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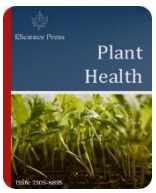

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In-Vitro Propagation of *Malus domestica* and its Conservation

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ABSTRACT

Conventional methods of propagation commonly used for commercially important apple varieties are time consuming, laborious and induce disease transmission from donor to propagating plant. To overcome these problems, a study was designed to optimize the procedure for micropropagation and conservation of Apple. For this purpose, the excised shoot tips of apple varieties were collected from the field of Biological Conservation Institute (BCI), NARC, Islamabad. The surface sterilization of shoot tips was done with 10, 15 & 20 % sodium hypochlorite for 15 minutes followed by ethanol application at 10, 15 and 20 % for 15 minutes. It had been observed that 10 % of sodium hypochlorite showed maximum survival percentage for ex-plant establishment. After culture initiation, ex-plants were transferred to MS media with the addition of different hormones i.e., BAP at the concentration ranging from 0.1 to 1.5 mg/L, GA3 from 0.5 to 2.5 mg/L and IBA from 0.5 to 3.5 mg/L under aseptic conditions. The results revealed that maximum number of leaves, shoots and plant height was attained with the addition of BAP 0.1 mg/L as compared to other hormonal concentrations. Cultures were incubated at 25 °C under 1000-1500 lux. The plants after propagation were shifted to conservation media for five months (sorbitol & mannitol at 10, 20 and 30 g/L each). The use of sorbitol at the concentration of 10 g/L showed slowest growth recorded after 35 days. This optimized method can be used for efficient mass scale production of true type apple varieties and breeders may get disease free plantlets of apples.

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INTRODUCTION

Apple (*Malus domestica*) is a dicotyledonous, low spreading woody plant, which belongs to the Rosacea family (Dirlewanger *et al*., 2002). It is used in making healthy food products like jellies, canned products, candies, fresh apple juice and cider or vinegar (Lyu *et al.,* 2020). Vegetative propagation via budding or grafting is the conventional apple propagation method which cannot ensure disease free and healthy plant (Dobresnski *et al*., 2010). There are diverse stages of apple micro-propagation or proliferation such as shoot multiplication, shoot elongation, rooting, regeneration, and acclimatization. The restoration of regenerated plant is related to its composition so the diseases must be eradicated, and the material must be cleaned for long-term preservation in germplasm banks (Panattoni *et al*., 2013). Gibberellins are recognized for its role in shoot elongation and germination, and it is utilized *in vitro* and

exogenous treatment of plant (Gantait *et al*., 2015). Whereas BAP and IBA play their role in shoot multiplication and root development as well. Micro-propagation and meristem culture has recently become an important technique, particularly for vegetatively propagated species (Ciccotti *et al*., 2008). Traditional propagation methods depend on the season and typically result in low multiplication rates. Micro-propagation method provides an efficient and alternative way for the commercial propagation of plants and among fruits, *in vitro* propagation of apple rootstock has been reported by many authors (Minaev *et al*., 2003; Kaushal *et al*., 2005; Dalal *et al*., 2006). A tree raised from seed showed desirable characteristics as the only way of multiplying it in large number is by asexual (vegetative or clonal) propagation (Hatipoglu *et al*., 2021).

The propagation of apple (*Malus domestica*) is becoming popular and the changes in traditional fruit propagation methods has become inevitable (Teixeira *et al*., 2019). reported that the production of disease free plantlets and conservation of threatened spp. could be possible through plant tissue culture technique (Shahzad *et al.,* 2017). Recently, tissue culture technology is being used in basic and applied sciences. Micro-propagation confers advantages that are not possible with conventional propagation methods (Abdalla *et al.,* 2022).The main aim of this study is to develop optimized micro-propagation and their conservation protocols for two selected commercially important apple varieties.

MATERIALS AND METHODS

The experiment was carried out at *in vitro* conservation laboratory of Bio-Resources Conservation Institute (BCI), National Agricultural Research Center (NARC), Islamabad, Pakistan.

Ex-plant Collection and Surface Sterilization

The apical portion of apple shoots was excised (4-5 cm) in two apple varieties i.e., Aina and Golden Delicious and further cut them into small segments (2 cm each). Then, they were surface sterilized with ethanol at different concentrations i.e., 10, 15 and 20% for 15 minutes followed by Clorox treatment for 15 minutes. To remove the traces of Clorox, washing of ex-plants was performed using sterilized water 3-4 times.

In-vitro Propagation & Conservation of Apple

Surface sterilized shoot tips were inoculated on MS Media having different hormonal concentrations i.e. BAP (0.1, 0.2, 0.5, 1.0 & 1.5mg/L), GA_3 (0.1, 0.5, 1.0, 1.5, & 2 mg/L) and IBA (0.5, 1, 1.5, 2 & 2.5mg/L) under aseptic conditions. Incubation of cultures at 25±3°C with the photoperiod of 16:8 hours. Five weeks old cultures of *Malus domestica* were then shifted into conservation media containing sorbitol and mannitol with different concentrations (10, 20 & 30g/L).

