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PERFORMANCE REACTION AND BIOCHEMICAL PROPERTIES OF BLACK CUMIN UNDER THE INFLUENCE OF DIFFERENT REGIMES OF NITROGEN, MENTHOL AND DROUGHT STRESS

Milad Soltanieh[1], Daryush Talei[1,2], Parisa Nejatkhah[1]

[1] Faculty of Marine Science and Technology, Islamic Azad University, North Tehran Branch
[2] Medicinal Plants Research Center, Shahed University, Tehran, Iran

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Abstract

The purpose of this study was to investigate the responses of growth indices and yield of the black cumin to nitrogen and methanol under drought stress conditions. The experiment was a split split plots based on randomized complete block design with three factors and three replicates in the research farm of Medicinal Plants Research Center, Shahed University, Tehran, Iran, during 2019–2020. The factors were drought stress with 4 levels (non-stress, mild, moderate, and severe) as the main factor, nitrogen with 4 levels (0, 30, 60, and 90 kg ha^{-1} nitrogen from the source of urea) as the sub-factor and methanol with 3 levels (0, 10, and 30%) as sub-sub-factor. Nitrogen treatments (except the 0 level) were each applied in three stages of black seed growth: 1) one-third at the same time as planting, 2) one-third at the stage of 4–6 leaves, and 3) one-third at the beginning of flowering. Methanol foliar application was done once at the first capsule formation stage and again at an interval of seven days later. Two days after the second time of methanol spraying, the data on biochemical traits (chlorophyll, proline and protein contents) and in the end of generative stage, agronomical (seed yield) and phytochemical compounds were measured. The results indicated that main effects and interactions significantly affected all biochemical traits and yield. The results showed that the yield under drought stress conditions decreased (11.67%) in comparison to the control, while the yield increased (5.7%) in comparison to control by applying the nitrogen and methanol. The interaction of methanol and nitrogen had significant effects on the amount of grain yield, chlorophyll, proline, protein, carotenoid contents and oil of seed. The results indicated that the interaction of drought stress-nitrogen, drought stress-methanol and nitrogen-methanol had significant effects on chlorophyll, proline, protein, carotenoid contents, oil seed and yield. The results showed the highest seed oil (717 kg/ha) in the interaction of without drought stress, 90 kg/ha nitrogen. Therefore, the application of 10% methanol and 90 kg/ha nitrogen under drought stress was the most effective treatments on increasing the yield and phytochemical compounds in black cumin.

Keywords: black cumin, drought stress, grain yield, growth index, phytochemical compounds

INTRODUCTION

Medicinal plants have a special place in traditional Iranian medical science. The seeds of Nigella sativa plant have been long used to protect health and combat different diseases with its frequent application in the pharmaceutical industry. Black cumin is a native plant of western Asia but is found wild in southern Europe, northern Africa and Asia Minor. It also grows in most parts of Iran (Srinivasan, 2018; Ghanavi *et al.*, 2022). Black cumin seeds contain oils, proteins, alkaloids (such as nigellicine and nigellidine), quinines (such as thymoquinone), sapiens and essential oil. The fixed and essential oil of black cumin contain various bioactive molecules such as thymoquinone, thymol, tocopherol, Tran's retinol and selenium (Hussain and Hussain, 2016; Yimer *et al.*, 2019). It is an annual plant and can tolerate different levels of drought originally grown in arid and semiarid regions. Also, black cumin has positive response on irrigation scheduling and irrigation levels (Hussain and Hussain, 2016; Habtewold *et al.*, 2017). Drought is one of the main inhibitors for crop production in arid and semi-arid regions. As an abiotic stress, it is multidimensional in nature and affects plants at various levels of their organization. Drought stress in plants reduces the plant-cell's water potential and turgor, which elevates the solutes' concentrations in the cytosol and extracellular matrices (Salehi-Lisar and Bakhshayeshan-Agdam, 2016; Bijalwan *et al.*, 2022). In response to water deficit, the physiological parameters of photosynthesis, stomatal conductance and transpiration decreased but increased the amount of proline and protein (Davazdahemami *et al.*, 2014; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Sprayed on plants methanol enters tissues rapidly. It can also be found in Serin structure following influence on plant carbon metabolism. Higher methanol concentration in plant issues positively affects carbon fixation efficiency and cause leaf enlargement via up-regulation of pectin methyl esterase gene (Badiger *et al.*, 2017). Nitrogen is an essential element required for successful plant growth. Nitrogen fertilizer application is an important issue for sustainable agriculture because it can reduce the negative effects of traditional farming on the surrounding environment (Anas *et al.*, 2020). In this respect, an agricultural system should include yield and environmental quality during management. Although there is substantial evidence of the effects of drought, methanol and nitrogen effects on plants, little is known about the effects of applied methanol and nitrogen on the growth traits of black cumin under drought stress conditions. Therefore, the purpose of this study was to evaluate the effects of methanol and nitrogen effects on yield and quality characters of black cumin under drought stress conditions.

