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Enhancing Acclimatization of Tissue Cultured Plants By Biotization- A Review

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ABSTRACT

The plants raised from tissue culture experience high mortality following laboratory to land transfer. Apart from the abiotic causes, one major cause of high mortality of such aseptically raised plants is their sudden exposure particularly the root system to microbial communities, including soil pathogens. Efficient antagonistic plant growth-promoting microbes have been used for hardening to increase the survival and to augment overall plant growth. Biotization is the metabolic response of *in vitro* grown plant material to microbial inocolum. Mycorrhization of tissue cultured plants is believed to provide an advantage to the transplanted propagules in terms of nutrient availability, soil pH, aeration and protection from water stress. The tissue culture raised plants when treated with bacterial inoculants produce plant growth promoting substances and some secondary metabolites which enhance nutrient uptake and provide resistance against pathogens. Multimicrobial biotization is inoculation of more than one microbial species to micro plants. Plantlet survival rate was maximum in dual inoculation, this must be due to the positive interaction between two or more species and their ability to enhance stress tolerance by protecting them from subsequent 'transplantation shock'. Thus biological hardening envisages physical, chemical and environmental conditioning of the micro propagated plantlets. Different parameters were reported for control and biotized plantlets to know the effect of microbes on plant performance. This review is focused on the effect of biotization on enhancing acclimatization of tissue culture raised plants.

KEY WORDS: Biotization, acclimatization, micropropagation, biotic factors.

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INTRODUCTION:

Tissue culture raised plants experience high mortality following laboratory to land transfer. One major cause of high mortality of aseptically raised plants apart from the abiotic factors is their sudden exposure, in particular, the root system to microbial communities, including minor and major pathogens, present in the soil. The primary target of several research groups attempting utilization of microbial inoculants in micropropagation is to induce stress resistance[1].

Biotization is the metabolic response of in vitro grown plant material to microbial inoculum, leading to the morphological and physiological development enhancing biotic and abiotic stress resistance of the derived propagules. Biotization is an emerging dimension of micropropagation technique where the growth of the host plant is promoted by the formation of secondary metabolites related to plant defense. Such systems allow for mutual adaptation between the host plant and the introduced bacteria. Bacterized plantlets not only grow faster than nonbacterized plantlets but they are sturdier and have a better-developed root system [2].

The inoculation of seeds with beneficial microorganisms has been practiced for many years, but the inoculation of tissue culture raised plantlets is an innovative aspect. Plant tissue culture is based on aseptic conditions, hence microorganisms including beneficial endophytes are treated as the problem causing contaminants. But nowadays microbial inoculants, primarily bacteria, and mycorrhizae are being evaluated as biopriming agents for successful transplanting. Biotization could be achieved during in vitro rooting or under ex vitro conditions. Thus efficient antagonistic plant growth-promoting bacteria used for biological hardening envisages physical, chemical and environmental conditioning of the micropropagated plantlets.

All beneficial microorganisms helped in the enhanced uptake of nutrients, nitrogen fixation, resistance to soil-borne diseases and improved plant water relations. Although the soils are not deficient in phosphorous, its unavailability in phosphorous fixation is found to be a major constraint in acidic soils. Effective microbes aids in the uptake of mineral nutrients in the available form where the normal plant roots fail. The microbial association helps in the improved hydraulic conductivity of the roots which contributes to the better uptake of water thereby resulting in the reduction of desiccation and wilting in immature plantlets. These favorable conditions help to increase the number of lateral roots and root length. Also, there was an increase in the activity of all the defense-related enzymes like phenylalanine ammonia lyase, peroxidase, and β-1,3-glucanase in the leaves of the plants under treatment [3]. Lower incidence of rotting and wilting diseases was

noticed in bioinoculant-treated plantlets. This suggests that bioinoculants were capable of eliciting systemic resistance.

Apart from bacteria and fungi, other endophytes like algae, amoebae, virus, archae, oomycetes also show a symbiotic association with plants[4]. For e.g., Green algae *Coccomyxa species* in *Ginkgo biloba*[5] and amoebal cysts in *Eleutherococcus sieboldianus*[6].