Data Collection

Data on morphological parameters (Plant height, number of leaves and number of shoots) were recorded on weekly basis for propagation experiment and on monthly basis for conservation experiment.

Statistical Analysis

The data collected was analyzed for analysis of variance (ANOVA) in Completely Randomized Design (CRD) for propagation and conservation experiment. Treatments means were compared for significant difference using Least Significant Difference (LSD) test. All the statistical analysis were carried out using statistic 8.1 software (Steel and Torrie,1997).

RESULTS

Optimization of Surface Sterilization Concentrations

For optimization study, different concentrations of ethanol and Clorox (10, 15 and 20 %) were used for surface sterilization of shoot tips of apple varieties for 15 minutes. Data were recorded up to five weeks. It has been revealed from the results that maximum survival rate (100 %) was observed with 10 % clorox followed by 10 % ethanol (98 %) as shown in (Figure 1). In contrast, lowest survival rate (75 %) was observed with 20% clorox and ethanol.

Optimization Studies of *Malus domestica In vitro* propagation

In vitro propagation was carried out by the amendment of different growth regulators (BAP, GA_3, and IBA) in MS media with varied concentration ranging from 0.5 to 3.5 mg/l. Morphological data of both varieties were recorded with weekly time interval and analyzed statistically. Results showed significant differences among treatments. Details are given below:

Plant height

The explants were cultured under sterile conditions in MS medium with different concentrations of hormones except control. Each treatment with three replicates was applied to both studied varieties. The height of each plant was recorded with interval of one week using measuring scale. Results depicted that golden delicious

showed higher plant height i.e. 3.5 cm with 0.1 mg/L BAP followed by 2.8 cm with 0.2 mg/L GA_3 and 0.5cm with 0.5ml/L IBA as compared to Aina as shown in (Figure 2(a) and 2(b). Plant height response was found highly significant by the addition of hormone.

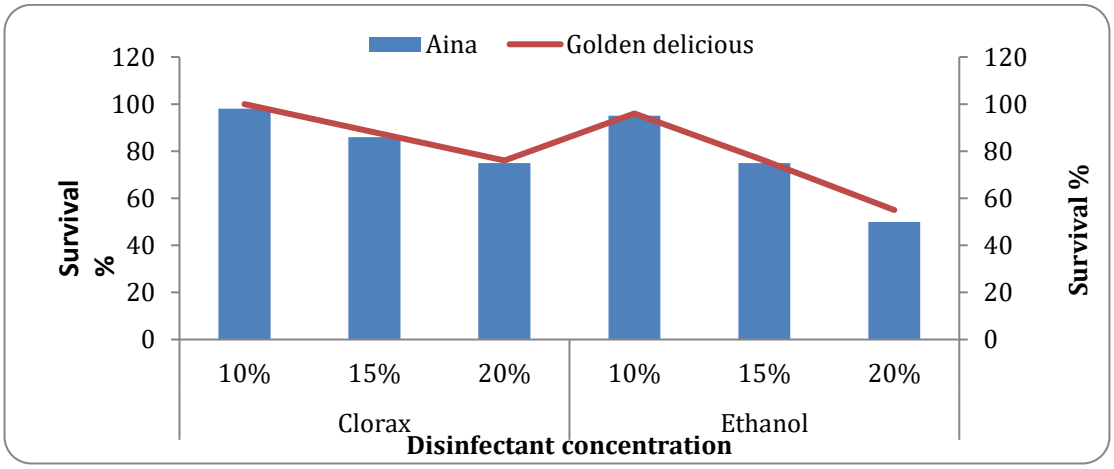

Figure 1 (a). *Optimization of disinfectants concentration for in vitro culture establishment of Malus domestica.*

Figure 1(b). Established *cultures of Malus domestica after ethanol treatment.*

Figure 1(c). Established cultures of *Malus domestica* after clorox treatment.

