The Journal of Animal & Plant Sciences, 28(2): 2018, Page: 589-596
ISSN: 1018-7081

ADDITIVE EFFECTS OF COCONUT WATER WITH VARIOUS HORMONES ON *INVITRO* REGENERATION OF CARNATION (*DIANTHUS CARYOPHYLLUSL*)

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ABSTRACT

Plant needs some essential nutrient as well as growth regulator for their growth and development. In the case of Carnation (*Dianthus caryophyllus* L.) micro-propagation, large scale production was not possible by using the available traditional media (MS media) and method. In this study we first demonstrated the *in vitro* effect of coconut water (CW) together with cytokinine hormone on efficient growth of Carnation. The highest percentage of shoot induction was observed when the medium was used with coconut water (cw) at 10% +BAP 1.0 mg/l. In this case multiplication rate of carnation increased more than four times when shoot tip and nodal segment were used as an explant. So our modified media could be useful for Carnation (*Dianthus caryophyllus* L.) micro propagation and be a powerful media for tissue culture of Carnation. The best Carnation growth was observed in half-strength MS supplemented with 1.0 mg/l NAA for root induction. The survival rate of the transplanted plantlets was 80% in potted soil condition.

Keywords: Micro propagation, Carnation, Media, Coconut water, Growth regulator.

INTRODUCTION

Carnation (*Dianthus caryophyllus L.*) is an important flower crop having great commercial value as a cut flower (Pralhad, 2009). The importance of this ornamental flower is due to its beauty, diversity of colours, excellent keeping quality and wide range of different forms (Santos-Hernandez *et al*., 2005; Ali *et al*., 2008; Kanwar and Kumar, 2009). Carnation a member of the family Caryophyllous has 88 genera and 1750 species (Ali *et al*., 2008). Caryophyllus means pink refers to the colour of blooms of the original species. From medicinal point of view, the Carnation flowers are considered to be cardiotonic, diaphoretic and alexiteric (Shiragur *et al*., 2004). Continuous development in production, imports and economic variables into account, has raised the consumption of carnation in world's market up to 35 billion US dollars in 2000 (Ali *et al*., 2008; Tarannum *et al*., 2014). Carnation flowers are sold as cut flowers round the year throughout the world and it is on the top three cut flowers traded in the international market (Mehmood *et al*., 2014). In the world, now it is estimated that more than 6000 ha of land is under cultivation of Carnation (Tarannum *et al.,* 2014). Considering the benefits of this crop and to fulfil the world's demand carnation breeders constantly seek new varieties with improved horticultural traits such as disease and pest resistance and long vase-life. Usually carnation varieties are maintained year after year by cutting or by other vegetative propagules (Karami, 2008). In this way the plants remain same phenotype and genotype but they may become internally infected by pathogen like fungi, bacteria and viruses which decrease their yield significantly. Vegetative propagation cannot eliminate the pathogen from the new plants. Plant tissue culture technique can play a key role to produce large number disease free plantlets which are true-to parental type.

Therefore, *in vitro* technique is considered the best alternatives method that may supply a large number of planting materials for commercial planting and further studies. Growth regulators are one of the most important components for large scale micro propagation of *in vitro* plants. Coconut water is widely used in the plant tissue culture industry due to its growth regulatory properties and cytokinin-type activity that support cell division and promote rapid growth (Arditti, 2008; Yong et al., 2009). The use of coconut water as a supplement to the culture medium started in the 1940 by Van Overbeek on *Daturas tramonium* micropropagation (George, 2008). It is also rich in magnesium, phosphate and high amounts of sugar which is around 2.5% (w/v).

There are many previous study published where researchers have worked with coconut water as a supplement in growth medium to improve regeneration of plant cells (Buah and Agu-Asare, 2014; Abdullahil *et al*., 2011; Nasib *et al*., 2008; Yoon *et al*., 2007; Krug *et al*., 2005; Pyati *et al*. 2002; Jayasih and Wattimena 1994; Boase *et al.,* 1993; Mathias and Simpson, 1986).

