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MICROSHOOT TIP CULTURE THERAPY FOR DISEASE ELIMINATION IN DIFFERENT VARIETIES OF GRAPES CVs. PRINCESS SEEDLESS AND GOLDEN ITALIAN MUSACT IN PAKISTAN

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ABSTRACT

Grape is considered as a vital commercial crop in Pakistan especially in Baluchistan. An effort has been made to establish a rapid protocol for virus free production of grapes varieties i.e. Princess seedless and Golden Italian musact through microshoot tip culture technology and callogenesis. The meristem (0.5mm) of grapes was cultured in Murashige and Skoogbasal medium provided with different levels of phytohormones (BAP, TDZ and NAA). The best results for shoot initiation were noted in MS+1.0mg/l BAP. In Princess seedless the shoot length was $1.9^{a}\pm0.06$ cm in $12^{c}\pm0.43$ days and in Golden Italian muscat the shoot length was $1.8^{a}\pm0.36$ cm in $13^{c}\pm0.48$ days. The extreme shoot multiplication was noticed inMS+BAP 1.0 mg/l+TDZ 6 µM for both varieties. The *in vitro* grown plantlets were then acclimatized in greenhouse in four different potting mixtures (redwood bark + coir + sand, sand+soil+ cocopeat, soil + cocopeat + leaf manure, sand + soil + leaf manure,).The frequency of plant survival was found best (95%) and (90%) in redwood bark-coir-sand for Princess seedless and Golden Italian muscat, respectively. For callogenesis, fresh leaves were inoculated on MS media supplemented with NAA, IAA, 2, 4-D and BAP. The highest percentages of callus formation i.e. 97% and 95% were obtained in MS+2,4-D 0.1mg/l+BAP 1 mg/l in Princess seedless and Golden Italian musact, respectively.In future, microshoot tip tissue culture therapy would be highly beneficial to produce virus free planting material and control spread of diseases in food crops at larger scale.

Keywords: Microshooting, virus eradication, disease free plants, Grape varieties and Acclimatization

INTRODUCTION

Grapes (*Vitis vinifera*) are the most significant plant species harvested with an area of about 9 million hectare globally. The foundation of domesticated grapes is supposed to be from Middle East (Alleweldt *et al.*1991). One of the most cost-effective vital crops all over the world is *V. vinifera* (Dev*et al*. 2019. On the basis of utilization and commercial importance *V. vinifera* are generally categorized as winegrapes, canning grapes, juice grapes, table grapes and raisin grapes. Different dietary, medicinal and commercial products such as vinegar, juice, jelly, jams and wine are made from grapes. Billions of dollars are represented by the global cost-effective influence of grape, grape juice and wine industries (Rehman *et al*., 2018). Grapes also inhibit numerous human ailments by antioxidant potential and exhibit antitumor potentials through blocking carcinogen-induced DNA adduct formation as a good source of basic food constituents, vitamins and minerals (Jung *et al.*2006).

In Pakistan, among fruits grapes rank at 10^{th} position (Ali *et al*., 2017). Grape is considered as a vital commercial crop in Pakistan especially in Balochistan. In 2009-10, total area for *V. vinifera* was 15300 hectare with total produce of 64700 t and 4.2 tons per hectare yield national wide (Ali *et al.*2014 and Rehman *et al*., 2018). *V. vinifera* L. belongs to the vitaceae, contains 60 species. Vitis is the only specie that attained noteworthy commercial importance with the passage of time (Rossetto*et al*. 2002). By using tissue culture therapy, multiplication of plant is done that are usually unable to produce seeds and didn't have ability to react well to customary vegetative propagation. Tremendous vital plants and plantlets have been produced by removing a small section of tissue from a plant and then grown it in media provided by nutrients under controlled aseptic physical conditions (Pedro *et al*., 2017). As this protocol is not dependent on weather conditions a huge quantity of uniform true copies of virus free plants have been manufactured in a comparatively lesser space and time (Hassan *et al*., 2018). Meristem, meristem tip cultures and axillary bud cultures have been used for proliferation of shoot. Nodal part of plant consisting of single axillary bud has been used to develop shoot cultures in *V. vinifera* (Mhatre *et al*.2000). For numerous grape species and hybrids the protocol of shoot redevelopment from patchy shoot apices has been found useful commercially (Barlass and Skene, 1978).

On the other hand, the monetary and medical benefits of *V. vinifera* could be damaged by numerous illnesses comprising parasitic (powdery mildew and gray rot), viral (corky bark, stem pitting) and fan leaf roll fleck) and bacterial infections (necrosis and pierces) which are responsible for the reduction in productivity and reduces life expectancy of *V. vinifera* (Jaskani*et al*.

2008). These ailments primarily begin from the contaminated spreading material acquired throughdifferent traditional methods of propagation. The large scale manufacturing of infection and malady free 'elite' planting material could only be possible by applying the procedures of unconventional propagation like microshoot tip tissue culture technique that has ability to wipe out the danger of contamination. It has now become necessity to apply *in vitro* techniques for conservation to overcome the problems of high mortality and low rooting in vegetative propagation of grapevines (Tehrim and Sajid, 2011). The current study was undertaken in order to optimize the conditions for protocol establishment for virus free grapes production through callogenesis and meristem tip tissue culture techniques.

MATERIALS AND METHODS

Explant source: Meristems from fresh *Vitis vinifera* explants from Princess seedless and Golden Italian muscat were taken from Lawrence garden, PHA, Lahore, Pakistan.

Surface sterilization of explant: The leaves of explant were removed followed by separation of apical meristem and washed with tape water to eliminate the dust. Afterwards explant were dipped in detergents (anti-bacterial liquid Max) and washed with distilled water to eradicate the particles of detergents. After that, all the explants were dipped in 10% sodium hypochlorite solution for 20 minutes. During this period the delicate tissues of *V. vinifera* were continually monitored in order to prevent browning of tissues. Then the explants were thoroughly washed with autoclaved distilled water 4-5 times to eradicate the tracesof commercial bleach (calcium hypochlorite).