MATERIALS AND METHODS

Plant Materials and Germination Conditions

Healthy seeds of black cumin were provided from Medicinal Plants Research Center, Shahed University. After harvest, the seeds were stored under optimum storage conditions (humidity (12%), temperature (0 °C) and ventilation).

Experimental Design

The experiment was a split split-plots study based on randomized complete block design with three factors and three replicates. The experiments were conducted in Tehran (35° 41' N latitude, 51°19' E longitude, and altitude of 1215 m) from 2019 to 2020. The factors were drought stress with 4 levels (non-stress, mild, moderate, and severe) as the main factor, nitrogen with 4 levels (0, 30, 60, and 90 kg/ha from the source of urea) as the sub-factor, and methanol with 3 levels (0, 10 and 30%) as the sub-sub-factor. Drought stress was applied 15 days after the emergence stage. Without stress, mild, moderate and severe stresses were respectively: irrigation once every three days, once every four days, once every eight days, and once every 12 days. Nitrogen treatments (except the 0 level) were each applied in three stages of black seed growth: 1) one-third at the same time as planting, 2) one-third at the stage of 4–6 leaves, and 3) one-third at the beginning of flowering. Methanol foliar application was done once at the first capsule formation stage and again at an interval of seven days later. In fact, for each level of methanol, two g/L glycine and one g/L tetrahydrofolate as catalysts and one g/L tween80 were added to increase the adhesion of methanol solution. In both areas, soil preparation, including plowing, leveling and paving was performed before planting. Black cumin seeds were weighed in the amount of 4 g and the seed was evenly distributed. The analysis of the field soil is given in Tab. I. Two days after the second time of methanol spraying, the data on biochemical traits (chlorophyll, carotenoid, proline, and protein contents) and in the end of reproductive stage, agronomical (seed yield) and phytochemical compounds were measured.

Chlorophyll Assay

Chlorophyll was extracted in 80% acetone from the leaf samples (0.5 gr), according to Arnon method (1949). The extracts were filtrated and then absorbencies of chlorophyll a, b were determined by spectrophotometer (UV-S, Sinco 2100) at 645 and 663 nm.

Carotenoid Assay

Carotenoid was extracted in 80% acetone from leaf samples (0.5 gr), according to Lichtedthaler method (1987). The extracts were filtrated and then the absorbance of carotenoid was determined at 470 nm. The carotenoids content was expressed as μmol/g FW and concentrations of carotenoids were calculated using an extinction coefficient ε = 33000 μM/cm.

I: *The soil characteristics of the experimental field*

Soil	pH	Soil organic matter (%)	N (%)	P (mg/kg)	K (mg/kg)
Loam	7.8	> 0.1	0.09	10.5	185.3

Proline Assay

Proline content was measured as described by Bates *et al*. (1973). The fully exposed leaves (0.2 g) were homogenized in a mortar and pestle with 5 mL of (3% w/v) sulfosalicylic acid. The extracts were then filtered through Whatman filter papers. Absorbance of the free proline content was measured at 520 nm. The proline concentration was expressed as mmolg^{-1} fresh mass (FM). The content of proline was calculated from a proline standard curve and was expressed as µg/g FW.

Protein Assay

To extract and assay the protein content, one gram of fresh leaf tissue was grounded in liquid nitrogen using pre-chilled mortar and pestle. The extracts were homogenized and mixed with 2.5 mL of the HEPES/KOH buffers according to the method of Talei *et al*. (2013). Finally, the samples were centrifuged for 15 min at 16,000 rpm at 4 ºC. The supernatants were collected and the total protein concentration was determined by the Bradford method (1976) at 595 nm using a spectrophotometer (Lambda 25, UV/VIS). The protein content was calculated using bovine serum albumin curve as a standard and expressed as µg/g FW.

Seed Yield Assay

At the end of the plant growth period and after the final ripening, one square meter is selected from each experimental unit after removing the margin effect and the seeds yield was measured.

Phytochemical Compounds Assay

To calculate seed oil, an area of the middle of each plot was harvested. Also, 20 g seed was selected from each plot to determine oil percentage by Soxcelle method (Dinari *et al*., 2013). The composition of fatty acids was determined by gas chromatography (GC-MS) (Dinari *et al*., 2013). A total of 100 µL of sodium methoxide (0.5 M) was added to 50 µL sample in one mL of hexane. The mixture was shaken vigorously for 15 min, allowed to stand. The gas chromatographic analysis of Younglin-Acme 6000 type XPB -70 was performed on Helium gas chromatograph equipped with a PIF detector. We used a 120-m column. Temperatures of the injector, detector and oven were 250 ºC, 280 ºC and 180 ºC respectively. FAMEs were identified based on the comparison of their relative RF (retention times) values with those of authentic standards.

