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Full Length Research Paper

In vitro adventitious shoot regeneration and acclimatisation of *Brassica oleracea* subsp. *italica* cv. Green Marvel

Seyed Ali Ravanfar[1], Maheran Abdul Aziz[1,2]*, Mihdzar Abdul Kadir[1], Azmi Abdul Rashid[1] and Fatemeh Haddadi[1]

[1]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia.
[2]Laboratory of Plantation Crop. Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia.

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Cotyledonary explants of *Brassica oleracea* subsp. *italica* (broccoli) cv. Green Marvel were cultured on Murashige and Skoog (MS) medium containing different combinations of the growth regulators 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) for shoot regeneration. The optimal medium for inducing shoots contained 3 mgl^{-1} BAP and 1 mgl^{-1} NAA, which produced a shoot induction percentage of 53.33% and a mean number of 0.43 shoot per explant. The shoots were subsequently rooted in MS medium that contained 0.2 mgl^{-1} of indol-3-butyric acid (IBA). Different potting media were assessed during plantlet acclimatization. The highest percentage of plant survival (83.33%) was on the medium that contained sand and soil (1:1), while maximum root length (4.37 cm) and plant height (7.87 cm) were attained in potting medium that consisted peat moss, perlite and vermiculite (3:1:1).

Key words: *Brassica oleracea*, broccoli, 6-benzylaminopurine, α-naphthalene acetic acid, indole-3- butyric acid.

INTRODUCTION

Brassica oleracea includes several important vegetable crops such as broccoli, cauliflower, kale and cabbage (Cardoza and Stewart, 2004). Broccoli (*B. oleracea* subsp. *italica)* is an important *Brassica* species that deserves greater breeding attention by seed companies with its increasing area of production in recent years and the anti-carcinogenic properties detected in some cultivars (Kirsh et al., 2007). *In vitro* plant regeneration system is essential for developing genetic transformation technology (Christey and Earle, 1991; Rafat et al., 2010). An efficient *in vitro* regeneration system in broccoli will facilitate the recovery of transgenic plants with improved characteristics such as abiotic stress tolerance and disease resistance. In broccoli, several types of explants have been used to regenerate plants, including the peduncle (Christey and Earle, 1991), hypocotyl (Puddephat et al., 2001), leaf tissue (Cao and Earle, 2003) and cotyledon (Qin et al., 2006). In most *Brassica* species, the success of *in vitro* regeneration is mostly dependent on the genotype and the influence of plant growth regulators (Ravanfar et al., 2009). The addition of cytokinins and auxins such as BAP and NAA respectively would enhance shoot multiplication in many species (Pierik, 1999; Razdan, 2005; Thorpe, 2007; George et al., 2008). This study reports the development of an efficient *in vitro* regeneration protocol and plantlet acclimatization of broccoli cv. Green Marvel.

MATERIALS AND METHODS

Plant material and sterilization protocol

Commercial seeds of broccoli, *B. oleracea* subsp. *italica* cv. Green Marvel were surface-sterilized for 2 min in 70% ethanol followed by continuous agitation for 15 min in 20% Clorox solution with two drops of Tween 20. The seeds were rinsed three to four times with

*Corresponding author. E-mail: maheran@agri.upm.edu.my or ramtin_ravanfar@yahoo.com. Tel: 03-89464115. Fax: 0389464146.

sterile distilled water and finally, germinated on a hormone-free half-strength MS medium(Murashige and Skoog, 1962) supplemented with 2.5 gl^{-1} phytagel and 30 gl^{-1} sucrose.

Medium composition and treatment

Cotyledonary explants, 5 to 8 mm in size, were excised from 7 day-old seedlings and cultured on MS medium incorporated with different concentrations of BAP (0, 1, 3, 5 and 7 mgl^{-1}) in combination with NAA (0, 0.5 and 1 mgl^{-1}) for shoot induction and multiplication. Subculture was performed at 4-week interval. MS medium supplemented with IBA at 0.2 mgl^{-1} was used for inducing root formation. The media were supplemented with 30 gl-1 sucrose and 2.5 gl-1 phytagel and the pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. MS medium without any plant growth regulator (MSO) was considered as a control. Finally, rooted shoots were transferred to different potting media [sand: soil (1:1), sand: perlite (1:1), peat moss: perlite (2:1), peat moss: vermiculite (2:1), peat moss: perlite: soil (2:1:1) and peat moss: perlite: vermiculite (3:1:1)] to determine the most suitable potting medium for the acclimatization of the broccoli plantlets.