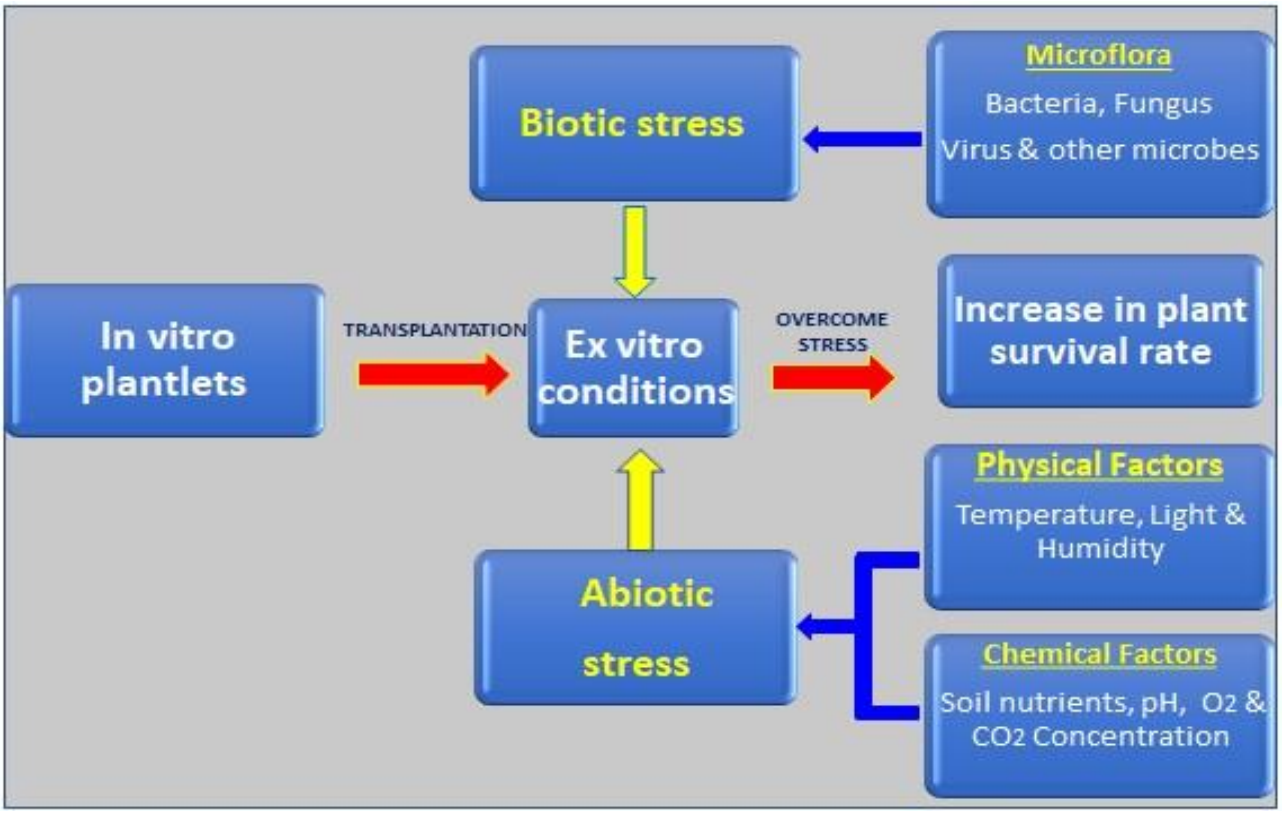

Fig.1: Effect of Abiotic and Biotic Factors on Acclimatization of Tissue Culture Raised Plants.

MYCORRHIZATION:

Acclimatization phase is a key step in the micropropagation cycle as it affects the survival and growth of the in vitro produced plantlets. There are many examples showing that the inoculation with Arbuscular Mycorrhizal Fungi (AMF), at the time of transplant from axenic to in vivo conditions, significantly improves plant survival and growth.Mycorrhization of tissue cultured plants is believed to provide an advantage to the transplanted propagules in terms of nutrient availability, soil pH, aeration and protection from water stress. An excellent review of factors affecting the result of mycorrhizal inoculation (timing, medium, fertilization, inoculation, fungus-host specificity, growth substrate, etc.) was published[7].