Although many researchers previously reported *in vitro* regeneration of Carnation (Danial *et al*., 2009; Khatun, 2013; Kharrazi *et al.*, 2011; Kanwar and Kumar, 2009; Daniel *et al.,* 2009; Ali *et al*., 2008; Karami*et al*., 2008; Thakur *et al.*, 2002; Altvorst*et al*., 1992; Miller *et al*., 1991; Leshem,1983). However, there is no study yet documented to observed the role of coconut water on *in vitro* regeneration of Carnation. Therefore, the aim of the present study was to determine the best doses of coconut water as an organic

supplement to promote the growth rate of *in vitro* culture of Carnation (*Dianthus caryophyllus L.*).

MATERIALS AND METHODS

Plant material and surface sterilization: Shoot tip and nodal segments were collected from garden grown plants of Institute of food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka, in winter season. The explant materials were kept in water, brought to the laboratory and washed thoroughly under running tap water for one hour in order to remove dirt on the stem surface. Subsequent sterilization was carried out in the laminar air flow cabinet. Plant materials were sterilized by rubbing with an aqueous solution of 0.1% $HgCl_2$ with two drops of Tween 20 for five minutes under aseptic condition and rinsed five times with autoclaved distilled water to wash out any trace of $HgCl_2$. After sterilization, Shoot tips and nodal segments were cut into small pieces (1.0-1.5 cm) for explants.

Preparation of coconut water: Coconut water was extracted in sterile chamber from fresh green fruits by drilling hole. The water was then filtered using a 0.45µm sterile mesh and stored at -20˚C until use without autoclaving to avoid degradation of the organic compounds by heat.

Nutrient medium and growth condition: MS basal medium (Murashige and Skoog, 1962) was used for regeneration of plantlets through *in vitro* culture. Shoot tip and nodal segment explants were cultured on MS supplemented different concentrations of cytokinins (Zeatin, BAP, Kn) and auxin (NAA) singly or in combination for shoot regeneration. Coconut water (CW) in doses of 5-20% (0 as control, 5, 8, 10, 15, 20% v/v) was added to the medium after autoclaving and before solidification for further development of shoot multiplication. In order to induce root system, shoots were excised individually and cultured on half-strength of MS supplemented with different concentrations and combinations of IBA, IAA and NAA. The pH of the media was adjusted to 5.8 before adding agar. All media were solidified with 0.7% agar. The cultures were incubated at 25±2ºC with a 16h photoperiod. Sub-culturing was done every three-week interval. The well rooted plantlets were taken out from the test tubes and gently washed them to free from medium. They were then transplanted to earthen pots containing a mixture of soil, sand and compost (Compost is a natural fertilizer made from cow dung) (2:1:1). Observations on cultures were carried out every alternate day.

Data recording and analysis: Each experiment was conducted at least twice. The percentage of shoot induction, length and number of shoots and roots were recorded. The mean values and standard deviations were calculated using computer software (Microsoft Office Excel Worksheet).

RESULTS AND DISCUSSION

As this research aiming to determine most effective media composition for large scale multiplication of carnation (*Dianthus caryophyllus L.*), the available media were used with various concentrations of plant hormones. At first step, very low concentration of Zeatin and Zeatin± NAA in MS medium was used to observe primary response of shoot tip and nodal segment explants (Figure:1). In these compositions the shoot induction was not satisfactory. Shoot induction percentage was not more than 50% for both shoot tip and nodal segment explants. Maximum number of shoots (6.08±0.19) for shoot tips and (8.25±0.17) for nodal segment were observed on MS medium supplemented with 1.0 mg/l Zeatin+ 0.5mg/l NAA respectively (Figure: 1, Table: Supplementary S1, Figure: Supplementary S1a, S1d).

