Article

Production of Black Cumin *via* Somatic Embryogenesis, Chemical Profile of Active Compounds in Callus Cultures and Somatic Embryos at Different Auxin Supplementations

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Citation: Higazy, A.E.; El-Mahrouk, M.E.; El-Banna, A.N.; Maamoun, M.K.; El-Ramady, H.; Abdalla, N.; Dobránszki, J. Production of Black Cumin *via* Somatic Embryogenesis, Chemical Profile of Active Compounds in Callus Cultures and Somatic Embryos at Different Auxin Supplementations. *Agronomy* **2023**, *13*, 2633. https://doi.org/10.3390/agronomy13102633

Academic Editors: Justyna Lema-Rumińska, Danuta Kulpa, Alina Trejgell and Moshe Reuveni

Received: 1 September 2023
Revised: 14 October 2023
Accepted: 16 October 2023
Published: 17 October 2023

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Abstract: Black cumin or *Nigella sativa* L. is a medicinal plant of the Ranunculaceae family that has enormous importance. It has traditionally been used to cure a lot of diseases since ancient times. In the current study, the effects of different auxins on callus induction and subsequent somatic embryo formation of *N. sativa* L. cv. Black Diamond were examined. The best result of callus induction was observed when cotyledon explants were incubated in a Murashige and Skoog (MS) medium supplemented with 1.0 mg L^{-1} α-naphthaleneacetic acid (NAA). The formation of somatic embryos was achieved efficiently from cotyledon-derived calli cultured on a 2 mg L^{-1} Indole-3-butyric acid (IBA)-containing medium. Furthermore, histological analysis of embryogenic calli was used to detect the presence of different developmental stages of somatic embryos. In contrast to the calli and embryos of *N. sativa* 'Black Diamond', which initiated in the dark, light was necessary for the complete differentiation of callus and embryo cultures into shoots/developed plants. Hypocotyl-derived calli and embryos were successfully differentiated on IBA at 2.0, 1.0 mg L^{-1}, and NAA at 2.0 mg L^{-1}. To the best of our knowledge, this work can be considered the first report on the differentiation of *N. sativa* 'Black Diamond' somatic embryos into developed plants. Moreover, the metabolic profiles of secondary products of *N. sativa* 'Black Diamond' callus and embryo cultures originated from the best auxin treatments identified and were compared with that of intact seeds. Callus cultures of *N. sativa* 'Black Diamond' contained thymoquinone (TQ) in a significant percentage of the peak area (2.76%). Therefore, callus cultures could be used as a perfect alternative source of TQ for pharmaceutical and therapeutic purposes. In addition, fatty acids and/or their esters were recorded as the major components in callus and embryo cultures. These vital compounds could be isolated and used for numerous industrial applications.

Keywords: black cumin; Ranunculaceae; medicinal plants; in vitro cultures; auxins; phytochemicals; gas chromatography; thymoquinone; antibacterial; anticancer; antioxidant

1. Introduction

Nigella sativa L. is classified as one of the most important medicinal plants containing volatile and fixed oils in its seeds. It is an annual herbaceous plant belonging to the Ra-

nunculaceae family [1]. It is widely grown for its black seeds in the countries bordering the Mediterranean Sea, Middle East, Southern areas of Europe, Pakistan, Iran, India, and Egypt due to its nutritional, medicinal, and industrial properties [2,3]. It originated from South and Southwest Asia, North Africa, and the Mediterranean region [4]. *N. sativa* seed is described as a medicinal herb. It has largely been used in folk medicine in Arabic and Asian regions for the remediation of numerous ailments, such as cough, fever, headache, toothache, gastrointestinal problems, diarrhea, rheumatism, influenza, diabetes, and hypertension [5]. Due to the highly valuable functional nutrients in black cumin seed, its extract can fortify yogurt [6], honey [7], can be used as a putative therapeutic agent [8], or a supplementary in the broiler industry [9].

Recently, *N. sativa* has drawn the attention of scientists to the therapeutic values and pharmacological effects of its seeds. The seeds have a wide range of biological active secondary metabolites, containing TQ, dithymoquinone (DTQ), thymohydroquinone (THQ), thymol, and carvacrol, which have pharmaceutical potential [10–12]. Analgesic, anti-inflammatory, anti-allergic, anticancer, anti-asthmatic, hypoglycemic, hypotensive, antioxidant activity, hepatoprotective effect, immunity stimulation, and antifungal potential have been reported for this important medicinal plant [13]. However, TQ, the essential component of *N. sativa* oil, is the most important one among the other isolated compounds. TQ exhibited significant antibacterial potential by inhibiting the bacterial biofilm formation against several human pathogenic bacteria, and it showed anticancer potential and hepatoprotective activity as well [14–16].

Medicinal plants are rich resources of naturally occurring bioactive compounds that are widely used as food additives, medicaments, agrochemicals, and perfumes [17]. However, secondary metabolites have various biological properties; their biosynthesis depends on genetics, geographical area, climate, and environmental conditions. In addition, their allocation is very restricted compared to primary metabolites, and many of these compounds occur in nature in very low quantities. Therefore, great efforts have been made via plant biotechnological approaches towards optimizing the culture conditions to maximize the secondary metabolite production needed to support industrial production [17]. Biotechnological investigations on this plant species have been carried out [10]. These studies focused on callus induction for secondary metabolite production [18–22]; callus differentiation into regenerated shoots [23]; phytochemical elicitation in callus cultures under salinity stress [4]; enhancing somatic embryogenesis (SE). However, the conversion of somatic embryos into shoots was not detected [24]. Callus and embryo cultures could be employed to produce valuable phytochemicals in a short period of time, under controlled and sterile conditions and even out of the growing season [18]. The extract obtained from callus cultures of *N. sativa* showed considerable antimicrobial activity against some bacterial strains. Moreover, thymol content in the extract of callus cultures was examined [10]. Somatic embryogenesis was proven to be an important technique that offers an alternative pathway for germplasm conservation, mass clonal propagation of elite plants in a short time, genetic transformation, and synthetic seed production [25].

The previous reports proved that the requirements of plant growth regulators (PGRs) needed for inducing somatic embryogenesis depend on certain cultivars or genotypes [26]. It is known that exogenously applied auxins could enhance somatic embryogenesis by affecting the endogenous content of auxins in the cultured explant or tissue such as IAA [26]. In addition, the mechanism of natural accrued auxin on SE is related to the type of exogenous auxin, which added to the medium [27]. The effect of synthetic auxins on SE could be observed as NAA in *Picea abies* and *P. omorika* [28], 2,4-D (2,4-dichlorophenoxyacetic acid) in Coffea [29], and IBA in *Digitalis lanata* [30].

Therefore, the present investigation aimed to study the influence of exogenous auxins (i.e., IBA, NAA, and 2,4-D), added separately in various concentrations to MS medium [31], on the induction of callus and somatic embryos in *N. sativa* 'Black Diamond' from hypocotyl and cotyledon explants. Moreover, we aim to evaluate the metabolomic profile of *N. sativa* 'Black Diamond' calli and somatic embryos compared to seeds, which are traditionally

used in the pharmacy. This is in order to examine and enhance the accumulation of the secondary natural products in these types of tissues.

2. Materials and Methods

This study was conducted in 2021 at the Physiology and Breeding of Horticultural Crops Laboratory, Department of Horticulture, Faculty of Agriculture, Kafrelsheikh University, Egypt.

2.1. Plant Material and Seed Germination

Seeds of pure diploid (2 n = 12) line, of *Nigella sativa* L. cv. Black Diamond, originating from more than five years self-pollination in breeding program at the Faculty of Agriculture, Kafrelsheikh University, Egypt, were used as starting plant material in this research [32]. Black Diamond is improved cultivar originating from local cultivar under registration as the first commercial cultivar in Egypt. Seeds were kept (cold stratification) at 4 °C for three weeks to break dormancy and in order to enhance germination. The cold treatment was reported to be the most efficient for optimal seedling growth [33]. After then, seeds were washed thoroughly using tap water that contained few drops of polyoxyethylene-sorbitan monolaurate ((Tween-20), Loba Chemical Company, Mumbai, India). In the laminar air flow, under aseptic conditions, the seeds were surface sterilized by using 70% ethanol for 2 min then dipped for 15 min in 0.1% mercury chloride ($HgCl_2$) containing 2–3 drops of Tween-20. After rinsing 3 times with sterile-distilled water, seeds were cultured for germination in 350 mL glass jars containing 50 mL of half-strength MS basal medium containing 3% (*w*/*v*) sucrose, 0.7% (*w*/*v*) Duchefa agar-agar (Hofmanweg 71, 2031 BH Haarlem, The Netherlands). The pH of the medium was set at 5.8, and then the medium was autoclaved for 20 min at 121 °C. The cultures were incubated at 22 $\pm$ 2 °C for 10 days under dark conditions followed by 16/8 h photoperiod supplied by cool-white fluorescent lights at photosynthetic photon flux density (PPFD), 30 μmol m^{-2} s^{-1}, for 10 days.