Mycorrhization has been proved beneficial for many other microplants of raspberry, wild cherry, apple, pear, grapevine, rose, oil palm, citrus, banana, asparagus, pineapple, common ash,

artichoke[8,9]. By enhancing access to the growth-limiting nutrients both ectomycorrhizae and endomycorrhizae can significantly increase carbon fixation of the plantlets10. This gain occurs primarily via increased photosynthetic rates. Mycorrhizal plants can also take up more carbon in drought periods than non-mycorrhizal ones, since they can maintain stomata open at lower soil water potentials.

Plants inoculated with ectomycorrhiza showed increased phosphorus and nitrogen uptake 11. In ectomycorrhizas, photosynthates move from the autotroph to the fungal mantle, where they are rapidly converted to metabolic intermediates, of which trehalose and mannitol are dominant. These carbon compounds are used for sustaining the fungal biomass existing in the mycorrhizal root tips of the soil mycelia network and also for producing new fungal biomass. As the photosynthetic rate is primarily limited by the accumulation of its end products in the leaf cells, the drain of carbohydrates by the mycobiont may offset end product limitation facilitating an increased photosynthetic rate. Due to this RuBisCO activity, chlorophyll and protein content of leaves of mycorrhizal plants, expressed in terms of fresh weight or of dry weight was reported significantly higher than in control plants. The increased photosynthetic rates were also due to increased N, P and K absorption[57]. Relationships between foliar phosphorus and net photosynthetic rate in non-mycorrhizal and ectomycorrhizal were compared in Pine seedlings[12].

Among endomycorrhiza, Vesicular Arbuscular Mycorrhiza (VAM) plays a significant role in phosphorus uptake and translocation in addition to uptake of zinc, sulfur, and copper. VAMs are exceptional to establish a symbiotic relationship in the roots of higher plants. A careful selection of functionally compatible host fungus substrate combination was essential for the early establishment of VAM in the nursery or in an open field of major horticultural crops.

The mycorrhizal dependency of banana has been extensively studied by various researchers, emphasizing its importance during hardening stages[13]. A marked increase in the uptake of P, Ca, Mg, Zn, Cu and reduced disease severity was observed in the mycorrhizal plants of banana[14]. It was reported that VAM fungi – Glomus fasciculatum inoculated at the hardening stage helped the banana plantlets to accumulate maximum plant height, root length, biomass, root colonization and nutritional quality [15]. Similar results on growth characters and root colonization of papaya plants with G. message and G. fasciculatum were reported. Inoculation of micropropagated sugarcane seedlings with G. diazotrophicus made the plants not only grow faster but also ensured efficient N2-fixing plants in fields[16]. It was also studied that the effect of inoculation of the fungus, Glomus intraradices increased the survival, growth, biomass production and nutritive status of cassava, grape and olive plants during hardening.

AMF can contribute to plant growth and survival by reducing the stress associated with

nutrition, water, aeration, soil structure, pH, salt, toxic metals, and pathogens. Biotization of AMF symbiotic endophytes also protect the juvenile axenic plants from an infestation of the harmful saprophytes. It was reported that AM fungi established a symbiotic association with hazelnut (Corylus avellana L.)[17]. Micropropagated plants of Ranunculus asiaticus inoculated by AMF showed an earlier flowering, an improved flower production and better rhizome yield[18]. The colonization of Glomus, P.indica and Trichoderma species is also known to reduce the osmotic potential of plants and also induced in vivo rooting in transplanted micro shoots of C. borivilianum giving rise to more than 50% establishment[19]. Mycorrhization enhanced the growth of micropropagated Chestnut plants, increased their protein content and photosynthetic rates, decreased respiratory rates and CO2 compensation point.