Next step, explants were cultured on MS supplemented media with different concentrations of BAP, Kn and NAA alone or in various combinations for multiple shoot regeneration. As, shown in figure: 2 and Table S2 all explants comprising shoot tips and nodal segments and were cultured for direct multiple shoot regeneration. Initiation of multiple shoots in most of the explants was observed within six weeks of culture. In both shoot tip and nodal segment explants, the highest percentage of shoot induction was observed in MS+1.0 mg/l BAP (figure: 2 and Table S2). In the case of nodal segments, 80% of cultures were found to regenerate shoots and the number of regenerated shoots per culture was 25.08±0.19on above medium (figure: 2 and Table S2 and, Figure: S1- e).

In the same medium, multiple shoot induction from the shoot tip explant was 20.58+0.28 per culture (Figure: 2 and Table S2, Figure: S 1-b). Previous study reported that MS supplemented with 1.0 mg/l BAP was the most effective to highest number of shoot regeneration of Carnation (Ali *et al.,* 2008; Pareek *et al.,* 2004; Kim and Kang, 1986; Leshem, 1983; Kovac, 1995; Thakur *et al.,* 2002). Our study also provides similar scenario in the case of shoot regeneration.

To see the enhancement of shoot proliferation, Coconut water (5-20% v/v) was added to the same multiplication medium (MS±1.0 mg/l BAP). In this case, the best regeneration was obtained using the medium with coconut water (cw) at 10% ± BAP 1.0mg/l which increased the number of shoots [nodal explants =113.83±0.40 (Figure: S1-f), shoot tip explants = 93.33±0.43 (Figure: S1-c)] per culture.

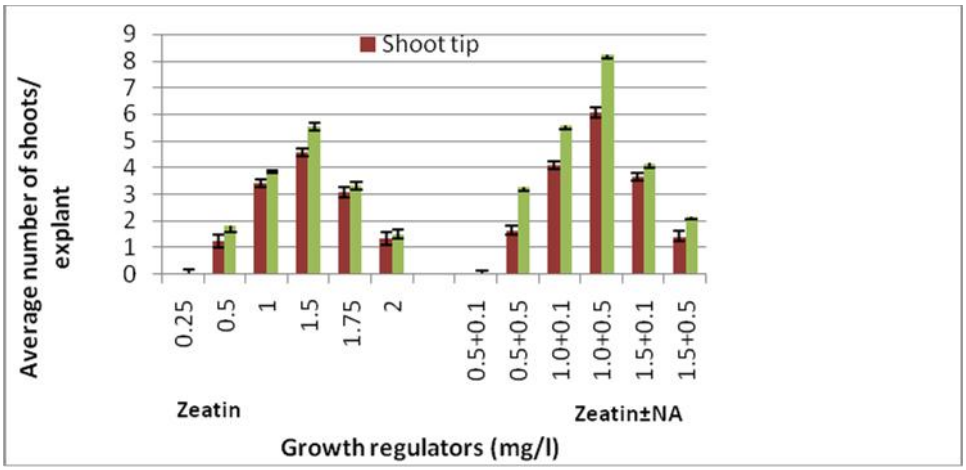

Figure 1. The effects of Zeatin and combination of Zeatin with NAA on shoot tips and nodal segment