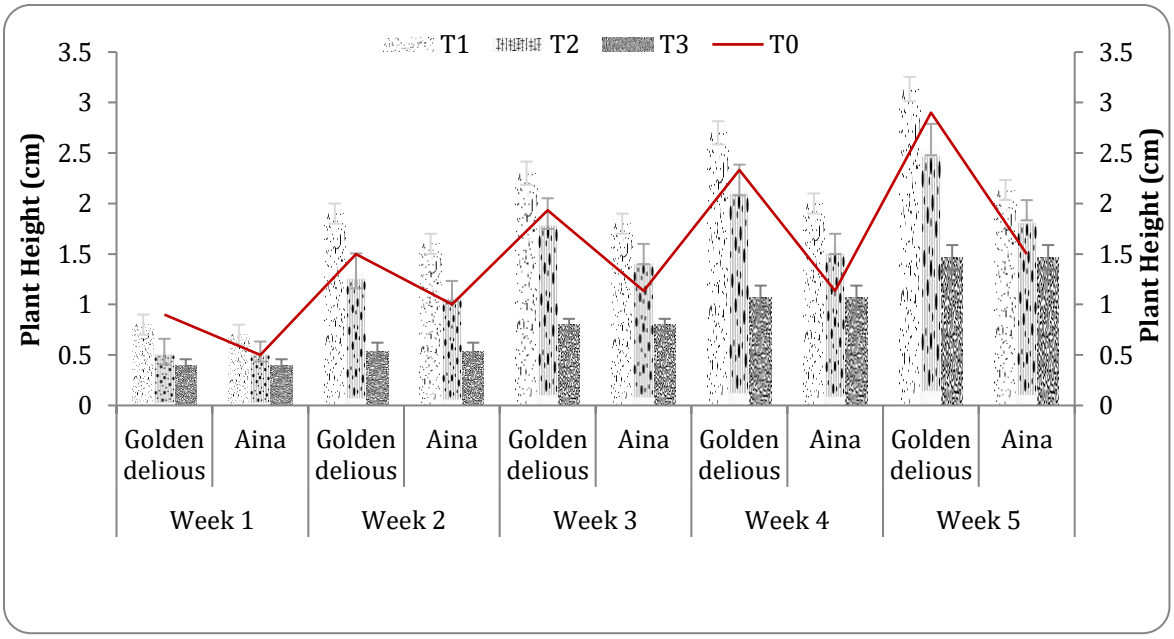

Figure 2(a). Effect of growth regulators on plant height of cv. Golden delicious and Aina under weekly interval.

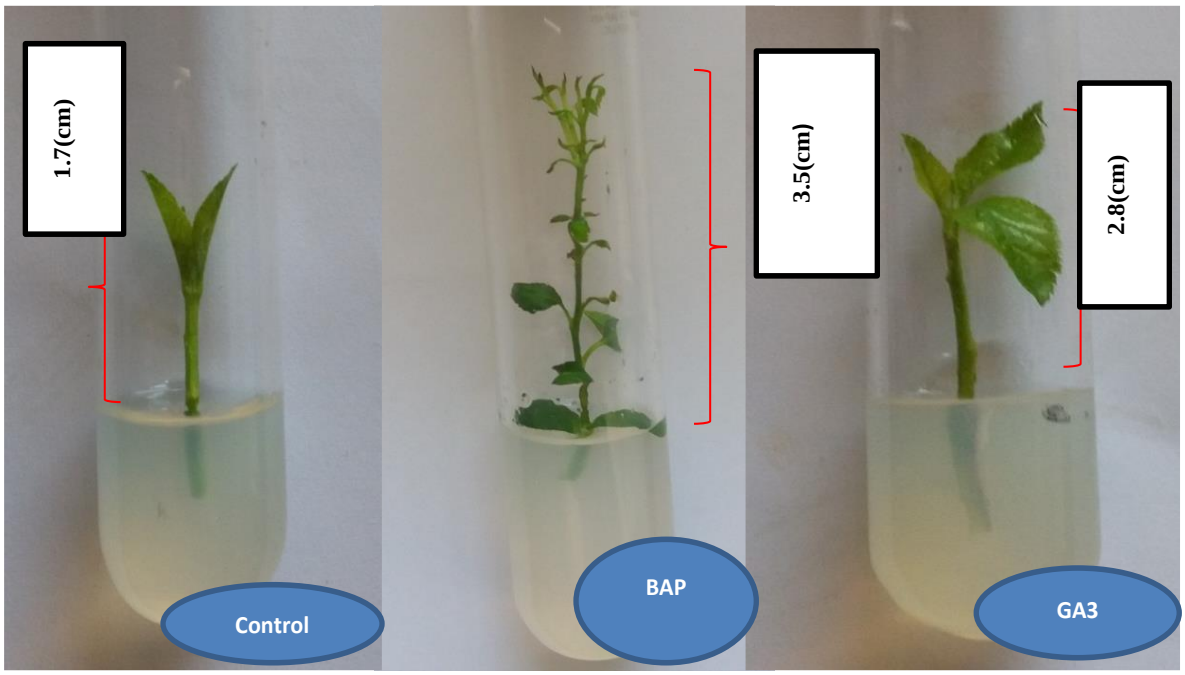

Figure 2(b). Effect of growth regulators on plant height of *Malus domestica*.

Number of leaves

After inoculation of explants in MS media with different concentration of growth regulators, the number of leaves was recorded with one week interval. It was noted that Golden delicious showed more number of leaves i.e. 6 to 4 with 0.1mg/L of BAP and GA_3 as compared to Aina. However, number of leaves was comparatively less (3) with 0.5 mg/L of IBA followed by 2 number of leaves. Aina showed less number of leaves in comparison of Golden delicious as shown in Figure 3(a), (b). Hormones showed significant effect on the number of leaves per plant.