Parameters recorded

The parameters recorded for shoot induction and multiplication were the percentage of explant producing shoots (%), and mean number of shoots produced per explant. Data were collected every three weeks until the eighth week of culture. The parameters recorded for the acclimatization experiment were the percentage of plant survival (%), root length (cm) and plant height attained (cm) after four weeks of acclimatization.

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). Data on the percentage of shoot formation from the cotyledonary explants were transformed to log (Y + 1). Duncan new multiple range test (DNMRT) at $\alpha = 5\%$ was used for comparison between the treatment means.

RESULTS

Multiple shoot induction from cotyledonary explants

The culture of cotyledonary segments on MS medium that contained only BAP at 0, 1, 3, 5 and 7 mgl^{-1} did not show any response in terms of shoot formation after eight weeks of culture (Figure 1). The explants began to expand after one week and were still green after eight weeks, with no shoot emergence and development (Figure 2a). However, the addition of NAA at 0.5 and 1 mgl^{-1} in the media that contained the different concentrations of BAP resulted in shoot formation (Figure 1; Figure 2b and c). The highest percentage of shoot formation (53.3%) was obtained on MS medium supplemented with 3 mgl^{-1} BAP and 1 mgl^{-1} NAA and showed significant difference when compared with all the other treatments after eight weeks of culture. Meanwhile, BAP at 3 and 5 mgl^{-1} combined with 0.5 mgl^{-1} NAA produced 37 and 34% of shoot formation, respectively. However, both treatments did not differ significantly from each other but differed significantly from the rest of the treatments (Figure 1a).

The highest mean number of shoots (0.43) per cotyledonary explant was also produced on the medium that contained 3 mgl^{-1} BAP and 1 mgl^{-1} NAA after eight weeks of culture (Figure 1b), and showed significant difference when compared with the rest of the treatments. This was followed by the medium that contained 3 mgl^{-1} BAP and 0.5 mgl^{-1}NAA that produced 0.34 shoot per explant and which also differed significantly from the rest of the treatments (Figure 1b). All the media with either BAP or NAA alone as well as the control did not produce any shoot and in terms of shoot formation, no significant difference was observed between those treatments (Figure 1). Cotyledonary explants in the control treatment produced roots instead, as shown in Figure 2d.

Effect of potting media on *ex vitro* performance of regenerants

After rooting in MS medium that contained 0.2 mgl^{-1} IBA, plantlets 3 to 5 cm in height and with well-developed roots were removed from the culture flasks and washed gently under running tap water. They were immersed in 5% Benlate (1 min) and then, transferred to pots (5 cm in diameter) containing six different types of potting media. The media was previously sterilized by autoclaving at 121°C for 20 min.

Prior to the transfer of plantlets from the *in-vitro* to *ex-vitro* condition, their leaf color was light green, in the first week after the transfer, the leaves became dark green. After four weeks of acclimatization, the highest percentage of plant survival (83.3%) was obtained in the potting medium that contained sand + soil (1:1) (Table 1). The percentage of plant survival in this medium differed significantly compared with the other media, except for that of the medium with peat moss+ perlite + vermiculite (3: 1: 1). However, the mean root length and plant height were comparatively low in the sand + soil (1: 1) potting medium at 1.5 and 3.16 cm, respectively. The lowest percentage of plant survival (13.3%) was obtained in the potting medium that contained sand + perlite (1: 1) and differed significantly from the rest of the treatments. A significantly high root length (4.37 cm) compared with that attained in other potting media was recorded on plants grown in peat moss + perlite + vermiculite (3: 1: 1) after four weeks of acclimatization. The potting media consisting of peat moss + perlite + soil (2: 1: 1) and peat moss + vermiculite (2: 1) produced root lengths that did not differ significantly from each other, but they differed significantly from that attained in the potting media that contained sand + soil (1: 1), sand + perlite (1:1) and peat moss + perlite + vermiculite (3: 1: 1). A maximum height of 7.87 cm was also obtained on plants grown in potting

