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IN VITRO PRODUCTION OF ACTIVE COMPOUNDS IN THE CALLUS OF THE CHIA PLANT (*SALVIA HISPANICA*)

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SUMMARY

The latest research aimed to study the effects of plant growth regulators on inducing callus from the chia plant (*Salvia hispanica*) and stimulating it to produce some medicinal compounds *in vitro*. The study transpired at the Plant Tissue Culture Laboratory, College of Agriculture, University of Anbar, Iraq. The use of Murashige and Skoog (MS) nutrient medium containing 2,4-D Auxin (dichlorophenoxyacetic acid) had concentrations of 1.0, 2.0, and 3.0 mg L^{-1} and cytokinin benzyl adenine (BA) with concentrations of 0.0, 0.5, and 1.0 mg L^{-1}. The use of their interactions continued to induce a perpetuation of callus. In another experiment, the salicylic acid at concentrations of 0, 10, 20, and 30 mg L^{-1} stimulated the callus to produce medicinal compounds. The results showed that for induction of callus and its sustainability in the leaves of the chia plant, the best concentration revealed 2,4-D at a rate of 3 mg L^{-1}, which achieved the highest fresh and dry weight and dry matter percentage with values of 0.5151 g, 0.0723 g, and 13.531%, respectively. The results also showed that the addition of salicylic acid at the concentration of 10 mg L^{-1} to the nutrient media stimulated the formation of the active compounds.

Keywords: Chia plant (*Salvia hispanica* L.), dichlorophenoxyacetic acid, benzyl adenine, salicylic acid, callus induction, secondary metabolites

Key findings: The MS media with 2,4-D (3.0 mg L^{-1}) revealed the best performance by showing the highest fresh and dry weight and dry matter percentage (0.5151 g, 0.0723 g, and 13.531%) for induced callus compared with other treatments. The results also showed that salicylic acid at 10 mg L^{-1} produced the maximum oleic acid compound content in the induced callus cultures created from the leaves.

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INTRODUCTION

Medicinal plants play an important role in human life due to their multiple uses, and the usefulness of these plants lies in the pharmaceutical industry (Abed, 2021). According to the World Health Organization (WHO), up to 80% of the world's population uses medicines prepared from medicinal plant genetic sources (Ramawat, 2008).

Chia (*Salvia hispanica*) is one of the essential medicinal plants. It is a herbaceous

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annual flowering plant belonging to the family Lamiaceae, native to Northern Guatemala and Southern Mexico (Gazem *et al.*, 2016). The chia plant in Central America served as a food and oil source, as well as, for pharmaceutical purposes, with its analysis showing that it contains the chemical components, i.e., unsaturated fatty acids, myricetin, quercetin, kaempferol, and caffeic acid (Lu and Foo, 2002). A study reported chia's richness in oil content (32%), of which 60% consists of alpha-linolenic acid and omega-3 fatty acids that have various therapeutic benefits (Rosamond, 2002). Likewise, its diagnosis displayed a variety of secondary compounds, such as, flavonoids, terpenes and phenolic compounds, used as significant pharmaceutical compounds in the treatment of many diseases, including heart and liver disease, diabetes treatment, anticoagulant, antimicrobial, antioxidant, cancer diseases, ulcers, and inflammation (Din *et al.,* 2021).

Medicinal plants may contain thousands of chemical compounds, such as, alkaloids, glycosides, terpenes, and phenols, which are of great medical value and are difficult to be chemically manufactured, with extraction from wild plants in limited quantities that do not meet the demand (Londhe and Joshi, 2020). Therefore, utilizing the plant cell culture technique to produce medicinal compounds proves very important and offers a promising alternative for acquiring medicinal substances from plants (Jain and Patil, 2020).

The increased benefits of the plant tissue culture technique demonstrated in quantity, quality, and output throughout the year under controlled settings (Aware *et al*., 2022), as well as, not being hampered by natural factors, such as, geographical location and environmental stresses (Arora and Meena, 2018). Similarly, the provision of optimal conditions or adding stimuli to raise production using this technique may be vital or not to the nutrient medium for the development of plant tissues (Islam *et al.*, 2015; Gopi and Jayaprakashvel, 2017). The addition of salicylic acid to a nutrient medium confirmed one of the methods used to induce the plants to produce active compounds *in vitro* (Alam and Prameela, 2016; Patel *et al.,* 2021).

The plant tissue culture means isolating a plant cell, tissue, and organ, sterilizing it, and cultivating it on a sterile artificial nutrient media completely free of pathogens. Then the cultivated part develops into a whole plant similar to the original from which it came. It is all carried out under controlled environmental conditions of heat, humidity, and lighting, as sterile cultivation of cells, tissues, organs, and their components under certain chemical and physical conditions *in vitro* (Gupta *et al*., 2006). The success of callus development and growth resulted to the state of equilibrium between the auxin and cytokinins indicated by Skoog (1957). Kazmi *et al*. (2015) found that using auxins and cytokinins in equal amounts stimulated cell division, growth, and callus formation.

The 2,4-D (2.0 mg L^{-1}) supplemented MS medium significantly contributed to callus induction of wheat plants and led to a fresh and dry callus weight (Mohammed *et al*., 2019). Al-Taha *et al*. (2020) also studied the possibility of inducing callus in *Zingiber officinale* Rosc using 2,4-D, adding benzyl adenine (BA) at different concentrations. The result indicated that MS nutrient medium, prepared with 2,4-D at a concentration of 1.0 mg L^{-1}, added with BA at a concentration of 0.5 mg L^{-1} + 500 mg L^{-1} PVP (polyvinyl pyridine), recorded the highest percentage of callus induction that reached 100%, with the largest diameter (2.26 cm) and the highest fresh and dry weight of callus (1.95 and 0.12 mg), respectively. Therefore, the present study aimed to make calli from the leaf tissue of the chia plant to obtain needed chemicals by adding different amounts of salicylic acid to the culture medium.