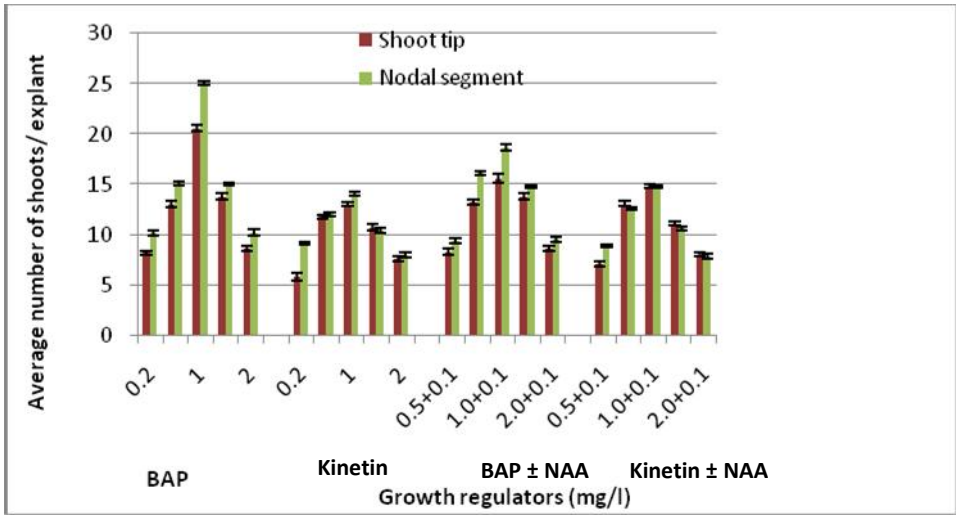

Figure 2. Effect of different Concentrations and combinations of growth regulators in MS media on shoot proliferation from shoot tip and nodal segment

Mean length of shoots were 5.33+0.14 (cm) for nodal explants and 5.66+0.14 (cm) for shoot tips explants respectively after 6 weeks of cultures. Similar phenomena were reported in previous study where the addition of 10% CW in the medium increased the number of shoots in *Boerhaavia diffusa* L. culture (Roy, 2008). In addition, (Nasib *et al*., 2008) observed 20% (v/v) coconut water combined with BAP during the *in vitro* propagation of Kiwifruit showed best shoot length and number of leaves. (Villa *et al*., 2010) also demonstrated the advantages of coconut water associated with BAP in olive cultivar. The author observed 25ml/l of coconut water associated with 500µg /l of BAP generated plantlets with higher shoot length and heavier fresh biomass than in plantlets cultivated without this compound. Working with the olive cultivar (Trevisan *et al.,* 2005) reported that coconut water is very useful for stem elongation and plant development *in vitro* culture of Passion fruit.

However, Coconut water alone is not sufficient to promote satisfactory multiplication of plants. The addition of 20% coconut water increased the number of adventitious shoots per explant with combination of 2mg/l BAP for *Corylus avellana* L. (Sandoval *et al.,* 2014). As reported in Grigoriadou *et al.,* (2002) the combination of CW with cytokinins is the most effective way to improve rates of multiplication. Also, Coconut water is a complex additive which contains many nutritional and hormonal compounds. Therefore, together with plant hormones the coconut water if very effective in plant regeneration. To observed the significant value of coconut water in the last years, there has been a significant increase in the use of coconut water in micro propagation protocols of economically important species such as passion fruit (Hall *et al*., 2000), coffee (Ismail *et al*., 2003), orchids (Santos-Hernandez *et al*., 2005) and Kiwifruit (Nasib *et al*., 2008).

