Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.7, 2016
www.iiste.org

Efficient Regeneration System for the Improvement of Kinnow mandarin (Citrus reticulata Blanco)

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Abstract
Kinnow mandarin (*Citrus reticulata* Blanco.) is a highly adaptable variety among citrus cultivars. An efficient system for *in vitro* regeneration by organogenesis starting from seed of (*C. reticulata* Blanco) was developed. Seeds were treated by Murashige and Skoog (MS) media supplemented with 2, 4-Dichlorophenoxyacetic acid (2, 4-D) to initiate callus induction. The best result (96%) were obtained when seeds were treated with MS basal media + 2,4-D (16.0) µM. The regeneration system tested allowed the attainment of highest shoots (90 %) with BA 13.0 µM. An average of 7.8 well-differentiated shoots per explant was obtained. Highest rooting (85%) was achieved in culture medium with 10.0 µM IBA. The well-developed plantlets were transferred to potting mixture. Of the rooted plant, 95% adapted well to soil conditions.
Keywords: *C. reticulata* Blanco, *In vitro*, Callus induction, Shoot formation, Explant, Rooting.
Abbreviations: µM = Micromolar, BA = Benzyl adenine, IBA = Indole-3-butyric acid, TSS = Total soluble solids, NAA = α-naphthalene acetic acid, KIN = Kinetin, GA = Gibberellic acid.

1. Introduction

Citrus is one of the world's major fruit crops with global availability and popularity contributing to human diets. Citrus fruits are regarded as major household items in more than 100 countries around the world. The genus citrus includes more than 162 species belonging to the Order Geraniales family Rutaceae and sub family Aurantoideae. Citrus fruits are native to southern China and Southeast Asia where they have been cultivated for some 4,000 years. Now grown in more than 100 countries in tropical, subtropical and Mediterranean climates, citrus (including oranges, grapefruit, tangerines and mandarins, and lemons and limes) is the leading fruit crop grown in the world. Kinnow, a King -Willow leaf mandarin hybrid, was developed at the University of California Research Center, Riverside by H. B. Frost in 1915 and released in 1935. The parental cross was made in 1915 and official release took 20 years. However, it took another period of more than 30 years before Kinnow became a successfully grown commercial cultivar in the Punjab region of Pakistan and India. It has several common names such as: Mandarin, Tangerine, Unshu orange, Satsuma Orange, Temple Orange, and Tangerine. Kinnow has inherited heat tolerance from the cultivar King which helps it to survive in ruthless hot summer of Punjab. This "easy peel" citrus has assumed special economic importance and export demand due to its high juice content, special flavor, and as a rich source of vitamin C. Citron is a scented citrus fruit; it is a small tree about 2.44 to 4.57 m, having large fruit (20 to 22.5 cm long) that resembles pineapple in shape. It is botanically classified as *Citrus medica L.* and was the first of the citrus fruits to come into view in the Mediterranean region. In Bangladesh mandarin is cultivated in Sylhet, Moulvibazar, Sunamgang, Habigang, Rangamati, Bandarban, Khagrachari, Panchagarh, Brahmanbaria, Tangail districts. Citrus species contain a wide range of active ingredients and research is still underway in finding uses for them. They are rich in vitamin C, Flavonoids, Acids and volatile oils. Nutrient Status of Kinnow is Average Vitamin C 31.0 mg/100 ml juice, Calcium 40 mg/100ml, Iron 0.4 mg/100ml, Phosphorus 18 mg/100ml and Average TSS/acid ratio 12-14:1. They also contain

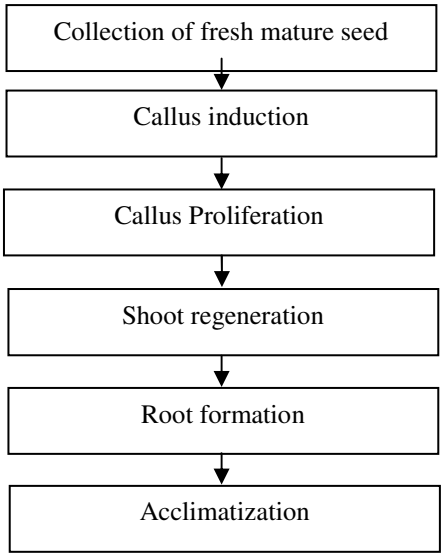
Coumarins such as Bergapten which sensitizes the skin to sunlight. Some of the plants more recent applications are as sources of antioxidants and chemical Exfoliants in specialized cosmetics. Because of the variability within the cultivar, the explants and the medium requirements are different, mentioned by various workers (Praveen et al. 2003; Singh et al. 2006; Gill et al. 1994; Jaskani et al. 2005; Bhatti et al. 2007).

