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Enhancing acclimatization of *in vitro* raised potato seedlings via biological hardening

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ABSTRACT

Potato is an autotetraploid tuber crop of global significance, widely propagated through tubers. However, conventional propagation methods face challenges such as low multiplication rates and disease susceptibility. Micropropagation *via* tissue culture enables large-scale production of disease-free plantlets, but high mortality during acclimatization remains a major limitation due to physiological and structural deficiencies. Biological hardening, involving beneficial microorganisms presents a promising strategy to enhance plantlet survival by improving rhizogenesis, nutrient availability, and stress tolerance. In this study, various biological hardening agents like *Bacillus subtilis*, *Pseudomonas flourescens* and *Trichoderma viride* with or without glycerol (0.5%), were tested to identify the optimal combination for maximizing survival rates during acclimatization. Out of all the treatment combinations application of *Trichoderma viride* along with glycerol (0.5%) gave highest plantlet survival (73.33%) after 3 weeks of acclimatization and all the treatments were found superior to the untreated control. Whereas, maximum increase in plant height was obtained when *Bacillus subtilis* + *Pseudomonas flourescens* (consortia) was applied with glycerol (0.5%). The results highlight the effectiveness of microbial inoculation in reducing stress, enhancing growth, and improving the establishment of tissue-cultured potato plantlets. This study underscores the potential of biological hardening as a sustainable approach for improving micropropagation efficiency and large-scale seed tuber production.

Key words: Biological hardening, anti-transpirant, bio-agents, micropropagation, tissue culture.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a natural autotetraploid (2n = 4x = 48) of the family Solanaceae and ranks as the fourth most important food crop globally after wheat, rice, and maize. Native to the tropical South American region, it exhibits wide genetic diversity. Potato is propagated both sexually (true potato seed) and asexually (tubers), with seed tubers commonly used for multiplication. However, conventional tuber propagation has limitations, including low multiplication rates and high disease susceptibility (Mirza *et al.,* 13). Micropropagation offers a biotechnological solution to produce disease-free, genetically uniform potato plantlets via clonal propagation, enabling large-scale production of healthy microtubers. Despite its efficiency, micropropagation faces challenges during the transition from in vitro to ex vitro conditions, where plantlets experience high sensitivity to abiotic (temperature, light, humidity) and biotic (soil microorganisms) stresses, often resulting in significant losses (Nguyen *et al.,* 16). Acclimatization is critical for survival, requiring gradual adaptation to greenhouse and field conditions. In vitro plantlets exhibit rapid wilting and high humidity dependency; the use of anti-transpirants such as glycerol, ABA, paraffin, or grease can enhance survival. Pre-hardening strategies, including sucrose adjustment, desiccant application, or improved ventilation, further improve plantlet establishment (El-Din *et al.,* 3).

Biological hardening represents a promising approach to mitigate acclimatization stress by introducing beneficial microorganisms into the growth substrate. In vitro plants are initially microbe-free and delicate, but inoculation with rhizospheric or endophytic bacteria (e.g., *Pseudomonas*, *Bacillus*) can promote root development, growth, and stress tolerance (Mirza *et al.,* 13; Lugtenburg and Kamilova, 12). These bacteria exhibit traits such as ACC deaminase activity, nitrogen fixation, phosphate solubilization, and production of indole-3-acetic acid (IAA), which regulates growth and development (Jimtha *et al.,* 10; Zhao *et al.,* 23). Additionally, some strains possess antagonistic activity against phytopathogens, enhancing plant resilience (Müller *et al.,* 14). Fungal agents such as *Trichoderma* spp.

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act as biocontrol agents and biofertilizers, improving root colonization, plant growth, and productivity (Vinale *et al.,* 22).

In this study, different combinations of biological hardening agents, with or without 0.5% glycerol, were evaluated to determine their effect on survival of in vitro raised potato plantlets during acclimatization, with the aim of identifying the most effective combination for maximum survival.

