

Regeneration of Broccoli (*Brassica oleracea* L. var. *bejo*) from Hybrid Mature Seed and Molecular Analysis of Regenerants

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Abstract. The objective of this research was to obtain broccoli plants through direct regeneration *in vitro*. Hypocotyls and shoot tips were used as explants *in vitro* plant regeneration of broccoli. 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 hypocotyls explants producing shoot (92.34%) on MS + 3 mgL⁻¹ BAP + 0.2 mgL⁻¹ NAA and the highest mean number of shoots produced per hypocotyls explants (5.76) were obtained on 3 mgL⁻¹ BAP. Meanwhile, the highest percentage of shoot tip explants produced shoot (100%) and highest number of shoot produced per shoot tip explants (3.56) were recorded on 3 mgL⁻¹ BAP. For rooting of shoots, NAA at 0-1 mgL⁻¹ were applied. RAPD analysis was used to identify the differences among regenerants from tissue cultures. The results of PCR products showed no difference between cultures and demonstrated that direct organogenesis is the most effective way to produce true-type regenerants in broccoli hybrid. A successful acclimatization to the free conditions was obtained by transferring of plantlets into pots contained a mixture of peatmoss and vermiculite (3:1) under high relative humidity conditions.

Key words: Broccoli, *in vitro* culture, hypocotyl, benzylaminopurine (BAP), naphthaleneacetic acid (NAA), RAPD.

1. Introduction

Broccoli (*Brassica oleracea* L. var. *bejo*) which originated from Holland and is related to the cabbage and cauliflower, is a very important vegetable crop. It is well known for its high vitamin, calcium and sulforaphane content.[1] *In vitro* regeneration offers a great opportunity for a rapid production of desirable and essentially genetically identical plants. [2] [3] An efficient *in vitro* regeneration system is also a crucial tool in genetic engineering of the crop for improved characteristics.[4] Broccoli, *Brassica oleracea* is one of the many valuable *Brassica* species, which is still less cultured under *in vitro* condition.[5] Some experimental results showed successful *in vitro* culture of *Brassica* species from hypocotyls segments, root segments, primary leaf discs, cotyledons and anther.[4] Among the *Brassica* species include kale, Brussels sprouts, cabbage and cauliflower.[6] 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.[7] 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.[5], [8]

This paper reports on the influence of BAP either singly or in combination with NAA, on adventitious shoot proliferation from hypocotyls and shoot-tips explants of broccoli cv. *bejo*, and the effect of α -naphthaleneacetic acid (NAA) on rooting of the *in vitro* regenerated shoots.

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2. Materials and Methods

2.1. 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 agar and 30 gL⁻¹ sucrose.

2.2. 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 (Murashige and Skoog, 1962) medium incorporated with different concentrations of plant growth regulators for shoot proliferation and root formation.[9] For shoot induction and multiplication from hypocotyls explants various concentrations of BAP (0, 1, 3, 5, 7 and 10 mg L⁻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. 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 agar and the pH adjusted to 5.8 prior to autoclaving at 121°C for 15 min. MS medium without any growth regulator (MS₀) was considered as a control.

2.3. Parameter Recorded

In the shoot induction and multiplication study from hypocotyls and shoot tip explants, parameters recorded were the percentage of explants 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 explants and the root length attained (cm). The data on rooting were collected after four weeks of culture.

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 $\alpha = 5\%$ for comparison between treatment means.

3. Results and Discussion

3.1. Multiple shoot formation from hypocotyls explants

In the first week of culture the hypocotyls explants began to expand and swelled in all BAP concentrations tested. Within three to four weeks of culture shoot appeared in the middle and distal end of the hypocotyls explants. By the eight week of culture, significant differences in the percentage of explants with shoot and mean number of shoot produced per explants were observed among the BAP treatments. The highest percentage of hypocotyls explants producing shoot (92.34%) on MS + 3 mgL⁻¹ BAP + 0.2 mgL⁻¹ NAA. BAP at 3 mgL⁻¹ induced the highest percentage of explants producing shoots (92.34%) followed by 5 mgL⁻¹ BAP (81.12%). There was no significant difference observed between the two treatments (3 and 5 mgL⁻¹ BAP) on percentage of explants 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 after eight weeks of culture. It is differed significantly compared to the rest of treatments (Fig. 1). BAP is most effective in enhancing shoot multiplication and triggering shoot elongation.[10] BAP also promotes differentiation of cell in to shoot initials followed by the formation of shoots. Above 5 mgL⁻¹ BAP the main number of shoots formed per explants decreased and become toxic to the shoot growth.

