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EX-SITU CONSERVATION AND MORPHO-BIOCHEMICAL STUDY OF EXOTIC GERMPLASM OF POTATO

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Abstract

To keep potato germplasm disease-free and available for use, it was conserved as *In vitro* micro-plants under tissue culture conditions using different concentrations of mannitol and sorbitol. Mannitol and sorbitol at 60 g/L effectively reduced growth of explants and kept healthy for 180 days. Whereas explants in Murashige and Skoog (MS) media alone or supplemented with 20-40 g/L of mannitol or sorbitol filled the growing tubes, and had to sub-culture after 110 and 150 days, respectively. Higher concentration of both mannitol and sorbitol (100 g/L) caused complete death of explants. Alike, explants in control treatments had higher chlorophyll while it was comparable in explants conserved in MS media along with 40-80 g/L sorbitol or mannitol. Higher proline and less sugar contents were depicted for explants conserved in MS media with 80 g/L of sorbitol compared to mannitol. Explants conserved in MS media along with 60 g/L sorbitol had maximum plant condition (4 out of 5) score than same level of mannitol (3.66). Genotype "Karuda" had maximum plant height, leaves and branches in both mannitol and sorbitol than genotype "Burna" and "Asterix". In short, sorbitol and mannitol play a similar role however sorbitol has more beneficial effects than mannitol.

Key words: *In vitro*, Potato, Conservation, Sorbitol, Mannitol.

Introduction

Germplasm of vegetatively propagated species are usually maintained as the whole plant and/or bulb and tuber in the field (Pathirana *et al*., 2016). However; maintenance of fields with plant materials can cause fungal, viral, bacterial diseases and pests attack. In addition, losses due to the environmental conditions such as high temperature, humidity, rainfall and drought, which lead to the damage of valuable germplasm materials (Barba *et al*., 2008; Carimi *et al*., 2011). Some plant species produce the seeds that are extremely heterozygous in nature and consequently inappropriate for conservation (Engelmann, 2011). The cultivated potatoes [(*Solanum tuberosum* L.); *Solanaceae*] are also a heterozygous and segregate on sexual reproduction and true seeds of this crop cannot be conserved for general plantation. Thus, elite parent lines and potato genotypes with good yield potential are maintained via vegetative propagation to sustain their genetic purity and yield potential. Although, the potato germplasm is maintained in the fields, however it needs large space and labor and moreover this practice is time consuming (Withers, 1991; Engelmann, 2011; Chauhan *et al*., 2019). Thus, germplasm of heterozygous species and threats related to field maintenance have directed the researchers to investigate secure, efficient, low-priced methods for successful germplasm conservation (Chauhan *et al*., 2019). In the world, potato clones and elite parental lines are conserved in the *In vitro* disease-free condition instead of field maintenance (Gopal *et al*., 2002; Engelmann, 2011). However; when these explants are kept under optimum *In vitro* conditions, the explants require sub-culturing after 4-8 weeks. In order to decrease the numbers of sub-culturing, the growth of these explants is retarded by using growth retardants or osmotic agents that cause stress decrease energy source to explants (Gopal *et al.,* 2002; Gopal & Chauhan, 2010).

Non-metabolizable sugar-alcohols decrease the availability of water to growing cultures by creating water stress conditions (Gopal *et al*., 2002). The water stress conditions may perhaps be responsible for slow growth of potato explants. Cruz-Cruz *et al*., (2013) reported that biological material could be conserved under *In vitro* conditions for months to years (2-3 years) without sub-culturing depending on plant material. Gopal *et al.*, (2002) used various levels of metabolically inactive polyols and sucrose for potato germplasm conservation in the *In vitro* conditions for 12 months. Further, they reported that application of sorbitol was more beneficial compared to mannitol as sorbitol resulted in higher survival rate. Alike, Sarwar & Siddiqui (2004) applied 2% mannitol with MS media for potato germplasm storage in the *In vitro* conditions and found lateral buds remained healthy for 165 days with survival rate of 75%. Marino *et al*., (2010) used MS media along with mannitol (58.4 mM) to conserve *Prunus* species for 7 months and documented 100% survival rate of *Prunus* species. Shatnawi *et al*., (2011) applied mannitol, sorbitol and sucrose with different concentration (3, 6, 9 and 12% w/v) to store micro-shoots of *Stevia rebaudiana*. They reported that after 32 weeks of storage, sucrose dose at 3 to 9% (w/v) recorded maximum survival rate (94.6%) compared to sorbitol or mannitol. Gopal & Chauhan (2010) determined the efficiency of sorbitol and mannitol for potato explants *In*

vitro conservation. They revealed that MS media with 20 g/L sucrose + 40 g/L sorbitol increased storage life of explants for 18 months without sub-culturing. Thus, application of different osmotica such as sucrose, sorbitol and mannitol to culture media for various plant species has been used to decrease growth and increase storage life in the *In vitro* condition (Shibli *et al*., 2006; El-Bahr *et al*., 2016). However, none of the studies regarding potato germplasm *In vitro* conservation is available. Therefore, the present study was planned to determine suitable concentration of sorbitol and mannitol for potato microplants in *In vitro* conservation.