MATERIALS AND METHODS

Sprouts and nodal segments of a medium maturing late blight resistant cultivar of potato 'Kufri Girdhari' were used as explants. Sprouts were excised from disease free healthy tubers. Nodal segments having single nodes were taken from pot grown plants and leaves were removed. Nodal segments and sprouts were first washed with tap water to remove any dirt and debris. Explants were first washed with few drops of tween-20 were added and the explants were placed under running tap water for 15-20 minutes. Afterwards they were treated with 2% Sodium hypochlorite for 5 min. Then the explants were washed with sterile water 4-5 times to avoid scorching effect on the explant and remove traces of sodium hypochlorite. MS (Murashige and Skoog) medium having 3% (w/v) sucrose and 0.8% (w/v) agar supplemented with 1mg/l TDZ and 0.5 mg/l BAP (Benzyl amino purine) was used for shoot regeneration. After surface sterilization nodal explants and sprouts are placed in the regeneration media maintaining the correct polarity of the explants. The cultures were incubated in a room maintained at 25±2 °C temperature, 16 hours of uniform light of 3000-3500 lux and 8 hours of dark period. The regenerated shoots (5-6 cm) were transferred to rooting media having half strength Murashige and Skoog salts, 3% (w/v) sucrose, 0.8% (w/v) agar and supplemented with 1 mg/l IBA (Indole butyric acid). The rooted plantlets were used for biological hardening experiments. Pre-hardening of the plantlets was carried out by removing the cotton plugs of the cultures with well-developed adventitious roots for 1 week before hardening treatment in the culture room. It was done to promote leaf adaptability to low humidity and water stress through stomatal regulation

Microbial cultures of *Bacillus subtilis, Pseudomonas flourescens,* consortia (*B. Subtilis* + *P. flourescens*) and *Trichoderma viride* were used as bioagents. For the bacterial bioagents a loopful of inoculum from each pure culture was added to 100 ml LB (Luria-Bertani) broth medium in a culture flask. The flasks were kept in an incubator at 28 ± 2 °C for 48 hours. The consortia of these bacteria were prepared by thoroughly mixing the both cultures and forming a homogenized culture. The culture broth of *T. viride* was prepared by adding a loopful of inoculum to 100 ml potato dextrose broth media and incubating the culture flasks at 28 ± 2 °C for 3 days. The culture broths of the bio-agents were first filtered through a filter paper and then used for hardening experiment. The uncapped well rooted plantlets were removed from the culture vessels using a forcep carefully to not damage the plantlet (Fig. 1). The agar adhering to the roots was washed gently under running tap water. The agar must be washed properly to prevent growth of disease-causing micro-organisms. The roots of the plantlets were then dipped in culture broths of the various bio-agents for 5 minutes. Anti-transpirant application was given to the shoot portion of the plants with and without bioagents by dipping the shoot portion (stem and leaves) of the plantlets in (0.5%) glycerol solution for 5 minutes. Immediately after microbial and anti-transpirant treatment the plantlets were transferred into cups with autoclaved potting mixture containing perlite, vermiculite and cocopeat in 1:1:1 ratio. The cups with plantlets were covered with perforated plastic bags and kept inside the hardening chamber. Humidity is maintained inside the bag by regular spray of water to protect the plantlets from moisture stress and for the quick acclimatization. Each treatment consisted of 30 plantlets and replicated thrice for statistical reliability. Plantlets were kept inside the hardening chamber for 15 days and humidity was maintained at 85-90% RH.

Fig. 1. Rooted plantlets used for hardening.

The plastic bags were removed after 15 days while still keeping them inside the hardening chamber for another week after which observations were made.

RESULTS AND DISCUSSION

The *in-vitro* propagation facilitates the mass production of large number of high-quality, uniform and disease-free plants but these plantlets must be acclimatized or hardened before being transferred to *ex-vitro* greenhouse conditions, as they are susceptible to a variety of stresses. Acclimatisation of tissue culture produced plants employing plant beneficial microorganisms can be done to increase their performance and survival. In the present study survival rates of micropropagated plantlets were assessed after 1st, 2nd, and 3rd week of biological hardening under partially controlled conditions. The micropropagated potato plantlets treated with different bio-agents alone or in combination with glycerol (0.5%) exhibited superior survival as compared to the untreated control plantlets during the hardening phase (Fig. 2). The superiority of bioagent treatments may be due to the fact that it initiates plant microbe interaction with the synthesis and expression of defense related proteins and enzyme stimulation in a controlled manner during the colonization process, resulting in a higher survival rate (Hazarika, 8). Moreover, micropropagated plants cannot tolerate abiotic stress as they have poorly developed stomata and weak root system. The application of bio-agents to micropropagated plants promotes the synthesis of lipids and abscisic acid, both of which play a key role in regulating transpiration rates (Bojan *et al*., 2). After the first week, the highest survival percentage (86.67%) was recorded in T_1 (*T. viride* + glycerol 0.5%) and T_5 (*T. viride*). By the second week, T_1 continued to show the highest survival (76.67%), followed by T_4 (Consortia (*P. fluorescens* + *B. subtilis*) + glycerol 0.5%) with 73.33%. After the third week, T_1 demonstrated the highest survival (73.33%), followed by T_4 (63.33%) and T_5 (60%). The superior survival percentage observed in T_1 (Fig. 3) might be due to the fact that *Trichoderma* colonised plants produce auxins, gibberellins, plant enzymes, antioxidants, phytoalexins, and phenols, which provide abiotic stress tolerance, root system branching capacity and its biocontrol property (Fiorentino *et al.,* 5). Similar results have been reported by Lincy and Sasikumar (11) in ginger and Sparta and Emilda (20) in banana. All bio-hardening treatments, particularly those involving anti-transpirant glycerol (0.5%) and bio-agents, were statistically superior to the control, indicating that these treatments significantly improved plantlet survival during the hardening process. Better survival was observed when glycerol (0.5%) was used in combination with bioagents as compared to use of bio-agents alone. Glycerol (0.5%) being an anti-transpirant reduces the water loss from the leaves and increases the water use efficiency (Gawad, 6). It has been reported that glycerol improves the survival rate of micropropagated plants by maintaining the turgor pressure during the acclimatization period (Gupta *et al.,* 7). *T. viride* shows best compatibility with glycerol (0.5%) as it demonstrates effective root colonization in micro propagated plantlets and/or a comparatively stronger ability of *T. viride* to suppress soil-borne pathogens.

