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Full Length Research Paper

Plant regeneration of *Brassica oleracea* subsp. *italica* (Broccoli) CV Green Marvel as affected by plant growth regulators

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Hypocotyls and shoot tips were used as explants in *in vitro* plant regeneration of broccoli (*Brassica oleracea* subsp.italica) cv. Green Marvel. The explants were excised from sterile germinated seedlings and placed on shoot induction medium containing basal salts of Murashige and Skoog (MS) and various concentrations of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA). The highest percentage of hypocotyl explant producing shoot (96.67%) and the highest mean number of shoots produced per hypocotyl explant (6.03) were obtained on 3 mgL^{-1} BAP. Meanwhile, the highest percentage of shoot tip explant producing shoot (100%) and highest number of shoot produced per shoot tip explant (3.76) were recorded on 5 mgL^{-1} BAP. For rooting of shoots, NAA, indoleacetic acid (IAA) and indolebutyric acid (IBA) at 0, 0.2 and 1 mgL^{-1} were applied. Highest percentage of shoots with roots (100%) and highest mean number of roots produced per shoot (6.5) occurred on medium with 0.2 mgL^{-1} IBA, while the maximum root length (2.46 cm) was attained on MS medium without plant growth regulator (MSO). Plantlets were successfully acclimatized in potting medium containing peatmoss, perlite, and vermiculite (3:1:1).

Key words: Broccoli, 6-benzylaminopurine, α-naphthaleneacetic acid, indole-3-butyric acid, *in vitro* culture, multiple shoot formation.

INTRODUCTION

In vitro regeneration offers a great opportunity for a rapid production of desirable and essentially genetically identical plants (Lazzeri and Dunwell, 1986; Msikita and Skirvin, 1989). An efficient *in vitro* regeneration system is also a crucial tool in genetic engineering of the crop for improved characteristics (Cao and Earle, 2003). Broccoli, *Brassica oleracea* subsp. *italica* is one of the many valuable *Brassica* species, which is still less cultured under *in vitro* condition (Widiyanto and Erytrina, 2001). Some experimental results showed successful *in vitro* culture of *Brassica* species from hypocotyl segments, root segments, primary leaf discs, cotyledons and anther (Cao and Earle, 2003). Among the *Brassica* species include kale, brussels sprouts, cabbage and cauliflower (Qin et al., 2006). In most *Brassica* species, the successful application of *in vitro* culture is mostly dependent on the genotype and the influence of growth regulators. The addition of cytokinins, such as kinetin or benzyladenine would enhance shoot proliferation and root formation (Arnison et al., 1990). Various concentrations of auxins such as naphthaleneacetic acid (NAA), indolebutyric acid (IBA) and indoleacetic acid (IAA) have been evaluated for rooting of *in vitro* regenerated shoots of broccoli and cauliflower (Vandemoortele et al., 1999; Widiyanto and Erytrina, 2001).

This paper reports on the influence of BAP either singly or in combination with NAA, on adventitious shoot proliferation from hypocotyls and shoot-tips explant of broccoli cv. Green Marvel, and the effect of various concentrations of auxins such as α-naphthaleneacetic acid (NAA),

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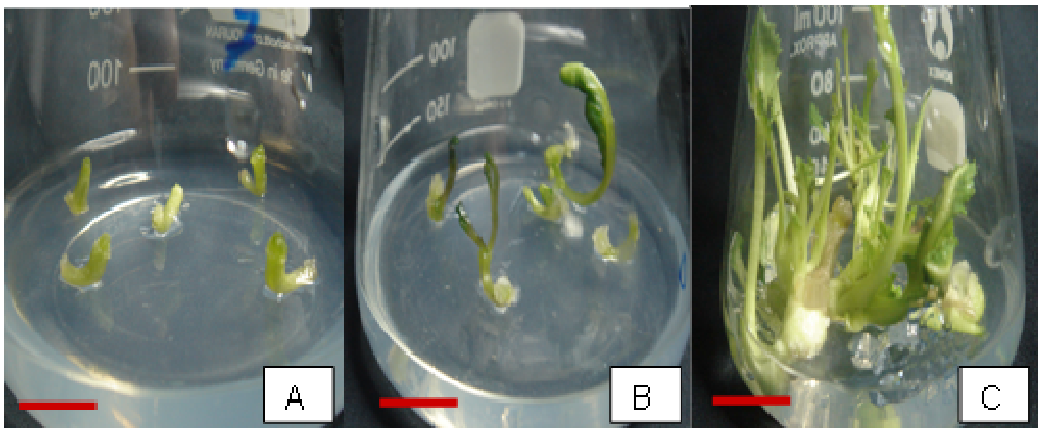