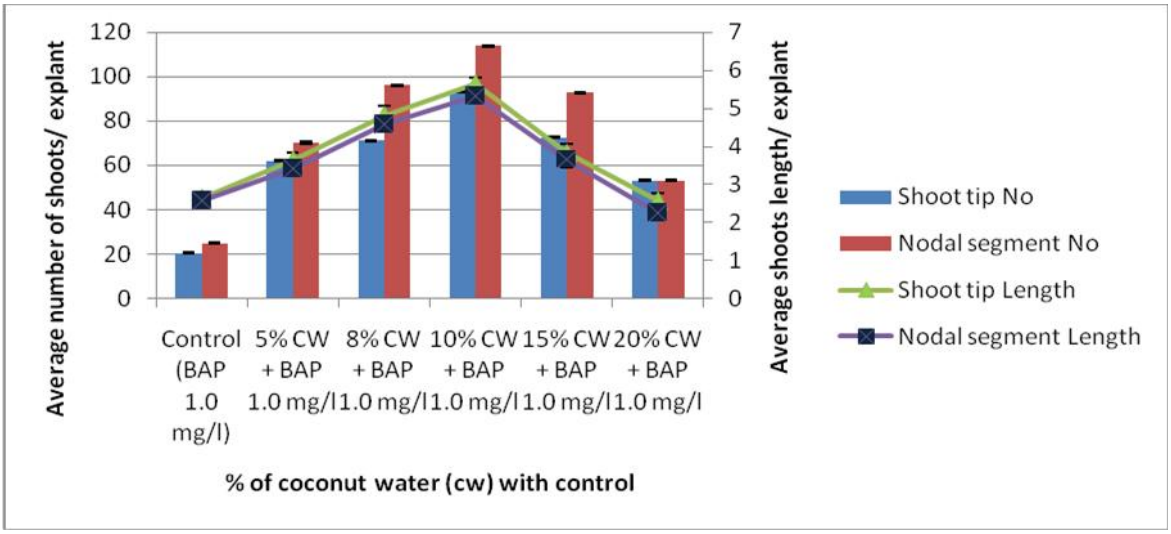

Figure 3. Effect of coconut water concentrations on proliferation of shoot number and length from shoot tips and nodal segments

Regenerated shoots need to form the root for their healthy growth. So, an experiment was conducted with half-strength MS supplemented with different types of auxins (IBA, IAA and NAA). Auxins were used individual or in combination with other auxins in various concentrations. Auxin plays a major role in root induction due to its effect on the rapid cell division (Farooq *et al.,* 2008). The best growth was obtained in half-strength MS supplemented with 1.0 mg/l NAA (Figure: 4 and Table: S4).

In this combination, it was observed that 90% shoots rooted well within 15.33±0.14 days of culture and each micro cutting section produced 10.16±0.20 roots. Mean length of the root was 6.58±0.14 (cm) (Figure: S1-g). (Kharrazi *et al.,* 2011) reported that NAA was more effective for *in vitro* rooting of carnation where MS with 0.5 mg/l NAA was suitable combination for best rooting. After sufficient roots development, plantlets were transplanted in small earthen pots containing a mixture of soil, sand and compost (2:1:1) (Figure: S1-h). The survival rate of the transplanted plantlets was 80% in potted soil condition.

The results of the study showed an efficient *in vitro* regeneration protocol for Carnation (*Dianthus caryophyllus* L.) by using coconut water as a supplement with growth medium. Effective media compositions for better shoot and root generation as well as acclimatization were described. The protocol can be exploited for large scale multiplication of Carnation and production of healthy plant for better floriculture. As there is other previous study already reported together with regeneration of Carnation that coconut water promotes significant growth of plant, therefore we are recommending using coconut water together with commercial available media. As coconut water is a natural compound, there is no any side effect or adverse effects found in using tissue culture. Therefore, coconut water could be used in plant regeneration unlike Carnation multiplication.

