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Original Article

Callus Induction, Shoot and Root Regeneration in *Hyssopus officinalis* Using Sodium Nitroprusside and Plant Growth Regulators

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ABSTRACT

The current study aims to assess the callus induction and regeneration of shoots and roots in *Hyssopus officinalis* L. using sodium nitroprusside (SNP) and plant growth regulators (PGRs). A significant impact was found in the callogenesis of leaf and petiole explants with different concentrations of SNP and 2,4-dichlorophenoxyacetic acid (2,4-D). SNP concentrations of 20 µm induced callogenesis to 72%, but when combined with 2,4-D concentrations, callogenesis increased to 100%. In the absence of SNP, no calluses were developed. A significant effect of 2,4-D and SNP was observed on callus fresh weight. Callus fresh weight from leaf explants was 0.165 g, while from petiole explants was 0.13 g. Furthermore, SNP enhanced shoot regeneration of leaf explants compared to petiole explants. When 20 µm of SNP was combined with 3 mg/L 6-benzylaminopurine (BAP), the maximum mean shoot number was obtained (84% in leaves and 54% in petioles). A combination of SNP and IBA enhanced rooting rate to 83% and root length to 8.3 cm. After acclimation, over 80% of the produced plants survived in greenhouse conditions. Several implications can be drawn from the study's findings regarding medicinal hyssop cultivation, the preservation of genetic resources, and pharmaceutical research on medicinal plants.

INTRODUCTION

In recent years, researchers have been working on formulations of plant growth regulators to enhance induced callus formation using SNP supplementation. As reported by Leterrier et al sodium nitroprusside (SNP) has been identified as a phytohormone and an important source of nitrous oxide (NO) [1]. Several plant species exhibited increased regeneration and callogenesis when SNP supplementation was applied [2, 3]. According to Kalra and Babbar NO is a gaseous, highly diffusible, and bioactive free radical [4]. Generally, NO donors are used exogenously instead of gas due to the technical difficulties with direct exposure to NO [4]. The function of NO in plant development and growth is well-documented [2,5]. Callus induction and regeneration are two critical steps in in vitro propagation [6]. The role of NO in germination and growth, decrease of seed dormancy, development of lateral roots, and senescence are demonstrated by studies [7,8]. SNP supplementation can enhance in vitro responses in a variety of plant species, including cherry rootstocks [9], *Ricinus communis* L. [10], *Hyoscyamus niger* L. [11], *Canscora diffusa* (Vahl) R. Br. [12], *Tagetes erecta* [13], Chrysanthemum (Dendranthema × grandiflorum) [14], *Citrus aurantifolia* [15] and *Haplophyllum virgatum* [16].

2,4-D is an essential growth regulator for embryogenic cells and in vitro plant culture systems [17]. A high concentration of 2,4-D, however, can inhibit development of embryo and affect SE [18]. It has been demonstrated that 2,4-D may influence endogenous IAA metabolism by physiological and molecular processes in SE [19]. In tissue culture systems, calluses are a form of unorganized tissue mass which are widely used in tissue culture.

The callus is either embryonic/organogenic or non-embryonic/organogenic, with the capacity to regenerate plant organs [20]. A wound on plant tissue or pathogens can also cause callus formation [21]. As a result of more than 50 years studied it is

revealed that the hormone concentration, the cultivation duration, and the number of passages undertaken in the cultivation process are affected on the callus formation. Even though callus cultures are useful for propagating clones, it is important to note that they do not initially produce hormones [22].

Traditional medicine has used hyssop benefits since ancient times due to both aromatic and medicinal properties. In addition to its usage in food and cosmetics, it is also used as a decorative plant. Furthermore, this herb is widely used in traditional medicine to treat intestinal and digestive disorders, respiratory diseases like tuberculosis, asthma, bronchitis, as well as rheumatic pains, bruises, burns, frostbite and wounds [23,24]. The most common use of hyssop (*Hyssopus officinalis* L.) is as an essential oil. The hyssop essential oil has been used in the pharmaceutical industry (as a powerful antioxidant, expectorant, antibacterial, antifungal, and antiseptic agent), in aromatherapy as a muscle relaxant containing the essential components of trans- and cis-pinocamphone, and in the perfume industry [25,26].

During the present study, we investigated the effects of SNP, 2,4-D, and different ratios of SNP and 2,4-D on callus induction in Hyssop. The results showed that SNP and 2,4-D can enhance Hyssop callus formation, the weight of fresh callus, and shoot regeneration.