Specific objective of this work was to develop tissue culture plants using seeds as explant source and the ultimate goal is to utilize natural and induced variability to obtain novel characteristics in Kinnow mandarin which might be used in genetic transformation system and/or efficient and suitable regeneration protocol in future. Plant tissue culture technology has been successfully used for the commercial production of microbe free plants (Parmessur et al. 2002 & Liao et al. 2004) and to conserve the germplasm of rare and endangered plant species to conserve them from extinction (Milkuk 1999; Chang et al. 2000; Jaime & Da Silva 2003). This technique involves callus induction from explants, morphogenesis, shoot development and finally root development to regenerate into a complete somaclone. All these steps require different sets of hormones and growth medium for developing somaclonal variants successfully. Although, tissue culture and micro propagation protocols have been described for a number of citrus species and explants sources (Grinblat 1972; Chaturvedi and Mitra 1974; Barlass and Skene 1982; Edriss and Burger 1984; Duran Vila et al. 1989). Micro propagation has many advantages over conventional methods of vegetative propagation (cutting or seed) and its application

in Horticulture. Embryogenic callus was successfully induced in six relatives of Citrus with combination of 5.0 mg/L BA, 2.5 mg 2,4-D and 600 mg/L malt extract in Murashige and Tucker (MT) medium (Jumin and Nito 1995).

2. Materials and methods

This research work was conducted at the Plant Genetic Engineering Laboratory of the Department of Genetic Engineering & biotechnology, Shahjalal University of Science & Technology (SUST), Sylhet 3114, Bangladesh.



The flow chart of method followed in this experiment.

The detail of methods employed during this study is given below:

2.1. Collection of explant

Healthy seeds of kinnow mandarin (*Citrus reticulata*) were used as explant for *in vitro* regeneration. Fresh

healthy seeds of kinnow mandarin (*Citrus reticulata*) plant were collected from BRAC, Gazipur, Dhaka and Citrus Research Institute, Jointapur, Sylhet.

2.2. Explant sterilization

Seeds of *C. reticulata* were washed by using detergents for 2 minutes. Then explants were immersed for 15 minutes with 2 or 3 drops of Tween-20. In order to remove all traces of detergents and Tween-20 from the surface, explants were washed by sterile-distilled water for 3-4 times.

2.3. Media for callus induction

For callus induction popular callus inducing hormone 2,4-D and different combination 2,4-D + BA + NAA was used. Seeds cultured on MS (Murashige and Skoog 1962) basal medium supplemented with hormonal concentration of 2,4-D varying in from 4.5 µM to 18.0 µM and combination of 2,4-D + BA + NAA were prepared separately in conical flasks then 0.7% agar was added and melted by boiling at 110ºC for 2-3 minute. After melting the agar, the media was autoclaved at 121ºC and 15 psi for 15 minute. Than media was allowed to cool down and coagulate in the Laminar Air Flow Cabinet. Visual observation was taken every 7 days and effect of different treatment was quantified on the basis of percentage of callus induction.

2.4. Shoot formation media

Shoot generation was carried out from calli. A healthy portion of the callus was taken and cut into pieces and these pieces were placed on shoot initiation medium. Full strength MS medium supplemented with BA varying from 6.0 µM to 18.0 µM alone and combination with other hormones BA + KIN (9.0+10.0) µM, BA + KIN (13.0+14.0) µM, BA + GA (6.0+6.0) µM, BA + GA (8.0+6.0) µM were used for shoot induction. Visual observation was taken every seven days and effect of different treatment was quantified on the basis of percentage of calli showing response for shoot regeneration.