MATERIALS AND METHODS

Preparation of MS medium and cultivation of chia seeds

The use of Murashige and Skoog (1962) - MS medium (Himedia - India) contained the weight of 4.4 g L^{-1} and added with sucrose at 30 g L^{-1}, the pH adjusted to 5.7 by adding drops of NaOH or HCl, and agar at 7 g L^{-1} to complete the volume to one liter. Heating the MS medium proceeded on a hot plate magnetic stirrer for homogeneity of the components found in this medium, then distributed in the culture jars and sealed. Afterward, the jar media sterilization used an autoclave device at a temperature of 121°C and a pressure of 1.04 kg cm^2 for 15 min.

The seeds underwent superficial sterilization inside the Laminar Air Flow Cabinet after soaking them in NaOCl (sodium hypochlorite) solution at a concentration of 3% for 15 min. Washing the seeds three times continued with sterilized distilled water to ensure the removal of remnants of the sterile material from the seeds. The sowing of

sterilized seeds took place on a solid MS medium with full salt force. The cultures received incubation in the culture room with a light intensity of 1000 lux for 16 h of light and eight hours of darkness, while the temperature was 25±2°C.

Callus induction

Cutting of the in vitro leaves then planted on MS medium equipped with different concentrations of 2,4-D (1.0, 2.0, and 3.0 mg L^{-1}) combined with benzyl adenine (0.0, 0.5, and 1.0 mg L^{-1}) happened with 10 replicates for each concentration. Incubation of the cultures ensued in complete darkness below 25±2°C. Five weeks after planting, the calculation of the fresh and dry weight of the callus and the percentage of dry matter took place according to the following (Soni *et al*., 2018):

$$\textbf{Dry matter percentage}\ (\%) = \frac{Dry\ weight}{Fresh\ weight} \times 100$$

Stimulation of callus

Planting 150 mg of induced callus from leaf tissue resulted from the induction stage on MS medium that was equipped with different concentrations of 2,4-D (1.0, 2.0, and 3.0 mg L^{-1}) and overlapping with BA at 0.0, 0.5, and 1.0 mg L^{-1} in 10 replicates for each concentration to find out the best medium for sustaining callus. Fresh and dry weight measurements progressed after four weeks of cultivation.

Salicylic acid effects on secondary metabolites

Acquiring a fixed weight (150 mg) of callus underwent culturing on an MS medium equipped with salicylic acid at different concentrations (0.0, 10, 20, and 30 mg L^{-1}) with 10 replicates for each concentration. The cultures received incubation in the growth chamber at a temperature of 25±2°C in complete darkness for four weeks.

Measurement of secondary compounds

The acquired 150 mg fresh weight of callus obtained 10 ml of 99.99% pure ethyl alcohol, then left for 24 h in a shaker (Tamura and Nishibe, 2002). Then the extract was heated to remove the alcohol and transferred for analysis using gas chromatography-mass spectrometry (GC/MS).

Statistical analysis

For statistical analysis, the study used a completely randomized design (CRD) with a factorial arrangement with 10 replicates for each treatment. The data were statistically analyzed using the Genstat program, comparing the averages and separated according to the least significant difference ($LSD_{0.05}$) test (Al-Rawi and Allah, 2000).

RESULTS

2,4-D and BA effects on the fresh weight of callus

After carrying out the study, an induced callus resulted from chia leaves after four weeks of planting (Figure 1). The results revealed that after adding 2,4-D Auxin with different concentrations to the nutrient medium, significant differences appeared in the characteristic of the fresh weight of the induced callus obtained from the leaves of the chia plant (Table 1). The concentration exceeding 3.0 mg L^{-1} provided the highest fresh weight (0.5151 g), followed by a concentration of 2.0 mg L^{-1} with a fresh weight rate of 0.4005 g. However, the concentration of 1.0 mg L^{-1} has the lowest average fresh weight of induced callus (0.1610 g). The nutrient medium added with benzyl adenine (BA) yielded no significant differences, as recorded among the various concentrations of BA for the fresh weight of the callus.

The interaction outcomes between the growth regulators 2,4-D and BA showed that 2,4-D (3.0 mg L^{-1}) and BA-free treatment had the highest fresh weight rate of induced callus (0.6043 g). The 2,4-D at the concentration of 3.0 mg L^{-1} and BA at the concentration of 1.0 mg L^{-1} followed with the second leading value for the fresh weight (0.4950 g). However, 2,4-D at the concentration of 1.0 mg L^{-1} with BA-free treatment produced the lowest average fresh weight of induced callus (0.1240 g).

2,4-D and BA effects on the dry weight of callus

The results showed significant differences among the different concentrations of the growth regulator 2,4-D in the average dry weight of the induced callus obtained from the leaves of the chia plant (Table 2). However, the 2,4-D at the concentration of 3.0 mg L^{-1} surfaced with the highest average dry weight (0.0723 g), followed by the concentration of

Figure 1. Induced callus obtained from chia leaves after four weeks of planting.