Microshoot tip cultures: In this technique, a meristem tip is excised and grown into a whole new plant. Actively growing shoot tips approximately 1 to 2 cm were collected from different varieties of grapes i.e. Princess seedless and Golden Italian muscat. Meristem excision of 0.5 mm size was done aseptically in laminar airflow hood. Meristematic dome and leaf primordial comprising the meristem tip was visible in the growing shoot tip after taking off several scaly leaves under stereo microscope (IRMECO).This meristematic portion was carefully cut under stereomicroscope in order to eliminate virus that could be present in protophloem. These meristems were cultured into MS medium in optimum conditions of culture room (8-16 hrs dark and light photo period, 3000lux light intensity and 20±1ºC culture room temperature) (Ilyas *et al*., 2014).

Acclimatization in Greenhouse: The plantlets were dipped in antifungal solution (diathine) for 1 minute. The *in vitro* grown plants were first shifted into autoclaved sand for a time period of at least 30 days with regular watering. Their survival rate was observed by calculating the percentage of survived plantlets. These pots were covered with plastic sheets to ensure higher humidity around the plants. Later on, the effect of different potting mixtures and fertilizers on plant growth and survival was observed under optimum conditions.

Callogenesis: Young leaves from different varieties of *Vitis vinifera* i.e. Princess seedless and Golden Italian muscat were collected and surface sterilized the explants under aseptic conditions. The young leaf sections were inoculated into suitable nutrient MS media under aseptic conditions (Javad *et al*., 2016).

Statistical analysis: A completely randomized design with at least 3-5 replicates was used for the experiments. The readings for each parameter were subjected to analysis of variance (ANOVA) by means of the COSTAT V.63: statistical software (Cohort software, Berkely, California). The averagevalue was analyzed with the LSD (least significant difference) test following Duncan's new multiple range (DMR) test at 5% significant level.

RESULTS AND DISCUSSION

Microshoot tip cultures: The effect of different concentrations of various plant hormones on shoot initiation of different varieties of grapes i.e. Princess seedless and Golden Italian muscat was observed. MS media supplemented with different concentrations of BAP (0, 0.1, and 1.5 mg/l) were tried to study the effect on shoot initiation in both varieties of grapes (Fig. 1). In Princess seedless the shoot length was $1.9^{a}\pm0.06$ cm in $12^{c}\pm0.43$ days and showed 91% shoot regeneration at 1.0 mg/l concentration of BAP as given in Fig. 7a. In Golden Italian muscat the shoot length was $1.8^{a}\pm0.36$ cm in $13^{c}\pm0.48$ days and showed 87% shoot regeneration at 1.0 mg/l concentration of BAP. It was noticed thatthe extreme percentage of shoot initiation and shoot length was noticed in BAP 1.0 mg/l for both varieties of grapes. These outcomes were also described in research experiments of Diab *et al.*(2011). They reported that BAP acts as promoter in case of plant establishment from meristems and rapid shoot initiation. Park *et al*. (2001) also reported similar findings and optimized BAP as the best media of shoot initiation for grapes. While Ali *et al*., (2017) reported that the cultivars responded differently to the different PGRs used for *in vitro* micropropagation. According to their research the best combination for efficient shoot induction and multiplication was MS medium supplemented with BAP 2.0 mg/L for Thompson, with KN1.0 mg/L for Crimson, with BAP 4.0 mg/L for Autumn Royal and with BAP 1.0 mg/L for Red Globe variety of grapes.

In present work, the potential of another cytokinin such as TDZ (µM) was also tested on shoot initiation of both two varieties. Various concentrations of TDZ (2, 4, 6 µM) were utilized to study the response of shoot induction of different varieties of grapes i.e. Princess seedless and Golden Italian muscat (Fig. 2). In Princess seedless the shoot length was 2.1^{ab}±0.22 cm in 16^{c}±0.89 days and showed 82% shoot regeneration at 6 µM concentration of TDZ. On the other hand in golden Italian muscat the shoot length was 2.2^{a}±0.21 cm in 14^{c}±0.75 days and showed 85% shoot regeneration at 6 µM concentration of TDZ (Fig. 7b). It was noticed that at high concentration of TDZ, maximum frequency of shoot induction with highest shoot length was observed and lesser time was utilized for shoot initiation in both varieties of grapes. The promotive effect of TDZ for shoot induction of grapes was also reported by Kumsa (2017).

The effect of several concentrations of BAP+TDZ on multiple shoot formation of two varieties of grapes i.e. Princess seedless and Golden Italian muscat were also studied (Fig. 3a-b). Different parameters were observed in combinations of BAP (0.5, 1.0, 1.0 mg/l) and TDZ (2, 4, 6 µM) at different concentrations. In Princess seedless the shoot length was 3.4^{a} ±0.14 cm, no of shoots were 3^{ab}±0.5 cm, the length of root was 3.0^{a}±0.51 cm and the no of roots were 2^{ab} ±0.45 in 20^{d} ±0.89 days and showed 90% frequency of multiple shoot formation at concentration of 1.0 mg/l of BAP and 6 µM TDZ of BAP+TDZ combination (Fig. 7c). Similarly in Golden Italian muscat, the shoot length was 3.5^{a} ±0.51 cm, no of shoots were 4^{a}±0.86, the length of root was 2.9^{a}±0.56 cm and the no of roots were 3^{a}±0.63 in 21^{cd} ±0.89 days and showed 86% frequency of multiple shoot formation at concentration of 1.0 mg/l of BAP and 6 µM TDZ in combination (Fig. 7d). Diab *et al*. (2011) found during their research that BAP 1.0 mg/l with high concentration of TDZ gave good results.

Cytokinin along with auxin also plays a vital role for shoot multiplication. Combinations of BAP+NAA were also tested for its effect on shoot multiplication of varieties of grapes as mentioned in Fig. 4a-b. In case of Princess seedless, the shoot length was 5.3^{a} ±0.29 cm and showed 86% frequency of multiple shoot formation at concentration of 1.0 mg/l of BAP and 0.1 mg/l of NAA in combination (Fig. 7e). In Golden Italian muscat the shoot length was 5.0^{a}±0.45 cm and showed 81% frequency of multiple shoot formation at concentration of 1.0 mg/l BAP and 0.1 mg/l BAP+NAA. Similar findings were also reported by Alizadeh *et al*. (2012). It is found that although both varieties of grapes showed good results towards BAP+NAA combination at concentration of 1.0 mg/l of BAP and 0.1mg/l of NAA but princess seedless somehow showed better results. It was noted that low concentrations of NAA i.e. 0.1mg/l with 1.0 mg/l concentration of BAP favored shoot multiplication and when the concentration of BAP was further increased it showed negative effect. It was further revealed in present study that various cytokinin combinations with auxins were found to be the best for shoot multiplication of both varieties of grapes.