MATERIALS AND METHODS

Plant Material

Hyssop seeds were harvested in the summer of 2022 in the mountainous regions of Rayen, Kerman province for the present study. After washing the seeds in tap water for 10 minutes, they were soaked in 70% ethanol for 2 minutes, then rinsed in sterile distilled water for 2 minutes to prepare the initial explants. In the next step, sterilizing the seeds for 7 minutes using 10% sodium hypochlorite, then seeds were rinsed 3 times with sterile distilled water. Germinating seeds were kept in sterilized Petri dishes with a diameter of 9 cm at 26 ºC in the germinator.

Culture Conditions

Following the production of sterile plants, the petioles and leaves were separated and cultivated on Murashige and Skoog (MS) medium [27]. To induce calluses, culture media was supplemented with combinations of SNP (0, 5, 10, 15 and 20 µM) and 2,4 D (0, 1, 2 and 3 mg/l) and maintained for two weeks at 25±1 ºC under dark conditions. Following that, cultures were transferred under 16/8 h photoperiod for two weeks.

Callus induction (%), and mean fresh weight (MFW) of calluses were measured one month after explant planting. Calculation of callogenesis rate (%) was performed using the following equation:

$$\text{Callogenesis rate (\%)} = \frac{\text{Number of explant that produce callus}}{\text{Total number of explants}} \times 100$$

Plant Regeneration, Rooting and Acclimation

Upon callus induction, they were transferred to a shooting medium (MS) containing varying concentrations of BAP (1, and 2 mg/l) and SNP (0, 5, 10, 15 and 20 µM) at 25±1 ºC under 16/8 light cycle. For the root induction, the shoots were placed on MS medium supplemented with different concentrations of SNP (0, 5, 10, 15 and 20 µM) and Indole-3-butyric acid (IBA) (0 and 1 mg/l). In the fourth week of culture, the average number of roots and root length (cm) were measured. For acclimation, the plants were placed into plastic glass (height 10 cm, diameter 6 cm) containing a 2:1 mixture of cocopeat and perlite.

Statistical Analysis

The callus induction test was conducted by cultivating 20–25 leaves and petioles on each medium with different combinations of SNP and 2, 4- D. A total of 30-40 calluses were tested for callus maintenance and shoot regeneration. A factorial experiment with three replications based on a completely randomized design (CRD) was performed to study the effect of factors on callus induction and plant regeneration.

SAS V.9.1 was employed for data analysis, and the Tukey Test, conducted at a 5% significance level ($P< 0.05$), was utilized to compare the means.

RESULTS AND DISSCUSSION

Callus Induction in Leaf and Petiole

For the micropropagation of plants, callus induction is a well-known and effective technique. There is a variation in the levels of endogenous plant growth regulators between species and tissues of plants [28]. Consequently, the choice of explants may have

a significant impact on the induction of calluses. The current study investigated the effects of different concentrations of SNP and 2,4-D on callogenesis (%) from leaf and petiole explants. Callogenesis rate (%) was significantly (P≤ %1) affected by SNP and 2,4-D in both leaf and petiole explants (Table 1). Between the different SNP concentrations, maximum callogenesis (72%) was obtained at a concentration of 20 µM SNP, however in combination with 2, 4-D the rate of callogenesis increased to 100% (Fig. 1 a and b). Conversely, leaf explants induced minimum callogenesis when treated with 5 µM SNP (Table 1). Similarly, when using 20 µM SNP and 3g/L 2, 4-D, petiole explants showed the highest callogenesis rate (86.3%). In both explants, no callus was developed on media without SNP or 2, 4-D (Fig. 2 a and b). By reducing cell wall lignification, cytokinins promote callus initiation and development in vitro. Callus growth typically starts on the cut surface of the explant and spreads to cover the whole explant [29]. It has been reported by Xu et al that SNP enhances the callus induction percentages in *Dioscorea opposita* plants [3]. According to Samsampour et al SNP increased callus induction in *Hyoscyamus niger* in contrast to the control [30]. Shen et al conducted a detailed investigation of NO effect on cell culture in Arabidopsis. They reported that, When NO was used as a regulator of cell proliferation and differentiation, it regulated cytokinin-induced dedifferentiation and proliferation of somatic tissues [31]. NO-deficient mutants were examined in response to cytokinin for callus formation and shoot regeneration. In hypocotyl and root explants, NO induces callus initiation and shoot regeneration following the action of cytokinin. The activation of the cell cycle gene CYCD 3;1 was also suggested to play a vital role in meristem maintenance. In addition, the authors stated that NO functions to restrict endocycles by enhancing the expression of CYCD 3,1 [31]. Callus fresh weight was another key index that was measured associated with callus induction in the current study. The present study found that 2,4-D and SNP significantly (P≤0.01) affected callus fresh weight. For example, a callus fresh weight of 0.165 g was obtained for the leaf, while a callus fresh weight of 0.13 g was obtained for the petiole (Fig. 3 and 4). Likewise, the SNP-free treatment resulted in the lowest callus weight for both explants in comparison with the treatment that contained SNP. Finally, in terms of leaf and petiole callus weight, different levels of hormone 2,4-D, except for those containing the highest concentration of SNP (20 µM) showed no significant difference (Fig. 3 and 4). There are a number of factors that influence SNP's impact on callus weight, including SNP concentration. It is possible that NO-mediated hormone signaling pathways promote cell division and elongation through low concentrations of SNP [32]. Lin et al showed that in vitro plant regeneration from in vitro cultures is often attributed to increased callus weight, indicating increased cell division and growth [33]. In addition, the increase in callus weight leads to the production of healthy, genetically stable plants [34].

