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Impact of Different Explants and Growth Regulators on *In vitro* Regeneration of Chrysanthemum

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MNHM and MR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KFNS, MHSR and MMAN managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Chrysanthemum is the world's second most economically important flower crop and commonly known as 'Autumn Queen'. It belongs to the family Compositeae (Asteraceae). It is native to Asia and northeastern Europe and has been cultivated for more than 2000 years. The present study within vitro regeneration of chrysanthemum was carried out to develop the standardized protocol for organogenesis. In this study, three types of explants viz. apical shoot tip, internodal segment and young leaf along with different concentrations and combinations of growth regulators were used for in vitro regeneration. BAP and KIN were used for in vitro microshoot regeneration and IBA along with 2, 4-D were used for in vitro microroot regeneration. Minimum days (7.00) for microshoot initiation, maximum microshoot initiation percentage (97.00), highest number of microshoot per plantlet (12.00), highest number of leaves per microshoot (14.60) and maximum microshoot length (4.60) at 28 DAC were recorded as best performances by apical shoot tip inoculated into MS medium supplemented with BAP 2.5 mg/L + KIN 0.5 mg/L. On the other hand, minimum days

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(5.00) for microroot initiation, maximum microroot initiation percentage (97.60), the highest number of microroots per plantlet (11.80) and maximum microroot length (6.20) were obtained from apical shoot tip inoculated into ½ strength MS medium supplemented with IBA 0.2 mg/L + 2, 4-D 0.1 mg/L. In case of microshoot regeneration, the best response was showed by apical shoot tip when inoculated into MS medium supplemented with BAP 2.5 mg/L + KIN 0.5 mg/L and the microrooting of plantlets were best from apical shoot tip inoculated into ½ MS medium supplemented with IBA 0.2 mg/L along with 2, 4-D 0.1 mg/L.

Keywords: In vitro regeneration; organogenesis; BAP; KIN; IBA and 2; 4-D.

1. INTRODUCTION

Chrysanthemum is the world's second most economically important flower crop and commonly known as 'Autumn Queen'. It belongs to the family Compositeae (Asteraceae). It is native to Asia and northeastern Europe and has been cultivated for more than 2000 years. It is mainly herbaceous, highly attractive short-day plant and perennial. The flowers bloom in early winter with a wide range of color, shape and sizes [1]. It is grown throughout the world both for cut flowers and as a potted plant having its long term vase life. Generally, suckers and terminal cuttings are used for its propagation system [2]. The conventional process of cutting is very slow and causes low multiplication rate, virus infection and often low quality plants, thereby increasing production costs [3,4]. These problems have been solved by applying micropropagation other *in vitro* techniques. Due to its high popularity and demand, it becomes one of the first commercial targets for micropropagation and thus tissue culture can be utilized for its large-scale production [5]. In this investigation, different types of explants and growth regulators were used to develop a suitable protocol for *in vitro* regeneration of chrysanthemum.

2. MATERIALS AND METHODS

The research activities for *in vitro* regeneration of chrysanthemum using different explants were conducted at the 'Plant Biotechnology Laboratory' and 'Germplasm Center', Department of Horticulture, PSTU, Patuakhali, Bangladesh.

2.1 Method of Culture

2.1.1 Collection and sterilization of explants

Healthy, disease free mother plant was selected as mother stock for collecting explants. The excision of explants from mother stock was ensured with the minimum injury of cells. After collection the explants were washed thoroughly with running tap water and then surface sterilized with 5-10 ml of Savlon followed by 2-3 drops of Tween-20 for 10-15 mins with gently shaking. Under laminar air flow cabinet the explants were then rinsed with 0.1% HgCl2 solution for several times.

2.1.2 Explants inoculation

The surface sterilized explants were then prepared carefully under aseptic conditions inside the laminar airflow cabinet to place them onto the sterilized culture media. For inoculation the shoot tip, internodal segments (1 cm) and leaves were cut carefully with scalpel by holding with the forceps. The excised explants were then inoculated in the culture vessels containing medium using sterilized forceps.

2.1.3 Explants incubation

The cultured bottles containing inoculated explants were then incubated both in dark and light in a temperature controlled growth room for 16 hours daily light of fluorescent Philip white tubes with intensified 2000-2500 LUX at 25 ± 10C temperature and the relative humidity of 70-75%.