Combined forms of TDZ+NAA (µM/mg/l) in MS media were also utilized for shoot multiplication from meristematic parts of both varieties (Fig. 5a-b). In case of Princess seedless variety, the shoot length was 3.9^{a}±0.24 cm and no. of shoots were 3^{bc}±0.37 at 6µM concentration of TDZ and 0.1 mg/l of NAA. In Golden Italian muscat the shoot length was 4.0^{a} ±0.37 cm, no of shoots were 3^{bc}±0.45, the length of root was 3.2^{a} ±0.36 cm and the no of roots were 2^{ab} ±0.37 in 25^{e} ±0.37 days and showed 85% frequency of multiple shoot formation at 6µM concentration of TDZ and 0.1 mg/l of NAA (Fig. 7f-g).The work of Jaskani *et al.* (2008) on grapes by using combination of TDZ+NAA revealed the same facts. The present work also highlights the acclimatization of regenerated plantlets of Princess seedless and Golden Italian muscat (Table 1). *In vitro* micropropagated plantlets of different varieties of grapes were shifted to greenhouse in different potting media which were soil + cocopeat + leaf manure and redwood bark + coir + sand,sand + soil + cocopeat and sand + soil + leaf manure. The mixture of redwood bark + coir + sand gave best (95%) survival percentage (Fig. 7h-i). After hardening, plants were well grown with treatments of Hoagland solution. Similar results were reported by Amancio*et al*. (1999).

Callogenesis: The another part of present workrevealed the role of various concentrations of 2,4-D on callogenesis in different varieties of grapes (Fig.6A). For Princess seedless and Golden Italian muscat, the optimum results were observed in MS medium having 2,4-D 3.0mg/l with maximum percentages of 93 and 95% respectively. It was also observed that callus was in brownish white colour in princess seedless and light brown colour in Golden Italian muscat (Fig. 7j). The effectof 2,4-D on callus formation of grapes and other plants is also described by many researchers. Similar findings were reported by Torregrosa *et al*. (1995) on *Vitis Muscadinia*.

Influence of cytokinin+auxin interaction wasplanned on callogenesis of grapes as well. MS medium + 2,4-D 0.1 mg/l + BAP 1 mg/l showed best results in Princess seedless, same concentrations proved to better than others in Golden Italian muscat with 97% (Fig. 7k) and 95% of callus formation respectively (Fig.6B). Das *et al*. (2002) also reported same findings during his research on grapes by using same combination. In present study, four various concentrations of 2, 4-D+NAA were utilized to calculate the bestcombination of auxin-cytokinin in MS basal media for the optimum induction of callus in grape varieties (Fig.6C). The high

frequency of callus induction i.e. 93% was calculated in concentrations of 2,4-D+NAA (3+0.5 mg/l). While for Golden Italian muscat the extreme rate of callus formation i.e. 91% was noticed in concentrations of 2,4-D+NAA (3+0.5 mg/l) (Fig. 7l). The same facts were proposed by Salunkhe *et al*. (1999). Some researcher reported that the combination of BAP 1 + NAA 1 + 2,4-D 2.5 (mg/L) produced friable green calli in some other plants like *Stevia rebaudiana*. By comparing the results of both varieties it was found that both varieties showed good results at this concentration. The present study also revealed the effect of different concentrations of 2, 4-D +IAA on callogenesis from explants of Princess seedless and Golden Italian muscat (Fig.6D). For Princes seedless the highest frequency of callogenesis i.e. 91% was obtained in 3+0.5 mg/l concentrations of 2,4-D+IAA(Fig. 7m). While for Golden Italian muscat the highest frequency of callogenesis was also obtained at the same concentration. Similar findings were reported in grapes by Lopez-Perez *et al*. (2015).It was concluded that microshoot tip tissue culture therapy was the best technique to produce maximum number of virus free plants of grape varieties.

Conclusion: The experimental results of current study revealed that micro shoot tip culture and callogenesis can be very effective techniques for maximum production of grapes varieties by adding different concentrations of plant growth regulators in MS basal medium. It is concluded from results that the best medium for shoot initiation among all combinations of plant hormones was MS + BAP 1mg/L and MS+BAP 1.0 mg/l+TDZ 6 µM was declared as best shoot multiplication in case of both varieties. Hardening of plantlets in green house was better accomplished in potting mixture of redwood bark + coir + sand. Thus, the study revealed that BAP and TDZ were more effective in maintaining the disease free plant production of grape varieties.

Table 1. Effect of different concentrations of potting media on hardening of plantlets of grapes varieties.

Sr. No.	Varieties	Potting mixture	Frequency of plant survival (%age)	Growth of plants after Acclimatization	
				Growth of plants treated with Hoagland solution	Growth of plants treated without Hoagland solution
1.	**Princess seedless**	Sand-soil-leaf manure	70	+	+
		Sand-soil-cocopeat	80	++	+
		Soil-cocopeat leaf manure	85	++	+
		Redwood bark-coil-sand	95	+++	++
2.	**Golden Italian muscat**	Sand-soil-leaf manure	71	+	+
		Sand-soil-cocopeat	79	++	+
		Soil-cocopeat leaf manure	80	++	+
		Redwood bark-coil-sand	90	+++	++