Table 1. Effect of 2,4-D and BA with different concentrations and their interaction on the fresh weight (g) of induced callus obtained from chia leaves after four weeks of planting.

Concentration BA (mg L^{-1})	Concentration 2,4-D (mg L^{-1})			Rate of BA
	1.0	2.0	3.0	
0.0	0.1240	0.3497	0.6043	0.3593
0.5	0.1594	0.4137	0.4458	0.3396
1.0	0.1997	0.4381	0.4950	0.3776
Rate of 2,4-D	0.1610	0.4005	0.5151	
$LSD_{0.05}$ 2,4-D = 0.04199, BA = 0.04199, Interaction BA × 2,4-D = 0.07272				

Table 2. Effect of 2,4-D and BA with different concentrations and their interaction on the dry weight (g) of induced callus obtained from chia leaves after four weeks of planting.

Concentration BA (mg L^{-1})	Concentration 2,4-D (mg L^{-1})			Rate of BA
	1.0	2.0	3.0	
0.0	0.0103	0.0376	0.0830	0.0437
0.5	0.0165	0.0474	0.0677	0.0439
1.0	0.0209	0.0512	0.0661	0.0461
Rate of 2,4-D	0.0159	0.0454	0.0723	
$LSD_{0.05}$ 2,4-D = 0.00576, BA = 0.00576, Interaction BA × 2,4-D = 0.00997				

2.0 mg L^{-1} with an average dry weight of 0.0454 g. Inversely, the 2,4-D at 1.0 mg L^{-1} concentration gave the lowest average dry weight of induced callus (0.0159 g). The same table also showed no significant differences among the various concentrations of BA in the dry weight of induced callus obtained from the leaves of the chia plant.

The interaction effects between the growth regulators 2,4-D and BA (2,4-D at the concentration of 3.0 mg L^{-1} with BA-free treatment) provided the highest average dry weight of induced callus (0.0830 g), followed by 2,4-D **(**3.0 mg L^{-1}) in combination with BA (0.5 mg L^{-1}) amounted to 0.0677 g. However, the concentration of 2,4-D (1.0 mg L^{-1}) with BA-free treatment gave the lowest average dry weight of induced callus obtained from the chia plant leaves (0.0103 g).

2,4-D and BA effects on dry matter (%) in induced callus

The results revealed significant differences as an effect of the 2,4-D various concentrations for the dry matter percentage of the induced callus obtained from the chia plant leaves (Table 3). The 2,4-D at the concentration of 3.0 mg L^{-1} achieved the highest percentage of dry matter for induced callus (13.531%), followed by 2,4-D at the concentration of 2.0 mg L^{-1} with a dry matter ratio (11.327%). The lowest percentage of dry matter (10.203%) occurred from the treatment of 2,4-D at the concentration of 1.0 mg L^{-1}.

The results also indicated significant differences in the dry matter percentage of the induced callus with the addition of the growth regulator BA to the nutrient medium (Table 3).

Table 3. Effect of 2,4-D and BA with different concentrations and their interaction on the dry substance percentage (%) of induced callus obtained from chia leaves after four weeks of planting.

Concentration BA (mg L^{-1})	Concentration 2,4-D (mg L^{-1})			Rate of BA
	1.0	2.0	3.0	
0.0	10,068	10,740	13.920	11.576
0.5	10.340	11,520	14.240	12.033
1.0	10.200	11.720	12.432	11,451
Rate of 2,4-D	10.203	11,327	13,531	
$LSD_{0.05}$ 2,4-D = 0.3763, BA = 0.3763, Interaction BA × 2,4-D = 0.6517				

Table 4. Effect of 2,4-D and BA with different concentrations and their interaction on the maintenance of induced callus (fresh weight - g) obtained from chia leaves after four weeks of planting.

Concentration BA (mg L^{-1})	Concentration 2,4-D (mg L^{-1})			Rate of BA
	1.0	2.0	3.0	
0.0	0.22020	0.3773	0.4957	0.3583
0.5	0.2778	0.3961	0.3979	0.3573
1.0	0.2845	0.3522	0.4107	0.3491
Rate of 2,4-D	0.2548	0.3752	0.4348	
$LSD_{0.05}$ 2,4-D = 0.03294, BA = 0.03294, Interaction BA × 2,4-D = 0.05705				

The BA at the concentration of 0.5 mg L^{-1} provided the highest dry- induced callus (12.033%), followed by BA concentrations of 0.0 and 1.0 mg L^{-1}, which provided at par dry substance ratios of 11.576% and 11.451%, respectively. Thus, the BA concentration of 0.5 mg L^{-1} has a preference over the rest of the BA growth regulator's concentrations by giving the highest dry matter of induced callus obtained from the chia plant leaves.

As for the interaction between 2,4-D and BA, results showed a significant effect in the percentage of the dry matter of induced callus (Table 3). The 2,4-D at the concentration of 3.0 mg L^{-1} exceeded the concentration of 0.5 mg L^{-1} with the highest dry substance percentage (14.240%). However, the interaction between the 2,4-D (1.0 mg L^{-1}) and BA gave the lowest percentage rate of dry substance of induced callus obtained from the chia plant leaves (10.068%).

2,4-D and BA effects on the fresh weight of maintenance callus

The 2,4-D various concentrations showed a significant effect for the fresh weight, with the 2,4-D at the concentration of 3.0 mg L^{-1} exceeding by giving the highest rate of the fresh weight (0.4348 g), followed by the 2,4-D (2.0 mg L^{-1}) at 0.3752 g (Table 4). However, the concentration of 2,4-D (1.0 mg L^{-1}) gained the lowest average fresh weight (0.2548 g) of the induced callus obtained from the chia plant leaves. The results also indicated no significant differences among the BA concentrations for the average fresh weight of induced callus.