The effect of various bio-hardening treatments on plant height was evaluated by measuring plantlet height at the time of transplanting and after 3 weeks of hardening (Table 1). *In vitro* raised plantlets when treated with various bio-agents alone or in combination with glycerol (0.5%) produced significant differences in the percentage increase in plant height. Plantlets treated with bio-agents demonstrated a notable positive response in terms of height increase. The highest increase in plant height was recorded in T_5 (Consortia: *P. fluorescens* + *B. subtilis* + glycerol 0.5%), with a 72.78% increase, where plantlets grew from an initial height of 11.43 cm to 19.75 cm

Fig. 2. Plantlets treated with *Trichoderma viride* + (0.5%) glycerol after 21 days of hardening period.

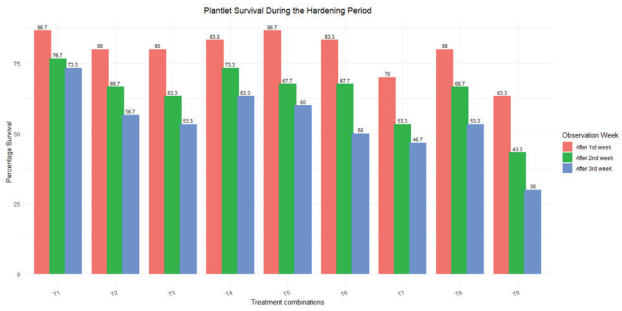

Fig. 3. Effect of different biological hardening treatments on percentage survival of micro propagated plantlets.

Table 1: Effect of biological hardening treatments on plant height (cm) and internodal length (cm) of *in vitro* raised plantlets.

Notation	Treatment combination	Plant height			Internodal length		
		Initial length (cm)	After 3 weeks (cm)	Percentage increase	Initial length (cm)	After 3 weeks (cm)	Percentage increase
T_1	*Trichoderma viride* + (0.5%) Glycerol	10.42	17.51	68.05	1.78	2.05	15.27
T_2	*Pseudomonas flourescens* + (0.5%) Glycerol	10.38	16.04	54.62	1.70	1.94	13.67
T_3	*Bacillus subtilis* + (0.5%) Glycerol	10.57	15.98	51.54	1.73	1.96	13.44
T_4	Consortia (*Pseudomonas flourescens* + *Bacillus subtilis*) + (0.5%) Glycerol	11.43	19.75	72.78	1.69	2.01	20.64
T_5	Trichoderma viride	10.77	17.78	65.15	1.67	1.9	13.77
T_6	Pseudomonas flourescens	10.89	17.35	45.98	1.70	1.91	12.35
T_7	Bacillus subtilis	10.36	15.07	45.41	1.65	1.84	12.31
T_8	Consortia (*Pseudomonas flourescens* + *Bacillus subtilis*)	9.74	16.48	69.12	1.61	1.85	15.82
T_9	Control (seedling dip in distilled water)	10.12	14.47	42.96	1.69	1.87	10.53
	SE(m)±	0.37	0.61	3.16	0.02	0.02	2.83
	$C.D._{(0.05)}$	1.12	1.82	9.26	NS	0.06	NS
	C.V. (%)	6.19	5.21	9.33	2.97	1.92	33.35

after 3 weeks. This was followed by T_8 (Consortia: *P. fluorescens* + *B. subtilis*), T_1 (*T. viride* + glycerol 0.5%), and T_5 (*T. viride*), with respective height increases of 69.12%, 68.05%, and 65.15%. These results clearly demonstrate that the application of bio-agents, particularly in combination with glycerol (0.5%), significantly enhances plant height during the hardening period compared to untreated controls. Similar increase in growth of micropropagated plantlets after treatment with bio-agents has been reported for *Capsicum annuum* (Estrada-luna *et al.,* 4) Ginger (Lincy and Sasikumar, 11) and black pepper (Anith *et al*., 1). The growth promotion by bio-agents is due to their ability to produce auxins, gibberellins and cytokinins, carry out phosphorus solubilization, sulphur oxidation, and increasing nitrate availability (Remans *et al.,* 17).