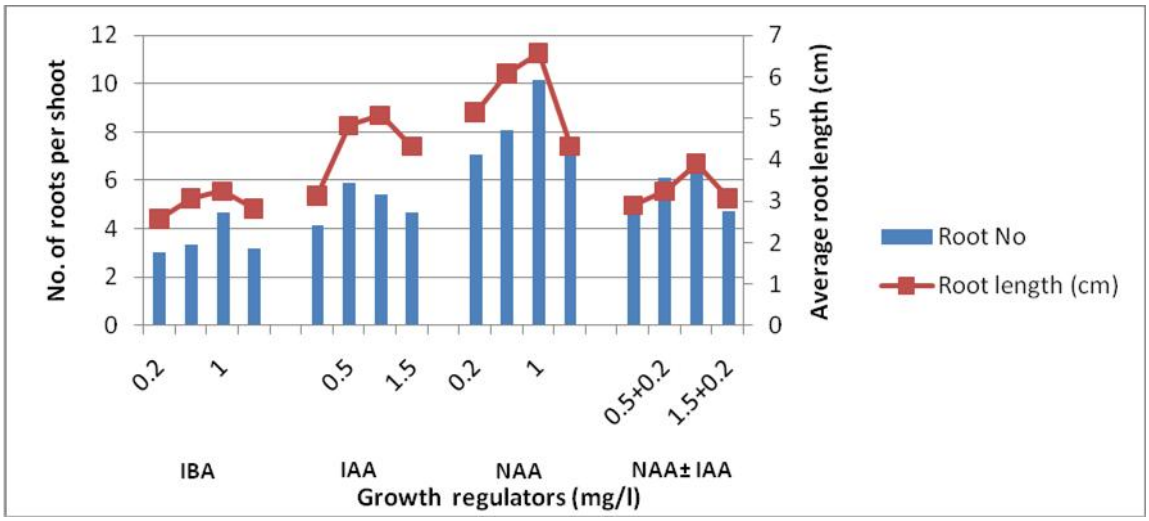

Figure 4. Effects of different concentration of IBA, IAA and NAAs in combination in half-strength MS on root induction from regenerated shoots of Carnation.

