Standardization of Micropropagation protocol in Rose (*Rosa* spp.) var. DIVINE

Abstract

The present investigation was carried out to establish a standardized sterilization protocol for explants and regenerate shoots from nodal explants. Results showed that sodium hypochlorite (1%) for 4 min., cefotaxime (0.10%) for 15 min., streptomycin (0.10%) for 15 min., carbendazim (0.20%) for 15 min. and mercuric chloride (0.10%) for 4 min were the most efficient treatment with the least contamination and highest survival rates and media containing 0.50 mg/L TDZ and 3.0 mg/L BAP favored the maximum percentage of shoot initiation (100%) with an average of 4.60 shoots per node within 8.38 days of the culture period resulting longest shoot with 1.88 cm and an average of 4.53 shoots per node within 9.00 days of the culture period resulting longest shoot with 1.86 cm after 30 days of inoculation, respectively. After first sub-culture, it was found that MS medium supplemented with TDZ (0.50 mg/L) and BAP (3.0 mg/L) achieved 100% success rate for shoot multiplication.

Keywords: *Rosa* spp., sterilization, multiplication, rooting, acclimatization

Introduction

Rose belongs to the family Rosaceae. They are mostly low or medium sized ornamental shrub, usually with prickly stems. Most of the cultivated roses are found in the temperate zones of the northern hemisphere and yet Asia seems to be the gene centre where the majority of species are found (Broertjes and Van Harten, 1988). The chromosome number of rose varies from 2n=2x=14 to 2n=8x=56, but most species are diploid or tetraploid. The commercially grown rose cultivars are generally triploid or tetraploid.

The Divine rose is a type of hybrid tea rose plant, known for its beautiful, bright red flowers. The chromosome number of Divine rose is 2n=2x=14. It is a relatively new rose variety that was developed by the French rose breeder Meilland International in 2012. The Divine rose plant typically grows to a height of about 1 to 1.5 meters and produces large, double blooms that are around 10 to 12 centimeters in diameter. The flowers have a classic rose shape and are deeply fragrant, making them a popular choice for cut flowers and bouquets. The Divine rose plants are also known for their disease resistance, which means they are less prone to

developing common rose diseases like black spot or powdery mildew. This makes them easier to care for and maintain than some other types of roses. Roses are loaded with anti-inflammatory and antibacterial property. Divine Rose's 5% essential oil is used in aromatherapy to relieve skin irritations, bruises, minor cuts and burns as well as to help relieve headaches and calm nerves (Britannica, 2023).

India is exporting cut roses to the more than 55 countries. However, the export of cut roses from India is less than 1.00 per cent of global export. India holds first position in cut roses production in the world followed by China, Ecuador, Colombia, Kenya, Mexico, Italy, Thailand, Japan and Netherlands. The total area in India under loose rose is 41.30 thousand hectares in the year 2022-23 with production of 210.40 thousand tonnes and productivity of 5092 kilogram per hectare. The total production in India under cut rose is 339.10 thousand tonnes in the year 2022-23. In Gujarat, total area under loose rose is 4.5 thousand hectares in the year 2022-23 with production of 76.4 thousand tonnes and productivity of 1684.7 kilogram per hectare (Anon.,2023).

Roses are the high demanded cut flowers all over India, due to its fragrance and attractive colour. It is widely known for its aesthetic purpose. It also holds major position in pharmaceutical industry. Rose extract and oil has extreme use in cosmetic industry as an ingredient of soap, perfumes and body spray etc. Some of the rose varieties are showing poor germination and slow growth habit if propagated by conventional propagation methods. In this regard, micropropagation techniques will be useful to obtain true-to-type and faster growing rose planting materials. Micropropagation in rose is carried out in order to produce a greater number of the healthy plants. For commercial exploitation on large scale, it is necessary to perform *in vitro* culture.

Material and Method

Plant material

The experimental material were collected from the *Motibaug* Nursery, Junagadh Agricultural University, Junagadh. Node was used for initation of *in vitro* culture in Tissue Culture Laboratory, Department of Genetics and Plant Breeding, JAU, Junagadh.