2.5. Rooting of In vitro grown Micro shoot culture

The *In vitro* grown Micro shoot were inoculated into the full strength MS media supplemented with different concentration of Auxin IBA, NAA alone and in combination were used for root induction. IBA (5.0, 7.0, 10.5, 12.0, 13.5, 15.0) µM, NAA (5.0, 8.5) µM and a combination of IBA + NAA (5.0 + 2.5, 5.0 + 5.5) µM.

2.6. Statistical analysis

All the data were recorded at regular interval for analysis and reckoned under statistical basis. Arithmetic mean (A.M.) and standard deviation (S.D.) were evaluated by analyzing data with Microsoft excel 2007. Standard error (S.E.) was calculated by dividing standard deviation by square root of the total 20 replications for a single variety in each hormonal concentration. In case of our experiment error related to contamination was calculated properly and expected values were taken from the calculation.

3. Results and discussion

3.1. Effect of different concentration of hormones on callus induction

The present study was designed to identify the ideal conditions for micro propagation of kinnow mandarin (*C. reticulata* Blanco, because not much work has been done on tissue culture and micro propagation of these plant in Bangladesh. *In vitro* callus induction from seed on MS basal media supplemented with various concentrations of 2, 4-D, BA and NAA were studied in this experiment. Altaf et al. (2009) reported that the seeds formed callus in MS medium supplemented with BA + 2,4-D each at 1mg/L. Callus induction occurred on half strength MS medium supplemented with BA at 1.0 mg/L and 2,4-D at 5.0 mg/1 were obtained by (Miah et al. 2002) for *Citrus macroptera*. MS medium supplemented with different concentration of BA & NA was found to be best callusing in case of *Citrus grandis* by (F. Begum et al. 2003). Callus formation in treatment with 0.5mg/L Kin, 2.0mg/L NAA and 2.0mg/L 2,4-Dwas also observed in rough lemon by (Sharma et al. 2009). F. Azim et al. (2011) reported that highest percent (68%) callus was obtained from 2, 4-D, 2mg/L in *Citrus sinensis*. Kiong et al. (2008) reported that 2, 4-D, 4.0 mg/L and 2,4-D 3mg/L showed highest percent callus induction. M. Ashrafuzzaman et al. (2011) found that 2, 4-D 1.0 mg/L and 3mg/L also showed a moderate response of callus induction in *Citrus sinensis.* In case of present experiment, after 2-4 weeks of inoculation high efficiency callus was produced. Among all the treatments seeds treated with 2,4-D (16.0) µM shows best response (96%) followed by (75%) on MS medium supplemented with 2,4-D + BA + NAA (4.5+2.0+1.0) µM (Fiure 1 & 2). The results of the treatments are summarized in the Table 1. Other concentrations of 2, 4-D, BA and NAA also showed various degree of response. But 2,4-D with concentration of 4.5 µM and 6.5 µM showed no response. Approximately 62% of the callus was nodular compact, while 38% was smooth and compact. The color of the callus produced was whitish and yellowish white.

3.2. Effect of plant growth regulators for optimal shoot proliferation

In this experiment, green healthy calli obtained from previous steps of experiment were cut into small pieces and these pieces were cultured on MS medium supplemented with different concentration of auxins. After 28 day's shoot appears on some culture and extended shoot become visible after 42 days (Figure 1). Inclusion of auxin in the medium has been found to be beneficial for shoot production in some cases (Chaturvedi and Mitra 1974; Bhansali and Arya 1978). A medium containing 22 µM BA with or without 5.4 µM NAA was optimum for shoot initiation in Citrus rootstocks (Moore 1986). Earlier also combination of BA and NAA have been shown to be favorable for shoot regeneration from calli of different Citrus species (Chaturvedi and Mitra 1974; Beloualy 1991). Some studies have shown use of BA alone to be better treatment for shoot regeneration in different Citrus species (Raman et al. 1992; Costa et al. 2002). Regeneration of different species of Citrus has been already investigated using MS medium supplemented with BA 3 mg/L or with BA 1 mg/L (Pena et al. 1995 & Dominguez et al. 2000). From the report of (Barlass and Skene 1982), In case of Mandarin (*C. reticulata*) stem explant cultured in MS medium supplemented with KIN (1.0mg/L) + NAA (1.0mg/L) + BA (2.0mg/L) for shoot initiation shows good response. From the report of (Mukhtar et al. 2005) shoot tip explants cultured in MS media supplemented with 1 mg/L of BA and 1.5 mg/L of kinetin showed highest shoot percentage. Te-Chato and Nudoung et al. (1998) reported that BA 0.5 mg/L gave the best results (75%) of shooting response in *Citrus reticulata* Blanco from different explants of *in vitro* raised seedlings. From the report of (Sharma et al. 2012) nodal explants were cultured on MS medium fortified with different concentrations and combinations of BA and NAA. Best shoot multiplication was obtained on MS medium supplemented with BA (3 mg/L) and KIN (0.5 mg/L). So in this experiment different combination of BA, BA + KIN & BA + GA were used. Maximum shoot regeneration response (90%) was observed on MS medium supplemented with BA 13.0 µM followed by (80%) on MS medium supplemented with BA 9.0 µM + KIN 10 µM (Table 2 & Figure 3). The lowest shoot regeneration response (32%) was observed on MS medium supplemented with BA 8.0 µM + GA 6.0 µM followed by (40%) on MS medium supplemented with BA 8.0 µM.