2.1.4 Subculture or multiplication of primary established explants

When the inoculated plants materials were developed with few leaf containing microshoots, then they were ready for subculture. Repeated subcultures were carried out at an interval of 28 days after microshoot initiation.

2.1.5 Microroot induction

Subcultured microshoot stimulated new shoot elongation on shooting medium. Near about 28 days the microshoots were excised from previous medium and placed on root induction medium containing rooting hormone named IBA and 2, 4-D.

2.2 Acclimatization

It was a critical stage for plantlets and extra care was ensured to prevent the loss of plantlets. The plantlets with 6-7 leaf and well developed root system (5-6 roots) were ready for acclimatization. At first the plantlets were kept in room temperature (300- 340) for 4 to 5 days [6]. After hardening the rooted microshoots were carefully removed from the test tubes and the roots were gently cleaned with tap water for removal of adhering media.

The plantlets were then covered with moistened filter paper to avoid drying and the effect of sudden shock. Each plantlet was then transferred into a small earthen pot containing soil, sand and well rotten cowdung at 1:1:1 ratio. It was done in the afternoon. Immediately after transplantation, the plantlets were irrigated with fine spray of water. The plantlets were kept into the shady area in order to harden in natural environment and irrigated regularly at 2 days interval for 1st two weeks and then at 1 day interval. The plantlets took 6 to 7 days to adopt in natural environment.

2.3 Experimental Design

The experiment was laid out in the Completely Randomized Design (CRD) with three replications.

3. RESULTS AND DISCUSSION

It was a two factorial experiment. MS medium containing different combinations of BAP (2.0, 2.5 and 3.0 mg/L) along with KIN (0.1 and 0.5 mg/L) and ½ MS medium supplemented with different combinations of IBA (0.1 and 0.2 mg/L) along with 2, 4-D (0.1 and 0.2 mg/L) were used for microshoot and microroot initiation respectively. The 2nd factor consisted with three types of explants viz. apical shoot tip, inter nodal segment and young leaf. Data of all parameters were analyzed statistically using analysis of variance technique (ANOVA) and means were compared by Duncan's Multiple Range Test [7].

3.1 Effect of Different Explants and Growth Regulators on Microshoot Regeneration

The explants, different combinations of growth regulator as well as their interaction effects had showed statistically significant variations in case of all parameter related to *in vitro* microshoot regeneration. Out of three explants, the apical shoot tip showed best results in case of all parameters.

Shorter period of time (13.83 days) for 1st microshoot initiation, maximum microshoot initiation percentage (88.67), the highest number of microshoot/plantlet (8.17), highest no. of leaves/microshoot (10.43) and maximum microshoot length (3.18) after 28 DOC were recorded from shoot tip explants (Table 1). In case of growth regulators, significant variations were also found in all parameters and the best response was showed by MS medium supplemented with BAP 2.5 mg/L + KIN 0.5 mg/L.

In case of interaction effects between explants and growth regulators, significant variations were also found in all parameters. The best response was showed by apical shoot tip inoculated in MS medium supplemented with BAP 2.5 mg/L + KIN 0.5 mg/L. The minimum days (7.00) for microshoot initiation, maximum microshoot initiation percentage (97.00), highest no. of microshoot/plantlet (12.00), highest no. of leaves/microshoot (14.60) and maximum microshoot length after 28 DOC (4.60) were obtained.

In case of microshoot initiation percentage, apical shoot tip with BAP 2.5 mg/L + KIN 0.5 mg/L showed highest percentage (97.00) of microshoot initiation followed by (95.20) using nodal explants with BAP 2.5 mg/L + KIN 0.5 mg/L. But the lowest percentage (54.67) of microshoot initiation was observed in case of young leaf segment with BAP 3.0 mg/L + KIN 0.5 mg/L and the other treatments showed intermediate results compared to the highest and lowest values of percentage of microshoot initiation (Figure 1). Faisal [6] conducted an experiment of multiple shoot proliferation from nodal segments and shoot tips of *C.morifolium* on MS medium with 1.0 mg/L BAP reporting maximum response of 95.00% and 91.00% respectively.