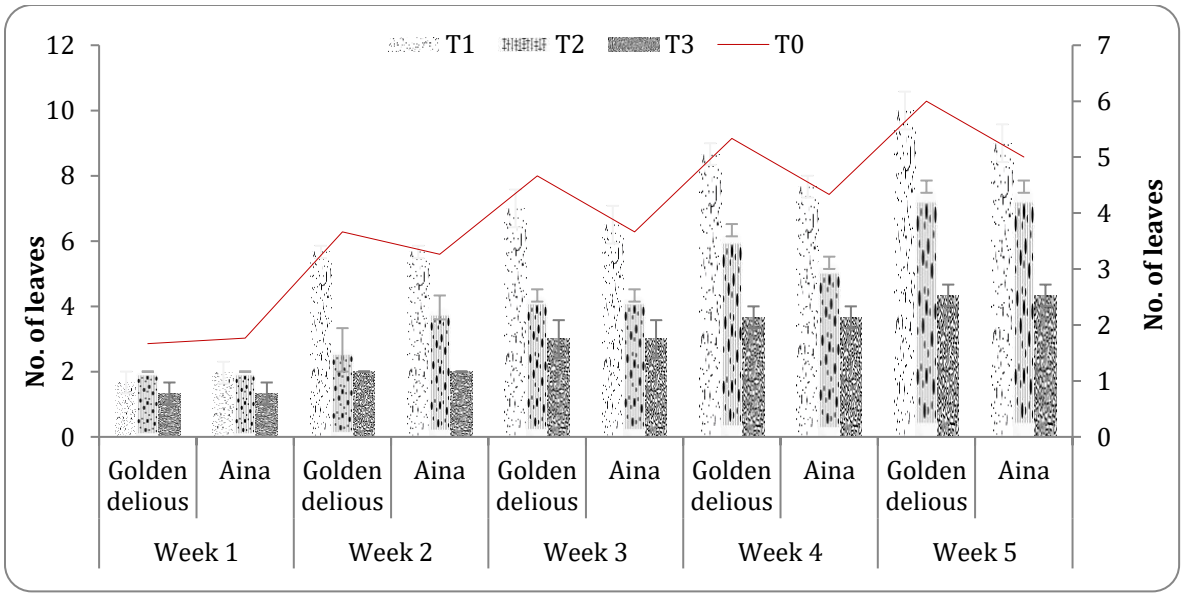

Figure 3 (a): Effect of growth regulators on number of leaves of cv. Golden delicious and Aina.

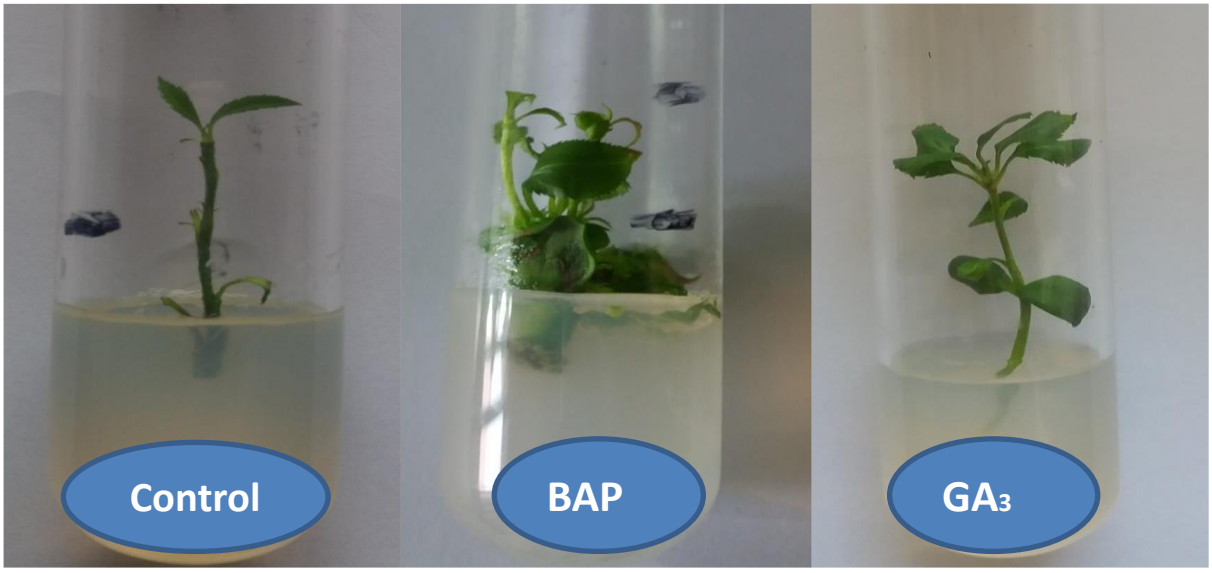

Figure 3(b). Effect of growth regulators on number of leaves (after 35 days).