2.2. Callus Induction and Differentiation, and Somatic Embryo Formation and Conversion to Plantlets

Hypocotyl and cotyledon explants were taken from 3-week-old in vitro seedlings of *N. sativa* 'Black Diamond', and were used for callus induction and somatic embryo formation. All explants were cut into 2–3 mm long segments and thereafter cultured on MS medium including sucrose (3%) *w*/*v*, 0.7% (*w*/*v*) agar, and supplemented with different concentrations of IBA, NAA, and 2,4-D at 0, 1, 2, and 3 mg L^{-1}, added separately to the different media. Plant growth regulator free (PGR-free) medium was considered as a control. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min. Explants were inculcated in sterile glass Petri dishes (70 $\times$ 15 mm) containing 25 mL of medium. Cultures were maintained at 22 °C in the dark. Callus percentage (%), callus fresh weight (g) and callus diameter (cm), somatic embryo percentage (%), and number of somatic embryos per callus were recorded after six weeks of culture. After then, the cultures were kept at 25 °C and 30 μmol m^{-2} s^{-1} PPFD light intensity and 16 h/d lighting period. Callus differentiation percentage and embryo conversion to plantlet percentage were recorded after 6 weeks of culture. All cultures were sub-cultured after three weeks on the same medium in dark and light conditions, accordingly.

Equations used in the current study:

Callus percentage (%) = (No. of explant gave callus/total No. of explants) $\times$ 100

Somatic embryo percentage (%) = (No. of explant gave embryo/total No. of explants) $\times$ 100

Callus differentiation percentage (%) = (No. of callus gave shoot/total No. of callus) $\times$ 100

Embryo conversion to plantlets percentage (%) = (No. of embryo conversion to plantlets/Total No. of embryo) $\times$ 100

The tissue culture experiments were factorial from three factors (auxin type and concentration, and type of explant), and they were organized in a completely randomized design.

Three auxins, four concentrations, and two explant types were examined in the current study. There were 10 explants per Petri dish (replicate) and 4 replicates per treatment.

2.3. Histological Study of the Embryogenic Callus

The histological analysis was carried out according to Boissot et al. [34]. Embryogenic calli were isolated from 6-week-old callus culture of *N. sativa* 'Black Diamond'. Embryos were fixed for 24 h in a solution of absolute alcohol, glacial acetic acid, and formaldehyde (90: 5: 5, *v*/*v*/*v*). Then, the samples were desiccated in a graded sequence of ethanol (70, 95, and 100%) for 1 h each; after that, they were embedded in paraffin wax. Sections of thickness of 15–20 µm were obtained using a rotary microtome (American optical rotary microtome, model 820, New York, NY, USA) and fixed to the slides with albumin. Sections were stained in toluidine blue for 12 h. Then, they were cleared in xylol and mounted in Canada balsam to be ready for microscopic examination. Ten sections were made for each sample on one slide, and then the best section was photographed. Observations were made using a Leica Aristoplan light microscope (Neu-Isenburg, Germany) with Leica DC 300 F digital imaging.

2.4. Gas Chromatography Analysis of Extracts from Seeds, Calli, and Embryos

The extracts were prepared from 2 g of seeds, callus, and somatic embryos of *N. sativa* 'Black Diamond' at globular stage; the latter ones originated from the best two treatments for callus induction (1 and 3 mg L^{-1} NAA) and embryo (2 and 3 mg L^{-1} IBA) developed from cotyledon explant, examined via gas chromatography (GC-MS) analysis. The samples were taken from three replicates. All samples were dried and finely powdered using an electrical grinder (Moulinex—French-DP706G Zerkleinerer La Moulinette Deluxe, France) and soaked in chloroform–methanol (C/M 2:1 *v*/*v*) in a ratio of 1:5 (*w*/*v*) at ordinary room temperature. The mixture of solvent and samples was covered with aluminum foil. After this, it was shaken for 24 h. The mixture was filtered using a Bucher funnel, and the residue was pressed to obtain a maximum amount of the filtrate. The combined filtrates were mixed with 2.5 g anhydrous sodium sulfate (Na_2SO_4) to remove traces of water and kept in a refrigerator for 2 h. After that, the extract was filtered through a Whitman filter paper (No. 1) and evaporated until dryness on a rotary evaporator (Heidolph—Laborota 4000 eco, Darmstadt, Germany) at 40 °C to remove chloroform, transferred into glass dark bottles, and kept upon completion of the oil for subsequent analysis. Finally, each extract was stored in a refrigerator at 4 °C [35]. Analysis of the extracts were carried out using Gas Chromatography GC-HP (Hewlett Packard, Palo Alto, CA, USA) 6890, with FID detector (flame ionizing) and DB-23 Column (50%—cyanopropyl—methylpolysiloxane), 30 m × 0.32 mm, ID = 0.25 µm film thickness. The carrier gas was nitrogen (1 mL min^{-1} gas flow).

2.5. Statistical Analyses

The obtained results of tissue culture experiments (callus and embryo induction, differentiation, and development) were analyzed using multiple-way ANOVA. ANOVA analysis was conducted using CoStat (version 6.311) statistical CoHort software (Berkeley, CA, USA). The mean separations were conducted using LSD and Duncan's multiple range tests, and significance was measured at $p \leq 0.05$.

3. Results

3.1. Callus Induction and Somatic Embryo Formation

The obtained data in Table 1 showed highly significant differences for the triple interaction between the three factors under study on callus induction measurements. Callus was induced on all tested media from both hypocotyl and cotyledon explants of *N. sativa* 'Black Diamond'. Callus was also initiated on the PGR-free medium in 40–50% from both studied explants. The highest value of callus percentage (100%) was recorded for the cotyledon explant when cultured on an MS medium supplemented with 2.0 mg L^{-1} IBA or 1.0 mg L^{-1} NAA. Callus diameter was the highest (2.7 cm) for hypocotyl explant

cultured on MS medium supplemented with 1.0 mg L^{-1} NAA. Explants produced the highest callus fresh weight (4.4 for hypocotyl and 4.5 for cotyledon, respectively) on the medium described above. The best treatment noticed for callus induction was the MS medium supplemented with 1.0 mg L^{-1} NAA followed by 3.0 mg L^{-1} NAA, and cotyledon was the superior explant (Figure 1A). Regarding embryo formation from calli derived from hypocotyl or cotyledon explants, the embryo percentage and number of embryos had high significant differences for the triple interaction between the three factors studied. Both the hypocotyl and cotyledon explants achieved a maximum value for the embryo percentage (98%) when were cultured on the MS medium supplemented with 2.0 mg L^{-1} IBA or NAA as well as 3.0 mg L^{-1} IBA. Calli derived from either hypocotyl or cotyledon explants failed to produce embryos on the MS medium supplemented with 1.0 or 2 mg L^{-1} 2,4-D. The number of embryos was significantly enhanced for the cotyledon explant cultured on IBA-supplemented media (2.9 or 2.8 embryos) and for hypocotyl on the 3 mg L^{-1} IBA-containing medium (2.8 embryos). The best results for embryo formation were obtained for cotyledon-derived calli on 2 mg L^{-1} IBA followed by 3 mg L^{-1} IBA-containing media (Figure 1D).

Table 1. Effect of some auxins added at different concentrations on callus induction and embryo formation from hypocotyl and cotyledon explants of *N. sativa* 'Black Diamond'.

Auxins (mg L^{-1})	Callus (%)		Callus Diameter (cm)		Callus Fresh Weight (g)		Embryo (%)		Number of Embryos	
	Hyp.	Cot.	Hyp.	Cot.	Hyp.	Cot.	Hyp.	Cot.	Hyp.	Cot.
IBA										
0.0	50 c	40 c	0.3 i	0.4 i	1.3 i	0.65 i	6.6 f	3.3 f	0.33 c	0.33 c
1.0	99 a	99 a	2.2 de	1.5 g	3.1 g	2.3 hi	67 d	77 c	1.9 b	2.8 a
2.0	99 a	100 a	1.7 f	1.6 fg	3.2 fg	3.3 ef	98 a	98 a	1.9 b	2.9 a
3.0	97 a	99 a	1.7 f	2.2 de	3.2 fg	3.2 fg	98 a	98 a	2.8 a	2.8 a
NAA										
0.0	50 c	40 c	0.3 i	0.4 i	1.3 i	0.65 i	6.6 f	3.3 f	0.33 c	0.33 c
1.0	98 a	100 a	2.7 a	2.4 b	4.4 a	4.5 a	89 b	67 d	2.8 a	1.9 b
2.0	99 a	98 a	1.3 h	1.6 fg	2.4 h	3.5 cd	98 a	98 a	2.7 a	1.9 b
3.0	98 a	98 a	2.4 b	2.3 bcd	3.4 de	4.2 b	78 c	88 b	2.9 a	1.9 b
2,4-D										
0.0	50 c	40 c	0.3 i	0.4 i	1.3 i	0.65 i	6.6 f	3.3 f	0.33 c	0.33 c
1.0	97 a	89 b	2.3 bcd	1.3 h	4.2 b	3.6 c	0 g	0 g	1.9 b	1.9 b
2.0	98 a	89 b	2.4 bc	1.2 h	3.5 cd	2.2 i	0 g	0 g	1.9 b	1.9 b
3.0	97 a	99 a	2.1 e	2.1 e	3.4 de	2.2 i	57 e	68 d	1.8 b	1.9 b
Significance										
A	***		***		***		***		***	
E	**		***		***		***		**	
C	***		***		***		***		N.S	
A × E	***		***		***		***		***	
A × C	***		***		***		***		***	
C × E	***		***		***		***		***	
A × C × E	***		***		***		***		***	

, * significant at $p \leq 0.01$, and 0.001, respectively according to Duncan's multiple range tests followed by ANOVA. Values followed by the same letters in the same column under the two explants were not significantly different. A = auxins; C = concentrations; E = explants. A × C × E indicates the significance of the triple interaction between the three factors (auxins, concentrations and explants); Hyp = hypocotyl and Cot = Cotyledon.

