Effect of the combination of NAA and BA on callus induction from hypocotyl explants in black cumin (Nigella sativa L.)

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Abstract. Black cumin (Nigella sativa L.) seeds contain the main bioactive compound thymoquinone, which is beneficial for health. Callus cell culture techniques are an alternative method for the production of plant bioactive compounds. Therefore, it was necessary to develop a method for producing callus in vitro. This study evaluated the effect of a combination of NAA and BA growth regulators on MS medium on in vitro callus induction using black cumin hypocotyl explants.

There were two variables used in the nature of the study: NAA (1 and 2 mg/L) and BA (1, 1.5, 2, 5 and 3 mg/L). All hypocotyl explants were able to induce callus on all medium used. The callus appeared between 8-11 days. A combination of NAA and BA influenced black cumin hypocotyl callus formation and development. With NAA 1 mg/L added and BA 1.5 mg/L added, MS medium was able to induce best hypocotyl explants to shape and develop callus with an average callus weight of 0.262 g/Explant. However, the time of callus emergence was not significantly affected by the combination of NAA and BA growth regulators.

1. Introduction

Nigella sativa L. plant commonly known as black seed or black cumin (English), Habbatul Barakah (Arabic) and Tikur azmud (Amharic) is an annual herbaceous plants native from the Mediterranean region and have been widely cultivated in other countries such as India, Pakistan, South Europe, Syria, Turkey and Saudi Arabia [1–3]. Black cumin seeds have primary benefit are those from essential oils with a content of 0.5-1.6% consisting mainly of monoterpenes; p-cymene, γ-terpinene, α-pinene, β-pinene, α-thujene, carvacrol, thymol and thymoquinone [4]. Thymoquinone functions as an antimicrobial, antiparasitic, anticancer, anti-inflammatory, immunomodulatory, antioxidant and hepatoprotective agent [5]. Also, thymoquinone is useful for preventing intestinal cancer and leukemia, antimicrobial and preventing damage to erythrocytes [6–8].

Domestic demand for black cumin seeds continues to increase, but it is not fulfilled from our country. Black cumin seeds are therefore imported from India, Egypt and the other countries of the Middle East. Based on the many benefits and high demand of black cumin seeds. Tissue culture techniques can be used as a means of producing secondary plant metabolites. The use of callus culture to increase the production of secondary metabolites, especially medicinal compounds, is considered to be more profitable than the production of whole plants because in callus culture a regular supply of nutrients can be guaranteed and it is also possible to regulate the process of metabolism so that
maximum results can be obtained [9]. Callus quality as a producer of secondary metabolites has the characteristics of color and texture following the desired secondary metabolite [10].

Callus formation can be induced by regulating the administration of growth regulators with the right type and concentration in the medium. The use of auxin and cytokinin will increase the callus induction process [11]. Callus induction can also be influenced by different ratio of auxin (NAA) and cytokinin (BA), so that the right combination is needed to induce optimal callus formation as plant bioactive material [12] The use of appropriate plant growth regulator (NAA and BA) concentrations is a major factor in the success of callus culture has never been done before. Therefore, This study aims to evaluate the effect of the combination of NAA and BA growth regulators on MS medium on callus induction from black cumin hypocotyl explants in vitro.

2. Material and Methods

2.1. Plant material
Black cumin (Nigella sativa L.) seeds collected from UPT Materia Medica Batu, East Java, Indonesia. N. sativa seeds are selected which are black, intact triangular and not moldy with sizes ranging between 0.3-0.5 mm.

2.2. Seed sterilization and germination
Seed washed for 1-2 hours with running water then the seeds were surface sterilized with alcohol 70% for one minute, and then 6% sodium hypochlorite (NaClO) for 5 minutes and rinsed three times with sterile distilled water each for 5 minutes. Sterile seed was grown on ½ MS medium without the addition of regulators for plant growth for germination. This method was modified from previous studies [13]. Seed cultured incubated at 24-25 oC in dark condition. 14-20 days old seedlings were transferred in light conditions to obtain seedling that are not etiolated.

2.3. Callus induction
Callus was induced from 14-day-old seedlings hypocotyl tissue. Hypocotyles have been decoupled to a segment of 0.5-1 cm and cultivated with NAA (1 and 2 mg/L) in combination with BA (1, 1.5, 2, 5 and 3 mg/L) in MS medium. The culture was incubated at temperature (25±1) oC with constant light conditions 1000 Lux for four weeks. Each treatment factor consisted of five replicates and each treatment was repeated five times.

The experimental design was completely randomised, in a 2 x 5 factorial scheme (two doses of NAA x five doses of BA). The effect of each plant growth regulator combination was calculated by observing explant survival percentage, callus formation time, percentage of callus-producing hypocotyls, and fresh callus weight. ANOVA analyzed quantitative data and compared the average with Duncan Multiple Range Test (DMRT) at a meaning level of 5 percent (P <0.05).

3. Results and Discussion
Black cumin seeds germination in this study using ½ MS medium without the addition of growth regulators and incubated for two weeks in dark conditions to accelerate the process of imbibition in the seeds and subsequently the sprouts have grown for 2 weeks in dark conditions moved to light conditions for 1 day (Figure 1). Hypocotyl explants were cultured on medium with various combinations of NAA and BA capable of inducing callus formation, with a percentage of 100%. Callus starts to form between 7-11 culture days. The first callus formed at the tip of the hypocotyl explant section is shown in Figure 2, callus begins to form in the first week of observation and callus growth can be observed in the second week to the fourth week cultured.
Observations after 4 weeks of incubation showed the black cumin callus in this study showed a yellowish green and brownish green color, a yellowish green color formed in the callus with the addition of NAA 1 mg/L and callus brownish green in addition to NAA 2 mg/L (Figure 3). The texture of all combination treatments in this study showed that the same callus texture was friable watery. Formation of fragile textured callus is influenced by the presence of endogenous auxin hormone in the already formed callus. The best callus requires a balanced combination of auxin and cytokinin [14].

