Smith SE and Read DJ (1997). Mycorrhizal symbiosis Academic Press, London.
[39]. Suthar RK and Purohith SD (2012). Biopriming of micropropagated *Boswellia serrate* Roxb plantlets role of endophytic root fungus P. indica. Ind J Biotech. 11: 304.
[40]. Verma S, Varma A, Karl-Heinz R, Hasse IA, Kost G *et al.*,(1998) Piriformospora indica gen. nov. a new root-colonizing fungus, Mycologia USA. 90: 895.