Statistical Analysis

All statistical analyses including analysis of variance and Duncan's multiple range tests were performed using SAS program version 9 (SAS Institute Inc., 2009a) at significance level of P ≤ 0.05. Excel software was used for drawing the graphs

RESULTS AND DISCUSSION

Chlorophyll Content

The results indicated that the chlorophyll content was significantly affected by the main effects and interactions. The results showed that applying the nitrogen and methanol fertilizers led to greater amount of chlorophyll contents. The significance

II: *Mean comparison of interaction of drought stress and nitrogen on some physiological traits in black cumin*

Drought stress	Nitrogen (kg/ha)	Chlo.a (mg/g FW)	Chlo.b (mg/g FW)	Total chlo. (mg/g FW)	Carotenoid (mg/g FW)	Protein (mg/g FW)	Proline (mg/g FW)
Control	0	0.259^{bc}	0.165^{d}	0.424^{bc}	0.171^{c}	2.584^{efg}	10.651^{i}
	30	0.227^{e}	0.129^{g}	0.357^{ef}	0.166^{d}	2.614^{ef}	8.861^{k}
	60	0.264^{ab}	0.159^{e}	0.423^{bc}	0.182^{a}	2.6505^{de}	8.514^{k}
	90	0.263^{b}	0.205^{a}	0.468^{a}	0.176^{b}	2.422^{gh}	6.492^{lm}
Mild stress	0	0.235^{de}	0.176^{b}	0.411^{bc}	0.135^{gh}	2.3965^{h}	11.947^{h}
	30	0.272^{a}	0.171^{c}	0.443^{ab}	0.1304^{hi}	2.0985^{jk}	10.657^{i}
	60	0.253^{c}	0.164^{d}	0.417^{bc}	0.153^{e}	2.696^{de}	12.123^{gh}
	90	0.205^{fg}	0.118^{i}	0.324^{fg}	0.153^{e}	2.324^{hi}	12.171^{gh}
Moderate stress	0	0.2002^{gh}	0.119^{i}	0.319^{g}	0.14^{f}	2.4185^{gh}	18.43^{b}
	30	0.228^{e}	0.146^{f}	0.374^{de}	0.135^{gh}	2.425^{gh}	17.068^{cd}
	60	0.256^{bc}	0.146^{f}	0.402^{cd}	0.141^{f}	2.233^{ij}	15.884^{e}
	90	0.197^{gh}	0.118^{i}	0.314^{g}	0.136^{g}	2.407^{h}	14.914^{f}
Severe stress	The plants treated with severe tension were unable to survive						

In each column, different letters indicate significant difference between the values of pair of treatments at the 5% probability level by Duncan's multiple range tests of the double interaction between irrigation and nitrogen in the chlorophyll content indicated the existence of a different reaction of nitrogen application at different levels of irrigation; so that in without drought stress, mild stress, and moderate stress conditions, the highest chlorophyll a content was obtained by consuming 60 kg N/ha, 30 kg N/ha, and 60 kg N/ha, respectively. Regarding chlorophyll b and total chlorophyll, in the without drought stress and moderate stress conditions, the highest amount resulted from the consumption of 90 kg N/ha and 60 kg N/ha, respectively (Tab. II). In total, the highest content of chlorophyll a (0.272 mg/g FW) was obtained under the interaction of mild stress and 30 kg/ha nitrogen application. The highest chlorophyll b and total chlorophyll content (0.205 and 0.468 mg/g FW) was obtained under irrigation conditions and 90 kg/ha nitrogen application (Tab. II). The obtained results showed that the dual interaction between nitrogen and methanol was significant in the content of chlorophyll a, b, and total, which indicated the different reactions of methanol application at different levels of nitrogen; so that in the non-application of nitrogen and the consumption of 60 kg N/ha, both levels of methanol decreased the content of chlorophyll a and total chlorophyll. In the application of 30 kg N/ha and 90 kg N/ha, the highest content of chlorophyll a was obtained by spraying 10% and 30% methanol, respectively (Tab. III). In the non-application of nitrogen, the highest content of chlorophyll b was related to the foliar application of 10% methanol. In the application of 60 kg N/ha, methanol application in both concentrations reduced the content of chlorophyll b. In the application of 30 kg N/ha and 90 kg N/ha, the foliar application of 30% methanol significantly increased the content of chlorophyll b and total chlorophyll (Tab. III). Interaction of 60 kg/ha nitrogen and 0% methanol had stronger effects on chlorophyll a (0.200 mg/g FW) but the interaction of 90 kg/ha nitrogen and 30% methanol had stronger effects on the chlorophyll b (0.131 mg/g FW) and total chlorophyll (0.328 mg/g FW) contents (Tab. III). Nevertheless, the application of 90 kg/ha N and 10% methanol showed the lowest chlorophyll a, chlorophyll b and total chlorophyll content (with a mean of 0.134 mg/g FW, 0.095 mg/g FW and 0.229 mg/g FW, receptively) (Tab. III). The chlorophyll a (0.197 mg/g FW), chlorophyll b (0.118 mg/g FW) and total chlorophyll contents (0.314 mg/g FW) decreased more under the interaction of moderate stress and 90 kg/ha nitrogen in comparison with the other interaction (Tab. II). Nitrogen is essential for chlorophyll synthesis and is involved in photosynthesis as part of chlorophyll molecules. Lack of nitrogen and chlorophyll means that the plant does not use sunlight as an energy source for basic tasks such as absorbing nutrients (Anas *et al.*, 2020). Researches have indicated that methanol plays a direct role in the chlorophyll content and photosynthetic processes, and since methanol efficiency reduces the efficiencies of carboxylation and photosynthesis, its efficiency can lead to significant increases in plant biomass (Wang *et al.*, 2012). In agreement with our results, Dinagaran *et al.* (2013) reported that drought stress leads to a significant decrease in photosynthesis due to chlorophyll detraction. There is evidence that drought stress causes damages to pigment and plastid (Fathi and Tari, 2016). It seems that this degradation is the result of chlorophyllase, peroxidase, phenol compounds and chlorophyll decomposition (Zahedi *et al.*, 2021). Reduced chlorophyll content was also observed at different