Materials and Method

Experiment was performed in two laboratories*; In vitro* conservation experiment was carried out in Bio Conservation Institute (BCI), while chemical tests were done in wheat wide cross laboratory in National Agriculture Research Council (NARC), Islamabad. For evaluating the suitable level of mannitol and sorbitol as osmotic agents on conservation of potato, explants of potatoes were transferred to MS media supplemented with 0, 20, 40, 60, 80 and 100 g/L of mannitol and sorbitol, and cultures were incubated at 24 ± 2°C. Experiment was laid out in CRD with three replicates. Micro propagation of potato was performed according to the standard procedure. The plantlets were 1.5-2 cm size with 1-2 nodes. These cultures were inoculated with MS medium (Murashige and Skoog) and various levels (0, 20, 40, 60, 80 and 100 g/L) of mannitol and sorbitol. The pH of culture was adjusted to 5.7 and and illuminated with 1000-2000 Lux of light and maintained under a photoperiod of 16 hours, and data was recorded on regular interval.

Observations: During the experiment, morphological traits such as plant height, number of viable shoots, leaves and roots were recorded. Similarly, chemical analysis i.e total chlorophyll, sucrose and proline were recorded. Plant condition score (0-5, 0 being very poor while 5 being very good) and number of days for sub-culturing was also recorded.

Proline estimation: For proline determination, fresh sample of potato (0.2 g) was mixed thoroughly in 5 ml salfosalycylic acid and then kept at room temperature for 12 hrs. After12 hrs of incubation, homogenized mixture was filtered and filtrate was used to determine proline concentration. Extract of each sample was poured in a test tube, which contained ninhydrin reagent and glacial acetic acid (2 ml). The mixture was kept in water bath and heated up to 100°C for 1 hr. After cooling, toluene (4 ml) was added to reaction mixture and then shifted to a separating funnel and vortex it. The chromospheres comprising toluene were separated and their absorbance was noted at 520 nm using UV visible spectrometer. The reading of blank toluene was also noted. Standard curve was drawn from known proline concentration (Bates *et al*., 1973).

$$\text{Proline (mg g}^{-1}) = \frac{\text{Absorbance of sample}_{x}\ \text{K value}_{x}\ \text{dilution factor}}{\text{Weight of sample}}$$

Soluble sugar: For soluble sugar, 0.2 g potato sample (fresh leaves) was used by following procedure of Dubois *et al*., (1956). The leaves were homogenized using 80% ethanol (10 ml). After homogenizing, it was heated at 80°C on a water bath for 1 hr. Phenol (5%) was poured into mixture and kept for 1 hr at 25±2°C then concentrated sulphuric acid (5 ml) was added. The reading of samples was noted at 490 nm on spectrometer.

$$\text{Sugar (mg g}^{-1}) = \frac{\text{Absorbance of sample}_{x}\ \text{K value}_{x}\ \text{dilution factor}}{\text{Weight of sample}}$$

Total chlorophyll content: To determine the chlorophyll content, 0.2 g fresh leaves were used by following method of Arnon (1949). These harvested leaves grinded in 80% ethanol (10 ml). After grinding, acetone was added and the caped the solution immediately. After few minutes, the extract was taken and boiled at 80°C for 10 min using water bath. After cooling in dark, the absorbance of mixture was read at 663 and 645 nm for chlorophyll *a* and *b*, respectively through spectrometer.

Results

In sorbitol, genotype “Karuda” had the maximum plant height followed by “Burna” while minimum was noted for "Asterix”. Karuda had more number of leaves while other two had at par with each other but less than Karuda. Alike, Karuda had maximum number of branches while two other genotypes had less number of branches.In mannitol, Karuda had more plant height compared to other two genotypes. After 180 days of *In vitro* storage, maximum height of explants was recorded in control treatment (MS media only) and explants conserved in MS media with 20-40 g/L of mannitol or sorbitol (Table 1, Figs. 1 & 2) followed by MS media with 60 g/L of mannitol or sorbitol. Minimum plant height was noted for explants conserved in MS media with 80 g/L of sorbitol followed by 80 g/L of mannitol. However higher concentration of both mannitol and sorbitol (100 g/L) caused death of explants. In genotypes comparison, Karuda had maximum height in both mannitol and sorbitol followed by Burna while Asterix showed minimum plant height (Figs. 1 & 2). Similarly, mannitol and sorbitol influenced number of branches in explants during storage (Table 1, Figs. 3 & 4). Explants in control treatments (MS media) had more number of branches but comparable with explants conserved in MS media with 20 g/L of mannitol after 180 days *In vitro* storage. Alike, branches of explants conserved in MS media with 40 g/L mannitol and 20 g/L sorbitol were similar to each other and these both were also at par with explants conserved in MS media with 20 g/L mannitol. Minimum number of branches was noted in explants which were conserved in MS media with 80 g/L sorbitol or mannitol and high concentration (100 g/L) of mannitol or sorbitol caused death of explants during storage. Karuda genotype showed greater number of branches on both mannitol and sorbitol conservation while Burna and Asterix had less number of branches than Karuda but Burna and Asterix at par with each other.