3.3. Effect of different plant growth regulators and their concentrations on rooting of shoots

For root induction, several combinations and concentrations of IBA and NAA were tried, but only those media that elicited favorable root induction are shown (Table 3 & Figure 1). Nel (1987) reported root development from shoot meristems of *in vitro* grown seedlings of Citrus species after 2 months in half-strength Murashige and Tucker (1969) medium supplemented with NAA only. A study with *Citrus grandis* showed maximal rooting at 2 mg/L NAA, and a decrease in the frequency of rooting below of NAA concentration 2 mg/L (Paudyal & Haq 2000). In the study by Parthasarathy and Nagarju (1996), MS medium supplemented with 0.05 mg/L NAA was found to be the best for rooting in many Citrus species. However, in our investigation, a combination of NAA and IBA was found to be essential for root development. On the hand, half strength MS medium supplemented with .1mg/L IBA shown significant response in *Citrus jambhiri* by (Rahman et al. 1987), *Punica granatum* (Khantharaj et al. 1998), *Citrus grandis* (Begum et al. 2003). Sandra & Morehart (1998) reported that increasing IBA concentration had no effect on induction of number of roots instead it reduced total root length and also promoted callus production. After four weeks of culture in rooting medium, the growth of roots was studied. In current study good root length and root number were found best on 10.0 µM IBA showed the highest percentage of cutting rooted 85% (Table 3 & Figure 4) followed by 80% by 5.5 µM IBA + 2.5 µM NAA.

3.4. Establishment of the plantlets in the soil

After the root formation, the plantlets were removed from the tubes and transplanted to plastic pots. The substrate was an autoclaved mixture containing equal parts of leaf mould, sand, peat moss and loam. The percentage of success during acclimatization was 95% and the plantlets exhibited normal growth with 3-4 leaves per plant. After 2-3 months in the greenhouse the plants were transplanted under the field conditions.

Acknowledgement

The authors are thankful to Ministry of Science and Technology of Bangladesh for financial support to accomplish this study.

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Table 1. Effect of different hormonal combination for callus Induction after 4 weeks of observation (20 replications for each combination).

Hormones	Concentrations (µM)	% of callus induction (A.M ± S.E.)	Color of callus	Types of callus
2, 4-D	4.5	0	N/A	N/A
2, 4-D	6.5	0	N/A	N/A
2, 4-D	9.0	20 ± 4.56	Yellowish whitish	Nodular compact
2, 4-D	11.0	44 ± 4.27	Yellowish whitish	Nodular compact
2, 4-D	13.5	70 ± 3.16	Whitish	Nodular compact
2, 4-D	16.0	96 ± 2.15	Whitish	Smooth compact
2, 4-D	18.0	50 ± 3.24	Yellowish whitish	Smooth compact
2,4-D+BA+NAA	4.5+2.0+1.0	75 ±2.56	Yellowish whitish	Nodular compact
2,4-D+BA+NAA	6.5+1.0+2.0	50 ± 2.36	Whitish	Nodular compact
2,4-D+BA+NAA	9.0+3.0+1.0	44 ± 4.47	Yellowish whitish	Smooth compact

Here, A.M. = Arithmetic Mean and S.E. = Standard Error

Table 2. Effect of different concentrations of hormone on Shoot proliferation after 4 weeks of observation (20 replications for each combination).