Shoot Formation

Callus cultures treated with SNP showed increased shoot regeneration. In this regard, NO plays a role in improving cell division, differentiation, and organogenesis [35]. The function of NO donors such as SNP in regulating shoot development may be explained by their ability to mediate some cytokinin effects in plants [36]. According to table 2, in calluses of both explant types, SNP increased average shoot induction, but leaf calluses produced more shoots than petiole explants. Using 20 µM SNP combined with 3 mg/l of BAP obtained 84 and 54% mean shoot numbers in leaf and petiole calluses respectively (Fig. 1a and 2c). The MS medium without SNP also produced in the lowest mean number of shoots compared to the MS medium with SNP. There was no significant difference in shoot induction in both explants between BAP 1 and 2 mg/l concentrations (Table 2). Our results with SNP 20 µM in the number of shoot increments were similar to the results reported by Han et al in *Malus hupehensis* plantlets [2]. In another study, Xu et al. reported that adventitious shoot regeneration in *Dioscorea opposita* was significantly increased by SNP [3]. Furthermore, several studies have shown that NO donors in MS media increase the induction of shoot regeneration such as in *Glycine max* [37], *Linum usitatissimum* [38], *Chrysanthemum morifolium* [39], *Arachis hypogaea* [40] and in *Echinacea purpurea* (L.) [41]. Plant cell cultures stimulated by cytokinins released NO which resulted in improvements in shoot differentiation and regeneration in vitro [42].

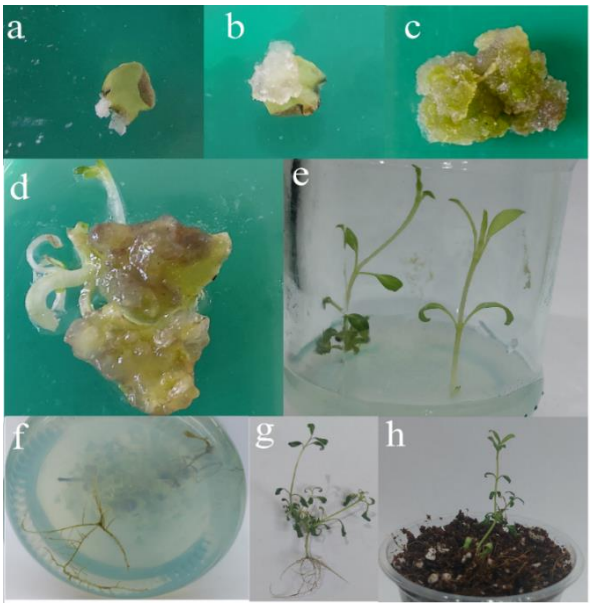

Fig. 1 Different combinations of SNP and PGRs induce calluses in *H. officinalis* leaf explants. a) Initial callus induction from leaf explant treated with 20 µM SNP with the combination of 2 mg/l 2,4-D b and c) callus development from leaf explant d) Growth of shoots from calluses augmented with SNP (20 µM) + BAP (2 mg/l) e) Multiplication of shoots on MS medium supplemented with SNP (20 µM) + BAP (2 mg/l) f and g) Induction of roots from shoots on MS medium supplemented with SNP (15 µM) and IBA (1 mg/l) h) Transplantation of regenerated plants.