III: *Mean comparison of interaction of nitrogen and methanol on sum physiological traits in black cumin*

Nitrogen (kg/ha)	Methanol (%)	Chlo.a (mg/g FW)	Chlo.b (mg/g FW)	Total Chlo (mg/g FW)	Carotenoid (mg/g FW)	Protein (mg/g FW)	Proline (mg/g FW)
0	0	0.188bc	0.109e	0.298abc	0.118bc	1.928efgh	9.728de
	10	0.165def	0.127b	0.292bcd	0.101e	1.839ghijk	8.677ghi
	30	0.168de	0.108f	0.275cd	0.117bc	1.782ijkl	12.366a
30	0	0.171d	0.103h	0.273cd	0.102e	1.732kl	9.427ef
	10	0.189bc	0.107f	0.296bc	0.106d	1.727l	9.104fg
	30	0.186c	0.126b	0.312ab	0.117c	1.894fgh	8.91gh
60	0	0.200a	0.120c	0.320ab	0.115c	1.837ghijk	8.087j
	10	0.194ab	0.119c	0.314ab	0.116c	1.982def	9.004ef
	30	0.185c	0.112d	0.297abc	0.126a	1.866ghij	9.903d
90	0	0.167de	0.105g	0.272cd	0.121b	1.454m	8.607hi
	10	0.134g	0.095i	0.229fg	0.108d	1.893fgh	9.703de
	30	0.198a	0.131a	0.328a	0.12b	2.018cde	6.873l

In each column, different letters indicate significant difference between the values of pair of treatments at the 5% probability level by Duncan's multiple range tests.

levels of water deficit stress in beans (Armand *et al.*, 2016) and marigold (Khalilzadeh *et al.*, 2020). Increased chlorophyll content by methanol was observed in sugar beet (Abido, 2012), soybean (Mirakhori *et al.*, 2009), Beans (Armand *et al.*, 2016), and lavender (Bagheri *et al.*, 2014). Amiri *et al.* (2010) showed that interaction between irrigation treatment and nitrogen fertilizer had a significant effect on chlorophyll content in plant.

Protein Content

The obtained results showed that the double interaction of irrigation and nitrogen was significant in the protein content, which indicated the existence of a different reaction of nitrogen application at different levels of irrigation; so that in non-stress, mild stress, and moderate stress conditions, the highest protein content was obtained by consuming 60 kg N/ha, 60 kg N/ha, and 30 kg N/ha, respectively (Tab. II). In total, the highest amount of protein content (17.067 mg/g FW) was achieved in 30 kg/ha N and 30% methanol spraying (Tab. II). The significance of the dual interaction between nitrogen and methanol in the protein content indicated the different reactions of methanol application at different levels of nitrogen; so that both levels of methanol decreased the protein content in the non-application of nitrogen while increased it in the application of 60 kg N/ha and 90 kg N/ha. In the application of 30 kg N/ha, the highest protein content was obtained by spraying 30% methanol (Tab. III). In total, the application of 90 kg/ha nitrogen and 30% methanol had stronger effects on the amount of protein (2.018 mg/g FW) in comparison with other interactions between methanol spraying and nitrogen application (Tab. III). However, 90 kg/ha N and 0% methanol application showed that the lowest protein content with a mean of 1.45 mg/g FW was related to 10% methanol foliar application and 90 kg/ha nitrogen application (Tab. III).