Table 1. Effect of mannitol and sorbitol on morphological traits of potato germplasm during *In vitro* conservation.

Treatments	Plant height (cm)	Number of leaves	Number of branches
Control (MS media)	9.50a	37.33a	21.0a
MS media + 20g/L mannitol	9.50a	32.33ab	17.66b
MS media + 20g/L sorbitol	9.50a	29.00bc	10.33d
MS media + 40g/L mannitol	9.16a	28.33bc	13.66c
MS media + 40g/L sorbitol	9.00a	25.00cd	8.33e
MS media + 60g/L mannitol	7.13b	21.66d	7.66e
MS media + 60g/L sorbitol	6.76b	20.66d	6.66ef
MS media + 80g/L mannitol	4.400 c	14.33e	3.66g
MS media + 80g/L sorbitol	5.50 d	12.66e	5.00 fg
MS media + 100g/L mannitol	Death of plants	Death of plants	Death of plants
MS media + 100g/L sorbitol	Death of plants	Death of plants	Death of plants
LSD at 5%	0.89	6.02	1.83

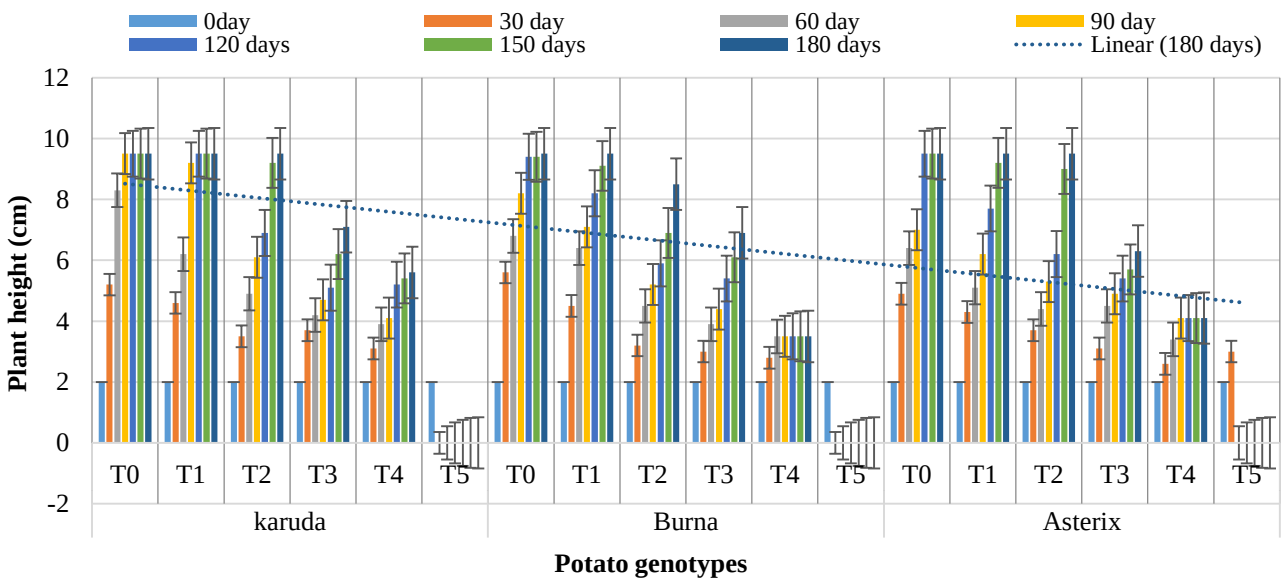

Fig. 1. Effect of mannitol on plant height of potato genotypes during *In vitro* conservation. T0 = control (MS media), T1 = MS media+ 20g/L mannitol, T2 = MS media+ 40g/L mannitol, T3 = MS media+ 60g/L mannitol, T4 = MS media+ 80g/L mannitol. T = MS media+ 100g/L mannitol.