3.2. Differentiation of Calli into Shoots/Plants and Embryo Conversion to Plantlets

Hypocotyl- and cotyledon-derived calli of *N. sativa* 'Black Diamond', initiated in the dark, were differentiated into shoots in light on all media under investigation, except 2,4-D-supplemented media (Table 2; Figure 1B,C). Moreover, the somatic embryos, produced in the dark on the callus originating from both hypocotyl and cotyledon explants, were differentiated into complete developed plants after they were put into light in all tested media, except 2,4-D at 1 or 2 mg L^{-1} (Figure 1D,F). Callus differentiation and embryo conversion to plantlets percentages showed high significant differences for the triple interaction between the three factors studied. Hypocotyl-derived calli were differentiated successfully with the highest significant value (97.7%) on the MS medium supplemented with 2.0 mg L^{-1} IBA. However, the embryos produced on hypocotyl-derived calli were converted to plantlets by percentage (37.7%) on the MS medium supplemented with either 1.0 mg L^{-1} IBA or 2.0 mg L^{-1} NAA, while embryos produced on cotyledon-derived calli were converted to plantlets at percentages of 35.0% and 37.7%, respectively, on the MS medium supplemented with 2.0 or 3.0 mg L^{-1} IBA. On the other hand, the embryos on

embryogenic calli originating from each of the hypocotyl and cotyledon explants did not differentiate on the MS medium supplemented with 1 or 2 mg L^{-1} 2,4-D.

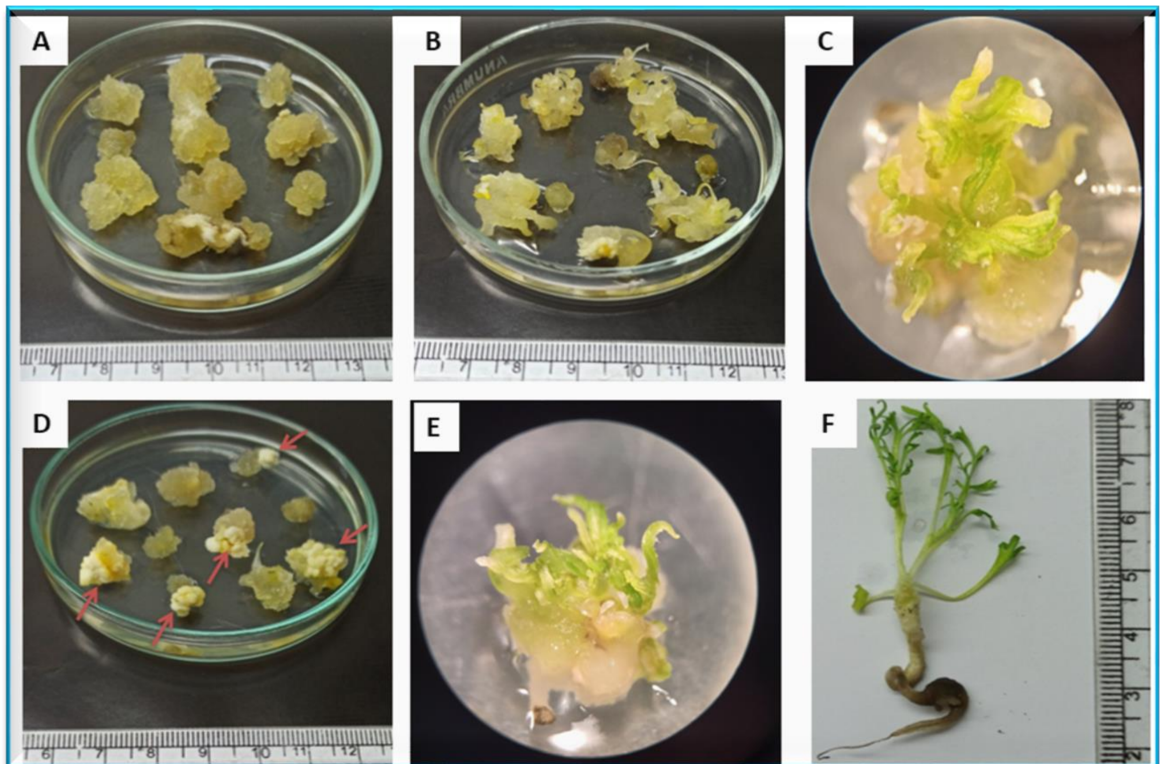


Figure 1. Callus and embryo formation of *N. sativa* 'Black Diamond'; (**A**) callus induction of cotyledon on 1 mg L^{-1} NAA; (**B**) callus differentiation; (**C**) callus differentiation in light conditions (2x * 10x); (**D**) somatic embryogenesis on 2 mg L^{-1} IBA (red arrows refer to embryos); (**E**) embryo conversion to plantlets (2x * 10x); (**F**) complete plantlet developed from somatic embryo.

Table 2. Effect of some auxins at different concentrations on callus differentiation percentage and somatic embryo conversion to plantlet percentage from each of the hypocotyl and cotyledon explants of *N. sativa* 'Black Diamond'.

Auxins (mg L^{-1})	Embryo Conversion to Plantlets (%)		Callus Differentiation (%)	
	Hyp.	Cot.	Hyp.	Cot.
IBA				
0.0	3.3 g	3.3 g	10.0 g	3.3 h
1.0	37.7 a	29.4 c	47.0 c	8.4 f
2.0	23.0 d	35.0 ab	97.7 a	77.7 b
3.0	23.0 de	37.7 a	79.7 b	77.7 b
NAA				
0.0	3.3 g	3.3 g	10.0 g	3.3 h
1.0	24.0 d	9.33 f	47.7 c	13.0 e
2.0	37.7 a	27.4 c	49.4 c	50.0 c
3.0	33.7 b	27.7 cd	77.7 b	27.7 d
2,4-D				
0.0	3.3 g	3.3 g	10.0 g	3.3 h
1.0	0 h	0 h	0 g	0 g
2.0	0 h	0 h	0 g	0 g
3.0	8.4 f	9.4 f	0 g	0 g
Significance				
A	***		***	
E	*		***	
C	***		***	
A × E	***		***	
A × C	***		***	
C × E	***		***	
A × C × E	***		***	

*, *** significant at $p \leq 0.05$, and 0.001, respectively according to Duncan's multiple range tests followed by ANOVA. Values followed by the same letters in the same column under the two explants were not significantly different. A = auxins; C = concentrations; E = explants. A × C × E indicates the significance of the triple interaction between the three factors (auxins, concentrations and explants).

3.3. Histological Analysis of the Embryogenic Calli

The histological micrograph of the embryonic calli of *N. sativa* 'Black Diamond' shows the ideal developmental stages of somatic embryos (i.e., globular, heart, torpedo shaped, and cotyledonary-stage embryos) (Figure 2).

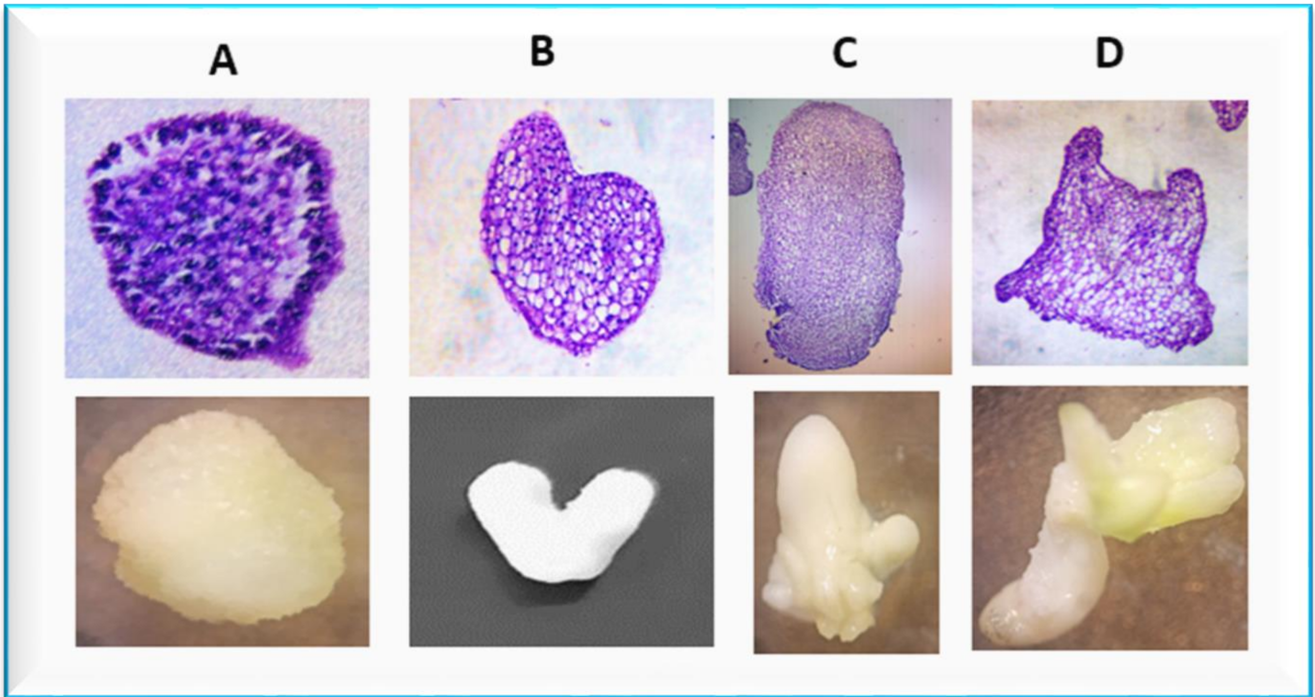


Figure 2. Histological micrograph of somatic embryos in *N. sativa* 'Black Diamond' at different stages of development: globular stage (**A**); heart stage (**B**); torpedo stage (**C**); cotyledon stage (**D**). The upper part of the figure shows a microscopic examination of embryonic tissues to confirm the different stages of growth and development of the embryos, and this was taken at 40× magnification. The lower part of the figure shows the different stages of living embryos separated from the explant studied under the binocular microscope at 15× magnification.