Proper root development is essential for the establishment of micropropagated plantlets and nutrient uptake from the soil. In the present study bio-agents treatment alone or in combination with glycerol showed a positive influence on root length. In the present study the root length of plantlets was measured at the time of transplanting and after 3 weeks of hardening (Table 2). The bio-hardening treatments, including the use of bio-agents and anti-transpirant (glycerol 0.5%), significantly influenced root growth during the hardening period. The highest root growth was recorded in T_1 (*T. viride* + glycerol 0.5%), with a 40.69% increase in root length, where the roots grew from 6.34 cm at transplanting to 8.92 cm after 3 weeks. This was followed by T_5 (*T. viride* without glycerol), which showed a 39.97% increase, with roots growing from 7.11 cm to 9.96 cm. Microbial association of micro propagated plantlets of potato is reported to have positive effect on root growth and development (Tkachenko *et al.,* 21). *Trichoderma* spp. positively impacts the plant growth and yield and helps to reduce drought stress by enhancing root growth, even in the presence of water scarcity, as indicated by the delayed rise in stress-related metabolites like proline, malondialdehyde (MDA), and hydrogen peroxide, as well as an elevated concentration of phenolic compounds (Shukla *et al.,* 19). It helps in improving soil structure, solubilize nutrients and secrete root stimulating enzymes such as auxins and gibberellins (Saba *et al.,* 18). Additionally, *Trichoderma viride* is known to induce systemic resistance in plants, enhancing their defense mechanisms against soil-borne pathogens and thereby promoting overall plantlet health and survival (Jamil, 9). Due to these properties that favor root elongation, the plantlets are able to establish a strong root system and perform better during the hardening period. Mukhongo *et al.* (15) reported improved survival and uptake of several micro-nutrients by tissue culture raised banana plantlets treated with *Trichoderma* Spp. and arbuscular mycorrhizal fungi (AMF).

Table 2: Effect of biological hardening treatments on root length (cm) of *in vitro* raised plantlets.

Notation	Treatment combination	Root length (cm)		Percentage Increase
		Initial length	After 3 weeks	
T_1	*Trichoderma viride* + (0.5%) Glycerol	6.34	8.92	40.69
T_2	*Pseudomonas flourescens* + (0.5%) Glycerol	6.76	8.57	26.79
T_3	*Bacillus subtilis* + (0.5%) Glycerol	6.41	8.06	25.71
T_4	Consortia (*Pseudomonas flourescens* + *Bacillus subtilis*) + (0.5%) Glycerol	7.28	9.24	27.04
T_5	Trichoderma viride	7.11	9.96	39.97
T_6	Pseudomonas flourescens	6.73	8.35	24.12
T_7	Bacillus subtilis	6.86	8.11	18.30
T_8	Consortia (*Pseudomonas flourescens* + *Bacillus subtilis*)	6.32	8.04	27.36
T_9	Control (seedling dip in distilled water)	6.52	7.56	15.96
	SE(m) ±	0.11	0.17	1.96
	$C.D._{(0.05)}$	0.31	0.51	5.74
	C.V. (%)	2.73	2.35	10.49

The results of this study reveal that biological hardening can significantly improve the survival of *in vitro* raised plantlets of potato. Out of all the treatment combinations *T. viride* with glycerol (0.5%) gave significantly higher plantlet survival than all the other treatments. It was also found that all the treatment combinations gave significantly superior survival percentage than the untreated control. Our study indicates that bio-agents in combination with anti-transpirants like glycerol (0.5%) have potential application in improving survival and growth promotion of micro propagated plants. However, more elaborative and exhaustive research in this direction can go a long way to improve the survivability of micro propagated plantlets under field conditions and thereby making plant tissue culture technology more efficient and productive.

AUTHORS' CONTRIBUTION

Conceptualization of research (RN, AB); Designing of experiments (RN, AB, BS); Contribution of experimental material (AB, BS); Execution of lab experiments and data collection (RN, AB, SS); Analysis of data (RN, RK, N); Data interpretation (RN, AB); Preparation of the manuscript (RN).

DECLARATION

The authors have declared to conflict of interest.

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