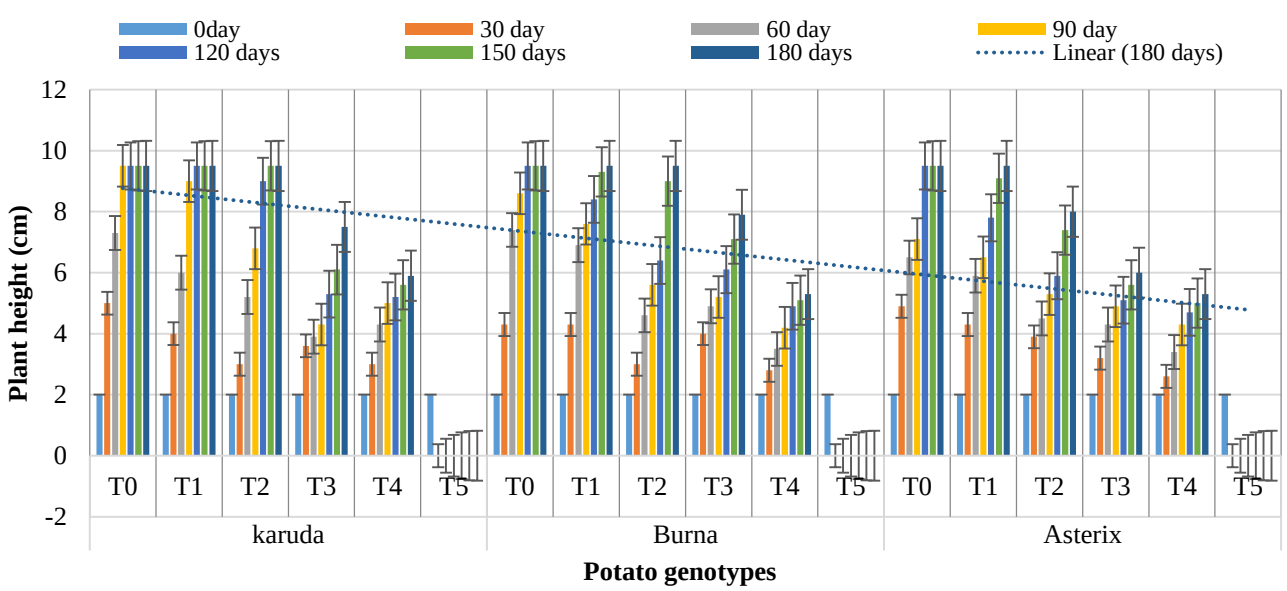

Fig. 2. Effect of sorbitol on plant height of potato genotypes during *In vitro* conservation. T0 = control (MS media), T1 = MS media+ 20g/L sorbitol, T2 = MS media+ 40g/L sorbitol, T3 = MS media+ 60g/L sorbitol, T4 = MS media+ 80g/L sorbitol, T5 = MS media+ 100g/L sorbitol.

Table 2. Effect of mannitol and sorbitol on total chlorophyll, proline and sugar of conserved potato germplasm during *In vitro* conservation.

Treatments	Total chlorophyll (mg/g)	Proline (mg/g)	Sugar (mg/g)
Control (MS media)	2.28a	0.18 bc	2.22b
MS media + 20g/L mannitol	2.41a	0.10 c	2.30b
MS media + 20g/L sorbitol	1.74ab	0.44 b	2.18b
MS media + 40g/L mannitol	1.20bc	0.11 c	2.54ab
MS media + 40g/L sorbitol	1.28 bc	0.44 b	2.56ab
MS media + 60g/L mannitol	0.67cd	0.13 c	2.73ab
MS media + 60g/L sorbitol	1.17bc	0.28 b	2.63ab
MS media + 80g/L mannitol	0.38c	0.08 dc	1.99b
MS media + 80g/L sorbitol	0.78bc	1.14a	3.22a
MS media + 100g/L mannitol	Death of plants	Death of plants	Death of plants
MS media + 100g/L sorbitol	Death of plants	Death of plants	Death of plants
LSD at 5%	1.06	0.30	0.82

Table 3. Effect of mannitol and sorbitol on days required for sub-culturing, plant condition score and revival percentage of potato explants during *In vitro* conservation.