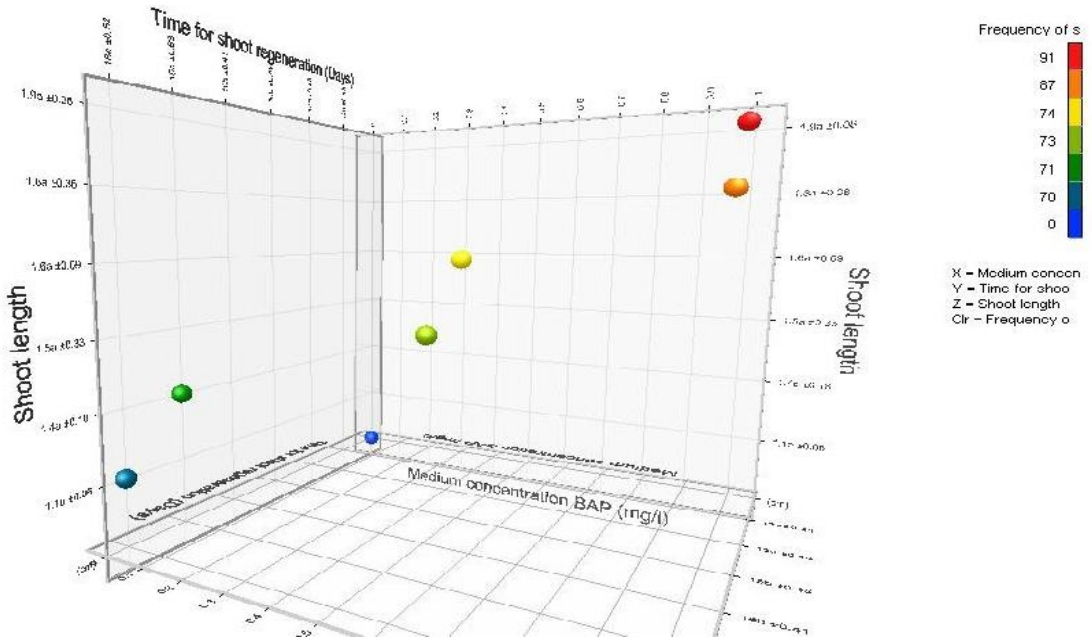


Fig. 1: Effect of different concentrations of BAP on shoot induction of grape varieties.

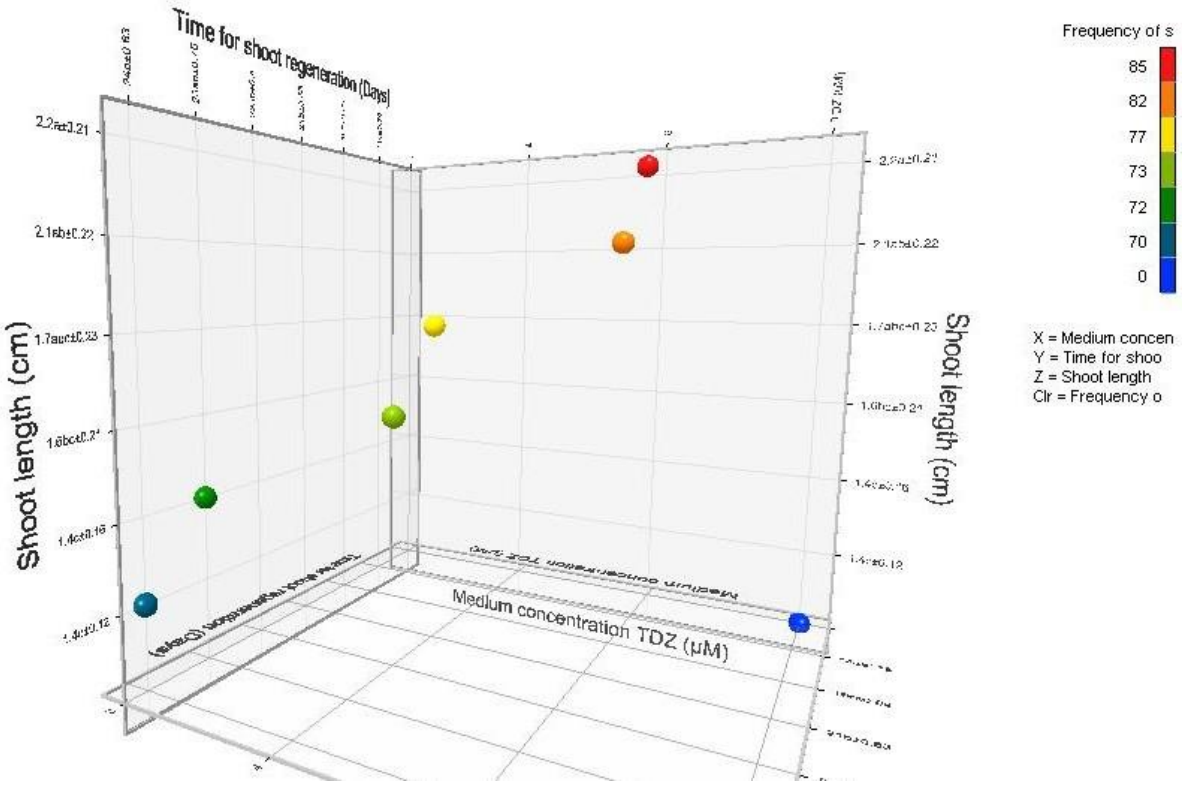
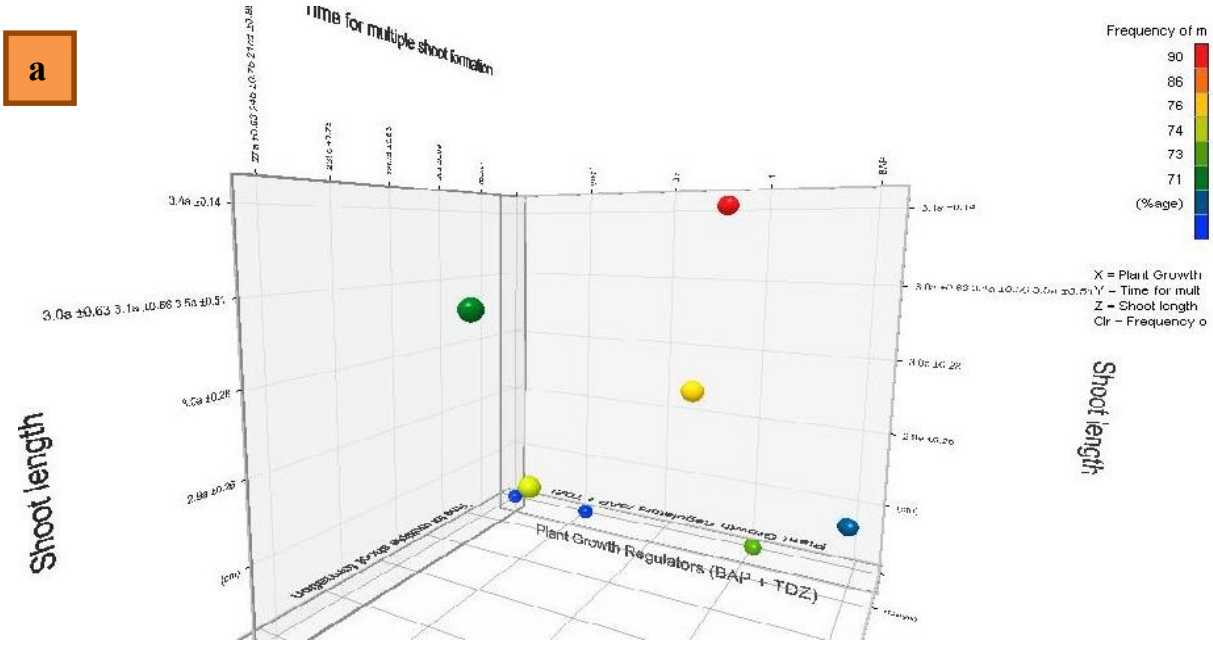