Culture establishment

Explant was first properly cleaned with tap water. All dead and contaminated portions were discarded from nodes and leaves. Then, these cut portions were placed in a clean culture bottle, the mouth of the bottle covered with muslin cloth and then kept under running tap water for 15 min. to remove dust particles and microbes adhering to the surface. Sterilization

of the explants was done outside the laminar flow with 2 times tap water wash then sterilized water wash for 2 times then wash with few drops of Tween-20 and carbendazim for 40 min. on shaker and sterilized water wash for 4 times. Explants were treated with four different treatments of sterilization and each treatment was repeated five times. Each repetition consisted of ten test-tubes. Treatment details for sterilization protocol were as per Table 1.

Table 1: Treatment inside laminar air flow

Sterilant / Treatments	Sodium hypochlorite 1%	Cefotaxime 0.1%	Streptomycin 0.1%	Carbendazim 0.2%	Mercuric chloride 0.1%
ST_1	-	10 min	10 min	10 min	5 min
ST_2	-	15 min	15 min	15 min	3 min
ST_3	-	20 min	20 min	20 min	5 min
ST_4	4 min	15 min	15 min	15 min	4 min

Note: **ST**= Sterilization treatment

After sterilization, explants were inoculated to Murashige and Skoog (1962) media (without hormones to check fungal and bacterial contamination and to standardize the sterilization protocol. The fungal and bacterial contamination were recorded after seven to fourteen days. The best treatment was selected and used further for experiment.

Multiplication stage experiments

The explants were transferred to sterile petri dish/autoclaved paper; fresh slant cut was given to the sterilized nodes to remove undesirable or dead portions and to prepare uniform-sized explants (1.0-1.5 cm length) with single node for inoculation. Explants were inoculated on three different treatments of TDZ, four different treatments of BAP and two kinetin along with control and each treatment was repeated four times. Each repetition consisted of twenty test tubes. Each test tubes consisted of single nodal explant (80 explants/treatment). The explants were inoculated vertically into MS medium for keeping the polarity of explant. The medium consists of full MS media with sucrose (3% w/v) and agar (0.8% w/v). The explants were inoculated into MS media devoid of hormones (control) and MS media supplemented with various concentrations TDZ (0.5-2 mg/L), BAP (1.0-4.0 mg/L) and kinetin (0.05-0.1 mg/L) (Table 2). Cultures were kept in the dark for four days and then exposed to 12 hrs

photoperiod. Periodical observations were taken for six weeks periods.

After 6 to 7 weeks periods, individual shoot buds of 1.5-2.0 cm long emerged from the axillary meristem of nodal explants were separated and sub-cultured. The shoot buds were sub-cultured in three different treatments of TDZ, four different treatments of BAP and two different treatments of kinetin (Table 2) for multiplication and each treatment was repeated four times. Each repetition consisted of five test tubes. Each test tubes consisted of four explants (80 explants/treatment). The emergence of new shoot bud from the base of the sub-cultured shoot bud was taken into consideration for calculating shoot multiplication frequencies.

Table 2: Media combination used for *in vitro* shoot regeneration and multiplication

Media code	Medium + Growth hormone
SM_0 (Control)	MS + 3% sucrose + 0.8% Agar
SM_1	MS + 0.5 mg/L TDZ + 3% Sucrose + 0.8% Agar
SM_2	MS + 1 mg/L TDZ + 3% Sucrose + 0.8% Agar
SM_3	MS + 2 mg/L TDZ + 3% Sucrose + 0.8% Agar
SM_4	MS + 1 mg/L BAP + 3% Sucrose + 0.8% Agar
SM_5	MS + 2 mg/L BAP + 3% Sucrose + 0.8% Agar
SM_6	MS + 3 mg/L BAP + 3% Sucrose + 0.8% Agar
SM_7	MS + 4 mg/L BAP + 3% Sucrose + 0.8% Agar
SM_8	MS + 0.05 mg/L kinetin + 3% Sucrose + 0.8% Agar
SM_9	MS + 0.1 mg/L kinetin + 3% Sucrose + 0.8% Agar

Note: **SM**= Shooting media

Statistical analysis

Statistical analysis was carried out as per standard statistical methods as suggested by Panse and Sukhatme (1995). Statistical analysis of data from various characters was carried out as per CRD (Completely Randomized Design).