Shoot multiplication

To study the effect of various concentrations of GA_3, BAP and IBA with MS medium on the shoot multiplication, the explants were cultured under sterile conditions. The number of shoots of each plant were recorded with weekly time interval. It was observed that maximum numbers of shoots (2 to 4) were obtained with 0.1mg/L BAP followed by 2mg/L GA_3 and 0.5ml/L IBA. Shoot multiplication of Golden delicious was more rapid than cultivar Aina as depicted in Figure 4(a), (b). Statistical analyses confirmed the significant effects of treatments for shoot multiplication.

***In-vitro* Conservation**

Established cultures (five weeks old cultures) of Golden delicious and Aina were shifted to MS medium amended with different concentration of sorbitol (10, 20 and 30 g/L) and mannitol (10, 20 and 30 g/L) under sterile condition. The culture was incubated at 25±3 °C under the light intensity of 1000 lux. The data were recorded on phenotypic parameters at monthly time interval. Results showed that 10mg/L sorbitol effect was found significantly different for both studied apple varieties' conservation as compared to other treatments and control as shown in Figure 5(a) and (b).

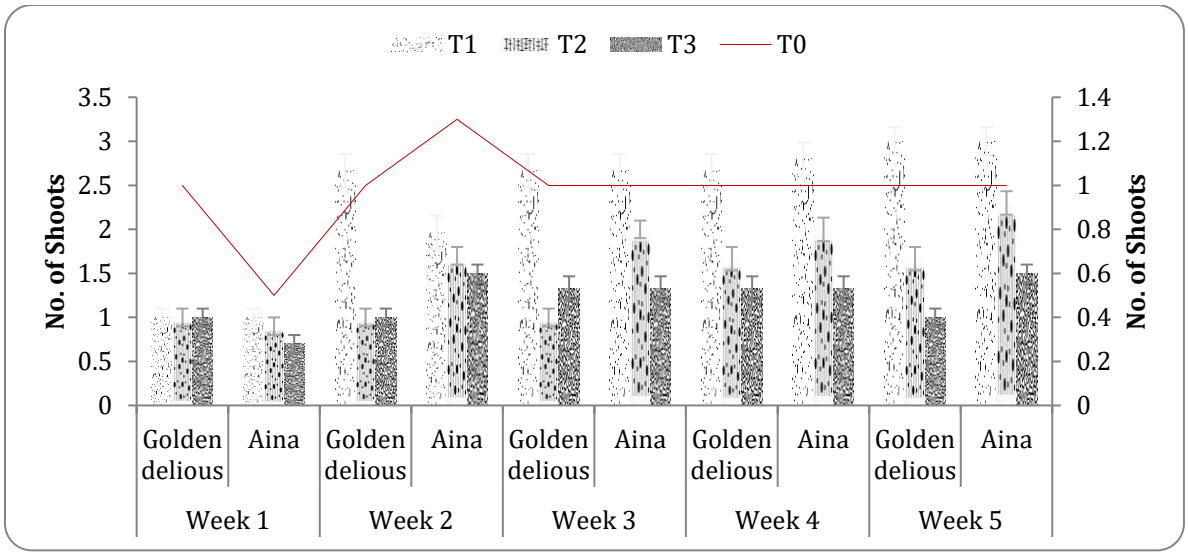

Figure 4(a). Effect of growth regulators on number of shoots of cv. Golden delicious and Aina.

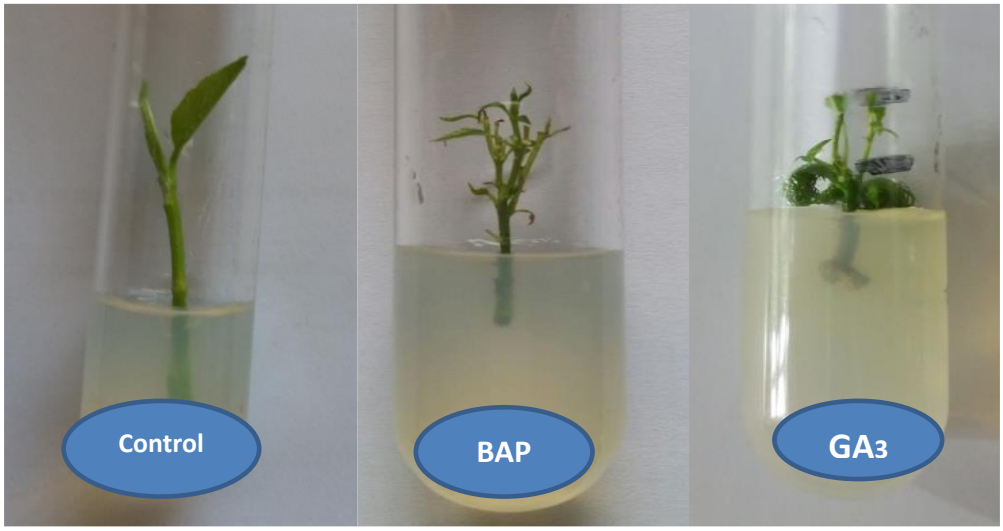

Figure 4(b). Effect of growth regulators on number of shoots after regular intervals of 5 weeks.