Treatments	Days of sub culturing	Plant condition score after 180 days
Control (MS media)	110 c	3.00 d
MS media + 20g/L mannitol	130 b	3.33 c
MS media + 20g/L sorbitol	110 c	3.33 c
MS media + 40g/L mannitol	160 a	3.66 b
MS media + 40g/L sorbitol	150 a	3.66 b
MS media + 60g/L mannitol	No sub-culturing required	3.66 b
MS media + 60 g/L sorbitol	No sub-culturing required	4.00 a
MS media + 80g/L mannitol	No sub-culturing required	2.66 d
MS media + 80g/L sorbitol	No sub-culturing required	2.66 d
MS media + 100g/L mannitol	Death of plants	Death of plants
MS media + 100g/L sorbitol	Death of plants	Death of plants
LSD at 5%	19.07	0.22

Explants conserved in sole MS media (control) produced more number of leaves followed by MS media with 20 g/L mannitol (Table 1, Figs. 5 & 6). Plants conserved in MS media with 40 g/L sorbitol had statistically similar number of leaves as explants conserved in MS media with 60 g/L mannitol or sorbitol. Less number of leaves in explants was noted when conserved in MS media with 80 g/L mannitol however it was at par with MS media with 80 g/L sorbitol while high concentration (100 g/L) of both osmotic agents in MS media caused death of plants after 180 days of *In vitro* storage (Table 1). Genotypes also showed significant differences for number of leaves. Karuda produced more number of leaves in both the osmotic agents while Burna and Asterix had less number of leaves than Karuda and these two were at par with each other (Figs 5 and 6). Total chlorophyll concentration was a maximum in explants after 180 days of storage, which were conserved in MS medium (control) however explants conserved in MS media with 20 g/L mannitol or sorbitol were also at par with controlled-explants (Table 2). Alike, explants conserved in MS media with 80 g/L mannitol had minimum total chlorophyll but it was at par with explants conserved in MS media with 60 and 40 g/L mannitol, 40, 60 and 80 g/L sorbitol (Table 2). Proline is generally accumulated in plants facing various stresses and it is important for tolerance of plants and their establishment. Results showed that explants conserved in MS media with 80 g/L of sorbitol had maximum concentration of proline while minimum was noted for explants conserved in MS media with 80 g/L mannitol (Table 2).

After 180 days of *In vitro* conservation, explants conserved in MS media with 80 g/L of sorbitol had maximum concentration of sugar however; statistically it was comparable with MS media plus 40 and 60 g/L of sorbitol or mannitol (Table 2). But explants conserved with MS media with 80 g/L of mannitol had significantly less concentration of sugar compared to sorbitol at 80 g/L. Plant condition score data (0-5 very poor to very good) showed that explants conserved in MS media with 60 g/L sorbitol had maximum score (4.0) however, it was at par with MS media plus 20 and 40 g/L of sorbitol or mannitol while explants conserved with MS media with 80 g/L sorbitol or mannitol had minimum plant condition score (2.66) which was also at par with control, MS media with 20 g/L sorbitol and mannitol (Table 3). Data regarding number of days required for sub-culturing during 180 days (Table 3) showed that explants conserved in MS media (control) and MS media with 20 g/L sorbitol required sub-culturing after 110 days followed by MS media with mannitol 20 g/L (130 days). Alike, explants conserved in MS media with 40 g/L sorbitol and mannitol required sub-culturing at 150 and 160 days after storage, respectively however statistically both had similar days for sub-culturing. No sub-culturing was required for explants conserved in MS media with 60 and 80 g/L of mannitol or sorbitol. Plants resist sub-culturing was assessed for their revival percentage (Table 3).