Result and Discussion

Sterilization stage

Analysis of variance revealed significant differences among the mean square due to treatments for surface sterilization (Table 3). The effect of different surface sterilization treatments on fungal and bacterial contamination (%) and survival (%) recorded for nodal explants was presented in Table 4 and graphically represented in Figure 1. The results and discussion related to various treatments tested for surface sterilization for nodal explant, ST_4 [sodium hypochlorite (1%) for 4 min., cefotaxime (0.1%) for 15 min., streptomycin (0.1%) for 15 min., carbendazim (0.2%) for 15 min. and mercuric chloride (0.1%) for 4 min.] was found as the best treatment with the least fungal contamination (11.00%) and bacterial contamination (2.50%) with highest survival (86.50%). Whereas, ST_1 [cefotaxime (0.1%) for 10 min., streptomycin (0.1%) for 10 min., carbendazim (0.2%) for 10 min. and mercuric chloride (0.1%) for 5 min.] was the least effective treatment in terms of fungal contamination (59.00%) and bacterial contamination (17.50%) with total survival (23.50%). The highest fungal contamination (59.00%) observed in ST_1 followed by ST_2 (43.50%) and ST_3 (29.00%) while the lowest fungal contamination (11.00%) observed in ST_4. In bacterial contamination, ST_1 treatment showed highest percentage of contamination (17.50%) followed by ST_2 (10.50%) and ST_3 (7.00%), while ST_4 treatment showed lowest percentage of contamination (2.5%).

Table 3: Analysis of variance for fungal and bacterial contamination and survival percentage for surface sterilization in *Rosa* spp.

Source of variation	d.f.	Mean sum of square		
		Nodal explant		
		Fungal Contamination (%)	Bacterial Contamination (%)	Survival (%)
Treatments	3	853.64**	202.96**	1367.47**
Error	16	8.51	2.14	13.50

Table 4: Percentage of fungal and bacterial contamination and survival for nodal explants

Treatments	Nodal explant		
	Fungal Contamination (%)	Bacterial Contamination (%)	Survival (%)
ST_1	49.97 (59.00)	24.65 (17.50)	29.24 (23.50)
ST_2	41.10 (43.50)	19.19 (10.50)	42.71 (46.00)
ST_3	31.42 (29.00)	15.54 (7.00)	53.20 (64.00)

ST_4	19.31 (11.00)	9.48 (2.50)	68.35 (86.50)
S.Em. ±	1.30	0.65	1.64
C.D. at 5%	3.91	1.96	4.93
C.V.%	8.17	8.50	7.59

Multiplication Stage

Analysis of variance revealed significant differences for *in vitro* shoot regeneration (Table 5) and multiplication (Table 6). Response of explants in media supplemented with varied concentrations of TDZ (0.5-2.0 mg/L), BAP (1.0-4.0 mg/L) and kinetin (0.05-0.10 mg/L) are evaluated and recorded under (Table 7) and graphically represented (Figure 2 and 3).

Table 5 Analysis of variance for experimental design for various observations for shoot regeneration in *Rosa* spp.

Source of variation	**d.f.**	**Mean sum of square**			
		Days to shoot initiation	**Shoot initiation (%)**	**Number of shoots per explant**	**Length of shoot (cm)**
Treatments	9	38.06**	939.17**	5.36**	0.66**
Error	30	0.19	12.50	0.03	0.01

'**' Significant at 1% level of significance

Table 6 Analysis of variance for experimental design for various observations for shoot multiplication in *Rosa* spp.

