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Research Article

A Rapid Method for *In Vitro* Plant Regeneration of Chia *Salvia hispanica* L.

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Abstract: Chia *Salvia hispanica* L. is an annual herbaceous plant. The seeds were shown to have significant macronutrient and micronutrient components. Most of these common macronutrients are proteins, lipids with an appropriate fatty acid composition, ash, carbs, and dietary fiber. This study aimed to find a simple and fast protocol for the regeneration of this plant. The hypocotyls, stem, cotyledons, and young leaves explants of seedlings were grown on Murashige and Skoog (MS) medium fortified with various concentrations of (NAA) and 6-benzyl adenine (BA). Moreover, 2, 4-diclorophenoxyacetic acid (2, 4-D) and kinetin (Kin) were used. Callus was successfully induced from all explants. The most interesting result was the regeneration of shoots from young leaves in one-step during the callus formation. The appropriate medium for shooting was MS enriched with 0.5mg L^{-1} NAA and 1.0 mg L^{-1} BA as the percentage of shoot regeneration was (112.5 %). The regenerated shoots were rooted successfully in five days following transferring to agar solidified MS medium. These results revealed the effectiveness of the protocol used in the current study, which is considered the first that was able to produce the important medicinal plant *S. hispanica* in one-step during callus induction in a short period.

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1. Introduction

The oilseed chia *Salvia hispanica* (Lamiaceae) is usually found in central and southern areas of America (Iannucci and Amato, 2021). Salvia is utilized for medicinal and ornamental benefits (Motyka et al., 2022). Chia is grown in tropical and subtropical environments. In some European regions (Brandan et al., 2019).

It was detected that seeds of chia have various significant constituents such as a high percentage of dietary fiber, protein, and fatty acids especially unsaturated omega-6 in addition, to omega-3 (Motyka et al., 2023). Moreover, different vitamins, minerals, and antioxidants that are found in chia seeds led to an increase in their production in the world (Rabail et al., 2021). The high ratio of natural products especially phenolic compounds of *S. hispanica* (Drużyńska et al., 2021), besides myricetin, and quercetin which are the major natural substances (Nyingi, and Mburu, 2021) have made this plant gained an important position to be exploited for many medicinal uses. Health usefulness comprises anti-inflammatory, anticancer, assisting healthier skin and strengthening bones and muscles (Ullah et al., 2016). Furthermore, decreases the hazard of cardiovascular diseases, and aging cues, (Grancier et al., 2019), and also decreases total cholesterol levels, and triglycerides condensation (Ullah et al., 2016).

The plant tissue culture technique, which is also called *in vitro* culture, has become a big interest in recent years. It became progressively opportunistic and positively affected contemporary agriculture, playing a major role in rapid plant propagation, variety amendment, and plant breeding (Ferris, 2023). This approach is built on "totipotency", in other words, each cell has a potential ability to regenerate a whole plant if it is supplied with the right conditions (Su et al., 2021). Different factors affect tissue culture technique, medium structure, type of explant, plant growth regulators, and culture circumstances (Sale et al., 2023). This method has various applications for instance, quick and efficient production of plants in a short time, the reservation of rare species in addition to commercial manufacture of secondary metabolites (Shahzad et al., 2017), and induction of polyploidy (Marangelli et al., 2022) as well as initiation of free- pathogen plants (Pe et al., 2020) and resistant plants (Soleimani et al.,2020).

Only a few studies have dealt with the tissue culture of chia plants (Patricia et al., 2013; Yadav et al., 2019; AL-Dabagh, and Salih, 2020). Therefore, the target of the present study is to find a quick and simple protocol for establishing callus cultures and *in vitro* high regeneration of this medicinal plant.

2. Material and Methods

2.1. Seed sterilization and germination

Chia seeds were surface sterilized using the method of Al-Dabagh and Salih (2020) with slight modification. The seeds were immersed in sodium hypochlorite solution (5% active chloride) for 10 minutes, then washed with sterile distilled water 3 times for three minutes. These sterilized seeds were transferred to solidified Murashige and Skoog (1962) (MS) medium, and placed in the dark at 25±2 °C for the first three days; then they were transferred to photoperiod 16-8 hours (light-dark) supplied with cool-white fluorescent lamps.