Callus with friable texture was shown in previous studies using shoot tip dates explants with the addition of NAA and BA to the medium, a combination of NAA 25 mg/L and BA 3 mg/L can induce a creamish white colored callus whereas by giving a higher NAA concentration a combination of NAA 50 mg/L and BA 3 mg/L can induce a brown callus [15]. The results showed that the plant growth regulator combination of NAA and BA were not significantly in the formation of callus from hypocotyl explants. The formation of black cumin callus began to be seen in the second week after culture with an average day of callus ranging from 8-11 days after culture (Figure 4). But the fastest black cumin induction hypocotyl induction is obtained by adding NAA 1 mg/L 8.3 days after culture and adding NAA 2 mg/L 9.1 days after culture. Both combined with cytokinins (BA) 1.5 mg/L can induce callus growth time quickly. Survival percentage of explants and percentage of hypocotyls producing callus in this study obtained all treatments 100% at four weeks culture.

The best callus requires a balanced combination of auxin and cytokinin[14] A friable textured callus has been shown in previous studies using shoot tip dates explants with the addition of NAA and BA to the medium, a combination of NAA 25 mg/L and BA 3 mg/L can induce a creamy white callus, while a higher NAA concentration can induce a combination of NAA 50 mg/L and BA 3 mg/L [15].

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Using a combination of growth regulators in the form of NAA and BA can induce 100% callus growth explants shown in previous studies using bean explants with a combination of growth regulators in the form of 1 mg/L BA+1.5 mg/L NAA and 1 mg/L BA+2 mg/L NAA can induce susceptible callus growth explants 7-9 days after culture. The interaction of NAA and BA may also influence the fresh weight of black cumin hypocotyl callus (Figure 5). The best callus was shown in the treatment of NAA 1 mg/L and BA 1.5 mg/L with callus weight 0.262 g/explants while the lowest callus was in the treatment of NAA 2 mg/L and BA 1.5 mg/L with callus weight 0.124 g/explant. The findings of the combining of Kinetin and NAA with *Nigella sativa* callus induction levels in various explants (leaves, hypocotyls, epicotyls and roots) relate to previous studies [16].

The study revealed MS media which were given growth regulators NAA 1 mg/L and Kinetin 2 mg/L can induce callus well, epicotyl explants can provide the best response to the time of rapid callus growth with callus whitish-green and fragile texture, whereas the leaf explants obtained green callus and compact textured. Previous research has been performed using black cumin callus explants from roots, stems and leaves with different concentrations of 2,4-D and Kinetin, which have been obtained 100% by increasing callous in leaf and stem explants, by adding 1 mg/l 2,4-D and 1.5 mg/L Kinetin [1]. We have used the highest callus weight and quick-callus days in explants of hypocotyl black cumin at the same concentration but with varying forms of auxin and cytokininine BA 1.5 mg/L and the same auxin concentration NAA 1 mg/L.
Figure 4. Impact of different NAA and BA combinations on the hypocotyl formation period of the *Nigella sativa* after 30 days of culture.

The best combination of BA with NAA 1 mg/L the fresh calf weight is NAA 1 mg/L + BA 1.5mg/L. The best fresh callus weight is NAA 2mg/L + BA 3mg/L for the combination of NAA 2 mg/L. There is a balance between the concentration of auxin and cytokinin for callus formation in this case the higher BA concentration is slightly compared to NAA, according to the theory of the ratio of auxin to balanced cytokinin spur callus formation. The interaction between auxin and cytokinin has been reported to play significant roles in inducing cellular differentiation and organogenesis in tissue and organ cultures. The fresh weight of the callus was influenced by water absorption and other components in the basal medium, which contributed to cell expansion, and cell division and new material synthesis were instrumental in increasing the dry weight of calluses. [17].

Optimization of callus induction of the *Nigella sativa* L. plant is expected to enable the development of protocols in producing secondary metabolites for commercially important black cumin species. The type and concentration of growth regulators such as auxin and cytokinin are known as important components in callus induction in producing secondary metabolites [18]. The results of previous studies showed that epicotyl explants and leaves of black cumin plants had excellent callus growth potential by using a combination of NAA and BA [19]. However, this study could induce 100 percent callus growth with different concentrations of NAA and BA in all treatments. This difference is caused by the response of different plant tissues differently to various growth promoters, nutrient media, hormonal balance, etc [20].

In the present study, Young purple–red leaves of mangosteen (*Garcinia mangostana* L.) used as explant can induced friable callus was highly on MS media with BAP + 2,4-D and BAP + picloram. However, the callus induced on medium with BAP + picloram was watery [21]. There are three factors were tested: combinations of BAP and 2,4-D, carbon source and inoculum size. Combinations of different concentration of plant growth regulator play important roles in cell division and enlargement [22].
Figure 5. Effect of various combinations of NAA and BA on callus induction (fresh weight) after 30 days of cultivation from *Nigella sativa* in vitro cultivated hypocotyls.

4. Conclusion

Black cumin hypocotyl explant will develop a 100 percent callus on MS media with a combination of NAA and BA in four weeks of culture. Callus formation from hypocotyl explants was influenced by a combination of NAA and BA on the media. The best callus formation and the fastest callus formation were obtained at a combination of NAA 1 mg/L and BA 1.5 mg/L on MS media. This protocol will promote the application of cell culture technology to facilitate production of bioactive compound.

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