Source of variation	**d.f.**	**Mean sum of square**		
		Shoot multiplication (%)	**Number of shoots after S.C.**	**Length of shoot after S.C.**
Treatments	6	1282.77**	12.68**	2.41**
Error	21	17.10	0.03	0.02

'**' Significant at 1% level of significance, **S.C.**= Sub-Culture

Table 7: Effect of various concentrations of TDZ, BAP and kinetin on initiation of shoots and on multiplication of shoots after sub-culture of *Rosa* spp.

Treatments	Concentration	Days to shoot initiation	Shoot initiation (%)	Number of shoots per explant	Length of shoot (cm)	Shoot-multiplication (%) after S.C.	Number of shoots after S.C.	Length of shoot (cm) after S.C.
SM_0 (Control)	-	18.43	58.75	1.33	0.50	55.00	2.41	0.80
SM_1	0.5 TDZ (mg/L)	8.38	100.00	4.60	1.88	100.00	7.50	3.50
SM_2	1.00 TDZ (mg/L)	9.68	97.50	3.35	1.50	96.75	6.50	2.80
SM_3	2.00 TDZ (mg/L)	11.13	87.50	2.48	1.33	85.00	5.00	2.14
SM_4	1.00 BAP (mg/L)	13.40	72.50	2.20	1.32	67.50	3.31	1.88
SM_5	2.00 BAP (mg/L)	10.38	96.25	3.32	1.37	88.75	4.32	2.23
SM_6	3.00 BAP (mg/L)	9.00	100.00	4.53	1.86	100.00	7.20	3.23
SM_7	4.00 BAP (mg/L)	9.38	98.75	3.35	1.80	97.50	6.37	2.40
SM_8	0.05 kinetin (mg/L)	12.28	78.75	2.29	1.30	66.25	4.26	1.98
SM_9	0.1 kinetin (mg/L)	14.40	67.50	1.33	1.22	58.75	3.34	1.75
	S.Em. ±	0.22	1.77	0.09	0.05	2.07	0.09	0.07
	C.D. at 5%	0.63	4.80	0.25	0.14	5.97	0.26	0.21
	C.V.%	3.73	4.12	5.92	6.78	5.07	3.58	6.35