Hoque et al. [8] reported that both shoot tip and nodal explants produced almost 100% microshoot regeneration when MS medium supplemented with 2.0-3.0 mg/L BAP. In case of number of microshoot, apical shoot tip with BAP 2.5 mg/L + KIN 0.5 mg/L produced maximum number of microshoot (12.00) followed by (10.20) microshoot from nodal explants at same concentration of BAP 2.5 mg/L + KIN 0.5 mg/L.

In contrast, the minimum number of microshoots (2.00) was produced by young leaf cultured into medium fortified with BAP 3.0 mg/L + KIN 0.5 mg/L followed by (2.20) microshoots with same explant at BAP 3.0 mg/L + KIN 1.0 mg/L but they were statistically similar with each other and ranked equally by using same letter (Figure 2). Lu et al. [9] found greatest average number of microshoots per explant (14.60) from the apical portion of the stem. Islam [10] reported the maximum number of microshoots when 2.0 mg/L BAP was used.

The interaction effects among different explants and different concentrations and combinations growth regulators also showed significant variations in microshoot length at 28 DAC. Apical shoot tip with BAP 2.5 mg/L + KIN 0.5 mg/L produced longest microshoot (4.60) followed by (4.10) microshoot using nodal explants at same concentration of growth regulators (BAP 2.5 mg/L + KIN 0.5 mg/L). In contrast, the shortest microshoots (2.00) was produced by young leaf with BAP 3.0 mg/L + KIN 0.5 mg/L followed by (2.07) and (2.20) from nodal explants at same treatment BAP 3.0 mg/L + KIN 0.5 mg/L and from young leaf explants at BAP 3.0 mg/L + KIN 1.0 mg/L respectively.

But those three interaction effects were statistically similar with each other and ranked equally by representing with same letter. And the rest of treatments showed intermediate results compared to the highest and lowest values of microshoot length after 28 DOC (Figure 3). Waseem [11] reported that the longest microshoot (5.00 cm) was recorded in 1.0 mg/L BAP from shoot tip explant. Vijaya et al. [12] also reported that BAP is the most effective plant growth regulator in relation to microshoot proliferation. Karim et al. [13] who stated that a combination of 1.0 mg/L BAP and 0.1 mg/L IAA had produced the longest microshoot of 4.50 cm in the shoot tips of chrysanthemum.

Table 1. Effect of different explants on the days required for microshoot initiation, percentage of microshoot initiation, number of microshoots per plantlet, number of leaves per microshoot and microshoot length (cm) after 28 DOC

Used Explants	Days required for microshoot initiation	Percentage of microshoot initiation	No. of microshoots per plantlet	No. of leaves per microshoot	Shoot length (cm) after 28 DOC
Apical shoot tip	13.83 c	88.67 a	8.17 a	10.43 a	3.18 a
Internodal segments	16.33 b	85.20 b	7.00 b	9.30 b	2.93 b
Young leaf	20.33 a	67.44 c	3.40 c	7.45 c	2.54 c
Level of significance	*	*	*	*	*
LSD0.05 value	0.1352	0.2191	0.1542	0.1352	0.1450
CV (%)	1.19	0.40	3.68	2.21	7.43

In a column, values having different letter(s) differ significantly at 5% level of probability.
** denotes significant at the 5% level of probability*

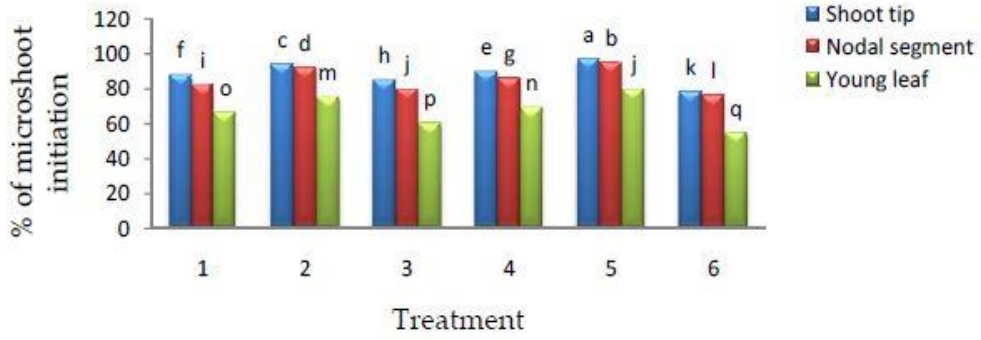

Figure 1. Combined effects of three explants and different concentrations of BAP and KIN in the percentage of microshoot initiation.