Hormones	Concentrations (µM)	No. of shoots per explant	% of shoot formation (A.M ± S.E.)
BA	6.0	5	55 ±2.67
BA	10.0	7	68 ±3.28
BA	13.0	9	90 ± 2.13
BA	18.0	6	45 ±4.65
BA+KIN	9.0+10.0	10	80 ±2.45
BA+KIN	13.0+14.0	7	65 ±3.24
BA+GA	6.0+6.0	5	47 ± 3.96
BA+GA	8.0+6.0	6	32 ±4.25

Here, A.M. = Arithmetic Mean and S.E. = Standard Error

Table 3. Effect of different concentrations of hormone on root initiation after 4 weeks of observation (20 replications for each combination).

Hormones	Concentrations (µM)	(%) Root Regeneration (A.M ± S.E.)	Average number of root
IBA	5.0	70 ± 1.36	11
IBA	7.0	40 ± 3.45	13
IBA	10.0	85 ± 1.27	12
IBA	12.5	65 ± 2.92	15
IBA	15.5	0	0
NAA	5.0	50 ± 3.26	7
NAA	8.5	0	0
IBA + NAA	5.5+2.5	80 ± .36	13
IBA + NAA	5.5+5.0	42 ± 4.15	12

Here, A.M. = Arithmetic Mean and S.E. = Standard Error

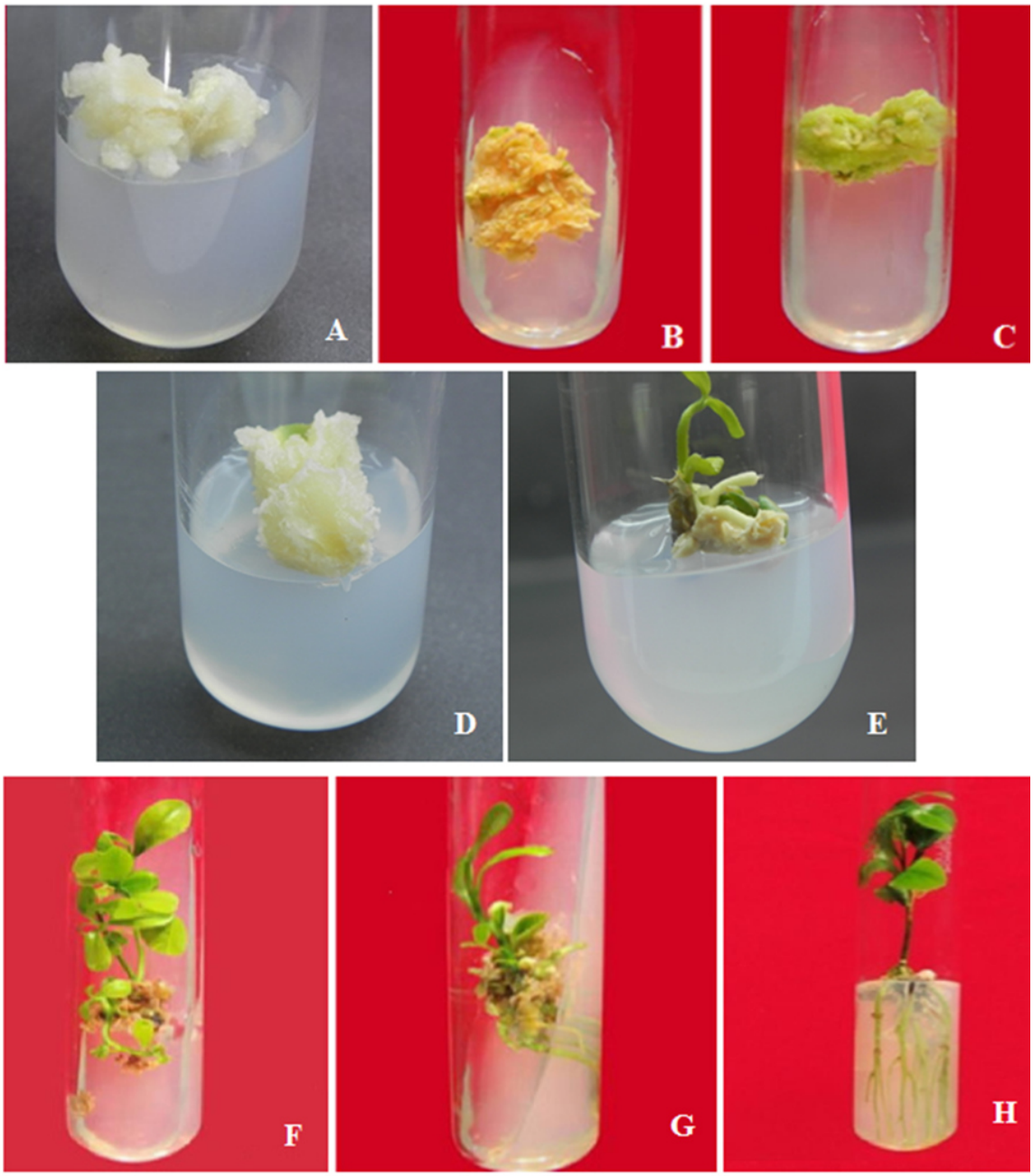

Figure 1. *In vitro* plant regeneration of *Citrus reticulata* Blanco
(A), (B) & (C) Callus induction from 16.0 µM 2, 4-D
(D), (E) & (F) multiple shoot proliferation from seeds cultured in MS + 13.0 µM BA
(G) & (H) *In vitro* rooting on MS + 10.0 µM IBA

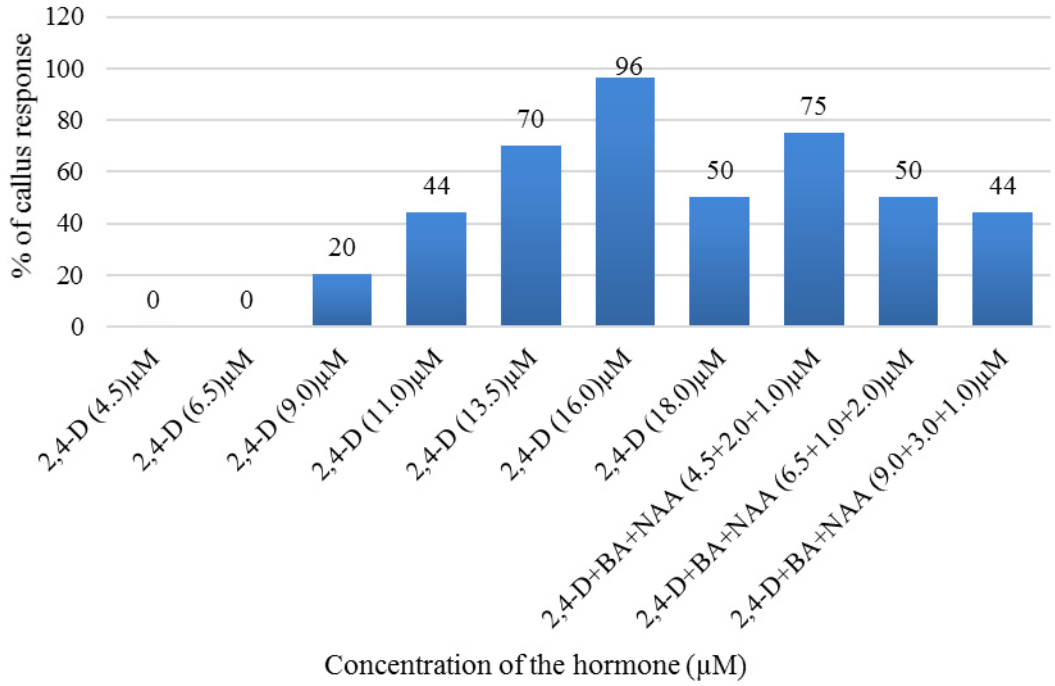

Figure 2. Effects of hormones in callus induction (callus response shown in percentage).