Piriformospora indica, a root endosymbiotic fungus, mimics AMF in many morphological, functional aspects and growth promotion. It was reported that *P. indica* treated plantlets of B. serrata showed an increase in total biomass production and more than 75 percent ex vitro survival in comparison to control plantlets[21]. In particular, P.indica helps in phosphorus acquisition and works as a biocontrol agent. A similar high degree of ex vitro survival of micropropagated plantlets of Artemisia annua, Nicotiana tobbacum, B. monnieri, T. bellerica, and F. limonia colonized with *P. indica* was reported[22]. A positive influence of root colonization was reported with *P. indica* on overall growth and development in micropropagated plantlets of T. bellerica[23]. P.indica were more resistant to pathogens and more tolerant to salt stress and showed higher yield [24].

The profound effect of T. viride on root initiation from in vitro micropropagated Neem shoots had been reported [25]. It was reported that T. viride when applied alone to Broccoli showed maximum N and P content in roots and shoots[26]. The enhanced vegetative growth of broccoli in Trichoderma treated plants could be due to the root-colonizing ability of the fungus that resulted in better nutrient absorption through increased root biomass.

BACTERIZATION:

The tissue culture raised plants when treated with bacterial inoculants produce plant growth promoting substances and few secondary metabolites that enhance nutrient supply and provide resistance against pathogens. These useful soil bacteria are named as plant growth promoting rhizobacteria (PGPR), which are preferentially related to the roots. PGPR include many bacterial genera like *Pseudomonas, Bacillus, Azospirillum, Azotobacter*, etc. Bacterial infection occurs through roots and they are translocated to all parts of the plant via the xylem, the aerenchyma and through interconnecting intercellular spaces[27]. These diazotrophs would have affected plant growth by the synthesis of phytohormones and vitamins, inhibition of plant ethylene synthesis and improved

nutrient uptake[28]. Bacterized plantlets were greener, had elevated levels of cytokinins, phenylalanine ammonia lyase, and free phenolics and contained more lignin[29]. Both in vitro and ex vitro benefits of bacterization relied on plant species, cultivar, and growth conditions.

Among the PGPR, *Pseudomonas* deserves a special mention because it improves the plant growth directly or indirectly by the production of plant growth substances, increasing the uptake of certain nutrients from the soil and additionally shows antagonistic effects against some important plant pathogenic microorganisms. In addition to this *Pseudomonas* species have been used to enhance tolerance to transplanting stress in potato[62]. In vitro co-cultivation of soybean cotyledon explants with two strains of *Pseudomonas maltophilia*, stimulated the development of nodular callus with high regeneration potential[30]. It was reported that *Pseudomonas* strain PsJN enhanced the tolerance to transplanting stress in potato and was found the most effective plant growth promoting bacterium under in vitro conditions[31]. The Oregano plantlets co-cultured with *Pseudomonas spp*. prevented vitrification and contained a lot of phenolics and chlorophyll than non bacterized controls[32]. Greenhouse experiments also demonstrated that plants derived from dual cultures of potato and the pseudomonad bacterium had a larger root system, and gave better tuber yield than control[33]. The tea plants inoculated with *Bacillus subtilis* and *Pseudomonas corrugate* acted as biocontrol agents and were able to defend pathogenic attack probably due to their antagonistic properties[34].

Three plant growth-promoting rhizobacteria viz. *Bacillus megaterium, B. subtilis and Pseudomonas corrugata* were used for biological hardening of micro-propagated plantlets of *Picrorhiza kurrooa*[35]. These bacterial isolates antagonized the pathogenic fungal species and positively influenced survival and growth parameters. The in vitro grown Oil palm plantlets inoculated with *Azospirillum brasilense* produced higher root and shoot biomass and more secondary roots[36]. Endophytic colonization in rice callus induced by using *Azorhizobium caulinodans* improved yield, grain weight and nutritional quality[37].

Thus the reports suggest the use of efficient antagonistic plant growth-promoting bacteria for biological hardening increase plant survival and augment overall plant growth of micro propagated plants.

MULTIMICROBIAL INTERACTION:

Multimicrobial biotization is inoculation of more than one microbial species to microplants. Root colonization by mycorrhizal fungi will have an affect the chemical composition of root exudates. The development of mycelium around roots (mycorrhizosphere) modifies their physical atmosphere and provides a new source of carbon to the microbial community. The development of a

mycorrhizosphere has both qualitative and quantitative repercussions on microbial populations in either the rhizosphere or rhizoplane[38]. Mycorrhization increases the dichotomy of roots and creates new compartments (mycorrhizosphere and microsphere), for the growth of symbiotic microbes.