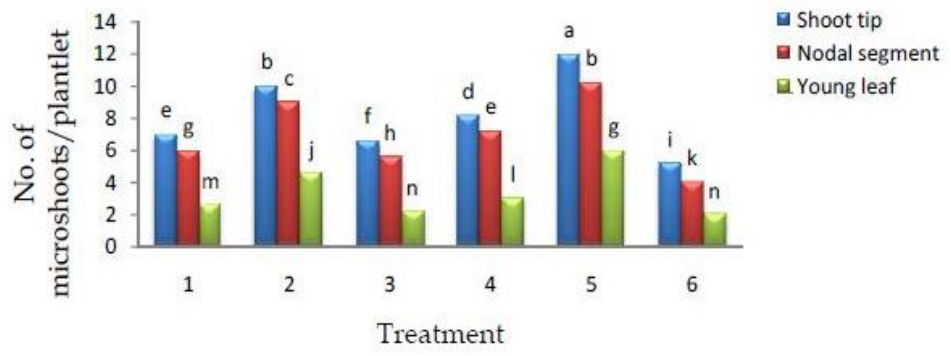

Figure 2. Combined effects of three explants and different concentrations of BAP and KIN on the number of microshoots per plantlet.

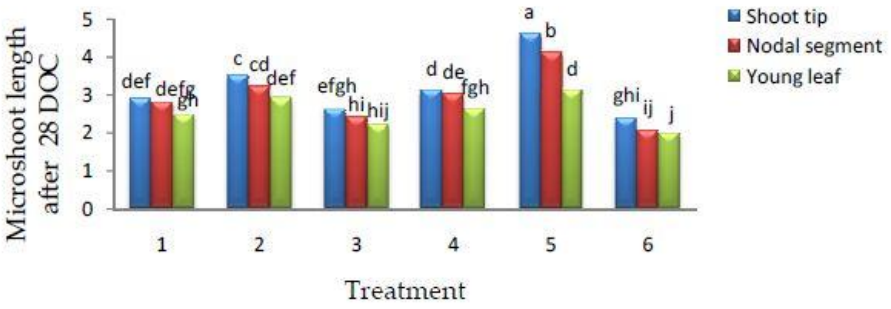

Figure 3. Combined effects of three explants and different concentrations of BAP and KIN on microshoot length after 28 DOC.

Treatment:

1= MS with BAP 2.0 mg/L + KIN 0.1 mg/L
2= MS with BAP 2.5 mg/L + KIN 0.1 mg/L
3= MS with BAP 3.0 mg/L + KIN 0.1 mg/L
4= MS with BAP 2.0 mg/L + KIN 0.5 mg/L
5= MS with BAP 2.5 mg/L + KIN 0.5 mg/L
6= MS with BAP 3.0 mg/L + KIN 0.5 mg/L

Table 2. Effect of different explants on the days required for microroot initiation, percentage of microroot initiation, number of microroots per plantlet and microroot length (cm)

Used Explants	Days required for micro-root initiation	Percentage of micro-root initiation	No. of micro-roots per plantlet	Micro-root length (cm)
Apical shoot tip	8.25 c	92.90 a	9.03 a	4.35 a
Inter nodal segments	9.50 b	88.75 b	7.48 b	3.20 b
Young leaf	16.00 a	72.50 c	6.23 c	2.50 c
Level of significance	*	*	*	*
LSD0.05 value	0.1685	0.1685	0.2148	0.2638
CV (%)	1.78	0.24	3.37	9.35

In a column, values having different letter(s) differ significantly at 5% level of probability
** denotes significant at the 5% level of probability*