The results of the double interaction between irrigation and methanol showed that both levels of methanol increased protein content in all irrigation levels (Tab. IV). In total, interaction of 10% methanol and control condition irrigation had stronger effects on the amount of protein in comparison with interactions of methanol and water stress condition treatment (Tab. IV). Interaction of moderate stress and 0% methanol (2.174 mg/g FW) decreased the amount of protein more in comparison with interactions of irrigation condition and methanol treatments (Tab. IV). Also, the amount of protein decreased more because of the interaction of 90 kg/ha N and mild tensions (2.098 mg/g FW) (Tab. II). Methanol has a direct role in increasing the amount of plant protein and by applying drought stress, the amount of plant protein increases to increase plant resistance to stress. In addition, nitrogen appears with the role of protein producing compound in the plant (Mirakhori *et al.*, 2009). Moreover, Nitrogen is part of the vitamins, amino acids, and energy systems within a plant that make up its proteins. Therefore, it is directly responsible for increasing the amount of protein in plants (Anas *et al.*, 2020). Drought stress is an important restricting factor that can damage photosynthetic pigment and plastid and reduce protein content. Also, droughts can severely affect plant growth, protein content, crop yield, and food production. Water deficit stress increased the amount of soluble proteins in pea leaves (Najaphy *et al.*, 2010). In the wheat, the concentration of protein also decreased with increasing drought stress (Abid *et al.*, 2018). Increases in total protein with methanol spraying have also been reported in other plants such as pea (Hossinzadeh *et al.*, 2014) and peanut (Babaei *et al.*, 2014), which is consistent with our results. The auxin and cytokinin hormones play an important

IV: *Mean comparison of interaction effect of drought stress and methanol on proline and protein contents in black cumin*

Drought stress	Methanol (%)	Protein (mg/g FW)	Proline (mg/g FW)
Control	0	2.471[e]	8.252[h]
Control	10	2.664[d]	9.842[fg]
Control	30	2.567[d]	7.795[i]
Mild tension	0	2.306[gh]	11.301[e]
Mild tension	10	2.415[ef]	11.084[e]
Mild tension	30	2.416[ef]	12.789[d]
Moderate stress	0	2.174[i]	16.297[b]
Moderate stress	10	2.362f[gh]	15.958[c]
Moderate stress	30	2.577[d]	17.467[a]
Severe tension	The plants treated with severe tension were unable to survive		

In each column, different letters indicate significant difference between the values of pair of treatments at the 5% probability level by Duncan's multiple range tests role in enhancing protein production in plants. Since foliar application of methanol enhances the activity of methylotrophic bacteria, they eventually provide some precursor constructs. Hormones such as auxin and cytokinin can increase the production of auxin and cytokinin in plants (Wu *et al*., 2013). Methanol stimulates continuous synthesis of soluble protein in plant leaves, delaying leaf senescence and ultimately enhancing photosynthetic function (Wu *et al*., 2013). The amount of soluble proteins of maize leaf under nitrogen treatment was higher than the control (Hossinzadeh *et al*., 2014). Nitrogen deficiency partially affected leaf protein changes during the stress period and reduced plant resistance to stress (Najaphy *et al*., 2010). Nitrogen is present in the structure of proteins, especially photosynthetic proteins such as the Rabisco enzyme, Therefore, low nitrogen levels partially affect leaf protein changes during drought stress and reduce plant resistance to stress, which was in agreement with the results of Hosseinzadeh *et al*. (2014).