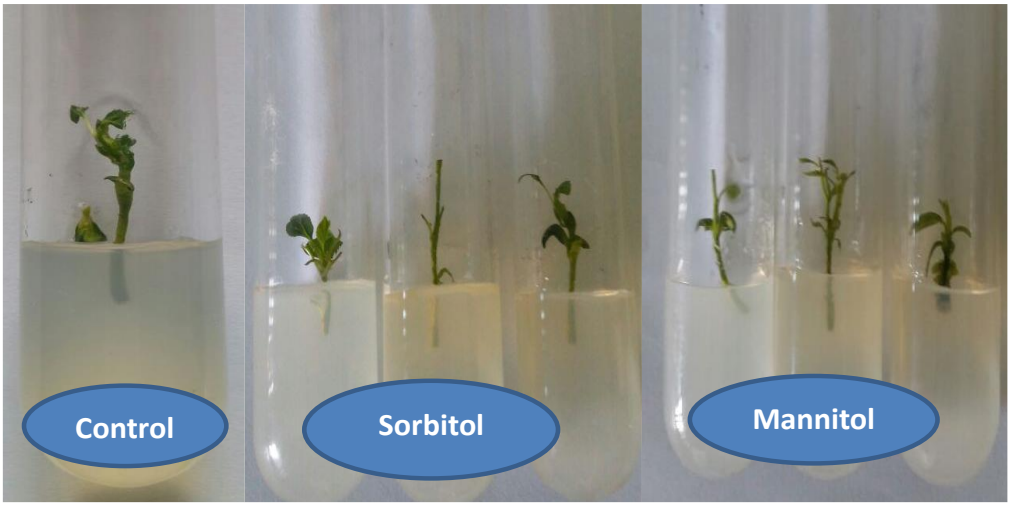

Figure 5(a). Plants cultured in conservation media at zero day.

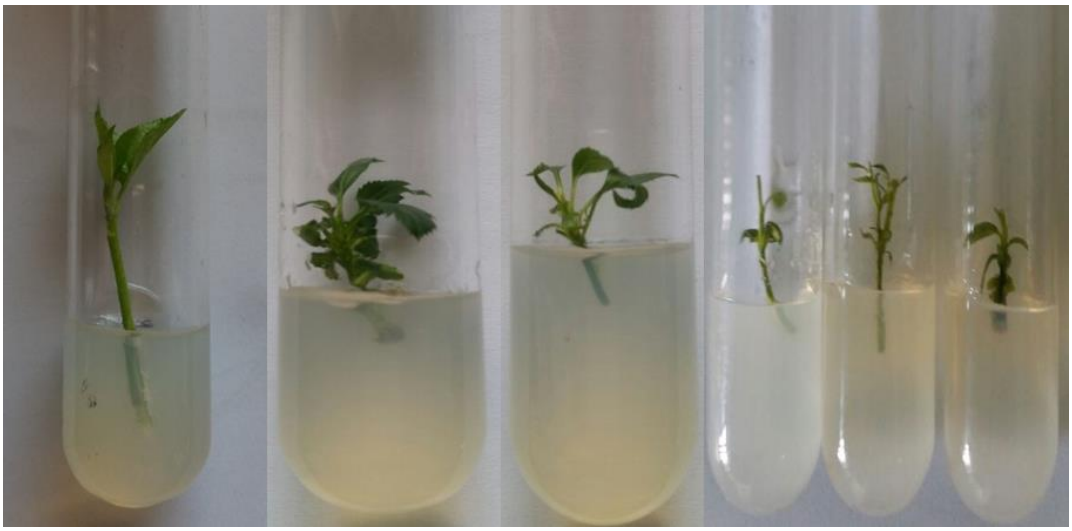

Figure 5(b). Effect of conservants after a month.