2.2. Establishment of callus cultures

MS medium enriched with 1.5, 1.0, 0.5, 0.1 mg L^{-1} naphthalene acetic acid (NAA), and 2.0, 1.5, 1.0, 0.5 mg L^{-1} of Benzyl Adenine (BA) respectively and 30 g L^{-1} sucrose was prepared, in addition to MS medium containing different levels of 2, 4-diclorophenoxyacetic acid 2, 4-D (1.5,1.0,0.1 mg L^{-1}) and kinetin (Kin) 2.0, 1.5, 0.5 mg L^{-1}. The pH of the medium was adjusted to 5.8 with NaOH or HCl before autoclaving at 121 °C. For callus cultures, one month old seedlings of *S. hispanica* were used (Figure 1A). Different explants: hypocotyls, stems with 1 cm length, while cotyledons and young leaves were cut into two fragments and then, transplanted on glass jars containing the previous combinations. All the samples were incubated in the growth room at 25±2 °C, with illumination 8 dark - 16 light hour, with a light intensity of 2000 lux from fluorescent tubular lamps.

$$\text{Callus initiation}(\%) = \frac{\text{Number of explants producing callus}}{\text{Number of inoculated explants in each treatment}} \times 100\ \% \quad (1)$$

For callus maintenance, 50 mg of developed calli were transferred to a fresh medium containing the best combination for callus growth after 28 days of culture.

2.3. Shoot regeneration and rooting

Shoot regeneration was observed during callus induction from young leaves of *S. hispanica* on a solidified MS medium, containing both BA and NAA. All regenerated shoots were transferred to 25 ml of MS medium without growth regulators in 100 ml flasks for rooting. All specimens were preserved in the same former conditions. The percentage of shoot regeneration was calculated according to the following equation:

$$\text{Shoot percentage} = \frac{\text{Number of regenerated shoots in each treatment}}{\text{Number of inoculated explant in each treatment}} \times 100\ \% \quad (2)$$

2.4. Statistical analysis

The data were analyzed according to the factorial experimental system, using a completely randomized design with five replications. Each replicate contains eight explants. The least significant difference test (LSD) was used to compare means. All data analyses were carried out with the Genstat program.

3. Results and Discussion

This study was carried out to explore the capability of *S. hispanica* in tissue culture, and it showed success in finding an easy and effective procedure for callus initiation and *in vitro* production of this important plant. All combinations of NAA and BA in MS medium were useful for starting callus cultures from all explants of chia since the percentage of callus initiation was between 50%-100% (Table 1).

Table 1. Percentage of callus initiation from different explants of *S. hispanica* L. in the presence of NAA and BA

NAA + BA (mg L^{-1})	Hypocotyls	Stem	Cotyledons	Leaves	Mean of combinations
0.1+ 0.5	75.00	75.00	62.50	87.50	75.00
0.5 + 1.0	100.00	100.00	87.50	100.00	96.88
1.0 + 1.5	100.00	100.00	100.00	100.00	100.00
1.5+2.0	50.00	50.00	50.00	62.50	53.13
Mean of explant	**81.25**	**81.25**	**75.00**	**87.50**	

LSD explant =7.9, LSD combinations=7.9, LSD explant ×combinations= 15.79 ($P<0.05$).
*The number of explants in each treatment was 8.

Furthermore, the best combination was MS fortified with 1.5 mg L^{-1} BA and 1.0 mg L^{-1} NAA for all explants as the efficiency for callus induction was 100%. Koçak et al. (2023) noticed that the addition of 1 mg L^{-1} NAA promoted callus induction from *Pelargonium quercetorum* Agnew. Hypocotyls and stem explants began to form greenish white friable callus after 10 days of culture in the medium having 1.5 mg L^{-1} BA and 1.0 mg L^{-1} NAA + (Figure 1B and 1C), while young leaves developed a light green compact callus after 10 days at the same medium (Figure 1D). Moreover, cotyledons responded successfully to callus induction and produced a green compact callus in this medium at the same period (Figure 1E). In contrast, supporting callus initiation medium with 2, 4-D, and Kinetin did not assist callus initiation (Table 2), as the callus initiation percentage for all explants was decreased. The difference in callus induction abilities among plant growth regulators can be attributed to different sensitivities to cellular mechanisms and incompatible lysis of chemical bonds in the plant cell wall (Gaspar et al., 1996; Adil et al., 2018). Patricia et al. (2013) indicated that the formation of callus in *S. hispanica* was 83% for stems and 29% for leaves in MS medium enriched with 2.25 µM 2, 4-D while the study by Bueno et al. (2010) which was performed to assess the *in vitro* behavior of *S. hispanica* explants (uninodal segments with 2 axillary buds, young leaves and cotyledons), stressed that the leaves and the cotyledons did not succeed in callus induction, but nodal segments showed the highest rate of shooting (78%).