The interaction effects between growth regulators, the 2,4-D (concentration of 3.0 mg L^{-1})and BA (with 0.0 concentration) gave the highest fresh weight induced callus (0.4957 g), followed by the interaction of 2,4-D (3.0 mg L^{-1})and BA (1.0 mg L^{-1}) with a fresh weight of 0.4107 g (Table 4). In contrast, the interaction of 2,4-D (1.0 mg L^{-1}) with BA (0.0 mg L^{-1}) displayed the lowest average fresh weight of the induced callus (0.220 g).

2,4-D and BA effects on the dry weight of maintenance callus

The results enunciated that the growth regulator 2,4-D concentrations revealed a significant effect on the average dry weight of the induced callus (Table 5, Figure 2). The 2,4-D concentration at the rate of 3.0 mg L^{-1} disclosed the highest average dry weight of the induced callus (0.04994 g), followed by the 2.0 mg L^{-1} concentration with an average dry weight of 0.04276 g. The 2,4-D concentration at 1.0 mg L^{-1} expressed the lowest average dry weight (0.02750 g). The growth regulator BA concentrations showed no significant differences for the dry weight of the induced callus.

The results further presented that the interaction effects between the various concentrations of 2,4-D and BA proved significant on the dry weight of the induced callus (Table 5). The interaction of 2,4-D (3.0 mg L^{-1}) and BA (0.0 mg L^{-1}) treatments had

Table 5. Effect of 2,4-D and BA with different concentrations and their interaction on the perpetuation of induced callus (dry weight - g) obtained from the chia leaves after four weeks of planting (starting weight: 150 mg).

Concentration BA (mg L^{-1})	Concentration 2,4-D (mg L^{-1})			Rate of BA
	1.0	2.0	3.0	
0.0	0.02191	0.04094	0.06020	0.04102
0.5	0.02816	0.04318	0.04331	0.03822
1.0	0.03242	0.04416	0.04630	0.04096
Rate of 2,4-D	0.02750	0.04276	0.04994	
$LSD_{0.05}$ 2,4-D = 0.004152, BA = 0.004152, Interaction BA × 2,4-D = 0.007191				

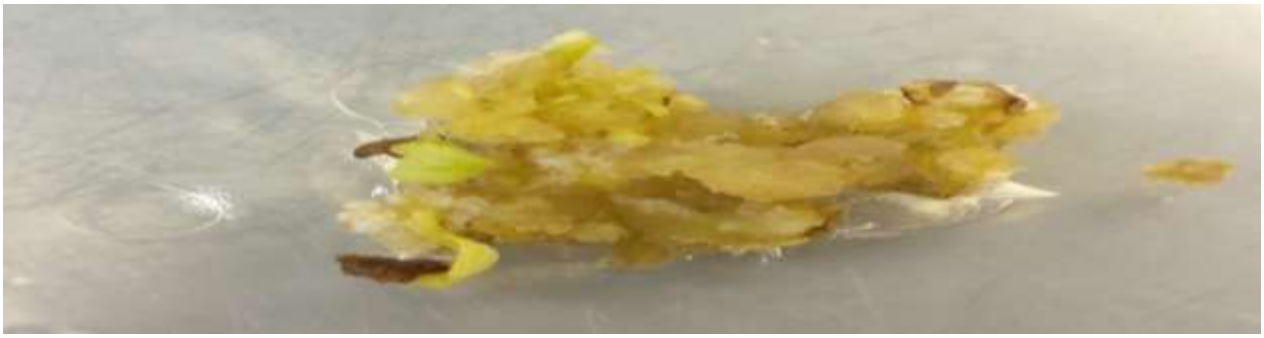

Figure 2. Callus obtained from the chia plant in the perpetuation stage.

the most effect with the maximum dry weight of the induced callus at 0.06020 g, followed by the interaction of 2,4-D (3.0 mg L^{-1}) with BA (1.0 mg L^{-1}) at 0.04630 g. On the other hand, the 2,4-D concentration at the rate of 1.0 mg L^{-1} combined with BA (0.0 mg L^{-1}) had the lowest average dry weight of the induced callus (0.02191 g).

Salicylic acid effects on the production of secondary compounds

The differences in the active ingredients and secondary metabolites extracted from the growing callus tissue on the nutrient medium free of salicylic acid (0.0 mg L^{-1}) appear in Table 6. The outcomes of the GC/MS analysis indicated the dominance of the compound cis-13-octadecenoic acid, methyl ester with an area of 28.12% and a retention time of 22.86, followed by oleic acid, with an area of 24.57% and a retention time of 23.31. The compound, Cyclohexane 1-(1,5-dimethyl hexane)-4(4-methyl pentyl-) provided the lowest area ratio of 0.46% with a time of 29.302.

Table 7 presents the differences in the secondary metabolites extracted from the developing callus tissue on the nutrient media supplied with salicylic acid (10 mg L^{-1}). The GC/MS analysis results showed the superiority of the oleic acid compound, with an area ratio of 57.61% and a retention time of 23.347, followed by the Octadecanoic acid compound, with an area of 7.38% and a retention time of 23.589. The n-Hexadecanoic acid closely followed the above compounds with an area ratio of 7.18% and a retention time of 21.218, pursued further by cis-Vaccenic acid with the lowest area ratio of 0.48% and a time of 29.743.