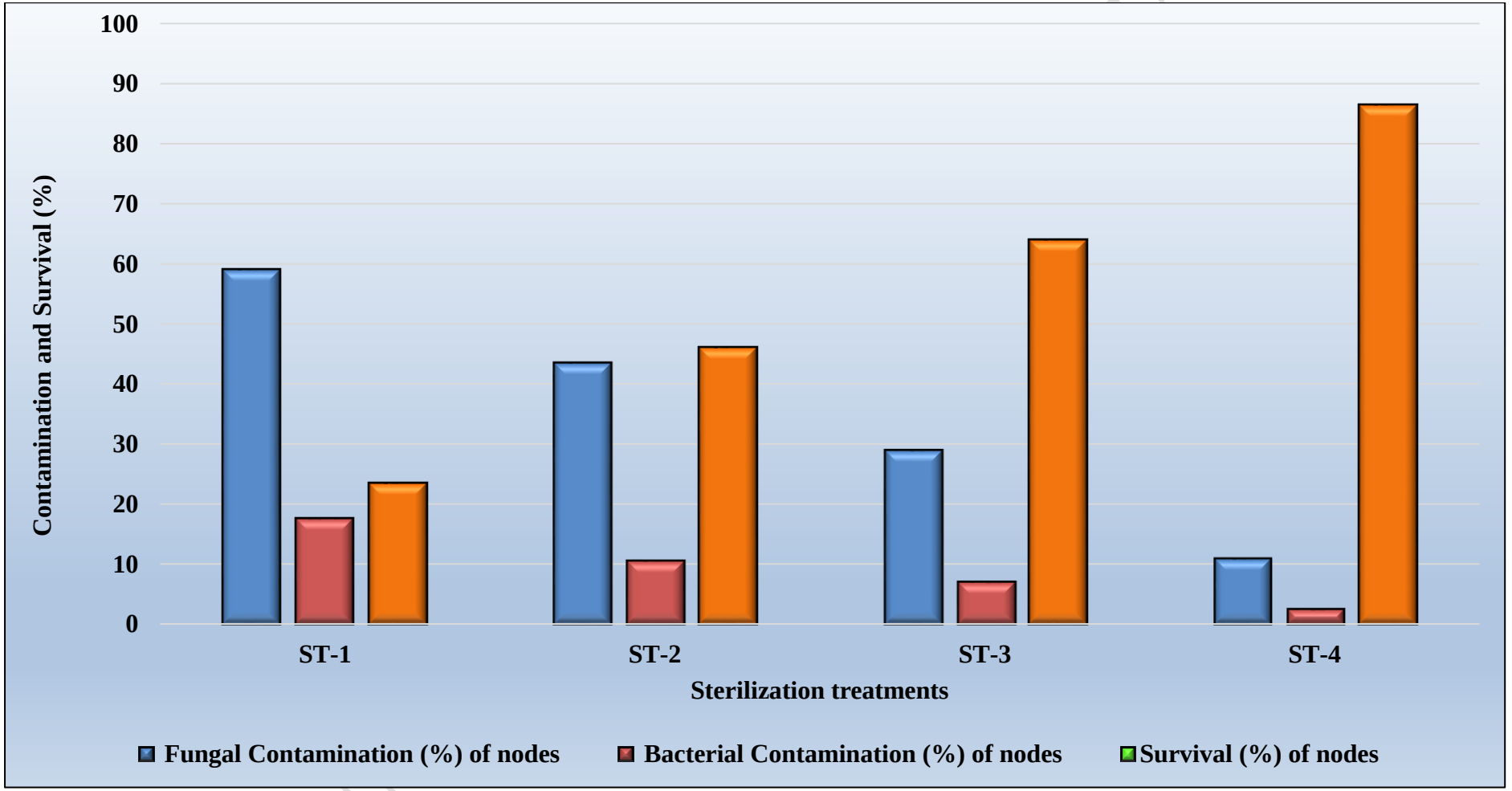

Figure 1: Percentage of fungal contamination, bacterial contamination and survival for nodal explants of *Rosa* spp.