Mycorrhizae-Helping Bacteria (MHB) enhance the growth of the plants. It was reported that rhizosphere strains of *Bacillus mycoides* and *Pseudomonas fluorescens* promoted AMF formation in various crop plants by improving susceptibility of roots to AMF[39]. Other rhizosphere microorganisms which are known to act as a phytostimulators or which possess antagonistic activities toward plant pathogens may be used in conjunction with AMF for biotizing microplants. They include bacteria like *Pseudomonas spp*. and Bacillus *spp*., and fungi such as Gliocadium *spp*. and *Trichoderma spp.* Plantlet survival rate was maximum in Trichoderma spp. inoculation, this must be due to the positive interaction among the species and their ability to enhance stress tolerance by protecting them from subsequent 'transplantation shock'.

It was found that the combined use of *Glomus mosseae* and *Pseudomonas fluorescens* caused a greater increase in plant growth of tomato as compared to the individual application[40]. Studies showed that plant-mediated interactions among *Pseudomonas fluorescens*, Rhizobium *leguminosarum*, and AMF on Pea enhanced nodulation by four fold[41]. *P. fluorescens* and ectomycorrhizal fungus, Suillus granulatus, used as dual inoculants for Pinus halepensis showed significantly high number of lateral rootlets as compared to single inoculations [42]. Better root system helped in more nutrient uptake, which resulted in healthy plants with more shoot biomass.

Agrobacterium rhizogenes causing the hairy-root syndrome in dicots is being applied successfully for the promotion of growth of roots and their effective mycorrhization 13. 100% plant survival was observed after biotization of micropropagated raspberry with Agrobacterium radiobacter and *Glomus mosseae* [64]. It was reported that dual inoculation of potato microplants with *P.fluorescens* and mycorrhizal fungi enhanced plant growth and protected against soil-borne potato pathogen Rhizoctonia solani[43]. Trichoderma sp. DB11and Gliocladium catenulatum were inoculated in the potting substrates of micro propagated strawberry in order to promote growth and protect against root and collar rot induced by *Phytophthora spp*[44].

Biotization of micropropagated *Chlorophytum sp*. with the fungus, *Piriformospora indica* and the bacterium, *Pseudomonas fluorescens*, improved plantlet survival rate, growth parameters, field performance, micronutrient acquisition, alkaloid and saponin content[45]. Significantly higher root-shoot biomass was reported in different crops like Maize, Bacopa, Poplar with dual *inoculation of P.fluorescens and P. indica* inoculation[65]. Maximum colonization of *P. indica* and bacteria in dual inoculated plants attributed the fact that mycorrhizal root tips tend to support slightly higher populations of *Pseudomonas* than non-mycorrhizal root tips, possibly due to the provision of

additional colonization sites or altered root exudation in mycorrhizosphere.

It was reported that acclimatization of in vitro rooted tea plantlets in soil amended with bioinoculants like *Pseudomonas fluorescens*, Azospirillum *brasilense*, and *Trichoderma harzianum*, either individually or in various combinations, promoted plantlet survival [46] .