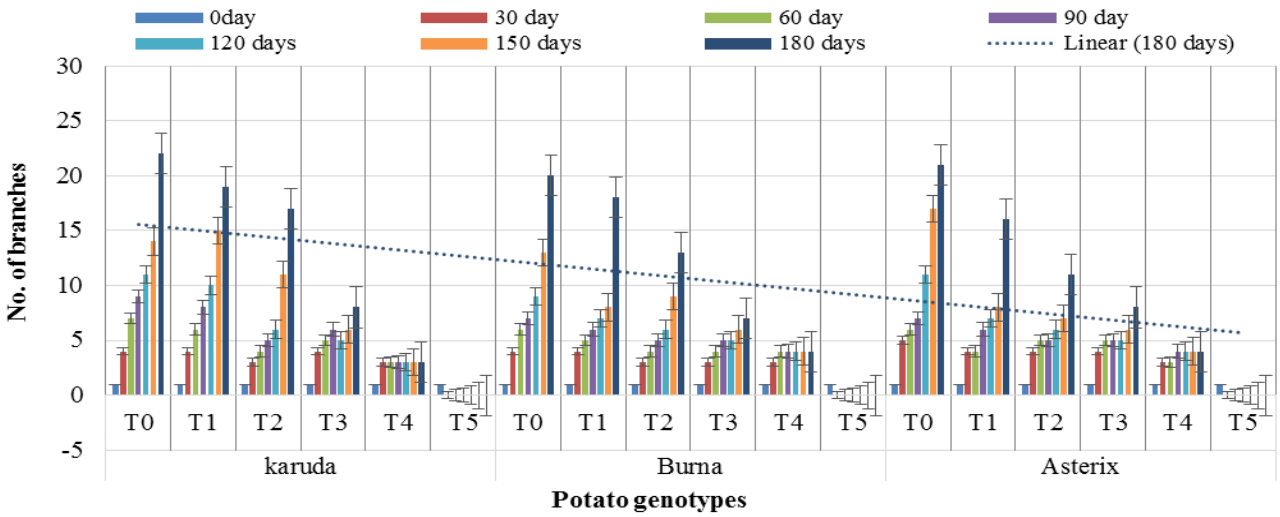

Fig. 3. Effect of mannitol on number of branches in potato genotypes during *In vitro* conservation. T0 = control (MS media), T1 = MS media+ 20g/L mannitol, T2 = MS media+ 40g/mannitol, T3 = MS media+ 60g/L mannitol, T4 = MS media+ 80g/L mannitol, T5 = MS media+ 100g/L mannitol.

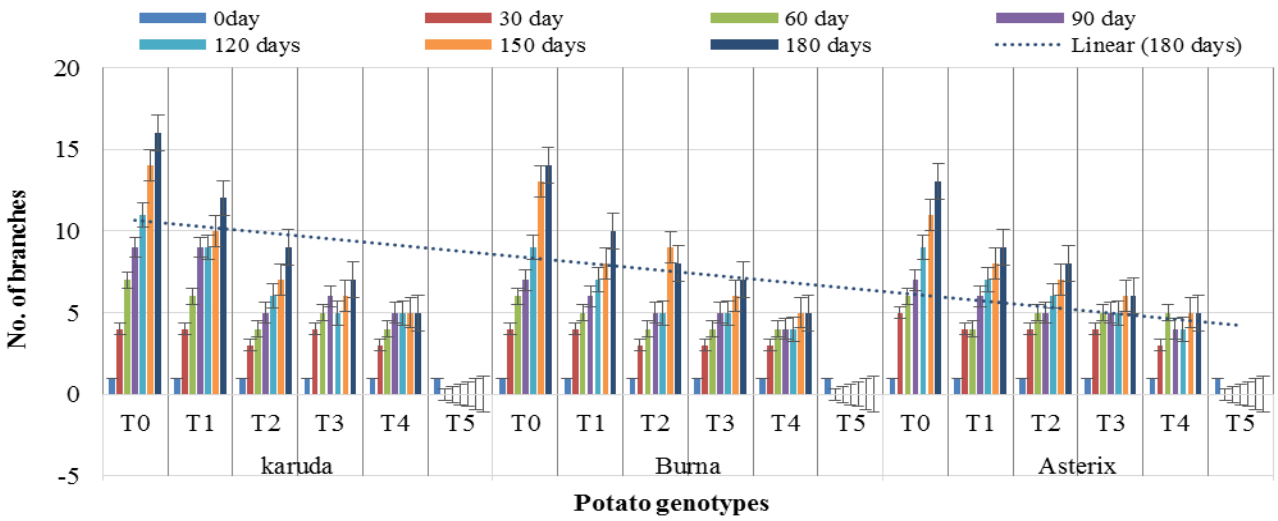

Fig. 4. Effect of sorbitol on number of branches in potato genotypes during *In vitro* conservation. T0 = control (MS media), T1 = MS media+ 20g/L sorbitol, T2 = MS media+ 40g/L sorbitol, T3 = MS media+ 60g/L sorbitol, T4 = MS media+ 80g/L sorbitol, T5 = MS media+ 100g/L sorbitol.