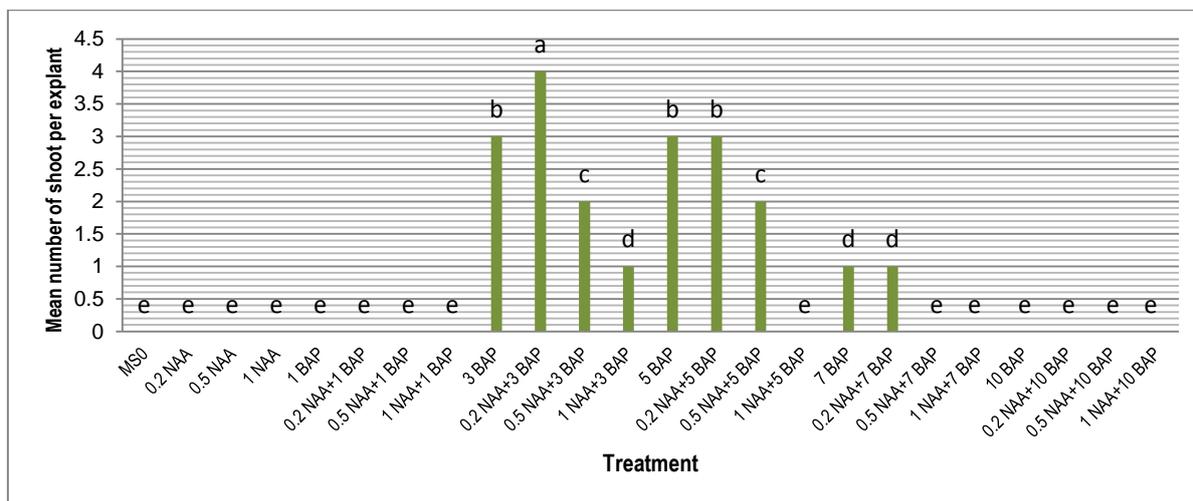


Fig. 1: Effect of different concentrations of BAP and NAA on mean number of shoot produced per hypocotyl explants after eight weeks of culture. Mean with the same letter were not significantly different at 0.05 probability level according to DNMR test.

3.2. Multiple shoot formation from shoot tip explants

Fig. 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. By the four week of culture multiple shoots were formed, showed significant differences with shoot cultured on the control medium. The highest percentage of explants forming shoots (100%) was obtained in treatment containing 3 mgL⁻¹ BAP which showed significantly difference compared to the control medium (MS medium without plant growth regulator, MS₀), 5 mgL⁻¹ BAP, 1 mgL⁻¹NAA and 7 mgL⁻¹ BAP + 1 mgL⁻¹ NAA

BAP at 3 mgL⁻¹ significantly enhanced the mean number of shoots produced per explants and was significantly different compared to the control medium treatments. The highest percentage of shoot tip explants produced shoot (100%) and highest number of shoot produced per shoot tip explants (3.56) were recorded on 3 mgL⁻¹ BAP (Fig. 2). The additions of 0.5 and 1 mgL⁻¹ NAA in the BAP containing media decreased the mean number of shoot produced. According to Widiyanto and Erytina (2001) highest shoot formation in broccoli was found in culture containing 13 μM (3 mgL⁻¹) BA. Cytokinin at relatively high concentrations promoted bud formation.[11] BAP alone was more effective on inducing shoot proliferation from the broccoli hypocotyl and shoot-tip explants.[12] The wide range of BAP concentrations without NAA influenced auxiliary shoot proliferation on shoot tips of broccoli. Callus formation was also observed on media containing combinations of BAP and NAA.