Proline Content

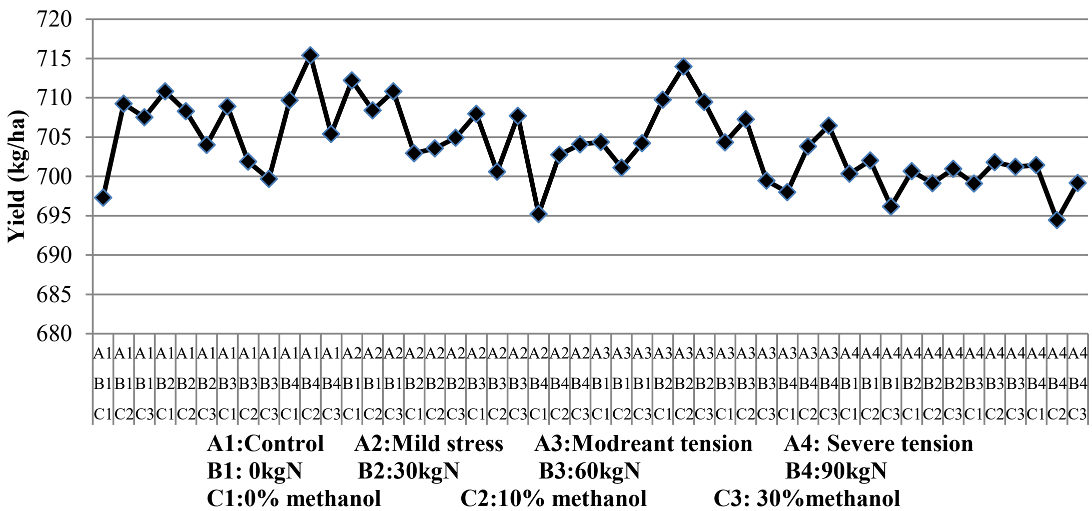
The significance of the dual interaction between irrigation and nitrogen in the proline content indicated the existence of a different reaction of nitrogen application at different levels of irrigation; so that in non-stress, mild stress, and moderate stress conditions, the highest proline content was related to the non-consumption of nitrogen, 90 kg N/ha, and the non-consumption of nitrogen, respectively (Tab. II). The results showed that the highest proline content (18.43 mg/g FW) was obtained in moderate stress conditions and 0 kg/ha N (Tab. II). Nevertheless, the result showed that the lowest amount of proline content (6.492 mg/g FW) was obtained as the effect of interaction treatment between 90 kg/ha nitrogen application and control stress condition (Tab. II). As a result of the dual interaction between nitrogen and methanol, both levels of methanol decreased the proline content in the application of 30 kg N/ha. In the non-application of nitrogen and the application of 60 kg N/ha and 90 kg N/ha, the highest proline content was obtained by spraying 30%, 30%, and 10% methanol, respectively (Tab. III). In total, more proline content was obtained between 30% methanol foliar application and 0 kg/ha N with mean of 12.366 mg/g FW in comparison with interactions of methanol spraying and Nitrogen application (Tab. III). The obtained results showed that the double interaction of irrigation and methanol was significant in the proline content, which indicated the different response of methanol application at different irrigation levels; so that in non-stress, mild stress, and moderate stress conditions, the highest proline content was obtained with the foliar application of 10%, 30%, and 30% methanol, respectively (Tab. IV). Totally, moderate stress and 30% methanol application increased the amount of protein content more with a mean of 17.467 mg/g FW in comparison with interactions between methanol spraying and irrigation condition (Tab. IV). Interaction of 30% methanol spraying and control condition of irrigation (7.795 mg/g FW) decreased the amount of proline content more in comparison with interactions of water stress and methanol percent (Tab. IV). In fact, the result showed that interactions between the application of 90 kg/ha nitrogen and 30% methanol decreased (6.873 mg/g FW) the amount of proline more in comparison with the interaction treatment between nitrogen application and methanol spraying (Tab. III). Proline is an important osmolyte to maintain inflammation that contributes to the continuous growth of plants under water deficit conditions (Takahashi *et al*., 2020). In fact, drought stress leads to a significant increase in proline due to its protective effect. The amino acid proline, as the most common and widespread osmolite in many plants, is responsible for osmotic regulation, water retention, and cell turbulence during drought stress (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Researches have indicated that water stress and nitrogen increased the amount of proline content, and its efficiency can lead to significant increases in plant drought tolerance (Srivastava *et al*., 2018). Increased proline content under drought conditions have been observed in plants such as Sorghum bicolor (Sarshad *et al*., 2021), coriander (Bagheri *et al*., 2014), Kalmatis from the darker Allalgan (Armand *et al*., 2016) and seedlings (Abido, 2012). Proline accumulation in many plant species has been reported due to high synthesis and less degradation within plants under stress conditions such as salinity, water deficiency and heavy metals (Jain *et al*., 2013). Increased proline content by methanol application has been reported in plants such as pea (Hossinzadeh *et al*., 2014), marigold (Khalilzadeh *et al*., 2020), and soybean (Akram, 2014). Methanol increases the activity of 5-carboxylate synthetase by producing an acidic environment and consequently develops the proline content in leaves (Jain *et al*., 2013). Proline is a compound containing nitrogen and as an osmotic compound stabilizes nucleic acids, proteins and membranes, so increasing nitrogen can elevate the amount of proline (Akram, 2014). The increase in proline by applying nitrogen was also in agreement with the results of Abido (2012) in pumpkin and Bagheri *et al*. (2014) in spring barley.