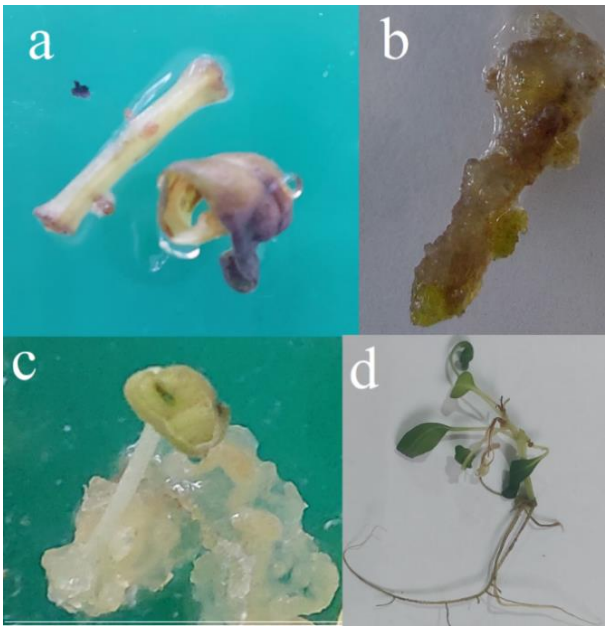

Fig. 2 callus induction from petiole explant in *H. officinalis* a) No callus induction from petiole in control treatment (MS medium without SNP and BAP) b) Callus induction from petiole supplemented with SNP (20 µM) and 2, 4-D (2 mg/l) c) Shoot regeneration from callus of petiole explants treated with SNP (20 µM) + BAP (1 mg/l) d) Rooting of shoots supplemented with SNP (15 µM) and IBA (1 mg/l).

Table 1 The effect of SNP and 2,4-D on the induction of callus in *H. officinalis* from leaf and petiole explants

SNP (µM)	2, 4- D (mg/l)	Leaves callogenesis (%)	Petiole callogenesis (%)
-	-	0 ± 0.00 j	0 ± 0.00 m
-	1	81.3 ± 0.88 e	47.2 ± 1.45 hi
-	2	87 ± 0.57 d	52.1 ± 1.20 hi
-	3	91.6 ± 0.80 bcd	54 ± 0.57 h
5	-	27 ± 1.45 i	17.1 ± 1.45 k
5	1	78 ± 0.87 e	55.4 ± 0.65 h
5	2	88.3 ± 1.65 cd	65.1 ± 0.87 fg
5	3	100 ± 0.00 a	72.2 ± 1.45 de
10	-	38.3 ± 2.40 h	26 ± 0.60 j
10	1	92.6 ± 1.45 bc	68.3 ± 1.00 ef
10	2	94 ± 1.20	67 ± 1.45 ef
10	3	100 ± 0.00 a	75.1 ± 0.33 cd
15	-	56 ± 1.30 g	12 ± 0.32 i
15	1	97 ± 1.00 ab	75 ± 0.57 cd
15	2	95 ± 0.65 ab	77.5 ± 0.31 cd
15	3	100 ± 0.00 a	78.6 ± 1.85 bc
20	-	72 ± 1.20 f	62.3 ± 1.45 g
20	1	100 ± 0.00 a	85 ± 0.32 a
20	2	100 ± 0.00 a	83.1 ± 0.31 ab
20	3	100 ± 0.00 a	86.3 ± 0.88 a

Based on three replications, the values represent the mean ± SD. There are no significant differences between means following the same letter within columns when compared with the Tukey test ($P < 0.05$).