Plate 1. Hypocotyl explants on MS medium containing 3 mgL^{-1} BAP after (A) one week, (B) after 4 weeks, and (C) after eight weeks of culture. Bar = 5 mm.

indolebutyric acid (IBA) and indoleacetic acid (IAA) on rooting of the *in vitro* regenerated shoots.

MATERIALS AND METHODS

Plant material and sterilization protocol

Seeds of broccoli were surface sterilized for 2 min in 70% ethanol solution followed by continuous agitation for 15 min in 20% Clorox solution (0.53% sodium hypochlorite) added with two drops of Tween 20. The seeds were rinsed three to four times in sterile distilled water and cultured on germination medium containing half-strength MS salts supplemented with 2.5 gL^{-1} phytagel and 30 gL^{-1} sucrose.

Medium composition and treatment

Hypocotyl and shoot tip explants, 5-8 mm in size, were excised from 6-day-old broccoli seedlings. The explants were cultured on MS medium incorporated with different concentrations of plant growth regulators for shoot proliferation and root formation. For shoot induction and multiplication from hypocotyl explants various concentrations of BAP (0, 1, 3, 5, 7 and 10 mgL^{-1}) were tested. While for shoot tip explants, BAP at 0, 1, 3, 5 and 7 mgL^{-1} either alone or in combination with 0.5 and 1 mgL^{-1} NAA were assessed. IAA, IBA and NAA at 0, 0.2, 0.5 and 1 mgL^{-1} were used for inducing root formation. The media were solidified with 2.5 gL^{-1} phytagel and the pH adjusted to 5.8 prior to autoclaving at 121^{o}C for 15 min. MS medium without any growth regulator (MSO) was considered as a control.

Parameters recorded

In the shoot induction and multiplication study from hypocotyl and shoot tip explants, parameters recorded were the percentage of explant producing shoots (%), and the mean number of shoots produced per explant. Data were collected after eight weeks of culture, while growth characteristics were observed every week. In the rooting study, the parameters recorded were the percentage of explants producing root (%), the mean number of roots produced per explant and the root length attained (cm). The data on rooting were collected after four weeks of culture.

Experimental design and statistical analysis

The experiments were arranged in a Randomized Complete Block Design (RCBD), with three replications and each replication per treatment contained 10 explants. Data were analyzed using the Analysis of Variance (ANOVA) and Duncan New Multiple Range Test (DNMRT) at α = 5% for comparison between treatment means.

RESULTS AND DISCUSSION

Multiple shoot formation from hypocotyl explants

In the first week of culture, the hypocotyl explants began to expand and swelled (Plate 1A) in all BAP concentrations tested. Within three to four weeks of culture shoot appeared in the middle and distal end of the hypocotyl explant (Plate 1B). By the eighth week of culture, significant differences in the percentage of explant with shoot and mean number of shoot produced per explant were observed among the BAP treatments. In Figure 1, BAP at 3 mgL^{-1} induced the highest percentage of explant producing shoots (96.7%) followed by 5 mgL^{-1} BAP (93.3%). There was no significant difference observed between the two treatments (3 and 5 mgL^{-1} BAP) on percentage of explant producing shoots. However, treatment 3 mgL^{-1} BAP showed significant difference compared to the control and the rest of the treatments.

BAP at 3 mgL^{-1} induced the highest mean number of transferable shoots per explant (6.03) after eight weeks of culture. It differed significantly compared to the rest of the treatments (Figure 1B; Plate 1C).

According to George et al. (2008), BAP is most effec-tive in enhancing shoot multiplication and triggering shoot elongation. BAP also promotes differentiation of cell into shoot initials followed by the formation of shoots. Above 5 mgL^{-1} BAP the mean number of shoots formed per explant decreased (Figure 1B) and became toxic to the shoot growth.