Fig. 2: Effect of different concentrations of TDZ on shoot regeneration of grape varieties.



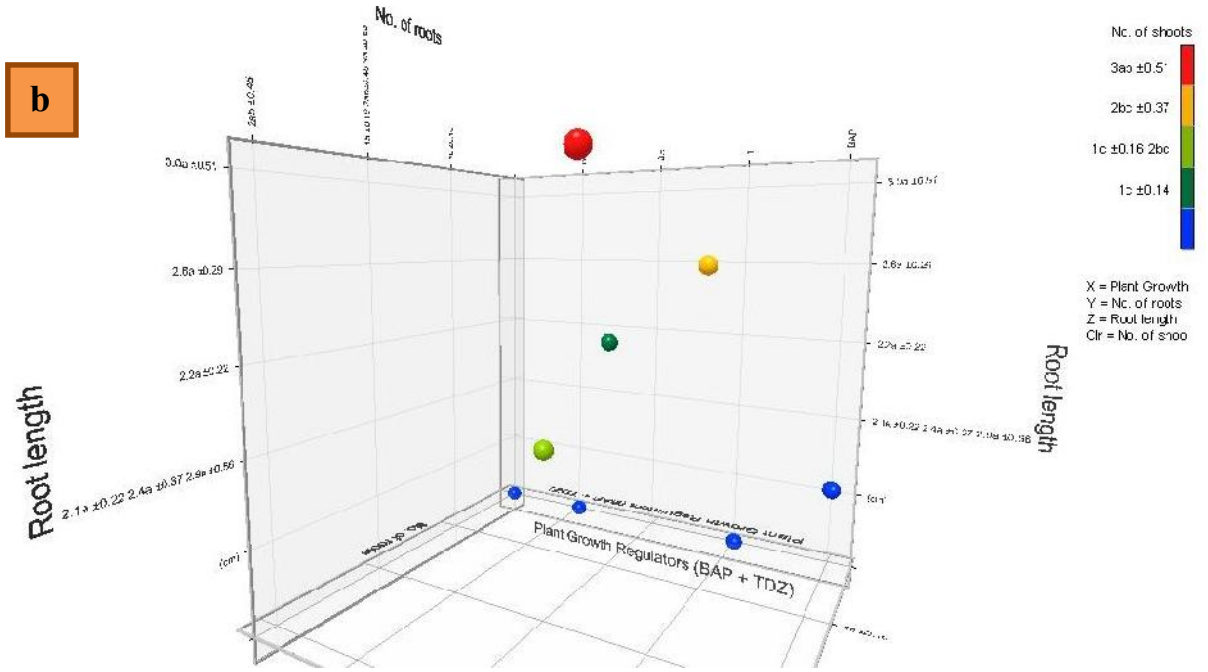
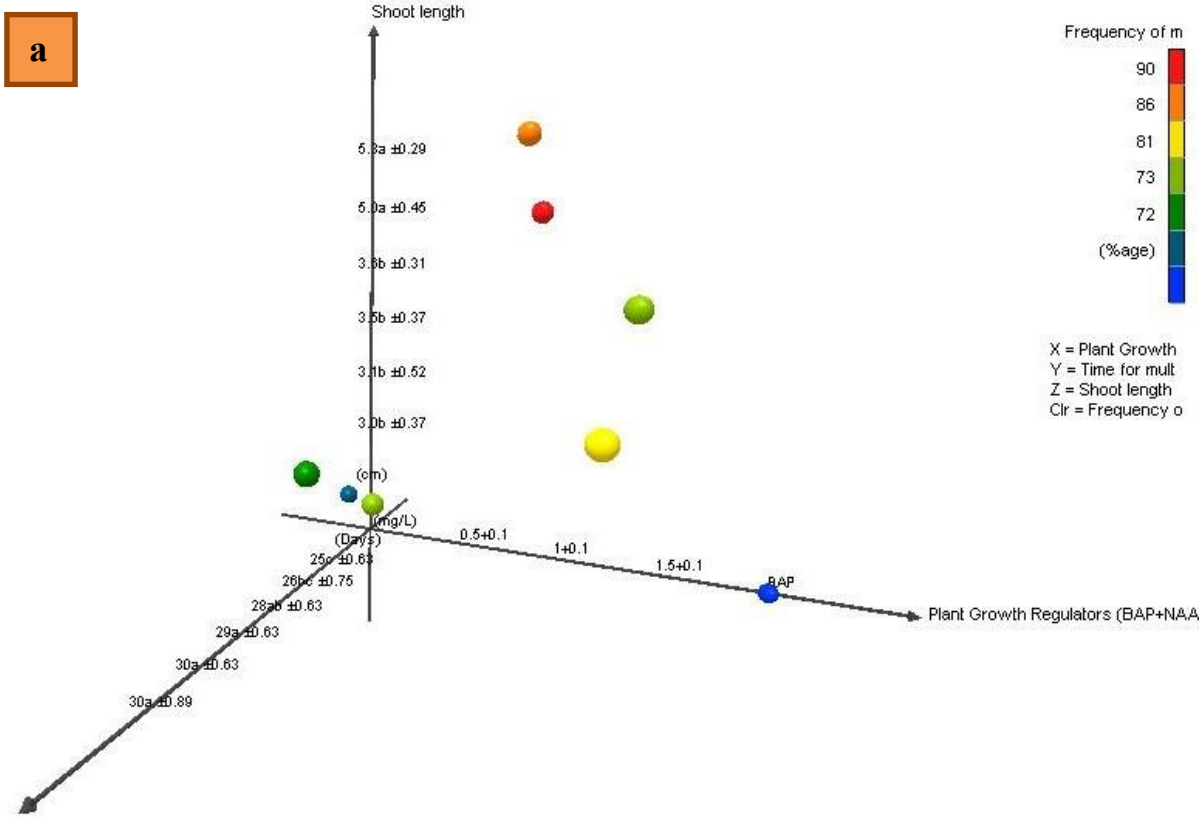