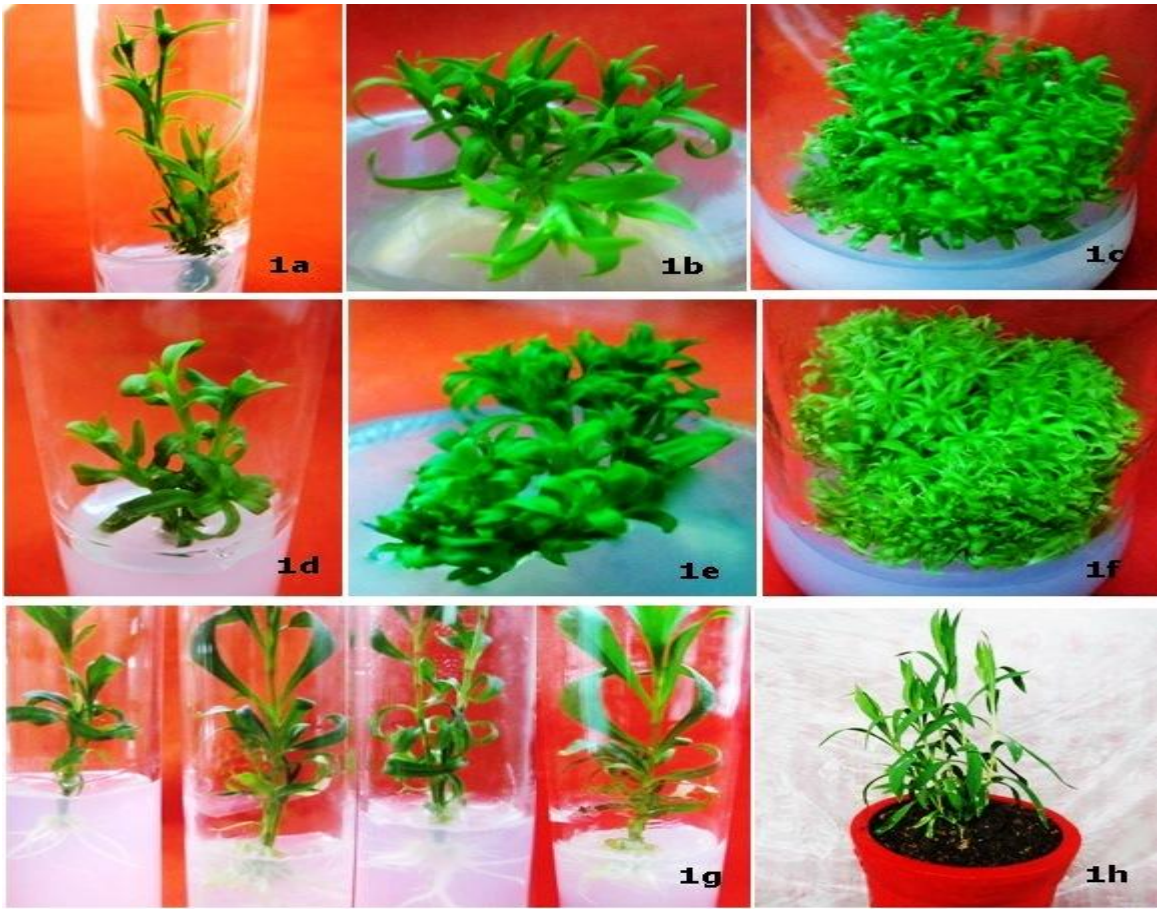

Figure:S1(a-h) ***In vitro*** **regeneration of** ***Dianthus caryophyllus*** (a) Initial shoot formation from shoot tip explant (b) Multiple shoot regeneration from shoot tip (c) effect of coconut water (10%) on shoot proliferation in shoot tip (d) Initial shoot formation from nodal segment explants (e) Multiple shoot regeneration from nodal segment (f) Coconut water (10%) effect on shoot proliferation in nodal segment (g) Root induction (h) *In vitro* regenerated plantlet in earthen pot containing a mixture of soil, sand and compost (2:1).

Supplementary data

Table S1. Effect of different concentrations and combinations of growth regulators in MS media on primary response of shoot tip and nodal segment explants of Carnation, *Dianthus caryophyllus* L.

Growth regulators	Conc of Growth regulators (mg/l)	% explants showing shoot regeneration		Average number of shoots / explant (Mean ± S.E.)	
		Shoot tip	Nodal segment	Shoot tip	Nodal segment
Zeatin	0.25	0	0	0±0	0±0
	0.5	16.7	16.7	1.25±0.25	1.83±0.27
	1.0	25	25	3.41±0.14	3.91±0.14
	1.5	33.3	41.7	4.58±0.14	5.55±0.14
	1.75	16.7	25	3.08±0.19	3.33±0.14
	2.0	8.33	16.7	1.33±0.22	1.5±0.19
Zeatin+NAA	0.5+0.1	0	0	0±0	0±0
	0.5+0.5	16.7	25	1.66±0.16	3.25±0.13
	1.0+0.1	25	25	4.08±0.14	5.58±0.14
	1.0+0.5	33.3	41.7	6.08±0.19	8.25±0.17
	1.5+0.1	25	33.3	3.66±0.14	4.16±0.16
	1.5+0.5	16.7	16.7	1.41±0.19	2.16±0.11

Data were recorded after 4 weeks of culture on the MS medium using 3% (w/v) sucrose. Values are the means ± standard error.

Table S2. Effect of different concentrations and combinations of growth regulators in MS media on shoot proliferation from shoot tip and nodal segment explants of Carnation, *Dianthus caryophyllus* L.