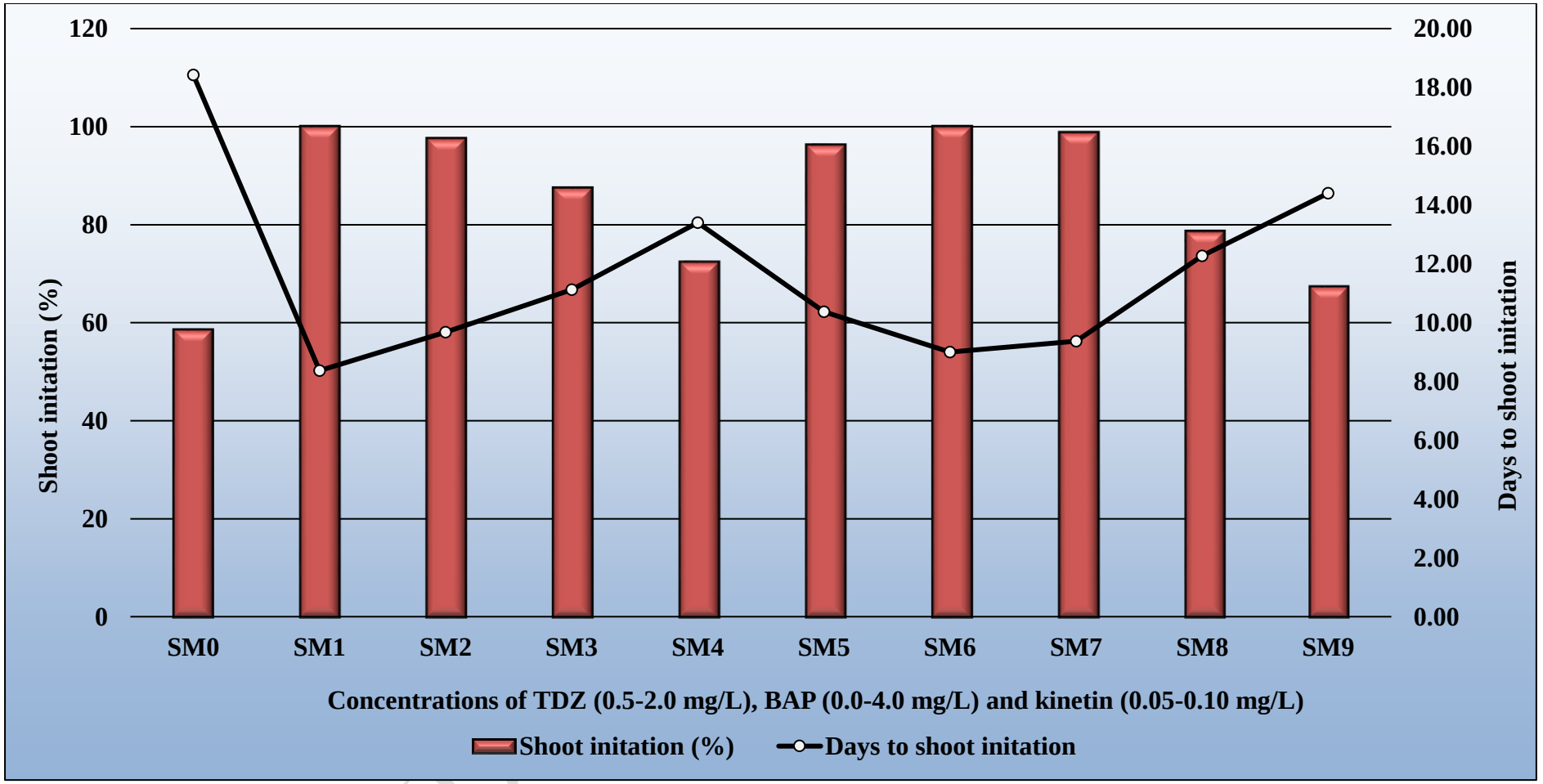

Figure 2: Effect of various concentrations of TDZ (0.5-2.0 mg/L), BAP (0.0-4.0 mg/L) and kinetin (0.05-0.10 mg/L) on shoot initation (%) and days to shoot initation of *Rosa* spp.