Carotenoid Content

The obtained results showed that the double interaction of irrigation and nitrogen was significant in the carotenoid content, which indicated the existence of a different reaction of nitrogen application at different levels of irrigation; so that the highest content was related to the application of 60 kg N/ha under non-stress, mild stress, and moderate stress conditions (Tab. II). The results indicated that the highest amount of carotenoid content (0.182 mg/g FW) was achieved in 60 kg N/ha and control irrigation (Tab. II). The obtained results showed that the dual interaction between nitrogen and methanol was significant in the carotenoid content, which indicated the different reactions of methanol application at different levels of nitrogen; so that the application of methanol, especially at 10% concentration, reduced carotenoid content in the non-application of nitrogen and the application of 90 kg N/ha. In the application of 30 kg N/ha and 60 kg N/ha, both levels of methanol, especially 30% level, increased carotenoid content (Tab. III). In total, nitrogen application by 60 kg/ha and 30% methanol had stronger effects on the amount of carotenoid content (0.126 mg/g FW) in comparison with interactions between methanol spraying and nitrogen application (Tab. III). However, 0 kg/ha N and 10% methanol application showed that the lowest carotenoid content with a mean of 0.101 mg/g FW was related to 10% methanol foliar application and no nitrogen application (Tab. III). Interaction of 90 kg/ha N and 10% methanol application decreased (0.101 mg/g FW) the amount of carotenoid more in comparison with interactions of nitrogen application and methanol application (Tab. III). Nitrogen is directly related to the synthesis of amino acids, carbohydrates, chlorophyll molecules and carotenoids, and plays a crucial role in plant growth and development (Sage *et al.*, 2013). Numerous studies have shown that leaf carotenoid concentration has a strong positive correlation with leaf nitrogen concentration (Rhein and Silva, 2017). Researchers have found that methanol and drought stress play a direct role in photosynthetic processes (Mirakhori *et al.*, 2009). Lower carotenoid content by elevating drought stress has also been reported in the medicinal plants, such as marigold (Khalilzadeh *et al.*, 2020), as confirmed by this study. Methanol application at all drought stress levels increased the carotenoid content of beans (Armand *et al.*, 2016), although enriching carotenoid content by the methanol consumption was consistent with the results of the above studies. Interaction between irrigation interval and nitrogen fertilizer had a significant effect on carotenoid content of sugar cane (Bassi *et al.*, 2018). Bagheri *et al.* (2014) reported that most of these photosynthetic pigments have nitrogen in their structure, so the application of nitrogen can greatly increase their content in the plant. Increasing carotenoid content with nitrogen application has also been observed in the bitter leaf (Tjhia *et al.*, 2018).

Seed Yield

The significance of the double interaction between irrigation and nitrogen in the seed yield indicated the existence of a different reaction of nitrogen application at different levels of irrigation; so that in non-stress and moderate stress conditions, the highest seed yield was obtained by consuming 90 kg N/ha and 30 kg N/ha, respectively (Fig. 1). The results showed that the average seed yield was 707.54 kg/ha, while in the case of severe drought stress, grain yield 1% decreased in comparison to the control. Also, the application of 30 kg/ha of nitrogen fertilizer had the highest grain yield per hectare. However, different levels of foliar application of methanol were not significantly different in the grain yield. The result showed that the highest grain yield (717.11 kg/ha) was obtained by the interaction of 90 kg/ha nitrogen and no drought in comparison with the other interaction. The more grain yield (715.41 kg/ha) in triple-interaction-treatments condition was obtained between the control irrigation, 90 kg/ha nitrogen and 10% methanol in comparison with the other triple interaction treatments. The lowest grain yield was also associated with severe stress conditions



1: *Mean comparison of interaction of nitrogen and methanol on yield under drought stress conditions in black cumin*

(Fig. 1). An increased biomass is usually recognized as a general response to drought and may improve salinity tolerance by restricting the instability of toxic ions to the shoots and roots, which consequently delays the onset of the tolerance threshold. Notably, drought tolerance can be measured by the grain yield (Pour-Aboughadareh *et al*., 2020). Increased nitrogen leads to greater dry matter production and grain yield, root development and greater moisture uptake. In addition, the increase in nitrogen accelerates the growth of greens, increasing the volume of the aerial part of the plant (Anas *et al*., 2020). The decrease in the grain yield with intensifying water deficit in our study was consistent with other studies on plants such as durum wheat (Pour-Aboughadareh *et al*., 2020), pumpkin (Aghaee and Ehsanzadeh, 2011), and coriander (Norzad *et al*., 2014). The effect of water deficit reduction on the grain yield is due to its negative effects on leaf area, photosynthesis, plant height, flowering period, and yield components such as the number of pods per plant, number of seeds per plant, grain weight and harvest index. Application of substances such as methanol that can increase CO_2 concentration in a plant will improve yield under water scarcity conditions (Armand *et al*., 2016). Under drought stress conditions, application of 14 and 21% methanol significantly increased grain yield in soybean (Mirakhori *et al*., 2009). Higher grain yield was also observed using methanol application in rice (Rezaeieh *et al*., 2015), soybean (Mirakhori *et al*., 2009), pea (Hossinzadeh *et al*., 2014), and peanut (Babaei *et al*., 2014). Increased grain yield with the consumption of 80 kg N/ha has been reported in black seed (Tulukcu, 2016). Increasing grain yield using low nitrogen levels in the study on soybean (Epie *et al*., 2022), black seed (Wako *et al*., 2022), barley (Armand *et al*., 2016), and pumpkin (Aghaee and Ehsanzadeh, 2011) has been reported. The increase in black seed yield with nitrogen consumption can be due to the effective role of nitrogen in the structure of chlorophyll, photosynthetic rate, and plant growth (Anas *et al*., 2020).