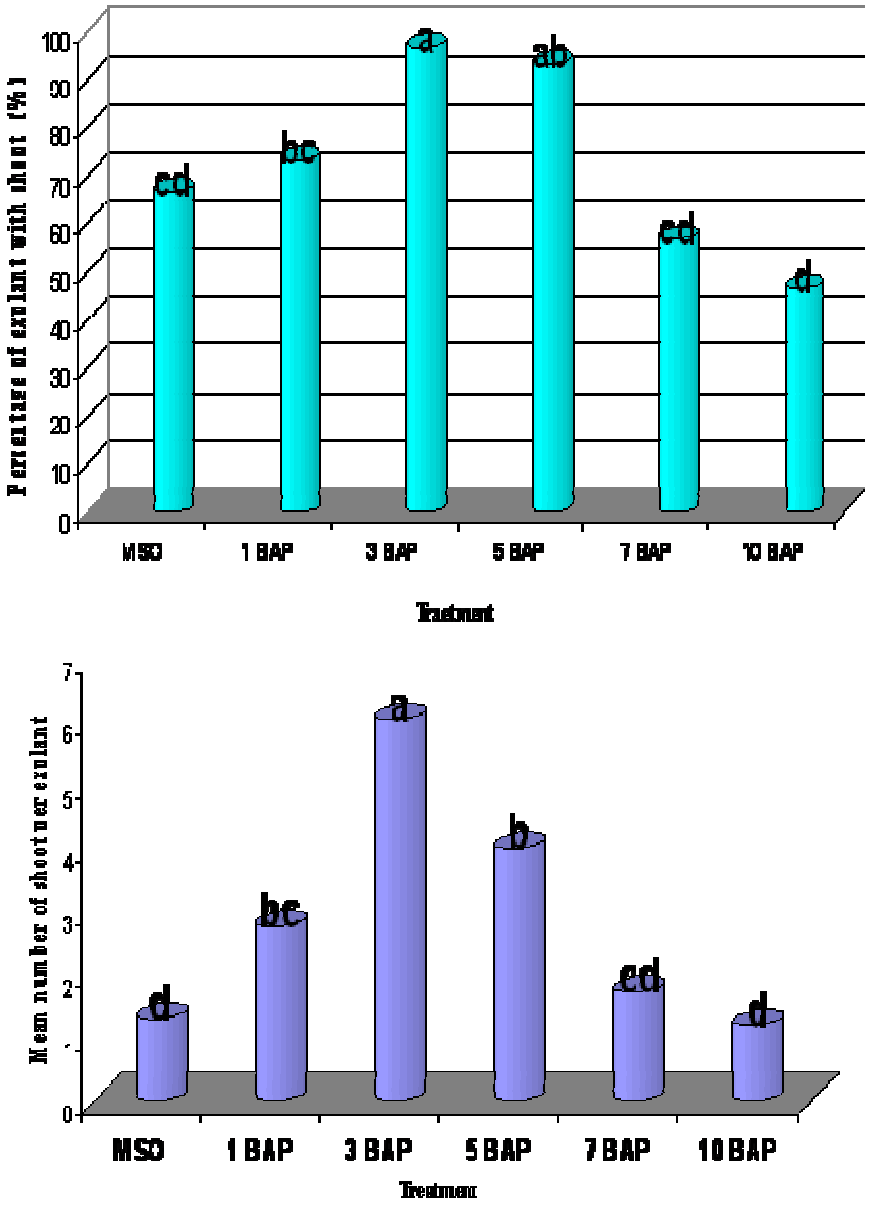

Figure 1. Effect of different concentrations of BAP on (A) percentage of hypocotyl explant with shoot and (B) mean number of shoot produced per hypocotyl explant after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

Multiple shoot formation from shoot tip explants

Plate 2 shows the stages of multiple shoot formation from shoot tip explants of broccoli. It was observed that the shoot tips elongated after the first week of culture (Plate 2B). The whole shoot tip began to swell after two weeks of culture, and by the fourth week axillary shoots emerged (Plate 2B). By the eighth week of culture multiple shoots were formed. There were significant differences among the treatments on the percentage of explant with shoot after eight weeks of culture (Figure 2 A). The highest percentage of explant forming shoots (100%) was obtained in treatment containing 5 mgL^{-1} BAP which showed significant difference with the control, 7 mgL^{-1} BAP, 1 mgL^{-1} NAA and 3 mgL^{-1} BAP+1 mgL^{-1} NAA.