Fig. 3a-b: Effect of different concentration of BAP+TDZon shoot multiplication of grape varieties.



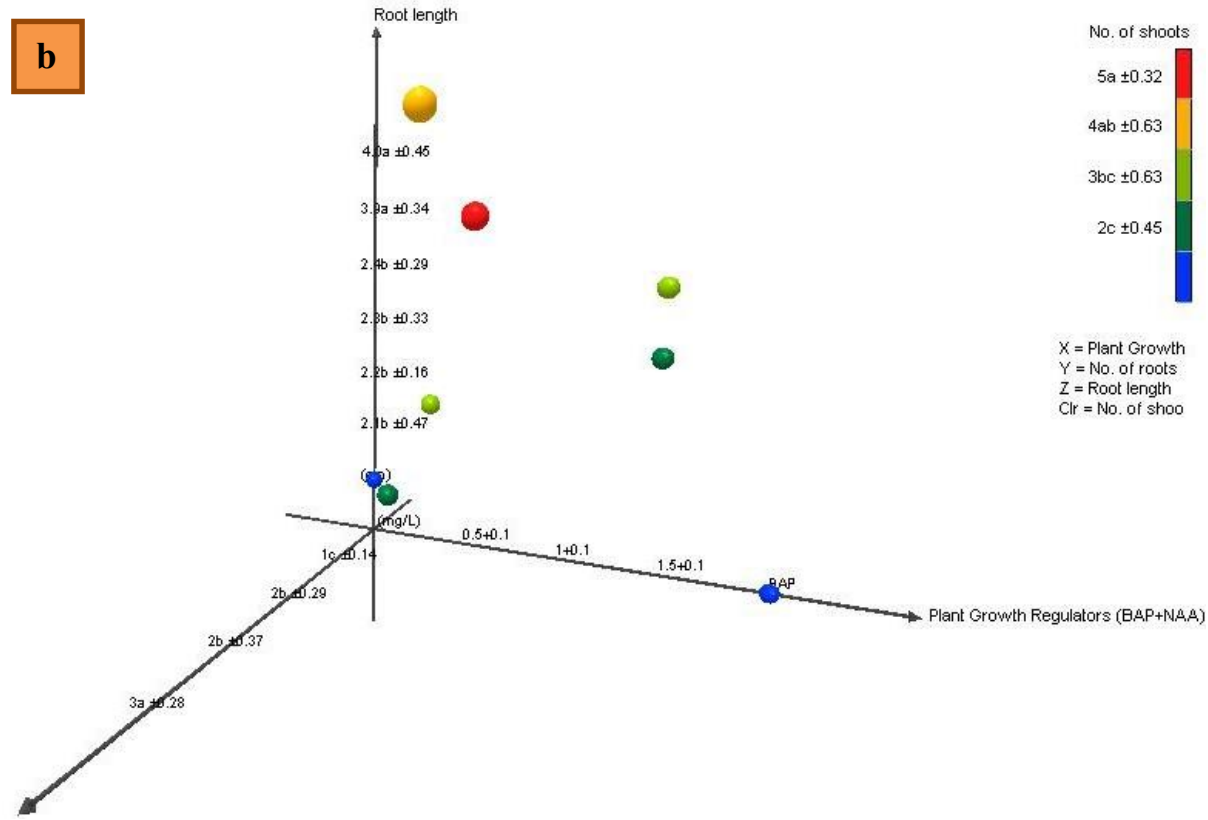
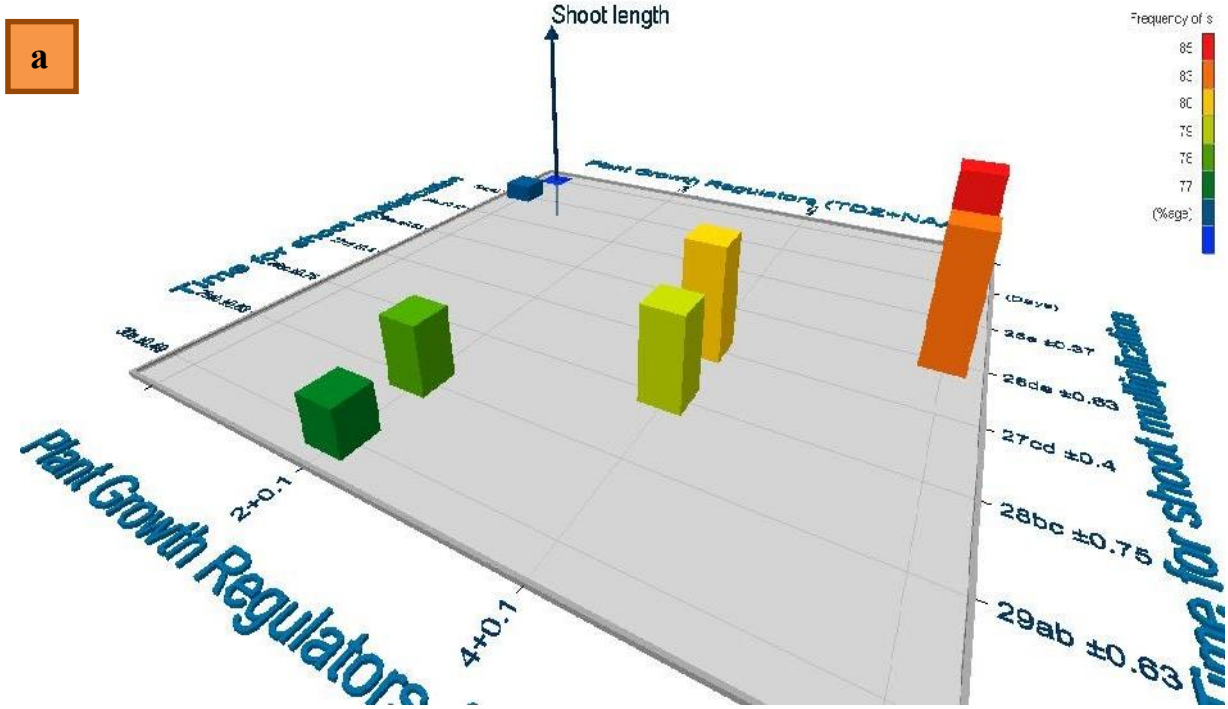