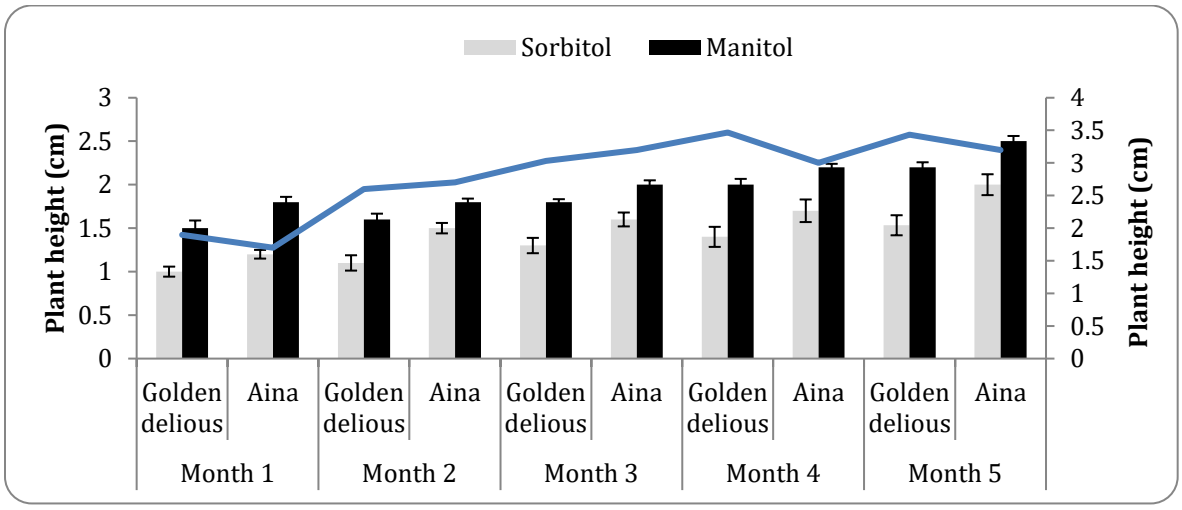

Figure 5(c). Effect of conservants on plant height of cv. Golden delicious and Aina under different time intervals.

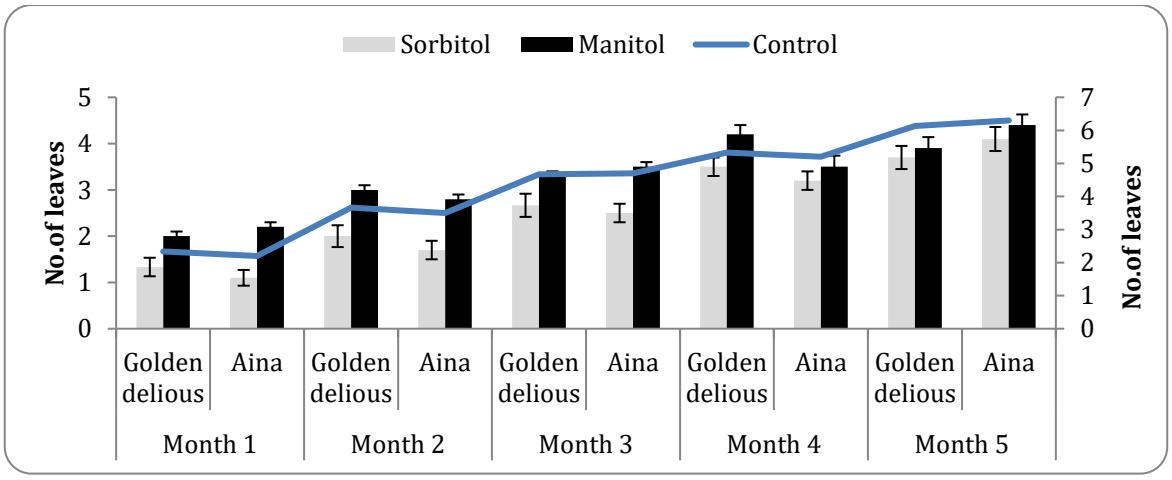

Figure 5(d). Effect of conservant on leaves number of cv. Golden delicious and Aina under different time intervals.

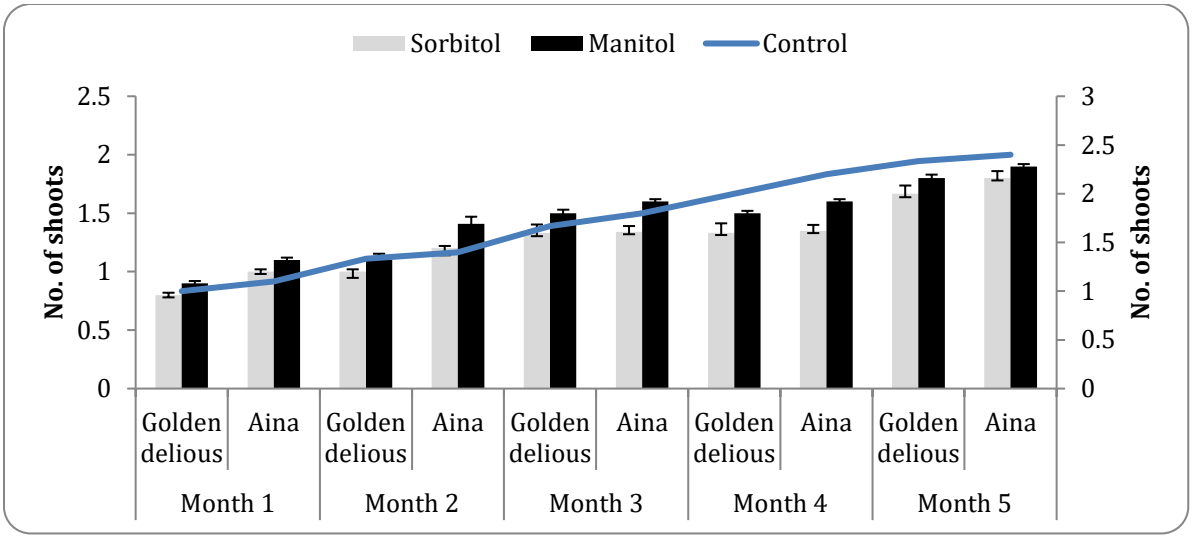

Figure 5(e). Effect of conservants on shoots numbers of cv. Golden delicious and Aina under different time intervals.