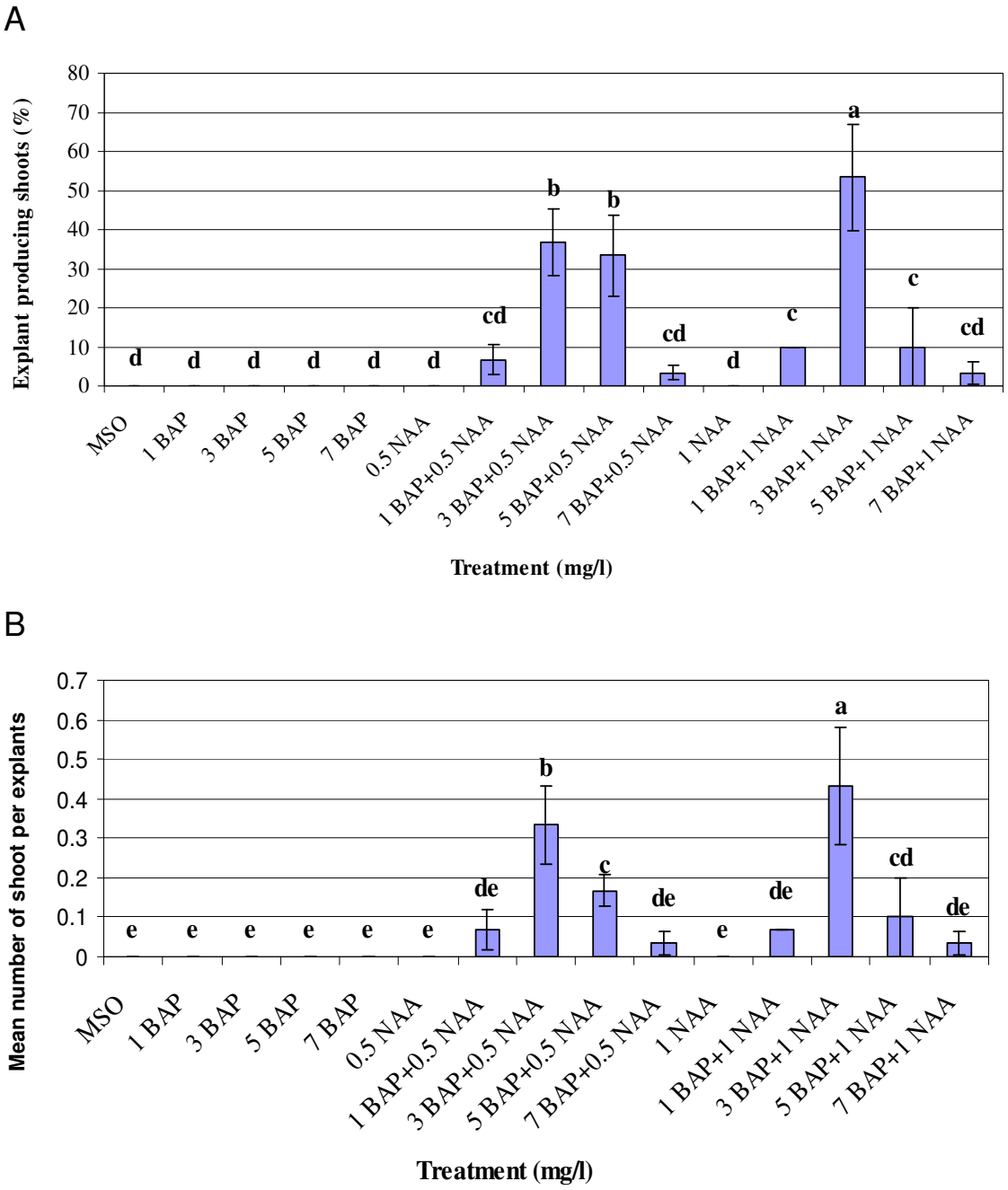

Figure 1. Effect of BAP in combination with NAA on shoot regeneration from cotyledonary explant of broccoli cv. Green Marvel (A) percentage of shoot formation and (B) mean number of shoots produced per explant after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test. Bar indicates the standard deviation of mean.

medium that consisted peat moss+ perlite + vermiculite (3: 1: 1) which differed significantly from the heights attained in other treatments. Nevertheless, no significant difference in height was observed between the plants grown in the medium with peat moss + perlite + soil (2: 1: 1) and that of peat moss + vermiculite (2: 1). However, both treatments showed significant difference when compared with the rest of the treatments.