Table 2. Percentage of callus initiation from different parts of *S. hispanica* in the presence of 2,4-D and Kin

2,4-D +Kin (mg L^{-1})	Hypocotyls	stem	cotyledons	Leaves	Mean of combinations
0.1+ 0.5	37.50	62.50	37.50	45.83	37.50
1.0 + 1.5	50.00	12.50	0.00	20.83	50.00
1.5+2.0	25.00	0.00	0.00	8.33	25.00
Mean of explant	**37.50**	**25.00**	**12.50**	**25.00**	

LSD explant =10.46, LSD combinations =10.46, LSD explant ×combinations = 18.12 ($P<0.05$).
*The number of explants in each treatment was 8.

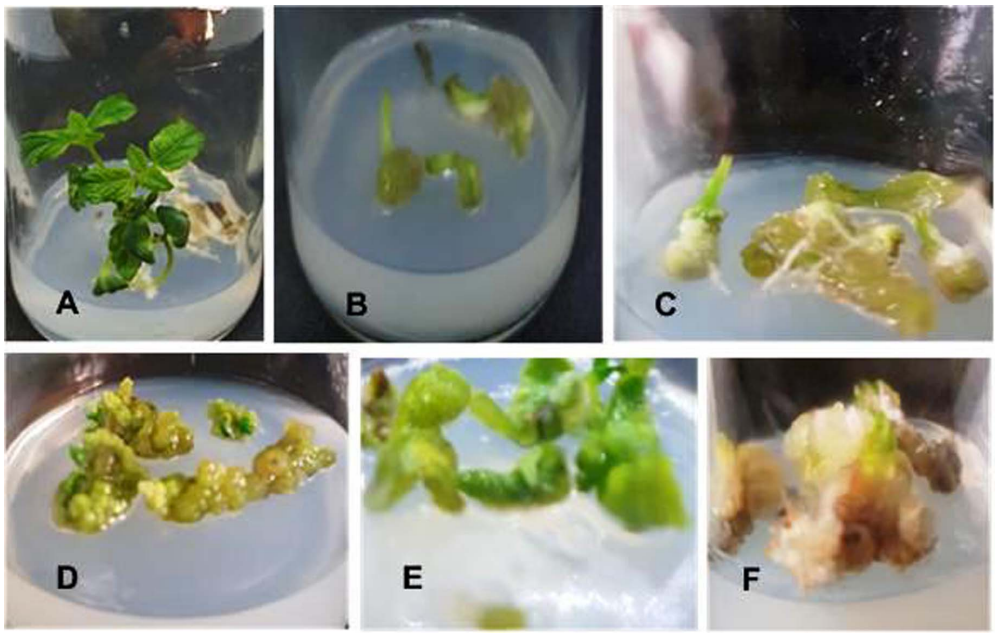

Figure 1. Callus initiation from different explants of *S. hispanica* L. A) one month old seedlings in MS free medium, B) hypocotyls explant after 8 days in MS medium enriched with 1.0 mg L^{-1} NAA and 1.5 mg L^{-1} BA, C) stem explants in the same medium of B, D) young leaves in callus induction medium, E) cotyledons after fifteen days of culture, F) maintained callus after 28 days.

Callus of different explants was maintained in MS agar containing 1.0 mg L^{-1} + 1.5 mg L^{-1} NAA and BA respectively every four weeks (Figure 1F). One of the interesting results of this study was the regeneration of shoots from young leaves in a one-step method during callus induction. The percentage of this phenomenon (112.5%) was recorded in combination with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA (Table 3). In addition, a mixture of 1.0 mg L^{-1} NAA and 1.5 mg L^{-1} BA was appropriate for shooting, since the percentage reached 75%. The capacity of plant regeneration is influenced by different factors, including the use of a plant growth regulator (Gerdakaneh et al., 2020), the composition of basal medium (Sundararajan et al., 2017), and the type of explant (Minutolo et al., 2020). Cytokinins and auxins are considered the most significant plant hormones because they regulate cell division and proliferation (Ashokhan et al., 2020). Exogenous plant growth regulators, such as cytokinins affect the dedifferentiation and differentiation in plant cells, and this explains why callus is initiated from the explants of *S. hispanica* and shoots regeneration (Su et al., 2014).

Table 3. One step shoots regeneration from leaves explant of *S. hispanica* L.

MS combinations NAA + BA	No. of shoots	Shoot regeneration (%)
0.1+ 0.5	0	0.0
0.5 + 1.0	9	112.5
1.0 + 1.5	6	75.0
1.5 + 2.0	0	0.0

*The number of explants in each treatment is 8.