Table: 1 Effect of Various Bioinoculants on Plant Performance

S.No.	Bioinoculant	Plant	Effect
I	**Monomicrobial Interaction - Mycorrhization**		
1	Ectomycorrhizae	Pine	Increase in phosphorus uptake and net Photosynthetic rate[11,12].
2	*Glomus fasciculatum*	Banana	Increase in plant biomass[14]
3	*Glomus message*	Papaya	Increase in nutritional quality[50]
4	*Glomus diazotrophicus*	Sugarcane	Efficient Nitrogen fixation[16]
5	*Glomus intradices*	Cassava,Grape,Olive	Increase in plant survival and nutrient status[51,58]
6	AM fungi	Hazlenut	Resistance towards soil pathogens[17]
7	AM fungi	*Ranunulus asiaticus*	Earlier flowering ,improved flower production and better rhizome yield[18]
8	*Piriformospora indica*	*C.borivilianum*	Invivo rooting[19]
9	*Mycorrhizae*	Chestnut	Increase protein content and photosynthetic rate[20]
10	*Piriformospora indica*	*B.serrata*	Increase in plant biomass and phosphorus uptake[21,23]
11	*Piriformospora indica*	*T.bellerica*	Increase in plant survival and photosynthetic rate[52]
12	*Piriformospora indica*	Barley	Resistance to pathogens and tolerance to salt stress[24]
13	*Trichoderma viride*	Broccoli	Maximum N and P content in roots and shoots[26]
14	*Trichoderma viride*	Neem	*In vitro* rooting[25]
II	**Monomicrobial Interaction - Bacterization**		
15	*Pseudomonas*	Potato	Enhance tolerance to transplantation stress[31]
16	*Pseudomonas*	Potato	Large root system and better tuber yield[53]
16	*Pseudomonas*	Soyabean	Increase in Plant survival[54]
17	*Pseudomonas*	Oregano	Prevents vitrification,Increase in phenolic and chlorophyll content[32]
18	*Bacillus subtilis*	Tea	Resistance towards pathogens[34]
19	*Bacillus megaterium*	*Picrorhiza kurrooa*	Induced systemic resistance[35]
20	*Azospirillum brasilense*	Oil Palm	Increase root and shoot biomass[36]
21	*Azorhizobium caulinodans*	Rice	Improved grain weight and nutritional quality[37]
III	**Multimicrobial Interaction**		
22	*Glomus mossae* + *P.fluorescens*	Tomato	Increase in yield[40]
23	*P.fluorescens* + AMF + *Rhizobium leguminosarum*	Pea	Increased nodulation by four fold[41]
24	*P.fluorescens* + Ectomycorrhizae	Pinus	Increase in root and shoot biomass[42]
25	*A.radiobacter* + *Glomus mossae*	Raspberry	Improved plant survival[55]
26	*P.fluorescens* + *AMF*	Potato	Protection against soil borne pathogens[43]
27	*Trichoderma* + *Gliocladium catenulatum*	Strawberry	Resistance against root rot and collar rot[44]
28	*P.fluorescens* + *P.indica*	Chlorophytum	Efficient nutrient uptake and increased secondary metabolite content[19,45]

29	*P.fluorescens* + *P.indica*	Maize,Bacopa,Poplar	High root -shoot biomass[56]
30	*P.fluorescens* + *A. brasilense* + *T. harzianum*	Tea	Prevented wilting and root rot[46]
31	*P.fluorescens* + *T.viride*	Tea	Increase in phosphorus uptake[47]
32	*P.fluorescens* + *T.viride*	Cotton	Increase in yield[49]
33	*P.fluorescens* + *T.viride*	Soyabean	Resistance against root rot and stem rot[48]

Root rot or wilting of tissue culture derived plants was not observed in bioinoculant-treated plants, as they possessed relatively higher activities of defense enzymes, including peroxidase and phenylalanine ammonia lyase. The synergistic effect of *T. viride* with *P. fluorescens* was that it can solubilize more P in the soil by producing organic acids[47]. Similarly, their combined effect on growth improvement was also reported by other workers[48,49] .

The application of multimicrobial biotization requires knowledge and understanding of the compatibility between different beneficial microorganisms in their interaction within the mycorrhizosphere, rhizosphere, and rhizoplane.

CONCLUSION:

Microplants represent an ideal material for developing basic research on microbial biotization and for producing results which can be easily applied in technology transfer. Microplants produced in vitro are usually transplanted into an inert substrate, a relatively simple environment, where the development of the introduced microbe and their impact on plant growth can be easily monitored. A drastic increase in our knowledge about microbial and microbial/plant interactions in the rhizosp here, and particularly of those that are considered beneficial to plant development, is necessary to produce the best results in micropropagation.

In the hardening technique, biotization economizes the production process and simplify the micropropagation technique, which could be adopted at village centres to extend the scientific technology from lab to land. Development of new culture methods allowing the establishment of stable associations between plants and beneficial organisms in vitro and ex vitro and understanding of mechanisms of signal recognition and transduction in plant-microbial associations under different environments are probably the most critical elements of this challenge.

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