DISCUSSION

Murashige and Skoog (MS) medium in the presence of different combinations of BAP and NAA significantly promoted shoot formation. In media with different concentrations of BAP alone, the cotyledon explant only swelled and expanded (Figure 2a). Meanwhile, the media with low concentration of 0.5 mgl^{-1} NAA affected callus

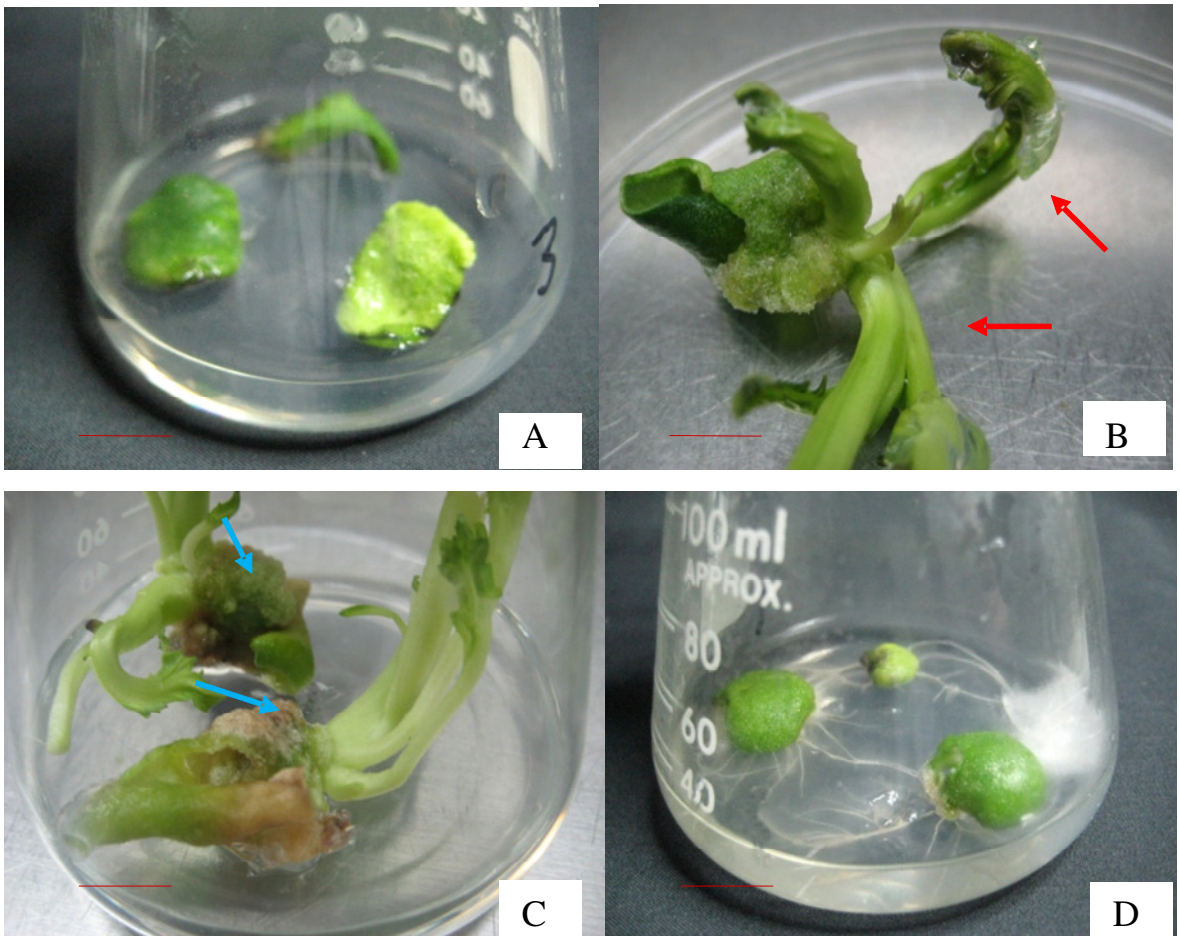

Figure 2. Multiple shoot formation from cotyledonary explant of broccoli cv. Green Marvel. (A), Expanded cotyledon segment with no shoot growth on the medium with 5 mgL^{-1} BAP after eight weeks of culture; (B) and (C) shoot regeneration and callus formation in the medium that contained BAP and NAA, respectively, after eight weeks of culture; (D), root formation on hormone free medium. Red arrow = shoot and blue arrow = callus. (A), (C) and (D) bars = 4 mm and (B) bar = 2 mm.

Table 1. Effects of different potting media on plant performance after four weeks of acclimatization.

Potting medium	Percentage of plant survival (%)	Root length (cm)	Plant height (cm)
Sand + soil (1:1)	83.33^{a}	1.50^{d}	3.16^{cd}
Sand + perlite (1:1)	13.33^{d}	0.90^{d}	2.33^{d}
peat moss + perlite (2:1)	50.00^{c}	2.00^{cd}	3.66^{c}
peat moss + vermiculite (2:1)	56.66^{bc}	2.66^{bc}	6.66^{b}
peat moss + perlite + soil (2:1:1)	63.33^{b}	3.16^{bc}	6.87^{b}
peat moss + perlite + vermiculite (3:1:1)	80.00^{a}	4.37^{a}	7.87^{a}

Means followed by the same letter (s) in the same column were not significantly different using DNMRT (p = 0.05).

formation. Adventitious shoot proliferation followed by callus formation occurred within four weeks of culture on the media that contained 3 mgl^{-1} BAP combined with 1 mgl^{-1} NAA and 3 mgl^{-1} BAP incorporated with 0.5 mgl^{-1} NAA (Figure 2b and c). The cotyledonary explant placed on the medium without any plant growth regulator only expanded and produced roots after eight weeks of culture (Figure 2d). Subculture was performed after four weeks of culture to produce the maximum number of shoots per explant. Nevertheless, phenotypic changes were ob served