Table 2 Differences in SNP and BAP concentrations affect shoot induction from callus in leaf and petiole explants

SNP (µM)	BAP (mg/l)	Leaf forming shoots (%)	Petiole forming shoots (%)
-	1	23.6 ± 0.91 f	12.5 ± 1.15 e
-	2	27 ± 0.57 f	12 ± 0.87 e
5	1	44.3 ± 0.30 e	22 ± 0.28 d
5	2	45.4 ± 0.55 e	23.6 ± 0.33 d
10	1	56.3 ± 0.88 d	32 ± 0.64 c
10	2	58 ± 0.57 d	33.2 ± 0.65 c
15	1	65 ± 0.65 c	42 ± 0.88 b
15	2	65.4 ± 0.33 c	40.6 ± 0.80 b
20	1	71.6 ± 0.88 b	53.6 ± 1.76 a
20	2	85 ± 0.57 a	54.3 ± 0.66 a

Based on three replications, the values represent the mean ± SD. There are no significant differences between means following the same letter within columns when compared with the Tukey test ($P < 0.05$).

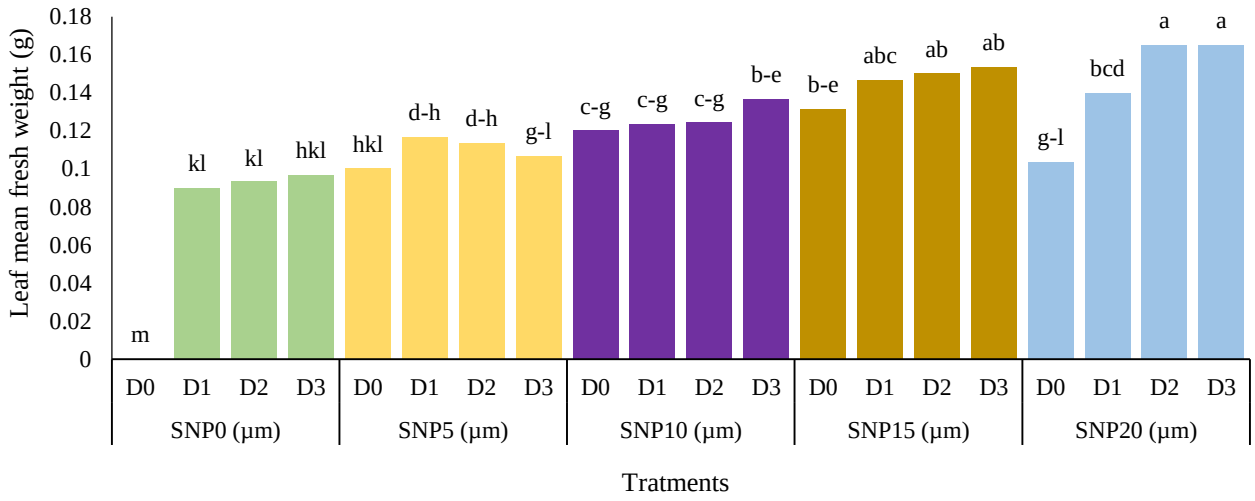

Fig. 3 Effect of SNP and 2,4-D combination on callus fresh weight from leaf explant. D0-D3 represented 2,4-D concentrations from 0- 3 mg/l, respectively. There are no significant differences between the means following the same letter when compared with the Tukey test ($P < 0.05$).