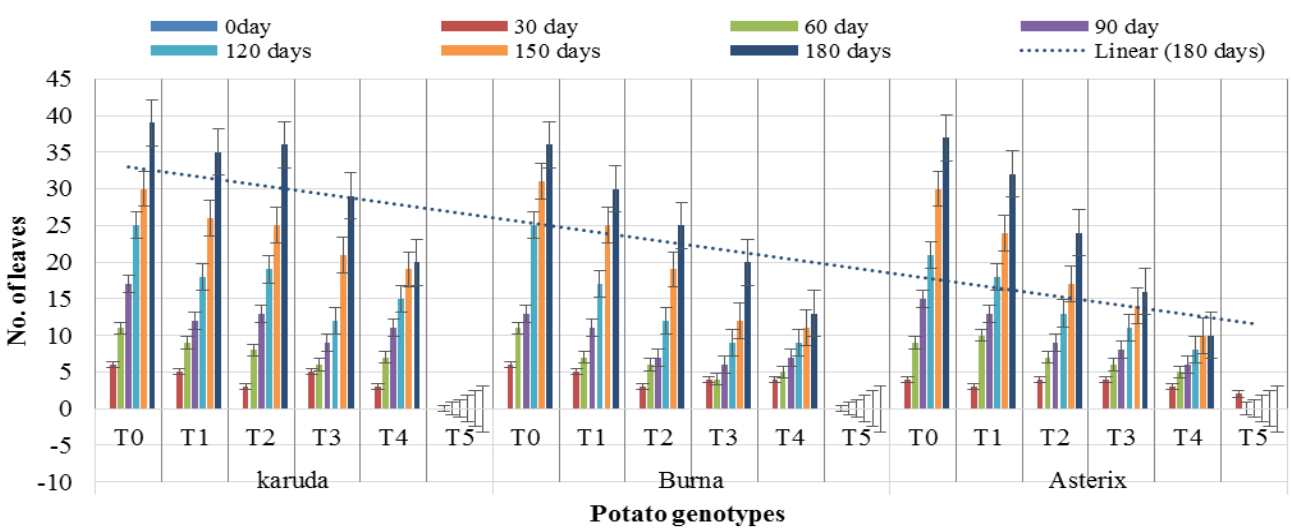

Fig. 5; Effect of mannitol on number of leaves in potato genotypes during *In vitro* conservation. T0 = control (MS media), T1 = MS media+ 20g/L mannitol, T2 = MS media+ 40g/mannitol, T3 = MS media+ 60g/L mannitol, T4 = MS media+ 80g/L mannitol, T5 = MS media+ 100g/L mannitol.

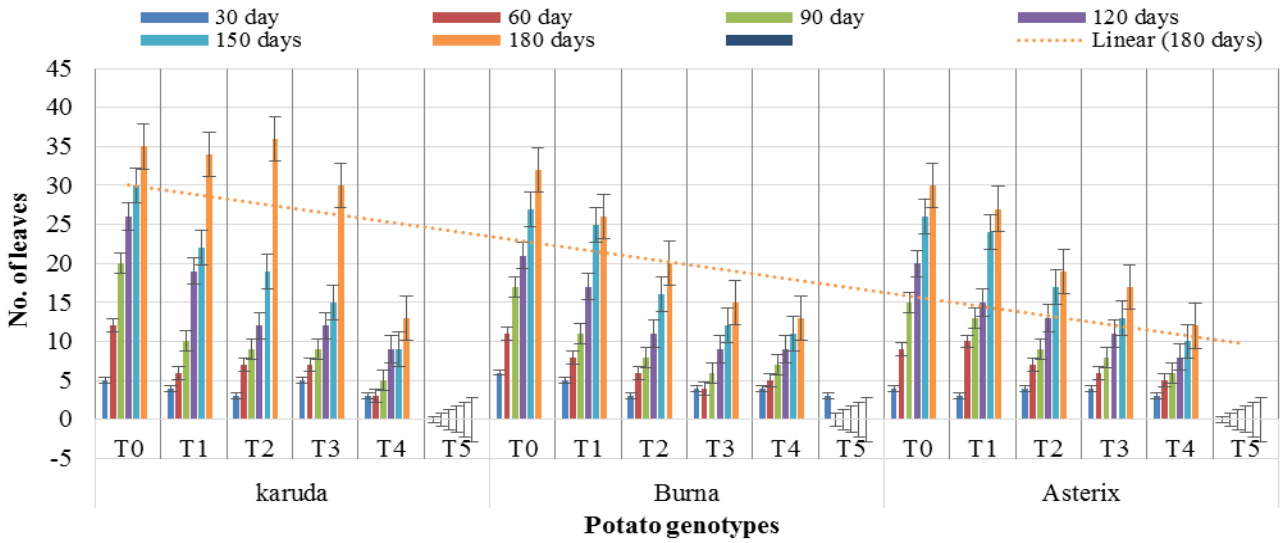

Fig. 6; Effect of sorbitol on number of leaves in potato genotypes during *In vitro* conservation. T0 = control (MS media), T1 = MS media+ 20g/L sorbitol, T2 = MS media+ 40g/L sorbitol, T3 = MS media+ 60g/L mannitol, T4 = MS media+ 80g/L sorbitol, T5 = MS media+ 100g/L sorbitol.