3.4. Gas Chromatography Analyses of Callus, Embryo, and Seed Extracts

We chose cotyledon-derived calli of *N. sativa* 'Black Diamond' embryos formed on them for phytochemical component analyses. Both types of samples originated from the best treatments of callus induction and embryo formation. Callus and embryo extracts were examined and compared to seed extract to determine their phytochemical components via GC–MS spectrophotometry (Tables 3–5). The analysis of the extract of cotyledon-derived calli, induced on MS medium with 1.0 and 3.0 mg L^{-1} NAA, showed the presence of a flavonoid, TQ from polyphenols, fatty acids (i.e., oleic, linoleic, and palmitic acids), their salts and esters (i.e., methyl palmitate, linoleol chloride, and oleic acid methyl ester), and amines and their oxides (benzyl amine, onamine 12, and myristamine oxide). The best results for all phytochemicals were mostly recorded for 3.0 mg L^{-1} NAA. The most important bioactive constituent in the callus culture extract was TQ. The percentage of this compound in the extract of calli produced on 3.0 mg L^{-1} NAA (2.76%) was nearly twice its value for calli initiated on 1.0 mg L^{-1} NAA (1.58%). Unsaturated fatty acids accompanied with their derivatives, salts, and esters were noticed to be the major component of the *N. sativa* 'Black Diamond' callus extract. They recorded a percentage of 83% from the total compounds from callus induced on 3.0 mg L^{-1} NAA. The other compounds were amines and their oxides; they represented the rest ratio of the callus extract (Table 3).

Table 3. Phytochemical compounds of *N. sativa* 'Black Diamond' calli derived from cotyledon explant cultured on 1 and 3 mg L^{-1} NAA.

No.	Compound	Molecular Weight	RT (min)	Area (%)	
				1 mg L^{-1} NAA	3 mg L^{-1} NAA
1	Thymoquinone	164	7.18	1.58	2.76
2	Onamine 12	213	11.96	8.80	8.14
3	Anastrozole	293	15.05	1.28	-
4	Myristamine oxide	241	15.76	2.98	-
5	Sulfobetaine 14	363	15.77	-	2.86
6	Methyl Palmitate	270	19.61	5.98	7.22
7	Hexadecanoic Acid (Palmitic acid)	256	20.30	4.00	4.32
8	2,3-Dehydro methyl linoleate	294	22.29	10.09	14.62
9	Oleic acid methyl ester	296	22.38	9.2	13.07
10	Elaidic acid methyl ester	296	22.48	2.11	2.75
11	Benzyl amine	234	22.56	2.18	1.87
12	Methyl stearate	298	22.80	1.72	2.41
13	Octadecadienoic acid (Linoleic acid)	280	22.98	5.49	6.85
14	Oleic Acid (cis-9-Octadecenoic acid)	282	23.05	8.21	-
15	Linoleol chloride	298	23.06	-	11.16
16	2,2′-methylenebis [4-methyl-6-tert-butylphenol]	340	27.02	1.17	1.33
17	Oleic acid	282	27.10	1.17	-
18	Diisooctyl phthalate	390	28.66	30.63	20.64
19	Oleic acid, 3-(octadecyloxy) propyl ester	529	34.29	1.57	-
20	4H-1-Benzopyran-4-One,2-(3,4 Dimethoxyphenyl)-3,5-Dihydroxy-7-Methoxy	344	35.25	1.83	-

Thymoquinone ($C_{10}H_{12}O_2$)

Onamine 12 ($C_{14}H_{31}N$)

Myristamine oxide ($C_{16}H_{35}NO$)

Oleic acid methyl ester ($C_{19}H_{36}O_2$)

Methyl Palmitate ($C_{17}H_{34}O_2$)

Sulfobetaine 14 ($C_{19}H_{41}NO_3S$)

Anastrozole ($C_{17}H_{19}N_5$)

Benzyl amine (C_7H_9N)

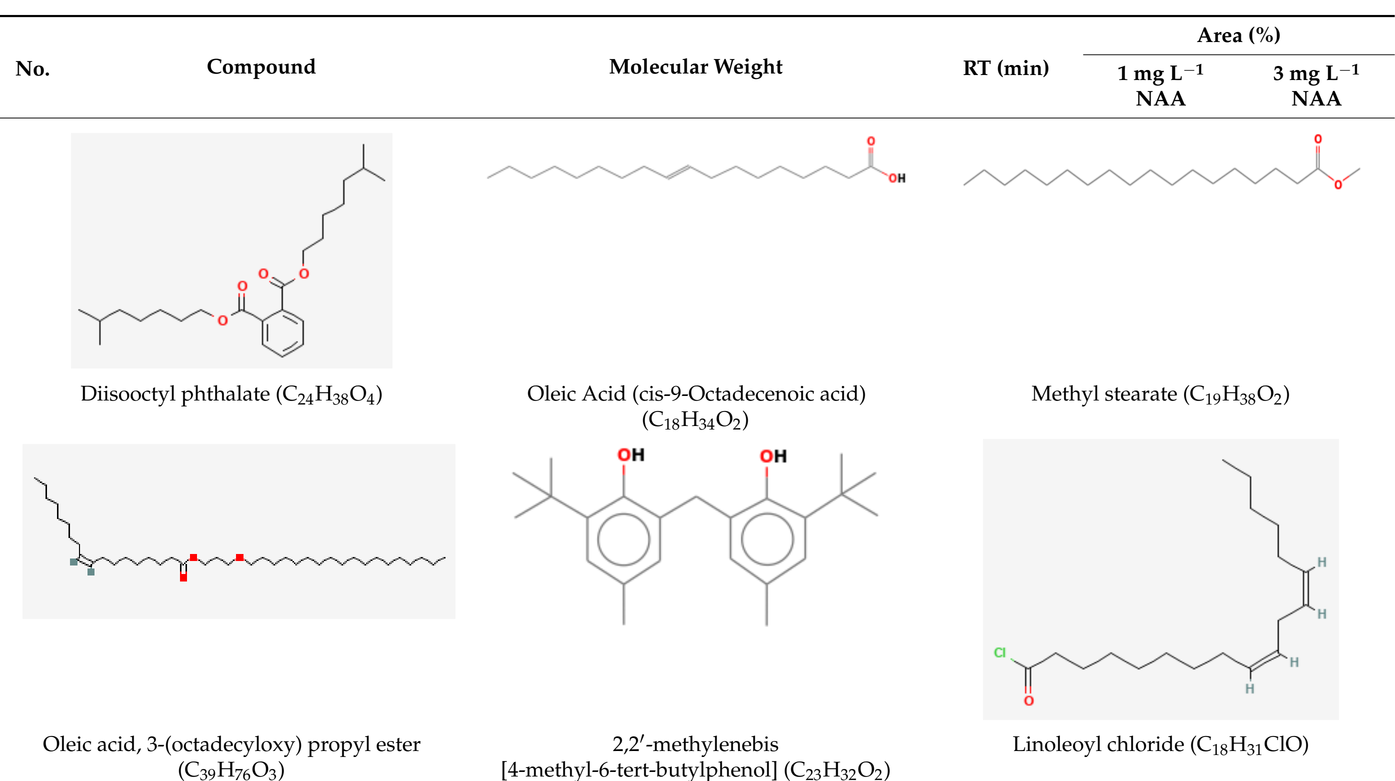
Hexadecanoic Acid (Palmitic acid) ($C_{16}H_{32}O_2$)

Oleic acid ($C_{18}H_{34}O_2$)

Octadecadienoic acid (Linoleic acid) ($C_{18}H_{32}O_2$)

Elaidic acid methyl ester ($C_{19}H_{36}O_2$)

Table 3. *Cont.*



No.	Compound	Molecular Weight	RT (min)	Area (%)	
				1 mg L^{-1} NAA	3 mg L^{-1} NAA
	Diisooctyl phthalate ($C_{24}H_{38}O_4$)	Oleic Acid (cis-9-Octadecenoic acid) ($C_{18}H_{34}O_2$)	Methyl stearate ($C_{19}H_{38}O_2$)		
	Oleic acid, 3-(octadecyloxy) propyl ester ($C_{39}H_{76}O_3$)	2,2′-methylenebis [4-methyl-6-tert-butylphenol] ($C_{23}H_{32}O_2$)	Linoleoyl chloride ($C_{18}H_{31}ClO$)		