Fig. 4a-b: Effect of different concentration of BAP+NAA on shoot multiplication of grape varieties.



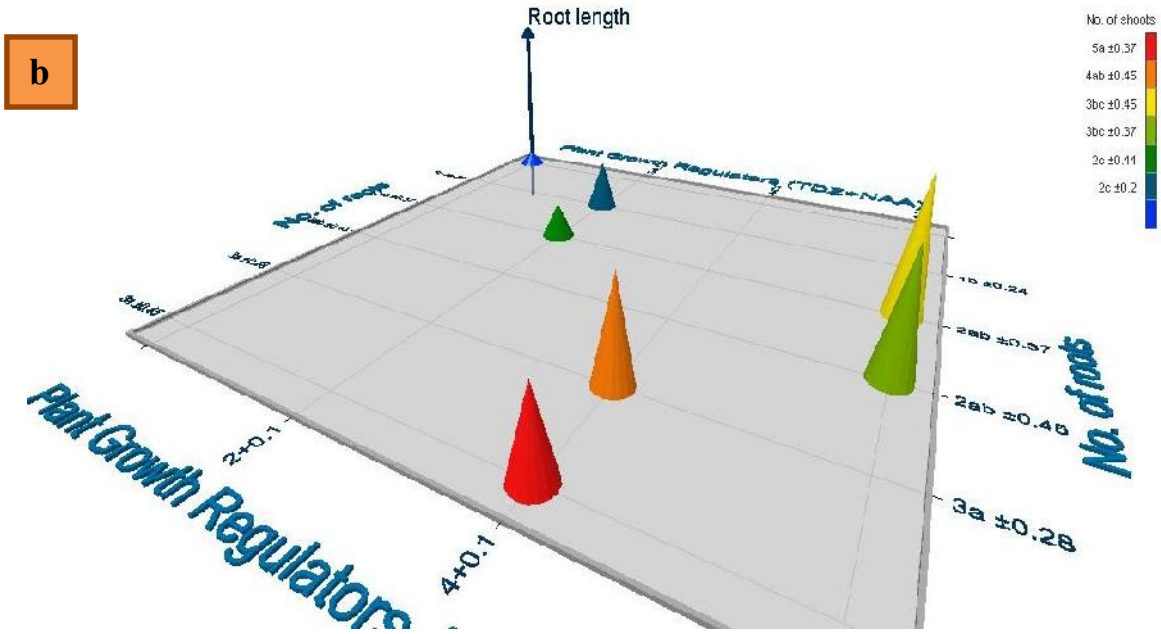


Fig. 5a-b: Effect of different concentrations of TDZ+NAA on shoot multiplication of grape varieties.