In tissue culture, plants are regenerated fundamentally by somatic embryogenesis and de novo organogenesis (Hill and Schaller, 2013). De novo organogenesis indicates the regenerative process that does not use a somatic embryo but instead, the differentiation of the meristematic core, reflecting the pluripotency of plant cells (Lardon and Geelen, 2020). In this study, the regeneration process occurred through indirect de novo shoot organogenesis after 30 days of culturing young leaves on the previous medium. It was started by the emergence of a single small leaf from the callus (Figure 2A) and then developed into distinct shoots over time (Figure 2B and 2C). There are four stages to the regeneration of a shoot from a callus: the creation of a pluripotent callus, the development of a shoot promeristem, the development of a shoot progenitor, and the regeneration of a shoot (Shin et al., 2020).

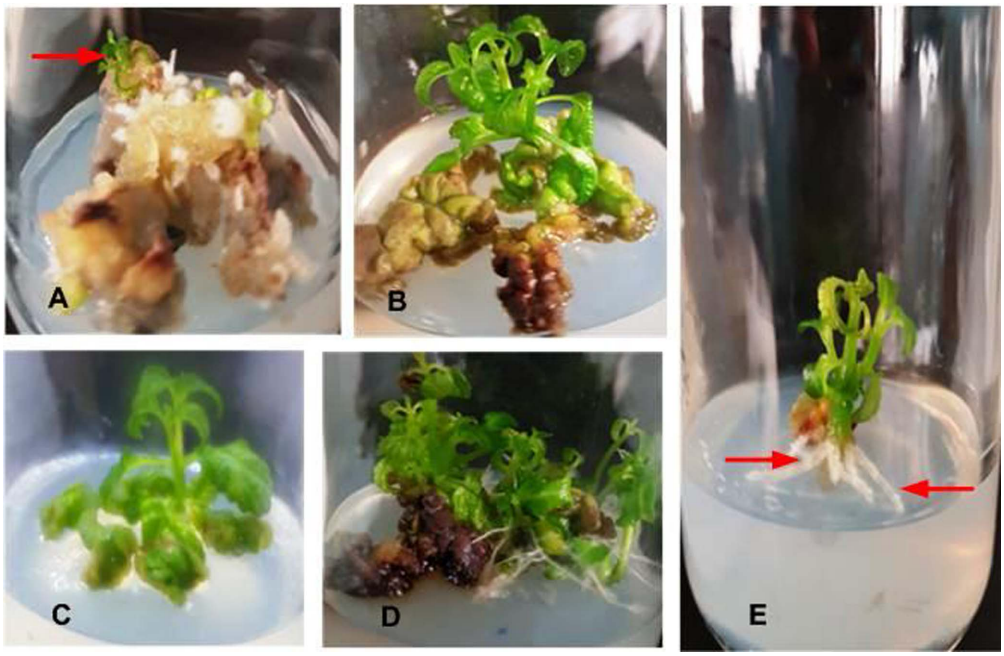

Figure 2. One-step regeneration of *S. hispanica* from leaves explant. A) beginning of shoot formation (red arrow) from leaves callus after 30 days, B, C) regenerated shoots after 40 days, D) browning of the callus after 50 days, E) rotting of the regenerated shoots (red arrows) after 7 days of transferring to MS free medium.

Even though numerous studies have been carried out on the *in vitro* establishment of *S. hispanica* (Patricia et al., 2013; Yadav et al., 2019; AL- Dabagh and Salih, 2020), so far, there is no published study concerning one-step shoot regeneration of this plant from callus. Hence, this study revealed the possibility of producing shoots in a high percentage. It was observed that after producing the shoots, the callus became dark brown (Figure 2D). This might be due to increasing the phenolic compound in the callus. According to several studies that have already been published, there are numerous chemicals and enzymes associated with the tissue browning phenomena, such as increasing the production of phenolic compounds (Irshad et al., 2017), that in turn increase the activity of various key enzymes like polyphenol oxidase and peroxidase (Chuanjuna et al., 2015; Lee et al., 2022). All the regenerated shoots began the rooting process successfully after 5 days of transferring to agar solidified MS medium free from growth regulators (Figure 2E). Moreover, some shoots were rooted at the same medium of callus induction. Number of roots for each shoot was 3-6, the range length of these roots was 1-3 cm.

Conclusion

The results of this study demonstrate that various explants of chia have the ability to produce callus on agar solidified MS medium enriched with NAA and BA. Moreover, the present investigation has established a simple and rapid approach for the regeneration of this important medicinal plant in a one-step method via indirect de novo shoot organogenesis at the same medium of callus induction. Furthermore, shoot regeneration occurred with high frequency. This method could be utilized as a tool for the propagation of chia plants within a short period of time. Another study should be established to acclimatize the regenerated plants, which may be exploited for the extraction of active compounds.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The author declares that there are no conflicts of interest.

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