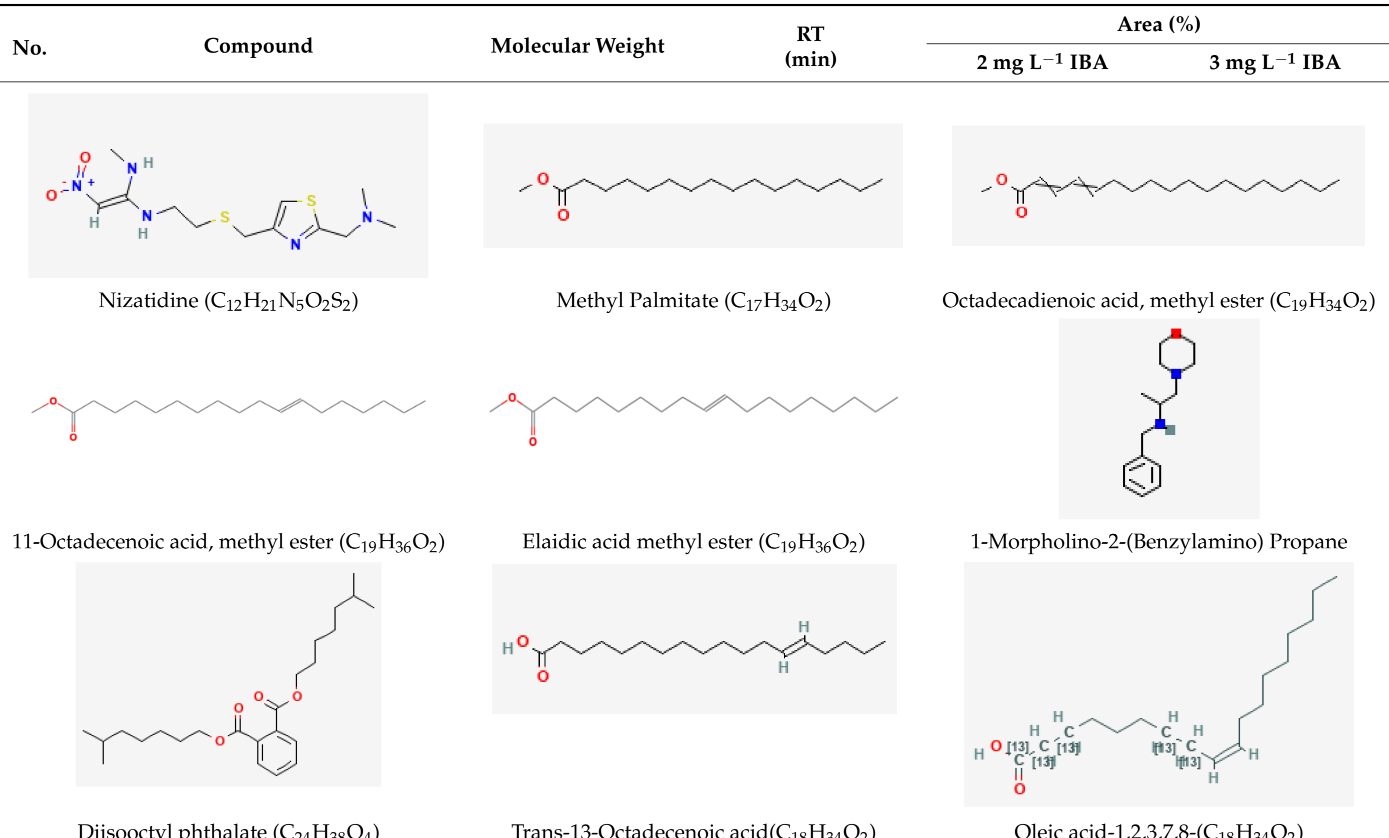
Sources: https://webbook.nist.gov/ and https://pubchem.ncbi.nlm.nih.gov/ accessed on 10 October 2023.

Table 4. Phytochemical compounds of *N. sativa* 'Black Diamond' embryos produced on calli derived from cotyledon explant cultured on 2 and 3 mg L^{-1} IBA, respectively.

No.	Compound	Molecular Weight	RT (min)	Area (%)	
				2 mg L^{-1} IBA	3 mg L^{-1} IBA
1	Nizatidine	331	11.96	6.42	5.59
2	Bisabolol oxide II	238	14.89	5.66	-
3	Bisabolone oxide A	236	15.40	4.91	-
4	Myristamine oxide	241	15.76	2.12	1.89
5	alpha Bisabolol oxide A	238	16.56	38.10	1.79
6	Methyl Palmitate	270	19.61	4.58	7.15
7	Palmitic Acid	256	20.30	2.72	3.75
8	Octadecadienoic acid, methyl ester	294	22.29	8.08	14.94
9	Elaidic acid methyl ester	296	22.38	8.72	14.72
10	11-Octadecenoic acid, methyl ester	296	22.48	-	3.20
11	1-Morpholino-2-(Benzylamino) Propane	234	22.56	-	1.20
12	Methyl stearate	298	22.79	-	2.86
13	Linoelaidic acid	280	22.98	3.77	6.85
14	Oleic acid -1,2,3,7,8-	282	23.05	6.31	8.72
15	Trans-13-Octadecenoic acid	282	27.10	-	1.50
16	Diisooctyl phthalate	390	28.66	8.61	25.84
	Alpha Bisabolol oxide A ($C_{15}H_{26}O_2$)	Bisabolol oxide II ($C_{15}H_{26}O_2$)	Bisabolone oxide A ($C_{15}H_{24}O_2$)		

Table 4. *Cont.*

No.	Compound	Molecular Weight	RT (min)	Area (%)	
				2 mg L^{-1} IBA	3 mg L^{-1} IBA



Nizatidine ($C_{12}H_{21}N_5O_2S_2$)

Methyl Palmitate ($C_{17}H_{34}O_2$)

Octadecadienoic acid, methyl ester ($C_{19}H_{34}O_2$)

11-Octadecenoic acid, methyl ester ($C_{19}H_{36}O_2$)

Elaidic acid methyl ester ($C_{19}H_{36}O_2$)

1-Morpholino-2-(Benzylamino) Propane

Diisooctyl phthalate ($C_{24}H_{38}O_4$)

Trans-13-Octadecenoic acid($C_{18}H_{34}O_2$)

Oleic acid-1,2,3,7,8-($C_{18}H_{34}O_2$)

Sources: https://webbook.nist.gov/ and https://pubchem.ncbi.nlm.nih.gov/ accessed on 10 October 2023.

Table 5. Phytochemical compounds of *N. sativa* 'Black Diamond' seed extract.

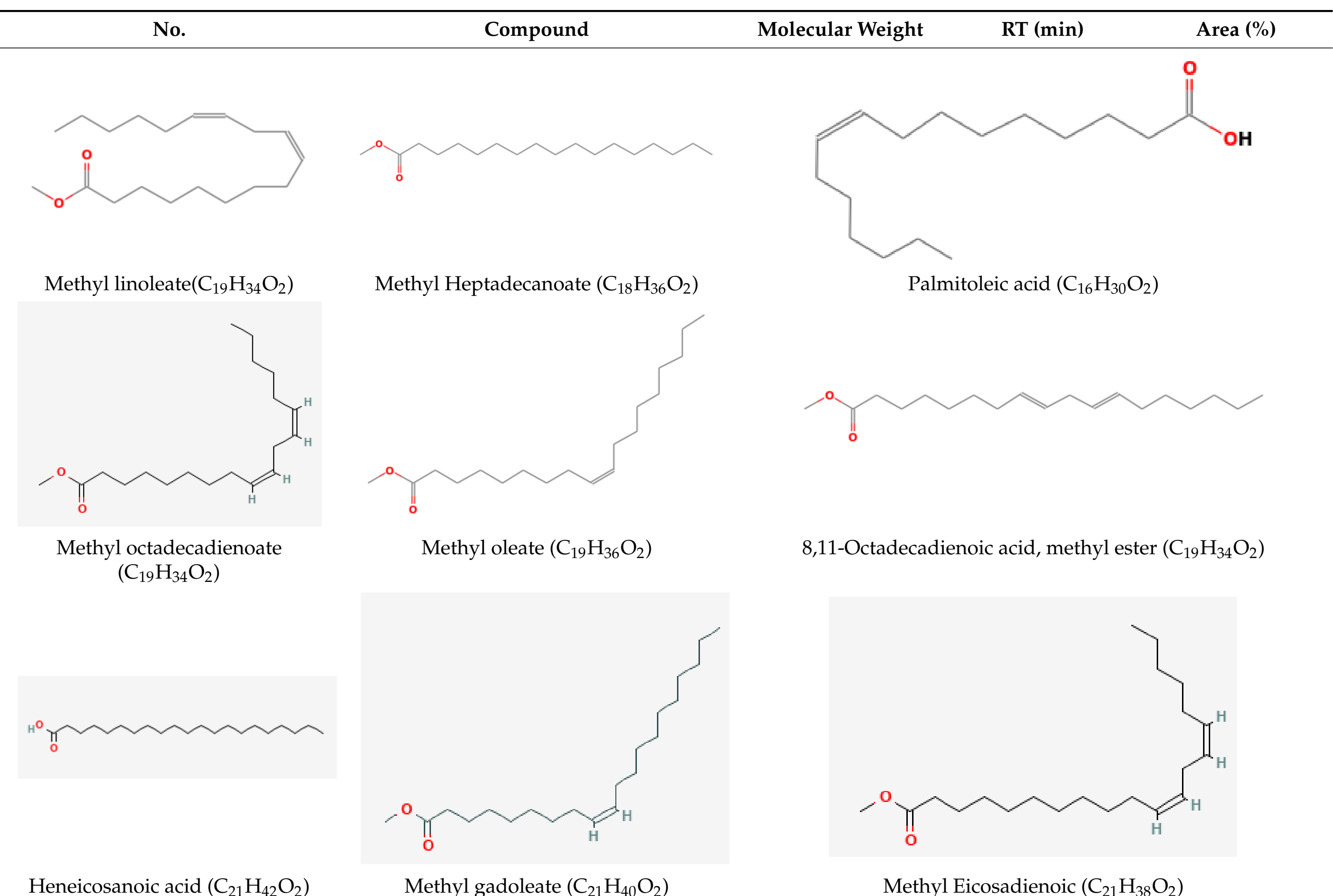
No.	Compound	Molecular Weight	RT (min)	Area (%)
1	Thymol	134	5.25	0.37
2	Thymoquinone	164	7.37	0.98
3	Methyl myristate	242	22.09	0.30
4	Palmitoleic acid	268	25.77	0.55
5	Methyl palmitate	270	26.27	16.00
6	Methyl Heptadecanoate	284	28.16	0.17
7	Methyl linoleate	294	29.42	38.38
8	Methyl oleate	296	29.60	25.03
9	Methyl stearate	298	30.04	8.01
10	8,11-Octadecadienoic acid, methyl ester	294	30.33	0.26
11	Methyl octadecadienoate	294	31.11	0.31
12	Methyl Eicosadienoic	322	32.92	7.09
13	Methyl gadoleate	324	33.02	1.45
14	Heneicosanoic acid	326	33.49	0.73
15	Pentacosylic acid	382	39.74	0.37