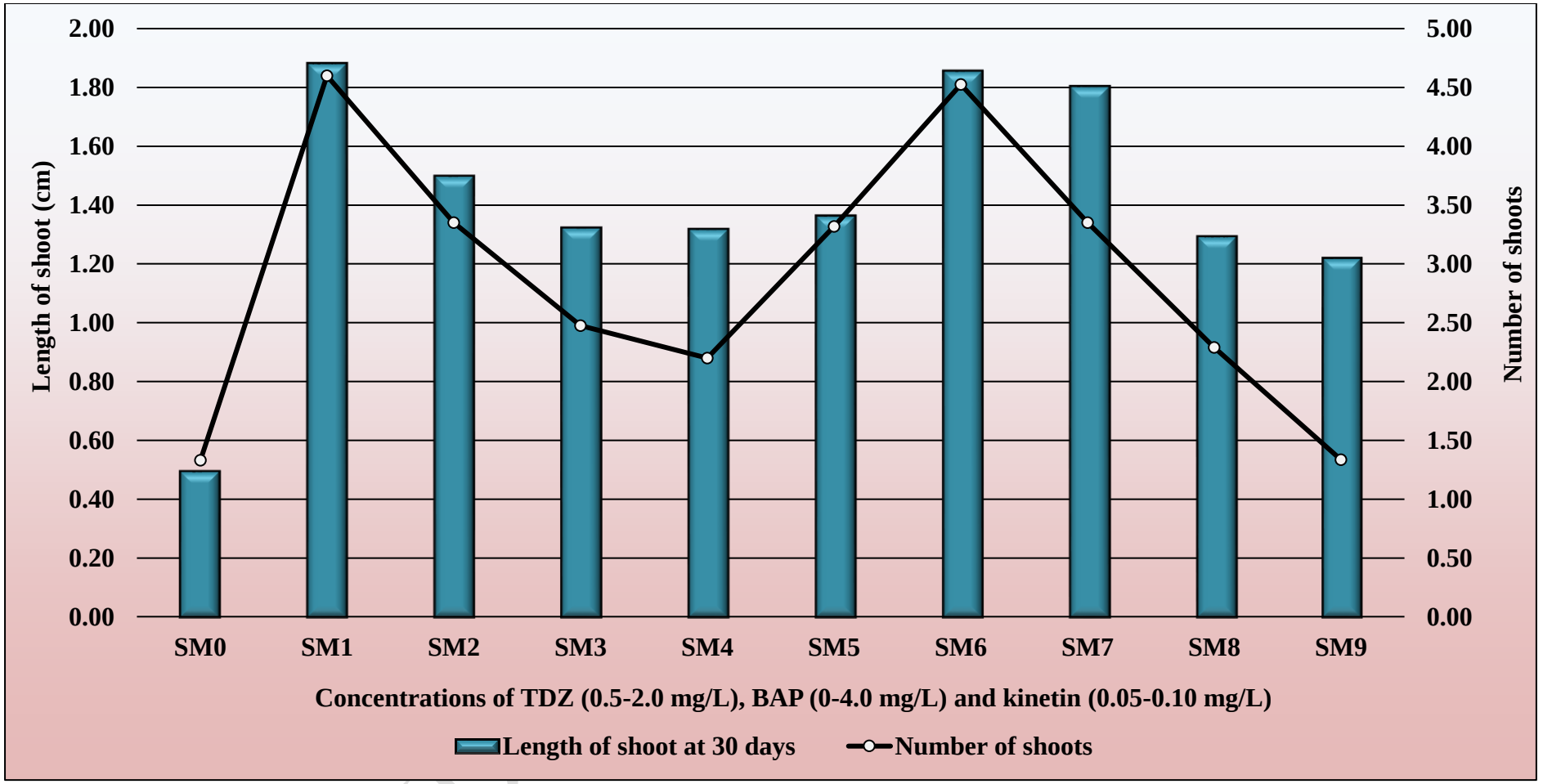

Figure 3: Effect of various concentrations of TDZ (0.5-2.0 mg/L), BAP (0.0-4.0 mg/L) and kinetin (0.05-0.10 mg/L) on length and number of shoots of *Rosa* spp.