DISCUSSION

Our study is the way forward for solving the issues of apple through tissue culture technique. This technology could be utilized for the commercial production of plants that are microbes free (Bhatti *et al*., 2010). It is a speedy and reliable method for the production of a huge number of homogeneous plantlets during the year. Micro-propagation is turned into an imperative technique in both basic and applied sciences and also used for commercial purpose(Kakimzhanova *et al*., 2023). In the present study, different disinfectants were used to establish the *in vitro* cultures of *Malus domestica*. Our results showed that 10% sodium hypochlorite was most suitable as it has given out 100% survival of *in vitro* cultures. Previous studies also confirmed our findings (Boudabous *et al*., 2013; Maira *et al*., 2010; Jahan *et al*., 2009; Modgil *et al*.,2017; Lizárraga *et al*.,2017). Micro-propagation depends on the time period of season due to which propagation rate of plants should be low otherwise tissue culture techniques gives an effective protocol for commercial propagation of plants and among fruits, in vitro propagation of apple has been reported by many authors (Minaev *et al*., 2003; Kaushal *et al*., 2005; Dalal *et al*., 2006; Ciccotti *et al*., 2008).

In the present study, MS media fortified with three hormones (BAP, GA_3 and IBA) were used to check their effect on propagation of *Malus domestica.* Selected plants showed significant phenotypic variations by using these hormones regarding to the morphological data. Significant highest number of shoots, leaves and plant height observed in BAP and GA_3 hormones treatments used in MS media as compared to control. These hormones were used in different concentration (0.1 mg/L to 3.5 mg/L). It was found that low concentration of these hormones provided good result on number of shoots, leaves and plant height as compared to higher concentrations. Similar to our findings, other data of researchers also support that hormones used in different concentration for micropropagation of apple have remarkable effects (Boudabous *et al.,* 2010; Jain *et al*., 2023). BAP is a synthetic cytokinin that elicits development plant responses (Siddiqui *et al*., 2011). Current observation showed that maximum numbers of shoots and leaves gave best outcomes in media with 0.1mg/L BAP followed by GA_3 media. These findings were similar with Geng *et al*., (2015). Depending upon mode and rate of shoot multiplication, the achievement showed a commercial utility of an apple by micropropagation protocol. Media composition and plant growth promoters are responsible for the effectiveness of shoot multiplication (Dobrnszki *et al*., 2010).

Depending on the storage or preservation, the duration required is different for *in vitro* culture techniques and have been extensively developed to preserve plant resources during previous times (Hao *et al*., 2001). In the present study, two conservants i.e., sorbitol and mannitol (10, 20 and 30 g/L) were tested for *in vitro* conservation of *Malus domestica*. Data were recorded for five months on morphological parameters. Results showed that best conservation treatment was found with sorbitol @ 10g/L followed by 20 and 30g/L,

respectively. Lowest conservation was found on control of MS media and mannitol @ 10g/L followed by 20 and 30g/L, respectively. Our outcome is in agreement with Homosany and Shimaa (2016). Our finding also agreed with the results of Salisbury and Ross, (1985), they reported that metabolic process of plants is interrupted by the low amount of nutrient supplies which might become the cause of protein synthesis reduction. So, the provision of accurate amount of conservants is very necessary to get long term storage.

In this research, mannitol and sorbitol decrease the growth of *Malus domestica* and maintain slow growth that might be due to the function of these chemicals, because according to Shibi *et al.,*. (2006), sorbitol and mannitol create the condition of osmotic stress inside the plants which causes reduction in growth. These osmotic regulators decrease the water potential, resulted in the form of reduction in the uptake of water and nutrients to the conserved plant. Our findings would be helpful for the breeders and farmers and also for the commercial production of *Malus domestica.* With the help of propagation, the farmers will get the fast growth of plant in short period of time. By conserving *Malus domestica*, its germplasm could be of good foreign exchange in return.

CONCLUSION

In the nutshell, 10 % sodium hypochlorite as a disinfectant is suitable for in vitro culture establishment of apple and for micro-propagation, 0.1mg/L BAP is optimal as it gives maximum number of leaves, shoots and plant height as compared to MS control. Golden delicious cultivar showed good response to treatments as compared to cv. Aina. For in vitro conservation, sorbitol @ 10 % showed best response towards vegetative growth in contrast to mannitol for 5 months. In future, the propagated as well as conserved apple (*Malus domestica*) could be used for further research work especially for proliferation and for re-growing them under *in vivo* (field) condition.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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