Thymol ($C_{10}H_{14}O$)

Thymoquinone ($C_{10}H_{12}O_2$)

Methyl myristate ($C_{15}H_{30}O_2$)

Table 5. *Cont.*



Sources: https://webbook.nist.gov/ and https://pubchem.ncbi.nlm.nih.gov/ accessed on 10 October 2023.

Embryos examined for phytochemical compounds were formed from cotyledon-derived calli on the MS medium supplemented with 2 and 3 mg L^{-1} IBA as the best two treatments for embryo formation. In contrast to the callus extract, the obtained data indicated the absence of TQ in the embryo extract (Table 4). However, some compounds were found in both the callus and embryo extracts, i.e., myristamine oxide, methyl palmitate, palmitic acid, elaidic acid methyl ester, methyl stearate, oleic acid, and diisooctyl phthalate. Moreover, fatty acids and their derivatives are considered to be the main ingredients of *N. sativa* 'Black Diamond' embryo extracts. They represent about 90% of the total compounds measured in embryos, which originated from the medium containing 3 mg L^{-1} IBA.

A rare occurrence of thymol was detected in the seed extract at 0.37% in all compounds (Table 5). Moreover, the seed extract contained 0.98% TQ. Fatty acids in the form of salts or esters represented the main component of the *N. sativa* 'Black Diamond' seed extract, as well as in callus and embryo extracts. The most important fatty acids that could be found in the seed extract were oleic acid and linoleic acid.

4. Discussion

Medicinal plants represent a spectacular store of bioactive compounds with various pharmacological properties. Folk medicine is a wealthy source of remedies. Traditionally, *N. sativa* seeds have been consumed to cure several health problems [36]. *N. sativa* seeds have been utilized for years as a food preservative and spice, flavoring in bakery products and cheese, in nutraceuticals and pharmaceutical products, and in functional foods [37].

In vitro plant cell culture techniques and biotechnological approaches constitute an invaluable, sustainable, and environmental substitute for the production of these bioactive compounds to diminish the use of compounds, which synthesize chemically, while decreasing the excessive usage of the available natural resources. Plant cell culture methods allow for the conservation of plant species, as well as the enhancement of metabolite biosynthesis, and the possibility of modifying the synthesis pathways [17]. Differences in the chemical composition of callus tissue and seeds of intact plants have been described for a number of plant species [22]. Nevertheless, very few studies have focused on metabolic comparisons between cell/tissue cultures (callus cultures and/or embryo cultures), and original plants have been reported, even though comparisons of the biological active metabolite content of cultured cells and tissues with that of the normal plants are of great importance.

Callus induction of *N. sativa* was reported for the first time by Banerjee and Gupta [38] but without metabolic identification and/or quantification of the phytochemical content. After years, callus cultures were proliferated from the stem, young leaf, petiole, and root of *N. sativa* plantlet on a solidified MS medium supplemented with 2.15 mg L^{-1} kinetin (Kin) and 1 mg L^{-1} 2,4-D in dark conditions [20], and the optimum growth rate of callus (115.4 $\pm$ 2.8 mg day^{-1}) was observed for the leaf explant. This result was in agreement with those obtained by Chand and Roy [39] and Al-Ani [19], who indicated that the leaf-derived calli had the highest growth rates. Moreover, the combination of 0.5 mg L^{-1} from both benzylaminopurine (BAP) and NAA resulted in effective callus induction from leaf explants of *N. sativa* [23]. In addition, callus cultures for *N. sativa* were successfully initiated on the MS medium supplemented with 1 mg L^{-1} NAA and 3 mg L^{-1} BAP, which recorded 80.41% for callus induction and 0.31 mm day^{-1} for the callus growth rate [4]. However, the hypocotyl explant was the superior explant for callus induction by percentage (81.78%), with a callus growth rate of 0.33 mm day^{-1}. Bibi et al. [18] reported an increase in the callus induction frequency up to 88%, when cotyledon explants were cultured on the MS medium containing 4.0 mg L^{-1} from both NAA and thidiazuron (TDZ). Chaudhry et al. [21] declared that the highest frequency of callus induction (82%) of *N. sativa* was observed for epicotyle explants on the MS medium containing 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kin. In another study on a medicinal plant of *Nigella damascena* L., Klimek-Chodacka et al. [40] reported that 83% and 100% callus formation were achieved from hypocotyl and cotyledon explants, respectively, on the MS medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. These findings are in agreement with our recently obtained results, which proved that cotyledon-derived explants of *N. sativa* 'Black Diamond' were more efficient at inducing callus than hypocotyl-derived ones and on the NAA-containing medium. In the current investigation, the supplementation of NAA at 1.0 mg L^{-1} gave the best results for callus induction of Black Diamond.

Callus cultures can serve as a means for the production of bioactive compounds in vitro as they have antioxidant potential or activity, due to the presence of flavonoids and phenolic compounds, like carvacrol, thymoquinone, and thymol. Because of its bioactivities, the industrial production of such health-promoting natural products is a main target through callus culture systems [4]. The callus obtained from the young leaf explant of *N. sativa* was proven to have great potential for TQ production [20].

Furthermore, callus can also be used as an explant to establish somatic embryogenesis, induce rhizogenesis, or can be differentiated into shoots, depending on the type and concentration of PGRs and the culture media [18,41]. Klimek-Chodacka et al. [40] found that shoots could regenerate and develop from 95% of hypocotyl-derived calli of *N. damascena* after transferring them on hormone-free media, regardless of the callus induction medium used before. However, in the present investigation, calli derived both from hypocotyl and cotyledon explants of Black Diamond and embryos formed on them could be regenerated into shoots/plantlets on the MS basal medium but in low percentages of 3.3–10% compared with the other auxin treatments (8.4–97.7%).

Somatic embryos are powerful biotechnological tools that can be employed for various applications, such as for clonal micropropagation, plant improvement, and germplasm

conservation. They also provide an excellent system to study the early development of plant morphogenesis and genetic transformation [40]. Few reports concerning the somatic embryogenesis of *N. sativa* have been published [24]. They examined some combinations of 2,4-D and NAA and they found that somatic embryos could be induced in *N. sativa*, but their conversion into plants has never been observed. In this work, somatic embryos could be initiated from cotyledon-derived calli of *N. sativa* 'Black Diamond' on the 2 mg L^{-1} IBA-supplemented medium.

Plant polyphenols are secondary metabolites with bioactivity, and they are produced in response to stress conditions in plants to mitigate the harmful effects of free radicals. The low-molecular-weight phenolic acids and flavonoids are important classes of polyphenols such as TQ. TQ has prominent antioxidant activity and is of pharmacological interest in the treatment of many human diseases [18,42]. So, plant in vitro technologies are widely applied to enhance the production of such high-value-added natural products as natural antioxidants [43].