Discussion

Slow growth is one of the most successful In vitro techniques for medium-term germplasm conservation enabling plant storage for a prolonged period without plant manipulation, while maintaining plant resources readily available (Cruz-Cruz *et al*., 2013; Peng *et al*., 2015). When sugar alcohols like sorbitol or mannitol are added to the culture medium, these cause osmotic agents reduce the hydric potential and restrict the water availability to the explants (Fortes *et al*., 2001). Mannitol, sorbitol or sucrose reduced mineral uptake by cells through differences in osmotic pressures thereby retarding plant growth (Dodds *et al*., 1992). In this scenario, sorbitol and mannitol were documented as osmolytes to strength the potato explants during storage. Our outcomes indicated that sorbitol and mannitol at 60 g/L recorded optimum plant growth, proline, sugar and plant condition score (4 out of 5) than control treatments or MS media with 20-40 g/L sorbitol or mannitol that had high growth, not fit for *In vitro* conservation. Alike, MS media with 80 g/L sorbitol or mannitol resulted in too short growth while MS media with 100 g/L sorbitol or mannitol caused complete death of plants. The high growth in control or MS media with 20-40 g/L sorbitol or mannitol might be due to the more availability of water and nutrients from media, while 60 g/L sorbitol or mannitol reduced water and nutrients uptake by causing osmotic stress during conservation. The death of explants showed that mannitol or sorbitol at 100 g/L stopped completely intake of water and nutrients, and consequently explants died after few weeks. Similar outcomes are reported by Sarkar & Naik (2004), who noted that 20 or 40 g/L mannitol plus suitable concentration of sucrose may increase of potato life in *In vitro*. Similarly, El-Bahra *et al*., (2016) reported that 40 and 60 mg/L of mannitol reduced the shoots in rose leadwort explants. Similarly, Shatnawi *et al*., (2011) used mannitol, sucrose and sorbitol with 3%, 6%, 9%, or 12%, w/v to store micro-shoots of *Stevia rebaudiana*. They found that sucrose level of 3 to 9% (w/v) was found to favor higher survival (94.6%) of micro-shoots after 32 weeks of storage. Similarly, Gopal and Chauhan (2010) applied mixture of sorbitol and sugar during *In vitro* conservation of potato microplants for 18 months storage without sub-culturing. They depicted that MS media with 40 g/ L sorbitol + 20 g/L sugar gave satisfactory results. However; Muñoz *et al*., (2019) reported that as the concentration increased from 20 to 60 g/L of sorbitol or mannitol, it reduced the growth rate in plants. There was more mortality of explants when level of sorbitol or mannitol was > 60 g L^{-1}.

Our results showed that high concentration of sorbitol or mannitol caused death of plants. Addition of mannitol to the medium reduced plant growth, confirming that mannitol, perceived by cells, acted as a chemical signal, suppressing growth while being metabolically inert (Yaseen *et al*., 2013). Our results corroborate those reported by Hammond *et al*., (2017) in the primary study where 4-month application of 30 g L^{-1} mannitol in culture medium suppressed the growth of ulluco microshoots. Similarly, Ozudogru *et al*., (2017) reported in cherry rootstock 'Gisela 5' that mannitol inclusion in the storage medium was effective at reducing the metabolic activity of the shoot cultures during the storage period. However, in *Cannabis sativa* L., mannitol considerably decreased the survival percentage as well as the plant re-growth ability of nodal cultures (Lata *et al*., 2012), proving that it could not be taken as universal medium supplement for the conservation of all species. Similarly depicted by da Silva & Scherwinski-Pereira (2011) demonstrated that high concentrations of sorbitol or mannitol might be injurious to plant and even death of plants might occur. This indicated that sorbitol had effects that allowed or even encouraged various types of cellular divisions. Feng *et al*., (2011) suggested that sorbitol might change physiological and molecular processes through chemical signaling, rather than by its function as an osmotic regulator or C source. Lemos & Baker (1998) observed that sorbitol caused callus formation and initiation of shoots in explants but mannitol did not do. By increasing sorbitol or mannitol concentration, proline concentration was higher and it might be due to that both sorbitol and mannitol increased osmotic stress condition during storage and in turns the enhanced accumulation of proline. Similar outcomes were reported by Al-Khayri & Al-Bahrany (2002) who noted highest content of proline when culture medium had high concentration of sorbitol.

Conclusion and Recommendation

In vitro conservation can serve to maintain the vitality of the potatoes, free from pest and diseases, and provide plant material immediately available at a lower cost than in field conservation. The addition of mannitol and sorbitol to MS media reduced the growth rate of potato germplasm. The rate of growing was drastically affected by the presence of these osmotic agents and among different concentrations in the MS media, 60 g/L sorbitol and mannitol was appropriated for 180 days storage of potato explants. However, sorbitol had better plant condition score. Thus, protocol represents a reliable conservation; suitable for potato explants under *In vitro* conditions. Karuda genotype has higher plant height, number of branches and leaves than Burna and Asterix. Use of mannitol and sorbitol at 60 g/L may also be tested in future studies. Further, this study also proposes further tests using more genotypes of potato species subjected to prolong durations. Research has shown the possibility of using other sugar alcohols as the slow growth factor in addition to sorbitol or mannitol.

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