Table 8 shows the different active ingredients extracted from developing callus tissue on the nutrient medium with salicylic acid (20 mg L^{-1}). The GC/MS analysis results showed the superiority of the 9-Octadecenoic acid compound, methyl ester, with an area ratio of 33.13% and a retention time of 22.862, followed by oleic acid with an area ratio of 22.64% and a retention time of 23.304. The two compounds, i.e., 2-Methyl-Z, Z-3, 13-octadecadienol, and Cyclopentadecanone, 2-hydroxy recorded the lowest area ratio of 0.47% with a retention time of 22.611 and 24.818, respectively.

The differences in the active ingredients extracted from the developing callus tissue on the nutrient medium supplied with salicylic acid (30 mg L^{-1}) emerge in Table 9. The GC/MS analysis findings identified the superiority of the oleic acid, with an area of 36.82% and a retention time of 23.312, followed by the compound 2-Amino-5-methyl

Table 6. Retention time, area, and height of secondary metabolites in chia leaf callus grown on MS medium supplied with salicylic acid (0.0 mg L^{-1}).

Sequence	Active compounds	Ret. Time (min.)	Area (%)	Height (cm?)
1	Acetic acid, hydroxy-, ethyl ester	4.054	0.50	409560
2	Silane, trimethyl(phenylmethoxy)-	4.686	1.32	2152707
3	3-Amino-5-(3-indolyl)-4-pyrazoleca rbonitrile	5.162	0.70	382673
4	2-Methoxy-2'-methyl-stilbene	6.045	0.47	482773
5	Cyclotetrasiloxane, octamethy	6.651	0.61	242591
6	Benzaldehyde, 2,5-bis[(trimethylsi lyl)oxy]-	8.849	0.61	427764
7	1-Piperidinecarboxaldehyde	11.662	0.77	721214
8	Dodecanoic acid, methyl ester	15,609	0.49	984556
9	Hexadecanoic acid, methyl ester	20,776	5.91	10507034
10	n-Hexadecanoic acid	21.209	2.15	2475191
11	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	22.767	5.80	8908028
12	Cis-13-Octadecenoic acid, methyl ester	22.862	28.12	35663216
13	9-Octadecenoic acid, methyl ester	22.931	6.59	4014290
14	Methyl stearate	23.122	3.40	4849824
15	Oleic Acid	23.312	24.57	13008513
16	n-Propyl 11-octadecenoate	23,572	4.83	2056527
17	8,11-Octadecadienoic acid	23.823	4.28	3054915
18	Cyclopropaneoctanal, 2-octyl	24.039	1.44	445557
19	Ethanol, 2-(9,12-octadecadienyloxy	24.273	1.95	596621
20	Cis-Vaccenic acid	24.550	0.68	237449
21	2-Methyl-Z,Z-3,13-octadecadienol	24.810	0.66	198496
22	Cis-13-Eicosenoic acid	25.043	1.14	723014
23	Cyclohexanecarboxylic acid	26.818	1.34	1507386
24	Oleic Acid	28.999	1.39	541086
25	Cyclohexane, 1-(1,5-dimethylhexyl)- 4-(4-methylpentyl -)	29.302	0.46	289960

Table 7. Retention time, area, and height of secondary metabolites in leaf callus of chia plant grown on MS medium supplied with salicylic acid (10 mg L^{-1}).

Sequence	Active compounds	Ret. Time (min.)	Area (%)	Height (cm?)
1	Cycloheptasiloxane, tetradecamethy	13.999	0.83	577578
2	Pentasiloxane, dodecamethyl	14.614	0.47	118673
3	Cyclooctasiloxane, hexadecamethyl	16.821	0.58	601666
4	Cyclooctasiloxane, hexadecamethyl	16.968	0.47	218816
5	Cyclononasiloxane, octadecamethyl	18.950	0.54	333917
6	9-Octadecenal, (Z)-	19.166	0.47	378434
7	Cis-9-Hexadecenal	20.439	0.89	805956
8	Methyl 9-methyltetradecanoate	20.759	0.82	795098
9	n-Hexadecanoic acid	21.218	7.18	5931269
10	13-Octadecenal, (Z)-	21.651	0.52	262846
11	2-Methyl-Z,Z-3,13-octadecadienol	22.611	0.52	426679
12	9-Octadecenoic acid (Z)-, methyl ester	22.854	3.59	2938910
13	Oleic Acid	23.347	57.61	19654086
14	Octadecanoic acid	23.589	7.38	4878580
15	9,12-Octadecadienoic acid (Z,Z)-	23.814	4.99	1658260
16	Z-4-Nonadecen-1-ol acetate	24.013	1.84	516935
17	9,12-Octadecadienoic acid (Z,Z)-	24.247	4.29	1998471
18	11-Dodecen-1-ol trifluoroacetate	24.515	0.60	329212
19	8-Methyl-6-nonenamide	25.684	1.17	852509
20	13-Tetradecenal	26.333	0.71	460074
21	Oleic Acid	26.826	1.56	1394531
22	Cinnamyl cinnamate	28.523	0.71	469656
23	Cyclopentane, 1-(2-decyldodecyl)-2,4-dimethyl	28.705	0.81	382597
24	6-Octadecenoic acid, (Z)-	28.990	0.95	231097
25	Cis-Vaccenic acid	29743	0.48	305417

Table 8. Retention time, area, and height of secondary metabolites in chia leaf callus grown on MS medium supplied with salicylic acid (20 mg L^{-1}).