Concerning the pharmaceutical importance of *N. sativa* seeds, the consumption of black cumin seed extract was confirmed to control many problems, such as cough, break up renal calculi, delay the carcinogenic process, and treat abdominal pain, diarrhea, and flatulence. It was also reposted to have anti-inflammatory and antioxidant effects [44]. This extract showed significant antioxidant and anti-inflammatory potential. Most of the pharmacological effects of *N. sativa* seeds are due to the quinine constituent, of which TQ is mainly abundant [45]. Several reports have confirmed TQ as one of the main ingredients of *N. sativa* seed extracts where it was found ranging from 8 to 27% [20]. Another study reported that TQ content in commercial black cumin seed oil (BSO) products varied from 0.07% to 1.88% wt/wt, where the TQ content in those products differed depending on both the oil source and extraction method [13]. In the current work, it was found that thymol could be found in the seed extract of Black Diamond but not detected in callus or embryo extracts. On the contrary, Al-Ani [19] confirmed the production of thymol in the leaf-derived calli of *N. sativa*. However, the major constituents in the *N. sativa* seed extract are esters of fatty acids, as reported by Mahmmoud and Christensen [36].

In the current study, GC-MS analysis was utilized to compare the secondary product metabolite profile of the extract from intact *N. sativa* 'Black Diamond' seeds with callus or embryo culture extracts. In this concern, HPLC analysis, using the standard TQ sample, indicated that the extract of the leaf callus of *N. sativa* 'Black Diamond' contained the highest amount of TQ (8.78 mg mL^{-1}). This content was 12-times higher than that measured in the seed extract (0.74 mg mL^{-1}) [20]. Therefore, callus cultures could be used as an alternative source of TQ, especially when seeds are not available. TQ is the major active compound in black cumin seed oil [13]. From the obtained results, TQ is one of the most active products that could be identified in callus and seed extracts of *N. sativa* 'Black Diamond', but it was not detected in the embryo extract.

Thirty-two fatty acids (99.9%) have been identified in the extracted fixed oil of *N. sativa*, while the major fatty acids were linoleic acid and oleic acid [46,47]. *N. sativa* seed oil was reported to contain a mixture of oleic and linoleic acids. They have a particularly significant role in lowering high blood pressure [36]. Oleic acid could be used in the industry as an emulsifying or solubilizing agent for the prevention of oxidation in oils [48].

5. Conclusions

Modern plant biotechnology techniques provide scientists with plant cells and tissue cultures, which allow for maximizing the production of active natural compounds from medicinal plants. There is increasing interest to study the biochemical properties of proliferated cell cultures under controlled artificial conditions and to compare the results with those of native plant species. An efficient protocol for enhancing the callus biomass of *N. sativa* 'Black Diamond' was developed. Callus cultures of *N. sativa* 'Black Diamond' were successfully induced from cotyledon explants cultured on the MS medium supplemented with 1.0 mg L^{-1} NAA. Callus cultures had potency for the further production of health-

promoting natural products. In addition to the applications of somatic embryos mentioned before, they should be reinvestigated to be employed for the production of secondary metabolites of this remarkable medicinal plant. The formation of somatic embryos was achieved from cotyledon-derived calli of *N. sativa* 'Black Diamond' on the 2 mg L^{-1} IBA-supplemented MS medium. This is the first report on the successful conversion of somatic embryos into plants in *N. sativa* 'Black Diamond'. Furthermore, studying the metabolic profile of callus, embryo, and seed extracts of *N. sativa* 'Black Diamond' to identify phytochemicals that might be found in the extract is not enough. But, also, the quantification of secondary products is considered urgent to elucidate the exact amount of these metabolites in in vitro cultures compared with the intact plant, in order to produce them on a large scale, industrially. Therefore, optimizing the culture conditions via biotechnological techniques is needed in the future to support the industrial production of the most valuable secondary products of *N. sativa* 'Black Diamond' through calli and/or embryo cultures.

Author Contributions: Conceptualization, M.E.E.-M.; methodology, A.E.H. and M.E.E.-M.; software, A.E.H.; validation, M.E.E.-M., A.N.E.-B. and N.A.; formal analysis, M.E.E.-M. and A.N.E.-B.; investigation, A.E.H.; resources, M.E.E.-M.; data curation, N.A.; writing—original draft preparation, N.A. and J.D.; writing—review and editing, N.A. and J.D.; visualization, M.E.E.-M. and J.D.; supervision, M.E.E.-M., A.N.E.-B. and M.K.M.; project administration, H.E.-R.; funding acquisition, H.E.-R. All authors have read and agreed to the published version of the manuscript.

Funding: Project no. TKP2021-EGA-20 has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA funding scheme.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the staff members of the Physiology and Breeding of Horticultural Crops Laboratory, Department of Horticulture, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt, for supporting the completion of this work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Telci, İ.; Özek, T.; Demirtaş, İ.; Özek, G.; Yur, S.; Ersoy, S.; Yasak, S.; Gül, F.; Karakurt, Y. Studies on black cumin genotypes of Turkiye: Agronomy, seed and thymoquinone yields. *J. Appl. Res. Med. Aromat. Plants* **2023**, *35*, 100494. [CrossRef]
2. Golkar, P.; Nourbakhsh, V. Analysis of genetic diversity and population structure in *Nigella sativa* L. using agronomic traits and molecular markers (SRAP and SCoT). *Ind. Crops Prod.* **2019**, *130*, 170–178. [CrossRef]
3. Dalli, M.; Azizi, S.; Kandsi, F.; Gseyra, N. Evaluation of the in vitro antioxidant activity of different extracts of *Nigella sativa* L. seeds, and the quantification of their bioactive compounds. *Mater. Today Proc.* **2021**, *45*, 7259–7263. [CrossRef]
4. Golkar, P.; Bakhshi, G.; Vahabi, M.R. Phytochemical, biochemical, and growth changes in response to salinity in callus cultures of *Nigella sativa* L. *Vitr. Cell. Dev. Biol. Plant* **2020**, *56*, 247–258. [CrossRef]
5. Hannan, A.; Rahman, A.; Sohag, A.A.M.; Uddin, J.; Dash, R.; Sikder, M.H.; Rahman, S.; Timalsina, B.; Munni, Y.A.; Sarker, P.P.; et al. Black Cumin (*Nigella sativa* L.): A Comprehensive Review on Phytochemistry, Health Benefits, Molecular Pharmacology, and Safety. *Nutrients* **2021**, *13*, 1784. [CrossRef] [PubMed]
6. Nazari, A.; Zarringhalami, S.; Asghari, B. Influence of germinated black cumin (*Nigella sativa* L.) seeds extract on the physicochemical, antioxidant, antidiabetic, and sensory properties of yogurt. *Food Biosci.* **2023**, *53*, 102437. [CrossRef]
7. Kemal, M.; Esertaş, Ü.Z.Ü.; Kanbur, E.D.; Kara, Y.; Özçelik, A.E.; Can, Z.; Kolaylı, S. Characterization of the black cumin (*Nigella sativa* L.) honey from Türkiye. *Food Biosci.* **2023**, *53*, 102760. [CrossRef]
8. Burdock, G.A. Assessment of black cumin (*Nigella sativa* L.) as a food ingredient and putative therapeutic agent. *Regul. Toxicol. Pharmacol.* **2022**, *128*, 105088. [CrossRef] [PubMed]
9. Fathi, M.; Hosayni, M.; Alizadeh, S.; Zandi, R.; Rahmati, S.; Rezaee, V. Effects of black cumin (*Nigella sativa*) seed meal on growth performance, blood and biochemical indices, meat quality and cecal microbial load in broiler chickens. *Livest. Sci.* **2023**, *274*, 105272. [CrossRef]
10. Kazmi, A.; Khan, M.A.; Ali, H. Biotechnological approaches for production of bioactive secondary metabolites in *Nigella sativa*: An up-to-date review. *Int. J. Sec. Metab.* **2019**, *6*, 172–195. [CrossRef]
11. Zaky, A.A.; Shim, J.H.; Abd El-Aty, A.M. A Review on Extraction, Characterization, and Applications of Bioactive Peptides from Pressed Black Cumin Seed Cake. *Front. Nutr.* **2021**, *8*, 743909. [CrossRef]