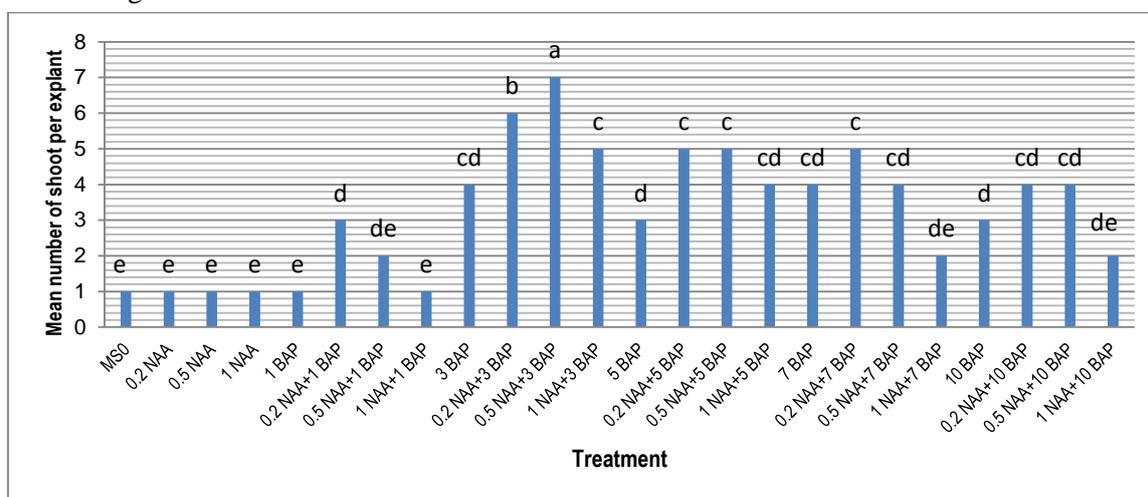


Fig. 2: Effect of different concentrations of BAP and NAA on mean number of shoot produced per shoot tip explants after eight weeks of culture. Mean with the same letter were not significantly different at 0.05 probability level

according to DNMRT test.

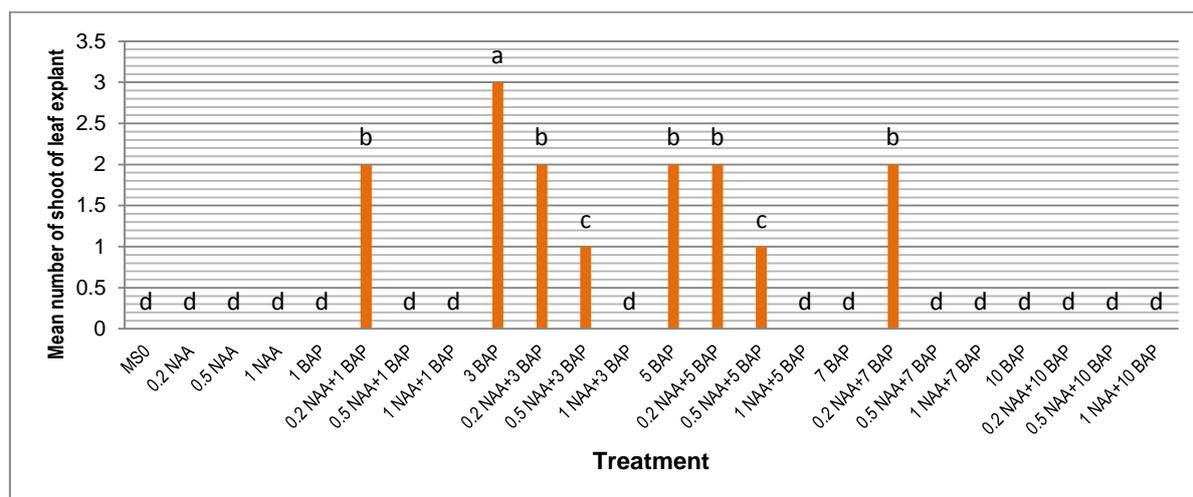


Fig. 3: Effect of different concentrations of BAP and NAA on mean number of shoot produced per leaf explants after eight weeks of culture. Mean with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