Sequence	Active compounds	Ret. Time (min.)	Area (%)	Height (cm?)
1	Acetic acid, hydroxy-, ethyl ester	4.175	1.28	104369
2	Silane, trimethyl(phenylmethoxy)-	4.591	1.03	511453
3	6H-Purin-6-one, 2-amino-1,7-dihydr o-1-methyl	4.721	2.29	640969
4	Oxime-, methoxy-phenyl	5.240	1.79	1639195
5	Acetic acid, N'-[3-(1-hydroxy-1-phenylethyl) phenyl]hydrazide	6.010	0.97	237896
6	Cyclotetrasiloxane, octamethyl	6.659	0.78	128,344
7	Phenanthridinium, 5,6-dimethyl odide	7.750	0.74	290770
8	1H-Trindene, 2,3,4,5,6,7,8,9-octah ydro-1,1,4,4,9,9-hexamethyl	8.841	1.20	590733
9	Hexanoic acid, 2-methyl-	9.360	0.62	147613
10	Hexadecanoic acid, methyl ester	20.776	5.85	6013238
11	Octadecanoic acid	21.244	2.70	1338606
12	2-Methyl-Z,Z-3,13-octadecadienol	22.611	0.47	223053
13	9,12-Octadecadienoic acid	22.758	3.89	2979475
14	9-Octadecenoic acid, methyl ester	.22.862	33.13	21077111
15	Methyl stearate	23.113	3.91	2872405
16	Oleic Acid	23.304	22.64	6382346
17	cis-Vaccenic acid	23,572	5.54	1188495
18	8,11-Octadecadienoic acid, methyl ester	23.823	4.66	1729184
19	9-Octadecenoic acid	24.031	1.56	250294
20	Cyclopropaneoctanal, 2-octyl-	24,282	1.99	300367
21	Butyl 9-tetradecenoate	24.567	0.68	105890
22	Cyclopentadecanone, 2-hydroxy	24.818	0.47	88343
23	9-Octadecenoic acid, (E)-	25.069	0.62	357771
24	1,2,5-Oxadiazol-3-amine, 4-(3-meth oxyphenoxy)-	29.007	0.71	179618
25	Oleic Acid	29.293	0.48	172259

Table 9. Retention time, area, and height of secondary metabolites in chia leaf callus grown on MS medium supplied with salicylic acid (30 mg L^{-1}).

Sequence	Active compounds	Ret. Time (min.)	Area (%)	Height (cm?)
1	Arsenous acid, tris(trimethylsilyl ester	3.821	1.93	353781
2	Oxime-, methoxy-phenyl-_	5.084	1.04	185012
3	2-Amino-5-methylbenzoic acid	5.552	21.15	11453748
4	10-Amino-10,11-dihydro-5-acetyldibenz[b,f]azepine	6.045	2.30	385127
5	Formamide, N-methylthio	6.504	0.78	915877
6	Cyclotetrasiloxane, octamethyl	6.772	2.79	507640
7	Tris(tert-butyldimethylsilyloxy) sane	8.304	0.82	400954
8	1,2-Bis(trimethylsilyl)benzene	8.443	4.02	3797993
9	1H-Trindene, 2,3,4,5,6,7,8,9-octah ydro-1,1,4,4,9,9-hexamethyl	8.849	2.46	1294800
10	4-Ethylbenzoic acid, 6-ethyl-3-oct yl ester	9.317	1.17	1368499
11	Naphthalen-1-yl(1-pentyl-1H-indol3-yl)methanone	9.421	0.48	319830
12	3,5-Dimethoxy amphetamine	9.741	0.47	490357
13	Benzoic acid, 4-methyl-2-trimethyl silyloxy-, trimethylsilyl ester	10.953	0.90	393421
14	Fluoren-9-ol, 3,6-dimethoxy-9-(2-henylethynyl)-	11.160	0.77	744528
15	Cyclotrisiloxane, hexamethyl	11.706	0.87	719115
16	(-)-Epinephrine, tris(trimethylsil yl) ether	13.246	0.78	283767
17	n-Hexadecanoic acid	21.201	4.25	2980086
18	3-Trifluoroacetoxypentadecane	21.642	0.48	107685
19	13-Octadecenal, (Z)-	22.862	0.98	484339
20	Oleic Acid	23.312	36.82	11 11783892
21	Oleic Acid	23.563	6.59	2398917
22	13-Oxabicyclo[10.1.0]tridecane	23.866	4.44	671755
23	9,12-Octadecadien-1-ol, (Z,Z)-	24.256	2.58	604239
24	cis-Vaccenic acid	28.757	0.64	108753
25	Bromoacetic acid, octadecyl ester	28.973	0.50	114,438

benzoic acid, with an area of 21.15% and a retention time of 5.552. Moreover, the compound 3,5-Dimethoxy amphetamine recorded the lowest area ratio of 0.47% with a retention time of 9.741.

DISCUSSION

The results showed the significant effects of the growth regulator 2,4-D treatments with different concentrations for fresh and dry weight and dry matter percentage of induced callus tissues obtained from the chia plant leaves. These results can have interpretation on the basis that the addition of Auxin to the nutrient media plays an important role in the development of calli from plant parts and the addition of Cytokinin to the media in the presence of Auxin leads to an enhancement in callus growth and cell division (Hartmann *et al*., 1997). The importance of Auxin in inducing callus comes through its effect on the enzymes responsible for building and degrading the cell wall, which affects the mechanical properties of the cell wall and the cell division, thus the formation of callus (Taiz and Zeiger, 2006). It could be due to 2,4-D as the most active auxin in cell division and callus formation on various plant parts (Fahmy, 2003; George *et al*., 2008).