12. Sakdasri, W.; Sila-ngam, P.; Chummengyen, S.; Sukruay, A.; Ngamprasertsith, S.; Supang, W.; Sawangkeaw, R. Optimization of yield and thymoquinone content of screw press-extracted black cumin seed oil using response surface methodology. *Ind. Crops Prod.* **2023**, *191*, 115901. [CrossRef]
13. Alkhatib, H.; Mawazi, S.M.; Al-Mahmood, S.M.A.; Zaiter, A.; Doolaanea, A.A. Thymoquinone content in marketed black seed oil in Malaysia. *J. Pharm. Bioallied Sci.* **2020**, *12*, 284–288. [CrossRef] [PubMed]
14. El-Sayed, S.A.E.-S.; Rizk, M.A.; Yokoyama, N.; Igarashi, I. Evaluation of the in vitro and in vivo inhibitory effect of thymoquinone on piroplasm parasites. *Parasites Vectors* **2019**, *12*, 37. [CrossRef]
15. Khurshid, Y.; Syed, B.; Simjee, S.U.; Beg, O.; Ahmed, A. Antiproliferative and apoptotic effects of proteins from black seeds (*Nigella sativa*) on human breast MCF-7 cancer cell line. *BMC Complement. Med. Ther.* **2020**, *20*, 5. [CrossRef]
16. Abd-Rabou, A.A.; Edris, A.E. Cytotoxic, apoptotic, and genetic evaluations of *Nigella sativa* essential oil nanoemulsion against human hepatocellular carcinoma cell lines. *Cancer Nanotechnol.* **2021**, *12*, 28. [CrossRef]
17. Mohaddab, M.; El Goumi, Y.; Gallo, M.; Montesano, D.; Zengin, G.; Bouyahya, A.; Fakiri, M. Biotechnology and In Vitro Culture as an Alternative System for Secondary Metabolite Production. *Molecules* **2022**, *27*, 8093. [CrossRef] [PubMed]
18. Bibi, A.; Khan, M.A.; Adil, M.; Mashwani, Z.U.R. Production of callus biomass and antioxidant secondary metabolites in black cumin. *J. Anim. Plant Sci.* **2018**, *28*, 1321–1328.
19. Al-Ani, N.K. Thymol Production from Callus Culture of *Nigella sativa* L. *Plant Tissue Cult. Biotech.* **2009**, *18*, 181–185. [CrossRef]
20. Alemi, M.; Sabouni, F.; Sanjarian, F.; Haghbeen, K.; Ansari, S. Anti-inflammatory effect of seeds and callus of *Nigella sativa* L. extracts on mix glial cells with regard to their thymoquinone content. *AAPS PharmSciTech.* **2013**, *14*, 160–167. [CrossRef]
21. Chaudhry, H.; Fatima, N.; Ahmad, I.Z. Establishment of callus and cell suspension cultures of *Nigella sativa* L. for thymol production. *Int. J. Pharm. Pharm. Sci.* **2014**, *6*, 788–794.
22. Landa, P.; Maršík, P.; Vaněk, T.; Rada, V.; Kokoška, L. In vitro antimicrobial activity of extracts from the callus cultures of some *Nigella* species. *Biologia* **2006**, *61*, 285–288. [CrossRef]
23. Hoseinpanahi, S.; Majdi, M.; Mirzaghaderi, G. Effects of growth regulators on in vitro callogenesis and regeneration of black cumin (*Nigella sativa* L.). *Iran. J. Rangel. Forest Plant Breed.* **2016**, *24*, 232–242.
24. Elhag, H.; El-Olemy, M.M.; Al-Said, M.S. Enhancement of Somatic Embryogenesis and Production of Developmentally Arrested Embryos in *Nigella sativa* L. *Hort. Sci.* **2004**, *39*, 321–323. [CrossRef]
25. Kim, D.H.; Kang, K.W.; Sivanesan, I. Influence of auxins on somatic embryogenesis in *Haworthia retusa* Duval. *Biologia* **2019**, *74*, 25–33. [CrossRef]
26. Vondráková, Z.; Krajňáková, J.; Fischerová, L.; Vágner, M.; Eliášová, K. Physiology and role of plant growth regulators in somatic embryogenesis. In *Vegetative Propagation of Forest Trees*; Park, Y.S., Bonga, J.M., Moon, H.K., Eds.; National Institute of Forest Science: Seoul, Republic of Korea, 2016; pp. 123–169.
27. Simon, S.; Petrášek, J. Why plants need more than one type of auxin. *Plant Sci.* **2011**, *180*, 454–460. [CrossRef]
28. Hazubska-Przybył, T.; Ratajczak, E.; Obarska, A.; Pers-Kamczyc, E. Different Roles of Auxins in Somatic Embryogenesis Efficiency in Two Picea Species. *Int. J. Mol. Sci.* **2020**, *21*, 3394. [CrossRef] [PubMed]
29. de Morais Oliveira, J.P.; da Silva, A.J.; Catrinck, M.N.; Clarindo, W.R. Embryonic abnormalities and genotoxicity induced by 2,4-dichlorophenoxyacetic acid during indirect somatic embryogenesis in Coffea. *Sci. Rep.* **2023**, *13*, 9689.
30. Bhusare, B.P.; John, C.K.; Bhatt, V.P.; Nikam, T.D. Induction of somatic embryogenesis in leaf and root explants of *Digitalis lanata* Ehrh.: Direct and indirect method. *S. Afr. J. Bot.* **2020**, *130*, 356–365. [CrossRef]
31. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
32. El-Mahrouk, M.E.; Maamoun, M.K.; Dewir, Y.H.; Omran, S.A.; EL-Banna, A.N. Morphological and molecular characterization of induced mutants in *Nigella sativa* L. using irradiation and chemical mutagens. In *The 9th Plant Breeding International Conference*; Faculty of Agriculture, Benha University: Benha, Egypt, 2015.
33. El-Mahrouk, M.E.; Maamoun, M.K.; Dewir, Y.H.; El-Banna, A.N.; Rihan, H.Z.; Salamh, A.; Al-Aizari, A.A.; Fuller, M.P. Synchronized Seed Germination and Seedling Growth of Black Cumin. *HortTechnology* **2022**, *32*, 182–190. [CrossRef]
34. Boissot, N.; Valdez, M.; Guiderdoni, E. Plant regeneration from leaf and seed-derived calli and suspension cultures of the African perennial wild rice, *Oryza longistaminata*. *Plant Cell Rep.* **1990**, *9*, 447–450. [CrossRef] [PubMed]
35. Folch, J.; Lees, M.; Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [CrossRef]
36. Mahmmoud, Y.A.; Christensen, S.B. Oleic and linoleic acids are active principles in *Nigella sativa* and stabilize an E(2)P conformation of the Na,K-ATPase. Fatty acids differentially regulate cardiac glycoside interaction with the pump. *Biochim. Biophys. Acta* **2011**, *1808*, 2413–2420. [CrossRef]
37. Hassanien, M.F.; Assiri, A.M.; Alzohairy, A.M.; Oraby, H.F. Health-promoting value and food applications of black cumin essential oil: An overview. *J. Food Sci. Technol.* **2015**, *52*, 6136–6142. [CrossRef]
38. Banerjee, S.; Gupta, S. Morphogenesis in tissue cultures of different organs of *Nigella sativa*. *Physiol. Plant* **1975**, *33*, 185–187. [CrossRef]
39. Chand, S.; Roy, S.C. Study of callus tissues from different parts of *Nigella sativa* (Ranunculaceae). *Experientia* **1980**, *36*, 305–306. [CrossRef]

40. Klimek-Chodacka, M.; Kadluczka, D.; Lukasiewicz, A.; Malec-Pala, A.; Baranski, R.; Grzebelus, E. Effective callus induction and plant regeneration in callus and protoplast cultures of *Nigella damascena* L. *Plant Cell Tissue Organ. Cult.* **2020**, *143*, 693–707. [CrossRef]
41. Khan, M.A.; Abbasi, B.H.; Ali, H.; Ali, M.; Adil, M.; Hussain, I. Temporal variations in metabolite profiles at different growth phases during somatic embryogenesis of *Silybum marianum* L. *Plant Cell Tissue Organ. Cult.* **2015**, *120*, 127–139. [CrossRef]
42. Khan, M.S.; Tabrez, S.; Rabbani, N.; Oves, M.; Shah, A.; Alsenaidy, M.A.; Al-Senaidy, A.M. Physico-chemical stress induced amyloid formation in insulin: Amyloid characterization, cytotoxicity analysis against human neuroblastoma cell lines and its prevention using black seeds (*Nigella sativa* L.). *Chin. J. Integr. Med.* **2015**. [CrossRef]
43. Abouzid, S.F.; El-Bassuon, A.A.; Nasib, A.; Khan, S.; Qureshi, J.; Choudhary, M.I. Withaferin A production by root cultures of *Withania coagulans*. *Int. J. Appl. Res. Nat. Prod.* **2010**, *3*, 23–27.
44. Majid, A. The Chemical Constituents and Pharmacological Effects of *Nigella sativa*—A Review. *J. Biosci. Appl. Res.* **2018**, *4*, 389–400. [CrossRef]
45. Forouzanfar, F.; Bazzaz, B.S.; Hosseinzadeh, H. Black cumin (*Nigella sativa* L.) and its constituent (thymoquinone): A review on antimicrobial effects. *Iran. J. Basic. Med. Sci.* **2014**, *17*, 929–938. [PubMed]
46. Radusheva, P.; Pashev, A.; Uzunova, G.; Nikolova, K.; Gentscheva, G.; Perifanova, M.; Maria Marudova, M. Comparative physicochemical analysis of oils derived from *Nigella sativa* L. and *Coriandrum sativum* L. *J. Chem. Technol. Metall.* **2021**, *56*, 1175–1180.
47. Albakry, Z.; Karrar, E.; Ahmed, I.A.M.; Oz, E.; Proestos, C.; El Sheikha, A.F.; Oz, F.; Wu, G.; Wang, X. Nutritional Composition and Volatile Compounds of Black Cumin (*Nigella sativa* L.) Seed, Fatty Acid Composition and Tocopherols, Polyphenols, and Antioxidant Activity of Its Essential Oil. *Horticulturae* **2022**, *8*, 575. [CrossRef]
48. Hernandez, E.M. Specialty Oils: Functional and Nutraceutical Properties. In *Woodhead Publishing Series in Food Science, Technology and Nutrition, Functional Dietary Lipids*; Sanders, T.A.B., Ed.; Woodhead Publishing: Sawston, UK, 2016; pp. 69–101. [CrossRef]

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