For days to shoot initiation, SM1 was found significantly earliest (8.38 days) among all as well as remained statistically at par with SM6 (9.00 days). Whereas, significantly delayed shoot initiation response was observed in SM0 (18.43 days). Nizamani *et al.* (2016) found similar result that the number of days for shoot initation was 12.66 days for BAP 0.5 mg/L and 10.00 days for BAP 2.0 mg/L. For percentage of shoot initiation, SM_1 and SM_6 (100%) were found significantly superior among all treatments as well as remained statistically at par with SM_7 (98.75), SM_2 (97.50%) and SM_5 (96.25%). But control was found least effective for percentage of shoot initiation (58.75%). In the number of shoots per explant, SM1 gave the best result among all the treatments with an average of 4.60 shoots per explant as well as remained statistically at par with SM6 with an average of 4.53 shoots per explant. Whereas, control and SM9 gave poor response with an average of 1.33 shoots per explant. The highest number of shoots per explant (4.53) was observed @ 3.0 mg/L BAP but after this level number of shoots decreased with increasing concentration of BAP (4.0 mg/L BAP, 3.35 number of shoots per explant). In 4.0 mg/L BAP, hyperhydricity was observed. The present findings concurred with Carelli and Echeverrigaray (2002). They also found that the number of shoots increased with increasing concentration of BAP in the media and the elongation decrease but to the specific level (3.0 mg/L) after this level number of shoots decrease with increasing concentration of BAP and hyperhydricity also observed at 5.0 mg/L. Rajendra *et al.* (2004) also found similar result that media which contain BAP observed more number of shoots than media which contain kinetin. Ozel and Arslan (2006) also found similar result that the number of shoots decreased with increasing concentration of TDZ in the media. Any increase in the concentration of TDZ in shoot induction media was found inhibitory and had negative effect on shoot regeneration. In SM_1 media, the length of shoot growth was found to be highest at 30 days (1.88 cm) as well as remained statistically at par with SM_6 (1.86 cm) and SM_7 (1.80 cm). In contrast, SM_0 media the length of shoot growth was found to be lowest at 30 days after inoculation (0.50 cm). The findings of the present study are consistent with research conducted by (Pati *et al.*, 2006), who reported that increase in length of shoots also increased with the concentration of BAP in media. Xing *et al.* (2010) also found similar result that 'Tang Red' cultivar of *Rosa* spp. showed more length at concentration of BAP 3 mg/L (2.00 cm) than 4 mg/L (1.16 cm).

After first sub-culture

Shoot buds of 1.55 to 1.85 cm long were subcultured individually into MS solid media supplemented with TDZ, BAP and kinetin at different concentrations showing multiple shoot

formation in four weeks periods. The experiment consisted of four repetitions of each treatment, with each repetition containing twenty test tubes. Each test tubes contained one explant, resulting in a total of 80 explants per treatment. The results of shoot multiplication after the first sub-culture are presented in Table 5. Media supplemented with 0.5 mg/L TDZ (SM_1) and 3.0 mg/L BAP (SM_6) were found to be the best for shoot multiplication (100%) as after the first sub-culture, but it remained statistically at par with SM_7 (97.50%) and SM_2 (96.75%). SM_0 was found to be the least effective in shoot multiplication with only 55.00 per cent of shoots multiplying after the initial sub-culture. The SM_1 media treatment resulted in the highest number of shoots with an average of 7.50 shoots per explant followed by SM_6 (7.20), SM_2 (6.50), SM_7 (6.37), SM_3 (5.00), SM_5 (4.32), SM_8 (4.26), SM_9 (3.34) and SM_4 (3.31), while the control treatment had the lowest number of shoots with an average of only 2.41 shoots per explant. The present findings concurred with Nizamani *et al.* (2016). They noted that the number of shoots increased with increasing concentration of BAP from 0.5 mg/L (5.00) to 2 mg/L (7.00). In the SM_1 media, the shoot exhibited the longest length, with an average of 3.50 cm per shoot SM_6 (3.23 cm), SM_2 (2.80 cm), SM_7 (2.40 cm), SM_3 (2.14 cm), SM_5 (2.23 cm), SM_8 (1.98 cm), SM_4 (1.88 cm) and SM_8 (1.98 cm), whereas the SM_0 media treatment resulted in the shortest length, with an average of 0.80 cm per shoot. The present findings concurred with Nizamani *et al.* (2016). They noted that the shoot length increased with increasing concentration of BAP from 0.5 mg/L (3.57 cm) to 2.0 mg/L (4.79 cm).

Conclusion

The conclusions drawn from the present study on the micropropagation of *Rosa* spp. through different treatments are that, for sterilization sodium hypochlorite (1%) for 4 min., cefotaxime (0.10%) for 15 min., streptomycin (0.10%) for 15 min.+ carbendazim (0.20%) for 15 min. + mercuric chloride (0.10%) for 4 min is best treatment among all treatments and for shoot regeneration and multiplication MS + 0.50 mg/L TDZ or 3.0 mg/L BAP + 3% Sucrose + 0.8% Agar is best treatment among all the treatments. So, the present findings developed a rapid and efficient protocol for shoot multiplication of *Rosa* spp.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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