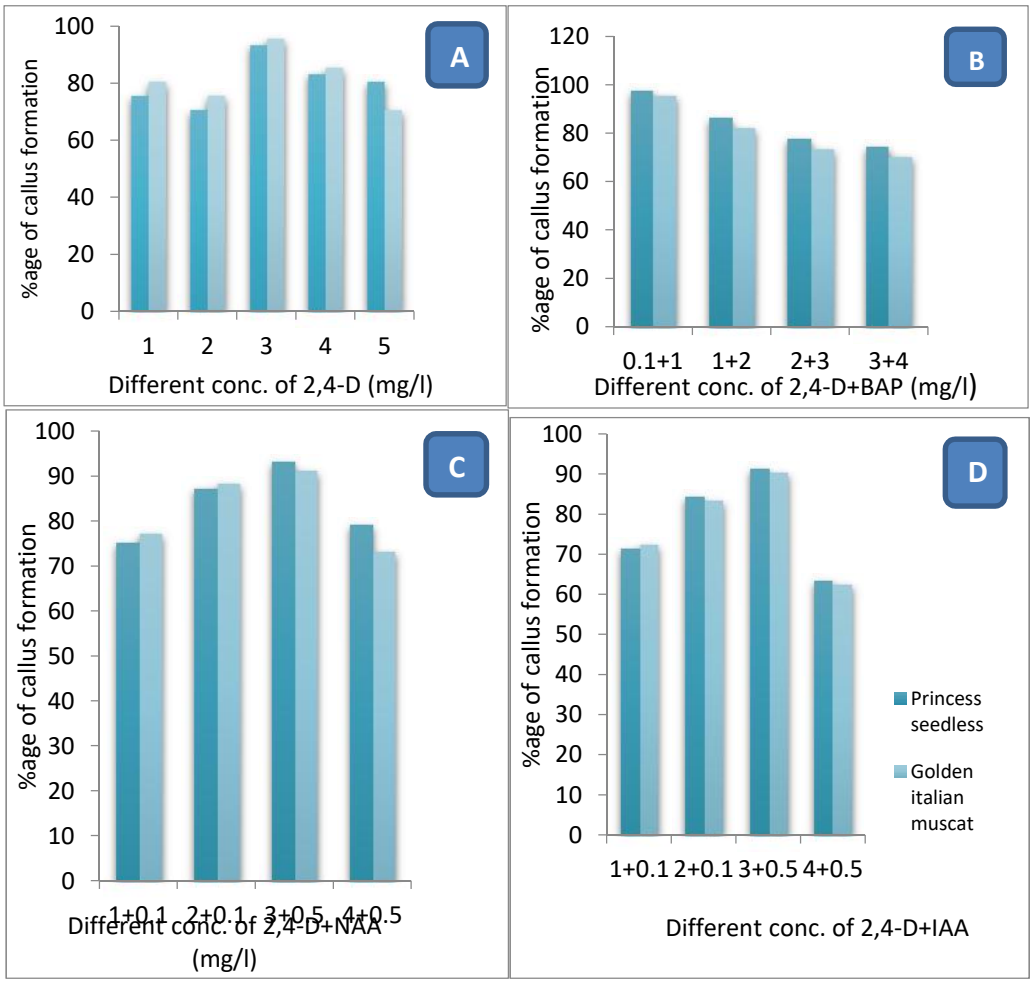


Fig.6A-D:Effect of different concentrations of auxin and cytokinin on callogenesis from explants of grapes. A). MS+2,4-D (mg/l), **B).** MS+2, 4-D + BAP (mg/l) **C).** MS+2,4-D + NAA (mg/l) **D).** MS+2,4-D + IAA (mg/l).

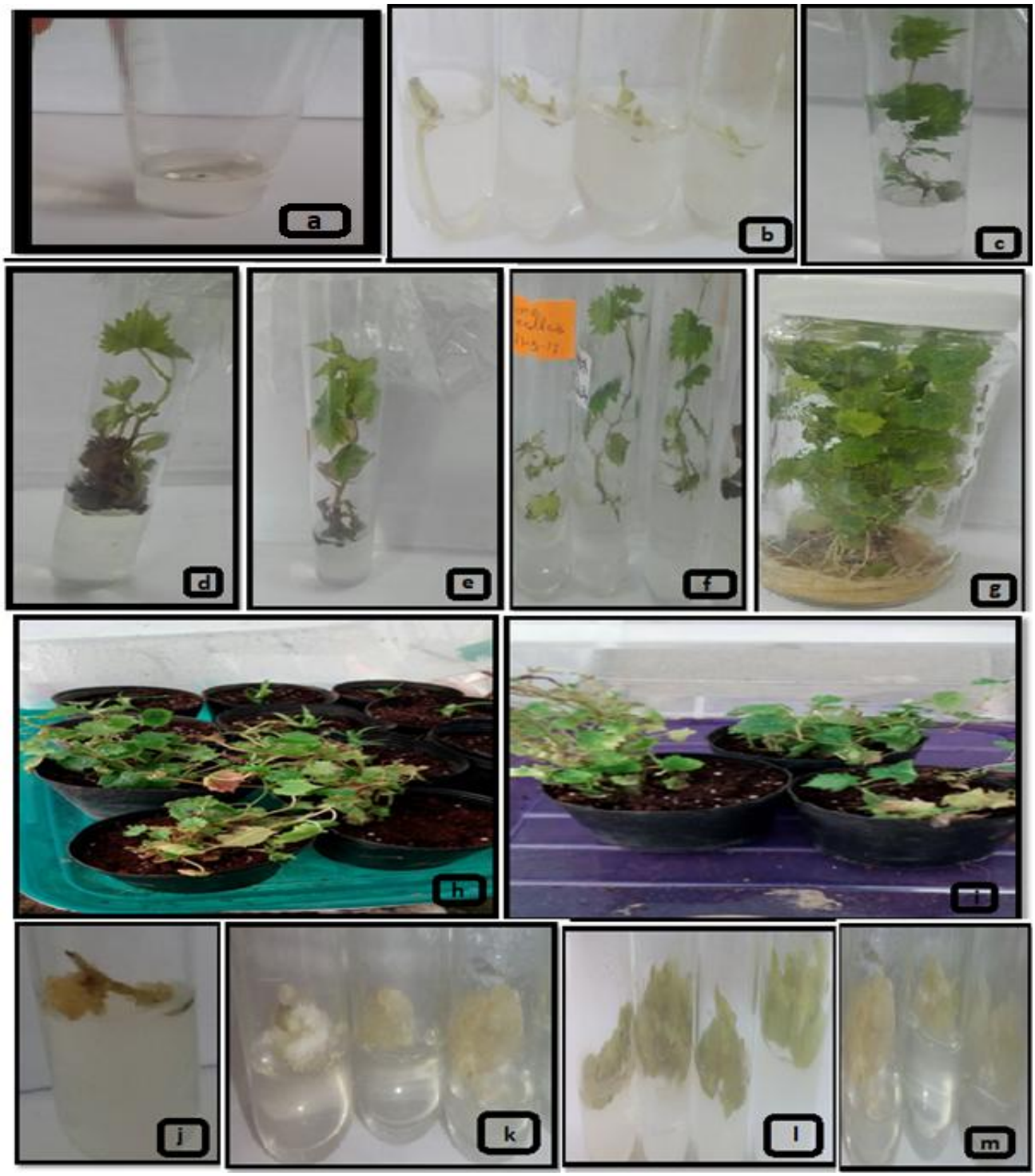


Fig.7a-m:Effect of MS media with different plant hormones on plant production technique and callogenesis of grape varieties. (a) Shoot initiation in MS+BAP 1mg/l in Princess seedless **(b)** Shoot initiationin MS+TDZ in Golden Italian muscat**(c)** Initiation of shoot multiplication in MS+BAP+TDZ in Princess seedless **(d)** Initiation of shoot multiplication in MS+BAP+TDZin Golden Italian muscat **(e)** Shoot length in MS+BAP+NAA in Princess seedless**(f-g)**Shoot length and shoot multiplication in MS+TDZ+NAA in Golden Italian muscat **(h-i)** In greenhouse acclimatization of plantlets of Princess seedless and Golden Italian muscat **(j)** Callus formation in MS+2,4-D 3mg/l in Princess seedless **(k)** Callus formation in MS+2,4-D 0.1mg/l+BAP 1mg/l in Golden Italian muscat **(l)** Callus formation in MS+2,4-D+NAA (3+0.5 mg/l) in Golden Italian muscat **(m)** Callus formation in MS+2,4-D+IAA(3+0.5 mg/l) in Princes seedless.

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