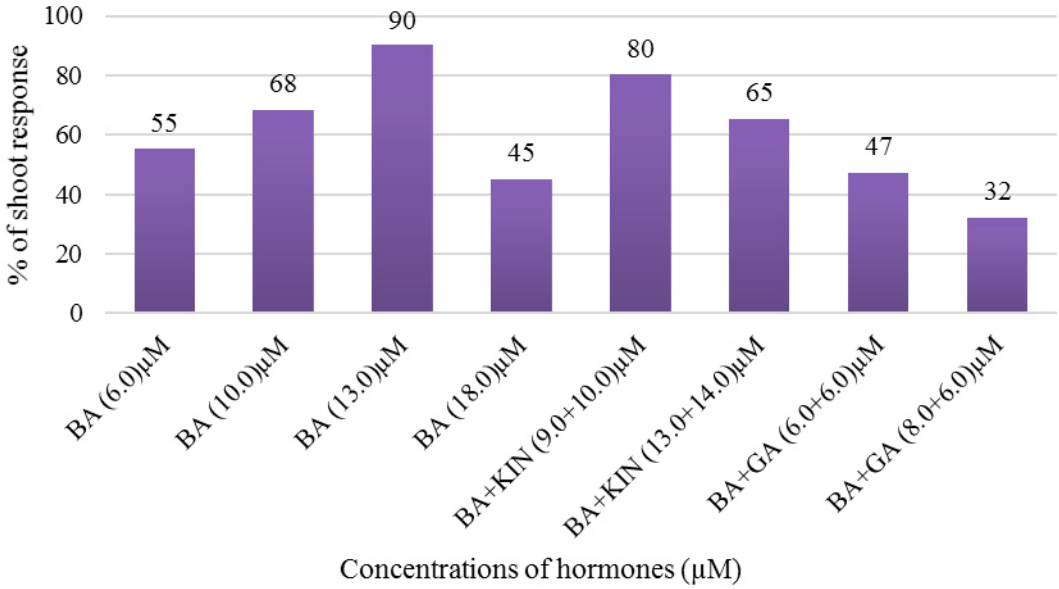

Figure 3. Effects of BA, KIN, GA and their combination on shoot proliferation (response shown in percentage).

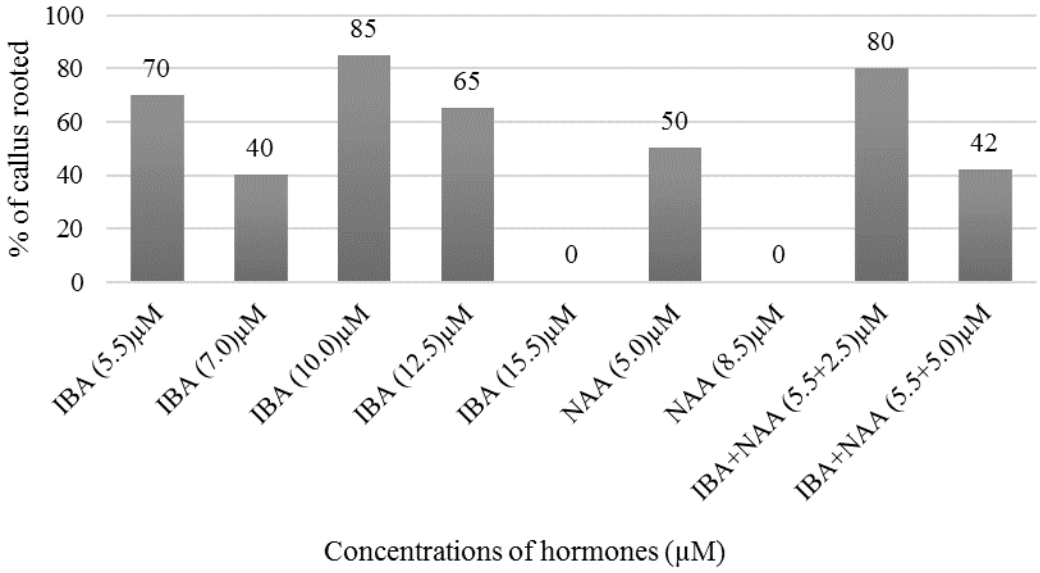

Figure 4. Effects of IBA, NAA and their combination on rooting (shown in percentage).