The addition of growth regulators to the MS nutrient medium attests to the importance of stimulating the callus and its physiological effect and biological activity in cell division and growth (Davies, 2004). The interaction between the auxin growth regulator 2,4-D and cytokinin BA is essential for the induction of callus, whereby cytokinin in the presence of auxin acts as a key to initiating cell division--the adenine in the cytokinin molecule may have an essential role in building the nucleic acids needed for mitosis (George *et al.,* 2008). These results also agree with the findings of AL-Arady *et al.* (2020), who found 2,4-D (3.0 mg L^{-1}) as the best growth regulator for the induction of callus from terminal buds of sugarcane plants. Al-Taha *et al*. (2018) also obtained the best callus from the stems of *Aloe vera* grown in the medium supplied with a 2,4-D concentration at a rate of 3.0 mg L^{-1} and 0.2 mg L^{-1} of BA.

The results of the separation using MS/GC technology showed differences in the percentages of the active compounds of the callus growing on the culture medium supplied with salicylic acid at different concentrations (0, 10, 20, and 30 mg L^{-1}) (Tables 6, 7, 8, 9, and Figures 3, 4, 5, 6). The results showed the analysis of the 25 secondary compounds in the callus and in percentages that vary from one concentration to another.

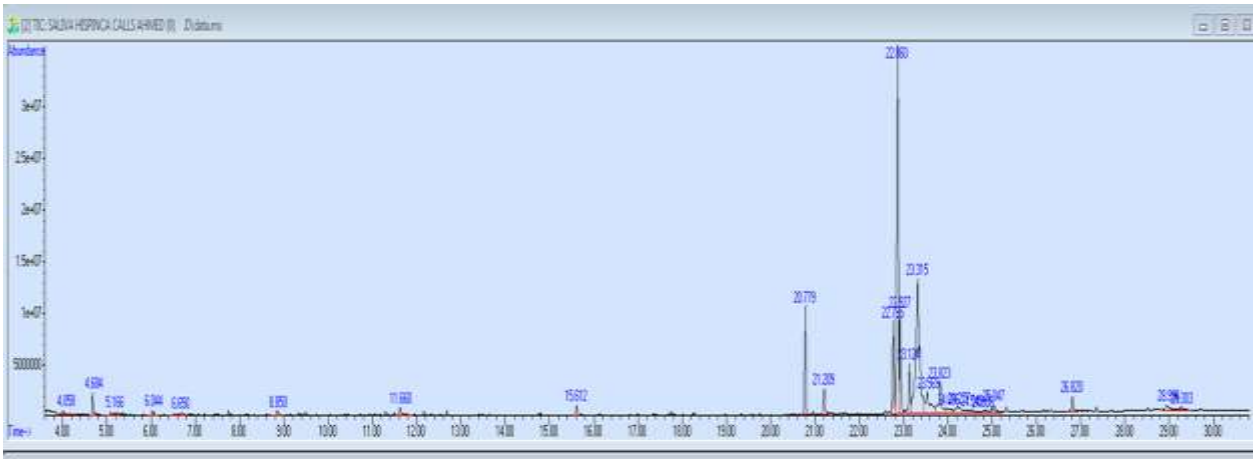

Figure 3. Effect of salicylic acid (0.0 mg L^{-1}) in the production of secondary metabolites from the callus of the chia leaf.

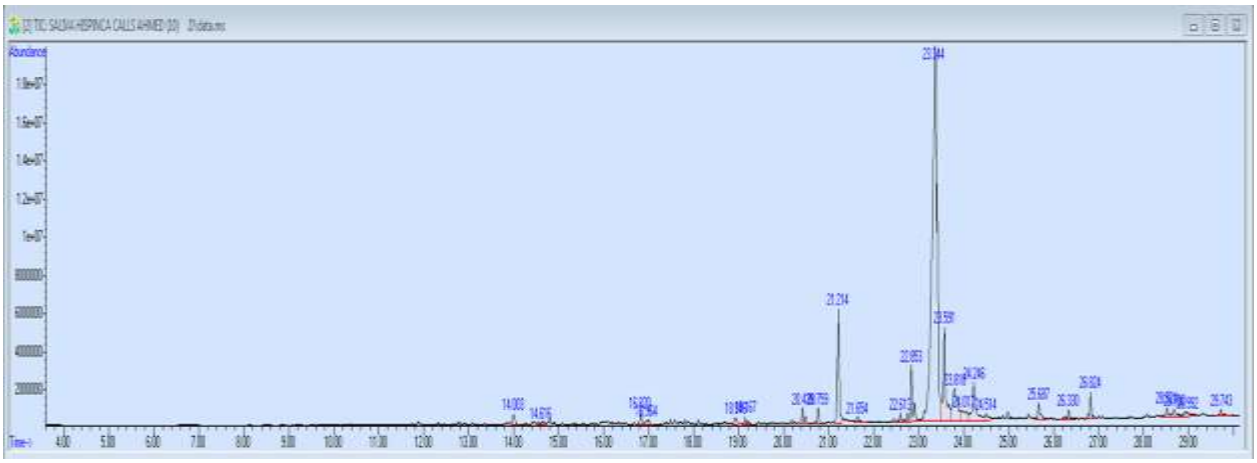

Figure 4. Effect of salicylic acid (10 mg L^{-1}) in the production of secondary metabolites from the callus of the chia leaf.