Growth regulators	Conc of Growth regulators (mg/l)	% Explants showing shoot regeneration		Average number of shoots / explant (Mean ± S.E.)	
		Shoot tip	Nodal segment	Shoot tip	Nodal segment
BAP	0.2	41.7	50	8.25±0.21	10.16±0.24
	0.5	58.3	66.7	13.08±0.28	15.16±0.20
	1.0	83.3	83.3	20.58±0.28	25.08±0.19
	1.5	66.7	66.7	13.83±0.27	15.08±0.14
	2.0	58.3	58.3	8.66±0.30	10.25±0.27
Kinetin	0.2	33.3	41.7	5.83±0.36	9.16±0.16
	0.5	41.7	50.0	11.83±0.20	12.08±0.19
	1.0	58.3	58.3	13.08±0.22	14.08±0.22
	1.5	50.0	50.0	10.75±0.30	10.41±0.25
	2.0	41.7	41.7	7.66±0.28	8.08±0.25
BAP+NAA	0.5+0.1	41.7	50.0	8.33±0.33	9.41±0.25
	0.5+0.5	41.7	58.3	13.25±0.21	16.16±0.16
	1.0+0.1	50.0	66.7	15.58±0.43	18.66±0.35
	1.0+0.5	66.7	58.3	13.83±0.27	14.75±0.13
	2.0+0.1	50.0	41.7	8.66±0.30	9.58±0.25
Kinetin+NAA	0.5+0.1	50.0	41.7	7.16±0.29	8.91±0.14
	0.5+0.5	58.3	50.0	13.08±0.25	12.58±0.14
	1.0+0.1	66.7	58.3	14.83±0.20	14.83±0.11
	1.0+0.5	66.7	50.0	11.16±0.20	10.66±0.22
	2.0+0.1	58.3	50.0	8.08±0.19	7.91±0.22

Data were recorded after 6 weeks of culture on the MS medium using 3% w/v sucrose. Values are the means ± standard error

Table S3. Effect of coconut water concentrations on proliferation of shoot number and length from shoot tip and nodal segment explants of Carnation, *Dianthus caryophyllus* L.

% of coconut water (cw)	Average number of shoots / explant (Mean ± S.E.)		Average shoots length / explant (Mean ± S.E.) (cm)	
	Shoot tip	Nodal segment	Shoot tip	Nodal segment
Control (only BAP 1.0 mg/l)	20.58±0.28	25.08±0.19	2.66±0.14	2.58±0.14
5%cw+BAP1.0mg/l	62.16±0.38	70.08±0.46	3.66±0.18	3.41±0.14
8%cw+BAP1.0mg/l	71.16±0.36	96.08±0.33	4.83±0.24	4.58±0.14
10%cw+BAP1.0mg/l	93.33±0.43	113.83±0.40	5.66±0.14	5.33±0.14
15%cw+BAP1.0mg/l	72.50±0.23	92.75±0.39	3.83±0.24	3.66±0.22
20%cw+BAP1.0mg/l	53.25±0.35	53.25±0.35	2.58±0.19	2.25±0.13

Data were recorded after 6 weeks of culture on the MS medium using 3% (w/v) sucrose. Values are the means ± standard error

Table S4. Effects of different concentration of IBA, IAA, NAA or in combination with MS media on root induction from regenerated shoots of Carnation

Growth regulators	Growth regulators (mg/l)	Days required for rooting	No. of roots per shoot	Average root length (cm)
IBA	0.2	20.91±0.22	3.0±0.24	2.58±0.14
	0.5	19.83±0.24	3.33±0.14	3.08±0.19
	1.0	19.58±0.19	4.66±0.18	3.25±0.21
	1.5	21.08±0.22	3.16±0.110	2.83±0.16
IAA	0.2	19.58±0.14	4.16±0.16	3.14±0.14
	0.5	19.16±0.27	5.91±0.08	4.83±0.24
	1.0	18.33±0.14	5.41±0.14	5.08±0.22
	1.5	19.75±0.21	4.66±0.18	4.33±0.22
NAA	0.2	18.41±0.19	7.08±0.25	5.166±0.20
	0.5	17.66±0.14	8.08±0.28	6.08±0.14
	1.0	15.33±0.14	10.16±0.20	6.58±0.14
	1.5	17.91±0.19	7.08±0.19	4.33±0.22

NAA+IAA	0.2+0.2	18.91±0.22	4.58±0.14	2.91±0.19
	0.5+0.2	17.66±0.25	6.08±0.08	3.25±0.27
	1.0+0.2	18.41±0.14	6.58±0.14	3.91±0.22
	1.5+0.2	19.75±0.21	4.75±0.17	3.08±0.19

Data were recorded after 4 weeks of culture on the MS medium using 3% (w/v) sucrose. Values are the means ± standard error.

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