3.3. Multiple shoot formation from leaf explants

In the first week of culture the leaf explants began to expand and swelled in all BAP concentrations tested. Within three to four weeks of callus culture appeared in the edge of the leaf explants. It was observed that the adventitious shoot showed after the first week of culture. By the ten weeks of culture multiple shoots were formed, showed significant differences with shoot cultured on the control medium. The highest percentage of explants forming shoots (100%) was obtained in treatment containing 5 mgL⁻¹ BAP which showed significantly difference compared to the control medium, 5 mgL⁻¹ BAP, 1 mgL⁻¹NAA and 7 mgL⁻¹ BAP + 1 mgL⁻¹ NAA (Fig. 3)

3.4. Effect of NAA on rooting of broccoli shoots

Shoot, 2 cm in height and with three or more leaves, were transferred to MS medium containing different concentration of NAA. By the four week of culture, it was observed that treatment containing 0.2 mgL⁻¹ NAA and control (MS₀) gave the highest percentage of explant producing roots and mean number of roots produced per explants. According to Ke Huang et al (2011) the best regeneration recipe for broccoli hypocotyl explant is on medium MS + NAA 0.107 μM + BAP 17.76 μM + 2% sucrose + 0.8% agar. Rooted plantlets were successfully acclimatized in potting medium containing peatmoss and vermiculite (3:1) and grew naturally in the greenhouse. The survival rate of regenerated plants was 100%.

3.5. Molecular Analysis

Molecular analysis by using RAPD (Rapid Amplified Polymorphic DNA) technique showed that UBC153, ROTH 480.01, and ROTH 480.03 primer have polymorphic band that can be used for linkage analysis. All polymorphisms regenerants independently of each other. Specific RAPD marker of Roth 480.03-3₁₂₅ and UBC 153-7₁₂₅ primers for hybrid of *Brassica oleracea* var. Bejo regenerants.

RAPD analysis was used to identify the differences among regenerants from tissue cultures. The results of PCR products showed no difference between cultures and demonstrated that direct organogenesis is the most effective way to produce true-type regenerants in broccoli hybrid.

4. Conclusions

The results indicated that hypocotyls, shoot-tips, and leaf are potential explants for *in vitro* shoot multiplication of broccoli (*Brassica oleracea* var. *bejo*). The highest percentage of hypocotyls explants producing shoot (92.34%) on MS + 3 mgL⁻¹ BAP + 0.2 mgL⁻¹ NAA and the highest mean number of shoots produced per hypocotyls explants (5.76) were obtained on 3 mgL⁻¹ BAP. Meanwhile, the highest percentage

of shoot tip explants produced shoot (100%) and highest number of shoot produced per shoot tip explants (3.56) were recorded on 3 mgL⁻¹ BAP. For rooting of shoots, NAA at 0-1 mgL⁻¹ were applied. Highest percentage of shoots with roots (100%) and highest mean number roots produced per shoot (5.5) occurred on medium with 0.2 mgL⁻¹ NAA, while the maximum root length (2.39 cm) was attained on MS medium without plant growth regulator (MS₀). BAP alone was more effective on inducing shoot proliferation from the broccoli hypocotyls, shoot-tip and leaf explants. The best regeneration recipe for the broccoli is on medium MS + BAP 3 mgL⁻¹. NAA 0.2 mgL⁻¹ is selected as the most suitable concentrations for the root initiation. Regenerated plants survived and grew normally in the green house. Based on this menu, the regeneration frequency of broccoli reached 100%.

5. Acknowledgements

This work was supported by Plant Biotechnology Research Project of Indonesian Government. I would like to thank PT Agrodata Bandung Indonesia who have given support broccoli seeds.

6. References

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