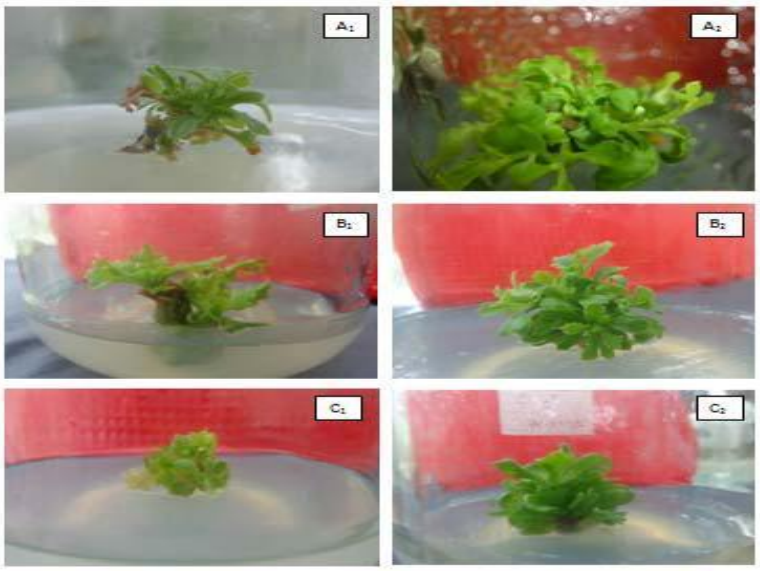

Photo plate 1. The effect of best treatment (BAP 2.5 mg/L + KIN 0.5 mg/L) on *invitro* microshoot regeneration using different types of explants viz.apical shoot tip (A1- A2), inter nodal segment (B1- B2) and young leaf explant (C1- C2)

3.2 Effect of Different Explants and Growth Regulators on Microroot Regeneration

In case of microrooting from *in vitro* plantlets the apical shoot tip showed best performances in all parameter and the results were minimum days (8.25) for microroot initiation, maximum microroot initiation percentage (92.90), highest no. of microroots/plantlet (9.03) and maximum microroot length (4.35) (Table 2). Significant variations were also found from different concentrations and combinations of IBA and 2, 4-D in case of *in vitro* rooting. The best results minimum days (8.00) for microroot initiation, maximum microrootinitiation percentage (91.20), highest no. of microroots/plantlet (10.03) and maximum microroot length (5.27) were recorded from ½ MS medium containing IBA 0.2 mg/L + 2, 4-D 0.1 mg/L.

The best results were obtained from combined effects between explants and treatment levels when apical shoot tip inoculated into ½ MS medium supplemented with IBA 0.2 mg/L + 2, 4-D 0.1 mg/L. The results were minimum days (5.00) for microroot initiation, maximum microroot initiation percentage (97.60), highest no. of microroots/plantlet (11.80) and maximum microroot length (6.20).

Chrysanthemum microshoots raised from tissue culture, developed microroots within 4-5 days ½ MS + 0.25 mg/L IBA [14,15]. The interaction effects between three explants and different concentrations of IBA and 2, 4-D also showed significant differences in required days for microroot initiation. The apical shoot tip in combination with ½ MS containing IBA 0.2 mg/L + 2, 4-D 0.1 mg/L required shorter period of time (5.00 days) for 1st microroot initiation followed by (6.00 days) from nodal explants at same concentrations of IBA and 2, 4-D. In contrast, young leaf explant inoculated into ½ MS with IBA 0.2 mg/L + 2, 4-D 0.2 mg/L needed longer period of time (19.00 days) (Figure 4). These results are also supported by the findings of Faisal and Amin [16], Sarkar and Shaheen [17] and Waseem et al. [18] who reported that 0.2 m g/L IBA produced maximum result s regarding the rooting of chrysanthemum microshoots. The culture response of explants, IBA along with 2, 4-D and their interaction were significant in case of microroots length.

Waseem et al. [19] found best responsemicroroot length (9.00 cm) at IBA 0.20 mg/L. Faisal [6] found best response of microroot length (3.76 cm) at half strength MS medium supplemented with 0.2 mg/L IBA. In regarding combined responses, shoot tip in ½ MS supplemented with produced tallest microroot (6.20) followed by (5.00) and (4.60) at same concentration (IBA 0.2 mg/L + 2, 4-D 0.1 mg/ L) using nodal explants and leaf explants respectively and they are ranked equally. In contrast, shortest microroot

(1.00) was produced by leaf explants at IBA 0.2 mg/L + 2, 4-D 0.2 mg/L followed by (1.2 0) from same explants at IBA 0.1 mg/L + 2, 4-D 0.10 mg/L and (1.50) were found from nodal explants cultured into IBA 0.2 mg/L + 2, 4-D 0.2 mg /L concentration b ut statistically similar with each. These three combinations were given same lettering mark. And the rest inter mediate levels of combination also showed favorable effect in micrroot length (Figure 5).