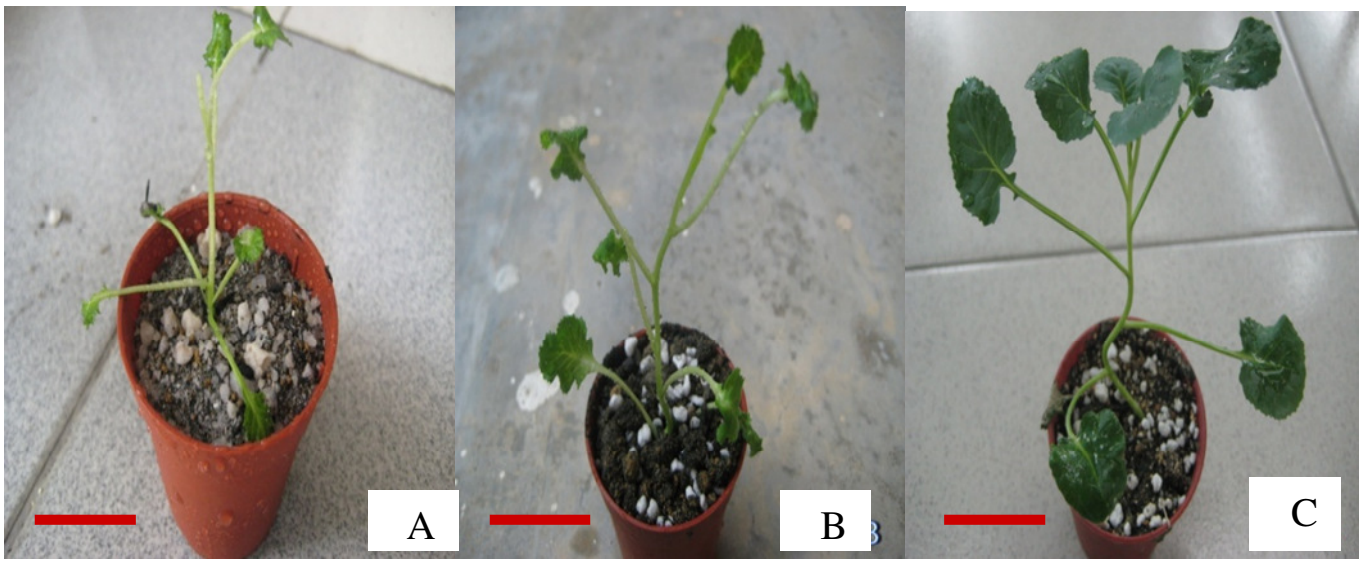

Figure 3. Plant survival in potting media consisting of (A) sand + soil (1: 1); (B) peat moss + perlite (2: 1) after four weeks of acclimatization; (C) plantlet survival in medium containing of peat moss + perlite + vermiculite (3: 1: 1) after eight weeks of acclimatization. Bar = 20 mm.

in the control plants and the plants derived from the cotyledon cultures. In this study, as many as six shoots were produced from a single cotyledonary explant of broccoli cv. Green Marvel placed on medium with 3 mgl^{-1} BAP and 1 mgl^{-1} NAA in successive subcultures, which is in agreement with the observation of Guo et al. (2005). Meanwhile, no shoots were regenerated from the cotyledonary explants placed on the medium that contained BAP alone. On the other hand, other cultivar of broccoli had responded on the medium with 3 mgl^{-1} BAP alone producing 3.5 shoots per explant as reported by Qin et al. (2006), indicating that the response was cultivar specific. The medium that contained high concentration of BAP (5 and 7 mgl^{-1}) in combination with 1 mgl^{-1} NAA produced few shoots but with intense callus formation.

The rooted plants were successfully acclimatized in different potting media and grew naturally in the greenhouse. Referring to Table 1, maximum root length and plant height were obtained on plants grown in peat moss + perlite + vermiculite mixture (3: 1: 1). The high response may be due to the ability of the mixture to provide enough moisture and aeration to the plants, thereby resulting in good root growth. Vermiculite possesses cation exchange properties, thus, it can hold and made available ammonium, potassium, calcium and magnesium to the growing plants. Vermiculite, when combined with peat moss promotes faster root growth and provides quick anchorage to young roots (Hartmann et al., 2007). Perlite is also an important commodity in the potting mixture when mixed with peat moss. The addition of perlite to peat moss increases the amount of air (oxygen) held in the peat moss, as well as the amount of water retained by the peat moss. This obviously improves the growing conditions for plants (Donahue and Miller, 1990).

The highest percentage of plant survival (83.3%) was on the medium that consisted sand + soil (1: 1) but was not significantly different when compared with the medium with peat moss + perlite + vermiculite (80%) (Figure 3). Morphologically, there was no obvious variation in the appearance observed on all the regenerated plants. In some pots, the number of leaves produced per plant and plant height were low which could be due to the low nutrient content of the sand. Nevertheless, the mixture with peat moss + perlite + vermiculite (3: 1: 1) resulted in a reasonably high per-centage of plant survival and the highest root length and plant height of broccoli cv. Green Marvel. It is therefore, a recommended potting medium for the growth of the cultivar during acclimatization.

Conclusions

The results of this study showed that, BAP together with NAA played a role in inducing shoot multiplication from cotyledonary explants of broccoli cv. Green Marvel. The use of BAP as a cytokinin and NAA as an auxin in an appropriate ratio was most effective for the shoot induction. Following acclimatization, the maximum root length and plant height were attained using medium that contained peat moss + perlite + vermiculite (3: 1: 1). The percentage of plant survival though highest (83.3%) on the medium that contained sand + soil (1: 1), it was reasonably high in the medium with peat moss + perlite + vermiculite (80%). Therefore, the mixture that contained peat moss + perlite + vermiculite was selected and is recommended for growth of broccoli cv. Green Marvel

plants during acclimatisation.

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