BAP at 5 mgL^{-1} significantly enhanced the mean number of shoots produced per explant (3.76) and was significantly different compared to the rest of the treatments (Figure 2B). The additions of 0.5 and 1 mgL^{-1} NAA in the BAP containing media decreased the mean

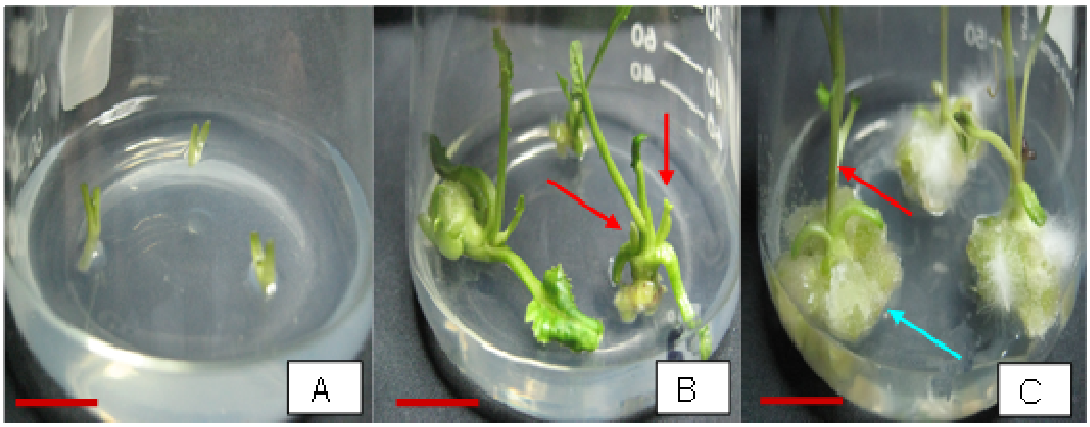

Plate 2. Multiple shoot formation from shoot tip explants of broccoli. (A) Shoot tips on 5 mgL^{-1} BAP after one week of culture. (B) Shoot emergence on 5 mgL^{-1} BAP after four weeks of culture. (C)Shoot and callus formation on 5 mgL^{-1} BAP in combination with 0.5 mgL^{-1} NAA after four weeks of culture. Bar = 5 mm. Red arrow = shoot and blue arrow = callus.

number of shoot produced. According to Widiyanto and Erytrina (2001) highest shoot formation in broccoli was found in cultures containing 13 µM (3 mgL^{-1}) BA. Hartmann et al. (2007) stated that cytokinin at relatively high concentrations promoted bud formation. Specifically, high levels of endogenous cytokinin have an important role in the initiation of proliferation centers in the explant and in turn can promote subsequent bud primordia formation (Valdes et al., 2001). The wide range of BAP concentrations without NAA influenced axillary shoot proliferation on shoot tips of broccoli (Figure 2A.). Callus formation was also observed on media containing combinations of BAP and NAA (Plate 2C).

Effect of NAA, IBA and IAA on rooting of broccoli shoots

Shoots, 2 cm in height and with three or more leaves, were transferred to MS medium containing different concentrations of NAA, IBA and IAA. By the fourth week of culture, it was observed that treatment containing 0.2 mgL^{-1} IBA and the control (MSO) gave the highest percentage of explant producing roots and mean number of roots produced per explants (Table 1). Both treatments showed significant difference with respect to the percentage of explant forming roots and mean number of roots produced per explant compared to treatments containing 0.2, 0.5 and 1 mgL^{-1} NAA, 1 mgL^{-1} IBA and 1 mgL^{-1} IAA. Maximum root length (2.46 cm) was attained on MSO medium. However, there was no significant difference between treatment MSO, 0.2 and 0.5 mgL^{-1} IBA on root length attained. The presence of NAA, IAA and IBA at 0.2, 0.5 and 1 mgL^{-1} induced root formation on shoots of broccoli. Shoots placed on medium containing 0.5 and 1 mgL^{-1} NAA produced fewer roots than that on 0.5 and 1 mgL^{-1} IAA or IBA. The highest concentration of NAA (1 mgL^{-1}) tested also caused callus formation at the base of shoots and produced stumpy and thick roots. Root formation occurred within 10-20 days after transferring shoots to the rooting treatments. Many investigators examined the use of auxins, such as NAA, IAA, or IBA on root induction. In some cases, high concentrations of NAA were more effective for root formation than high concentrations of IAA or IBA (Lazzeri and Dunwell, 1986). Nevertheless, the influence of NAA, IAA or IBA on root induction was highly dependent on genotype (Arnison et al., 1990; Vandemoortele et al., 1999). Rooted plantlets were successfully acclimatized in potting medium containing peatmoss, perlite, and vermiculite (3:1:1) and grew naturally in the greenhouse. The survival rate of regenerated plants was 90-95%. No obvious variation in appearance was observed among the regenerated plants.