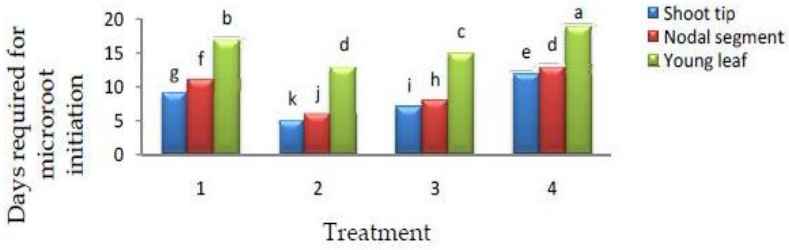

Figure 4. Combined effects of three explants and different concentrations of IBA and 2, 4-D on the required days for microroot initiation.

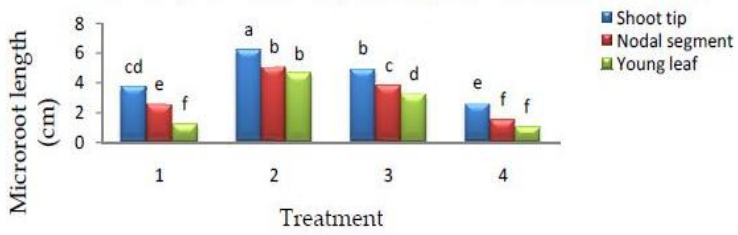

Figure 5. Combined effects of three explants and different concentrations of IBA and 2, 4-D on microroot length.

Treatment:

1= ½ MS with IBA 0.1 mg/L+ 2, 4-D 0.1 mg/L
2= ½ MS with IBA 0.2 mg/L+ 2, 4-D 0.1 mg/L
3= ½ MS with IBA 0.1 mg/L+ 2, 4-D 0.2 mg/L
4= ½ MS with IBA 0.2 mg/L+ 2, 4-D 0.2 mg/L

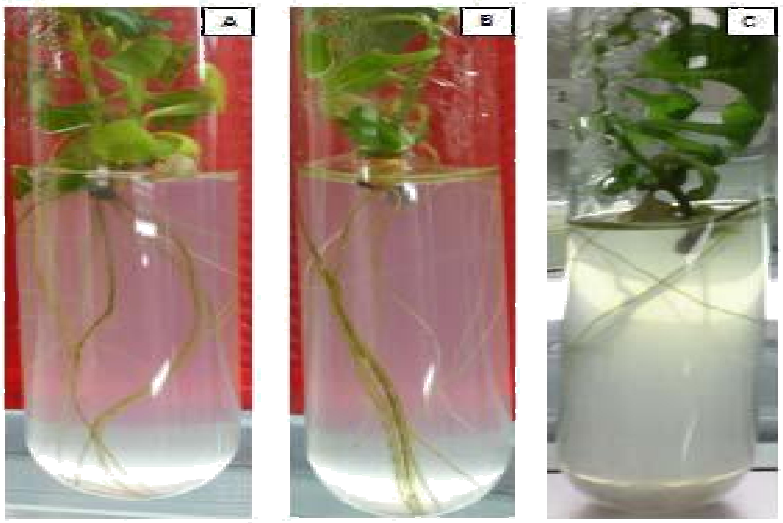

Photo plate 2. The effect of ½ strength MS medium supplemented with IBA 0.2 mg/L + 2, 4-D 0.1 mg/L on *in vitro* microroot regeneration using different microshoots initiated from different explants viz. apical shoot tip (A), inter nodal segment (B) and leaf explant (C)

4. CONCLUSIONS

The explants, different combinations of growth regulator as well as their interaction effects had showed statistically significant variations regarding all parameters related to *in vitro* regeneration. In case of microshootre generation, the best response was showed by apical shoot tip when inoculated into MS medium supplemented with BAP 2.5 mg/L + KIN 0.5 mg/L and the microrooting of plantlets were best from apical shoot tip inoculated into ½ MS medium supplemented with IBA 0.2 mg/L along with 2, 4-D 0.1 mg/L.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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