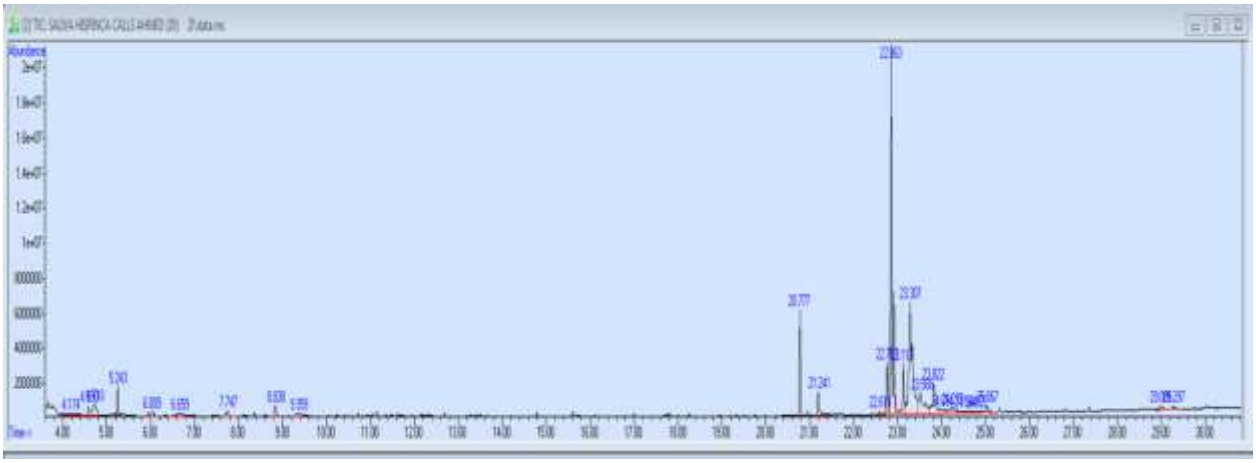

Figure 5. Effect of salicylic acid (20 mg L^{-1}) in the production of secondary metabolites from the callus of the chia leaf.

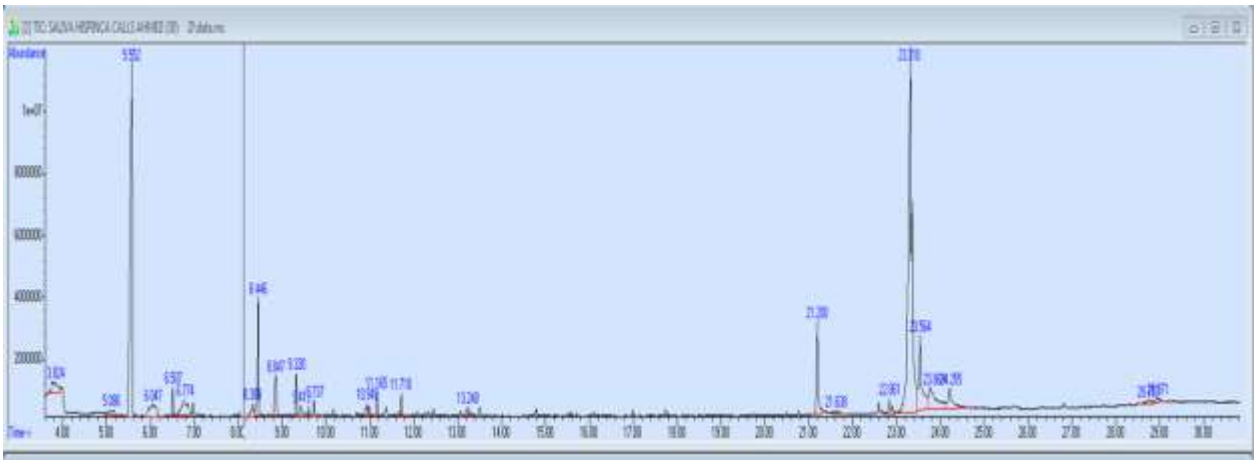

Figure 6. Effect of salicylic acid (30 mg L^{-1}) in the production of secondary metabolites from the callus of the chia leaf.

This study shows the possibility of using salicylic acid to stimulate the production of some secondary metabolic compounds with a fundamental difference in the production of compounds, especially for oleic acid. The salicylic acid at 10.0 mg L^{-1} notably records with the maximum increase in the compound, oleic acid. The increase in secondary compounds was associated with an increase in the concentration of salicylic acid to a certain level, as it acts to regulate various physiological and biochemical processes in plants (Orabi *et al*, 2015). Salicylic acid also plays a vital role in the signal transduction process that stimulates the biosynthesis of phenolic compounds. It may lead to an increased tissue content of defense-related compounds, such as, total phenols, flavonoids, and alkaloids. As a result, it further increases the levels of gene expression responsible for the biosynthesis of these compounds (Al-Khafaji, 2014).

CONCLUSIONS

The results confirmed the superiority of the growth regulator 2,4-D (3.0 mg L^{-1}) by giving the highest rate of the fresh and dry weight of induced callus from chia leaves without adding BA. The interaction of BA (0.5 mg L^{-1}) and 2,4-D (3.0 mg L^{-1}) achieved the highest dry matter percentage. Results also favor the value of adding cytokinin in the induction stage and the possibility of using salicylic acid to stimulate the active compounds in the induced callus obtained from the chia plant leaves.

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