Conclusion

The results indicated that hypocotyl and shoot-tips are potential explants for *in vitro* shoot multiplication of broccoli cv. Green Marvel. BAP alone was more effective on inducing shoot proliferation from the broccoli hypocotyl and shoot-tip explants. BAP at 3 and 5 mgL^{-1} were selected as the most suitable concentrations for the shoot initiation and multiplication from both explants. For inducing root formation, medium supplemented with 0.2 mgL^{-1} IBA and medium devoid of growth regulators (MSO) were the most suitable. Regenerated plants survived and grew normally in the greenhouse. The procedure, developed in this study, is recommended for rapid and efficient *in vitro* shoot regeneration of broccoli cv. Green Marvel.

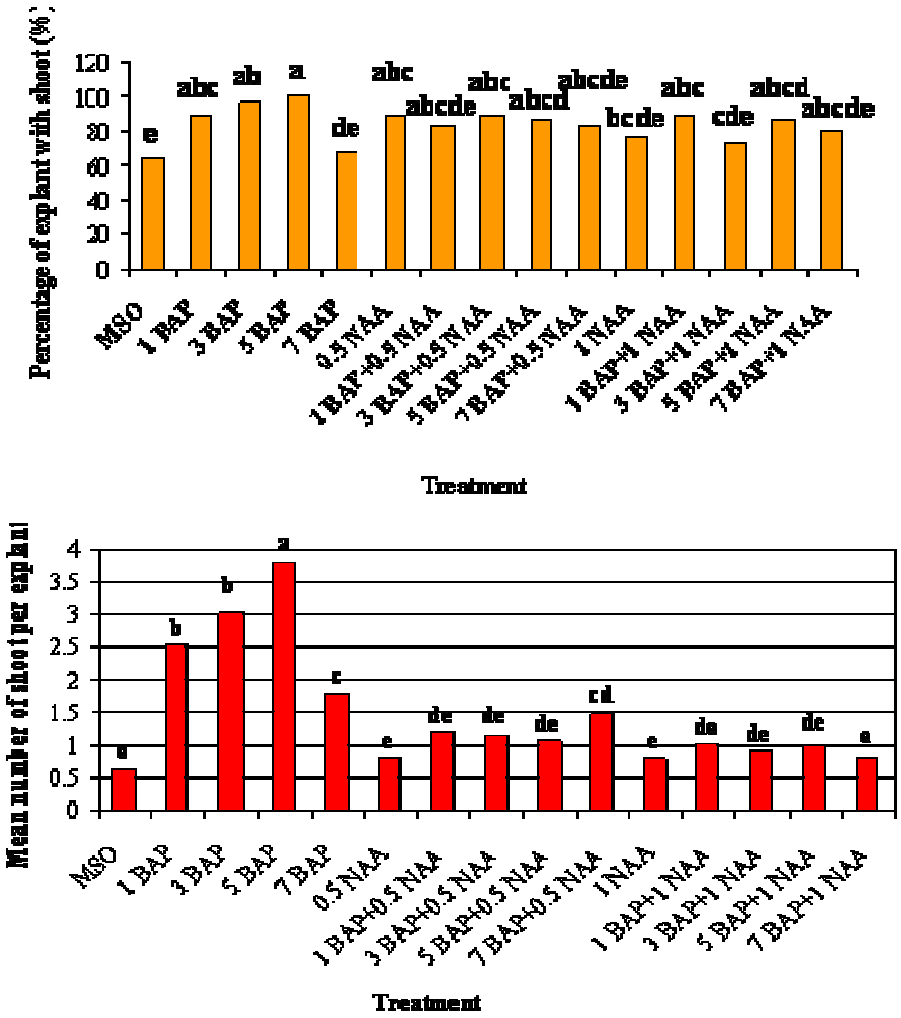

Figure 2. Effect of different concentration of BAP and NAA on (A) percentage of shoot tip explant producing shoot and (B) mean number of shoot produced per shoot tip explant after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

Table 1. Effect of auxin types on percentage of explant with root (%), mean number of roots produced per explant and root length attained (cm) of broccoli shoots after four weeks of culture.