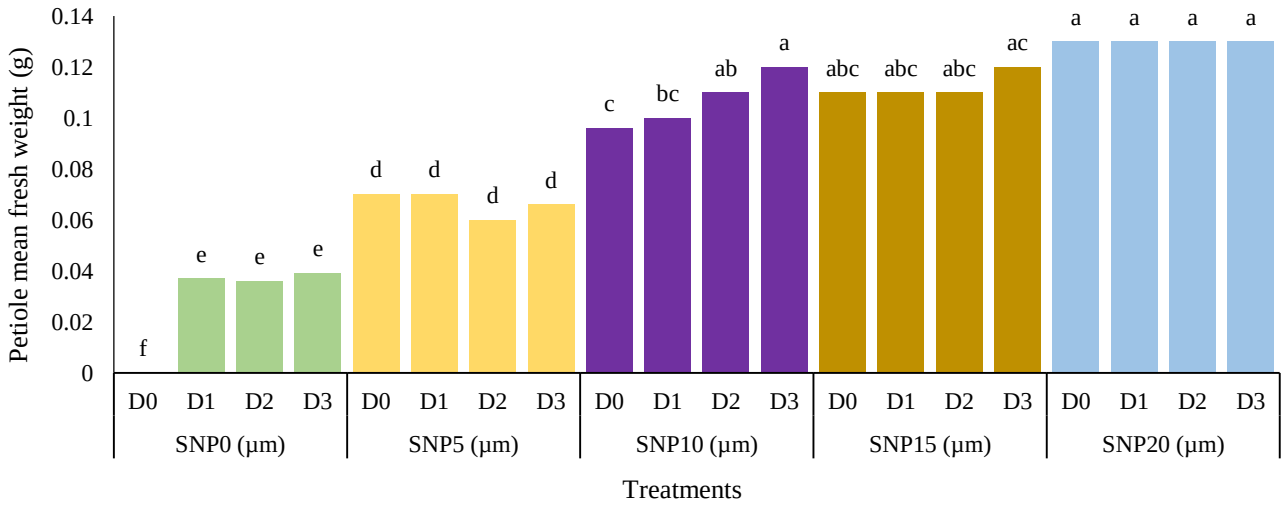

Fig. 4 Effect of SNP and 2,4-D combination on callus fresh weight from petiole explant. D0-D3 represented 2,4-D concentrations from 0- 3 mg/l, respectively. There are no significant differences between the means following the same letter when compared with the Tukey test ($P < 0.05$).

Table 3 SNP and IBA combination influence root induction in H. *officinalis*

SNP (µM)	IBA (mg/l)	Rooting (%)	Root length (cm)
-	-	0 ± 0.00 g	0 ± 0.00 d
-	1	45.3 ± 0.87 d	3.4 ± 0.43 bc
5	-	25 ± 0.56 f	2.7 ± 0.35 c
5	1	56 ± 0.57 c	4.7 ± 0.37 b
10	-	35.2 ± 0.33 e	3.5 ± 0.28 bc
10	1	62 ± 1.15 b	3.7 ± 0.35 bc
15	-	63 ± 1.14 b	4.4 ± 0.30 b
15	1	83 ± 1.52 a	8.3 ± 0.31 a
20	-	42.3 ± 1.20 d	3.7 ± 0.29 bc
20	1	52.2 ± 1.25 c	3.7 ± 0.30 bc

Based on three replications, the values represent the mean ± SD. There are no significant differences between means following the same letter within columns when compared with the Tukey test ($P < 0.05$).

As a result of this observation, NO was found to be a significant signal transducer in the cytokinin-mediated signal pathway.

Root Induction and Acclimation

It has been demonstrated that auxins and NO (SNP) act synergistically to regulate various plant responses, including the formation and extension of lateral, adventitious and hairy roots [43,44]. Among all the combinations tested in the current study, the combination of SNP (15 µM) and IBA (1 mg/l) produced the highest rooting (83%) and root length (8.3 cm) (Table 3) (Fig. 1 f, g and 2 d). Between all treatments, the lowest rooting (25 %) and root length (2.7 cm) were obtained in SNP (5 µM) without IBA. Rooting rate improved with an increase in SNP concentration until to 15µM. However, at 20 µM, rooting percentage and root length decreased. SNP and IBA combinations were the most effective treatments for hyssop rooting (Table 3). For acclimation, rooted plants were transferred to cocopeat and perlite. After the rooted plants were transferred, more than 80% survived under greenhouse conditions (Fig. 1 h). According to Correa-Aragunde et al NO stimulates auxins-mediated lateral root growth, and Correa-Aragunde found that NO modulates genes involved in cell cycle regulation in tomato pericycle cells, leading to the formation of Lateral Roots [8,45]. Additionally, NO may influence gene expression associated with root meristem identity. NO regulates CYCD 3;1 gene initiation and CDK inhibitor KIPP-related protein 2 (KRP2) gene expression during lateral root primordial initiation and auxin-dependent cell cycle gene expression. The link between cell division rate and differentiation may be established by NO interaction with auxins and cytokinins during de-differentiation and re-differentiation of plant cells [3,46].

CONCLUSION

In the present study, callus formation and regeneration of shoots and roots in *H. officinalis* were investigated using SNP and growth regulators. When the leaf explant is cultured on MS media containing 20 µm SNP combined with 1-3 mg/L 2, 4-D, maximum calluses can be induced. As a result of applying 20 µM SNP and 2 mg/L IBA, the callus from the leaf explant produced the maximum number of shoots on MS media. Further research might investigate the effect of SNP and 2,4-D on callus compositionThis study offers a valuable framework for propagating medicinal hyssop through micropropagation. Additionally, the study results could also prove useful in the field of cell suspension culture, genetic resource conservation, and pharmaceutical research related to medicinal plants.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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