Auxin types and conc. (mgL^{-1}) in MS medium	Percentage of explant with root (%)	Mean number of root produced per explant	Root length attained (cm)
MSO	96.66 ab	5.93 a	2.46 a
0.2 NAA	70 cde	2.16 bcd	0.75 e
0.5 NAA	53.33 e	1.23 cd	0.6 e
1 NAA	26.66 f	0.33 d	0.63 e
0.2 IBA	100 a	6.5 a	1.93 abc
0.5 IBA	76.66 bcd	4.53 ab	2.1 ab
1 IBA	63.33 ed	2.4 bcd	1.06 de
0.2 IAA	83.33 abcd	4.53 ab	1.3 cde
0.5 IAA	90 abc	4.43 ab	1.46 bcd
1 IAA	53.33 e	2.9 bc	1.06 de

Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

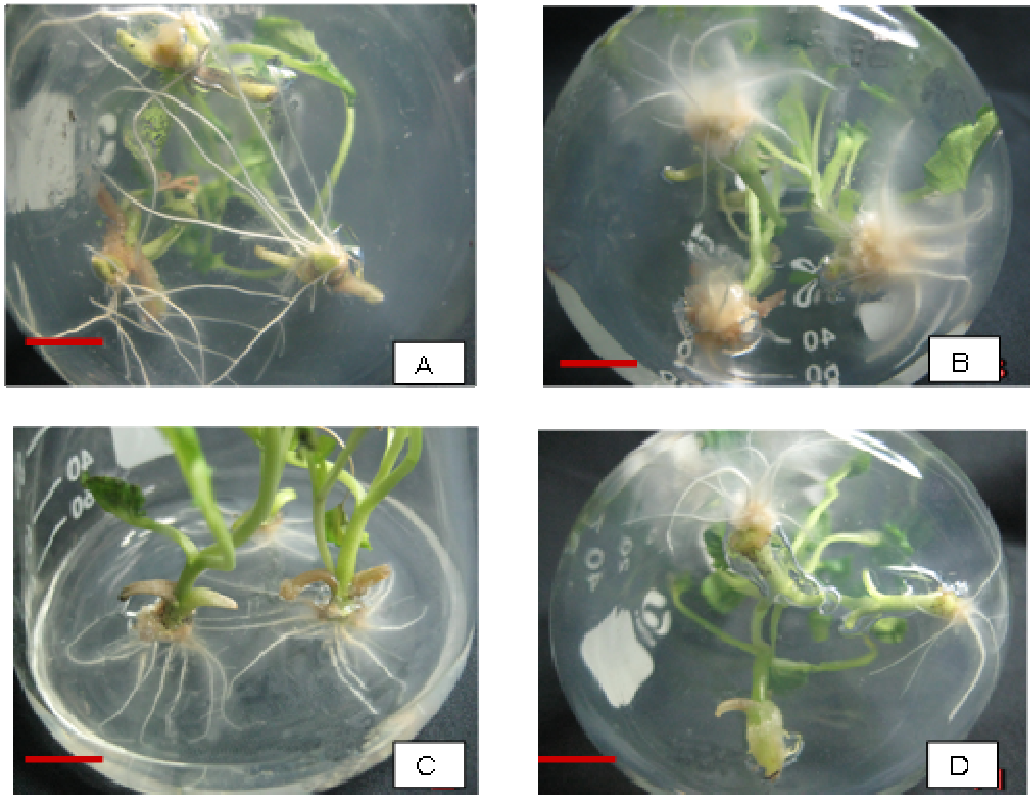

Plate 3. Root formation of broccoli shoots placed on different concentration of NAA, IBA and IAA. (A) 0 control (B) 0.2 mgL^{-1} NAA (C) 0.2 mgL^{-1} IBA (D) 0.2 mgL^{-1} IAA after four weeks of culture. Bar = 10 mm.

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