Phytochemical Compounds Analysis

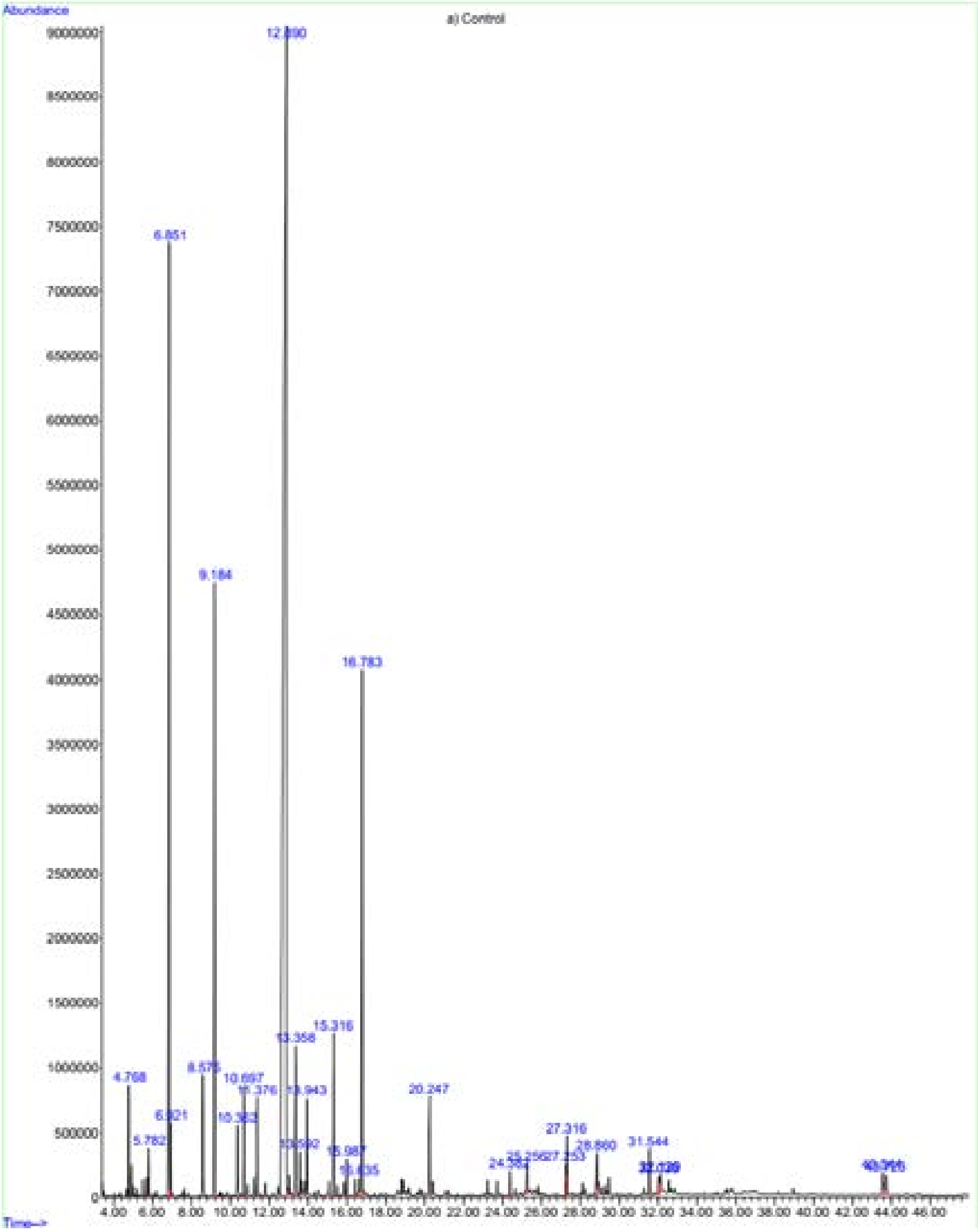
The results showed higher nitrogen level led to an increase in oil weight and oil volume so that the highest oil weight (12.7 g) and oil volume (12.9 mL) were obtained under 90 kg/ha nitrogen treatments (Tab. V). Nevertheless, spraying the methanol reduced the oil weight and oil volume. Drought stress had negative effects on the oil weight and oil volume. The largest reduction of the oil weight (8.2 g) and oil volume (10 mL) was obtained at moderate stress (Tab. V). A total of 24 to 35 phytochemical compounds were obtained in the GC-MS analysis results under different nitrogen and methanol treatments under drought stress condition (Tab. VI). The highest (57.98%) and the lowest (22.47%) Thymoquinone percentages were obtained in 10% methanol spraying and application of 90 kg/ha nitrogen, respectively. By elevating the drought stress, the Thymoquinone and symol percentages were decreased, while the moderate stress led to an increase in Thymoquinone and Symol percentages. The highest (35.55%) and the lowest (5.82%) Symol percentages were obtained under 30% methanol spraying and severe tension condition, respectively. The result showed that the amount of Thymoquinone decreased by augmenting the nitrogen levels (Tab. VI and Fig. 2). Drought stress decreased the oil content in black cumin (Bayati *et al*., 2020). Drought stress also enriched seed oil content (Armand *et al*., 2016), as confirmed by our results on increasing oil content under drought stress. Aghaei and Ehsanzadeh (2011) reported that irrigation program before the harvest stage had no significant effect on the oil percentage of the oil seed.

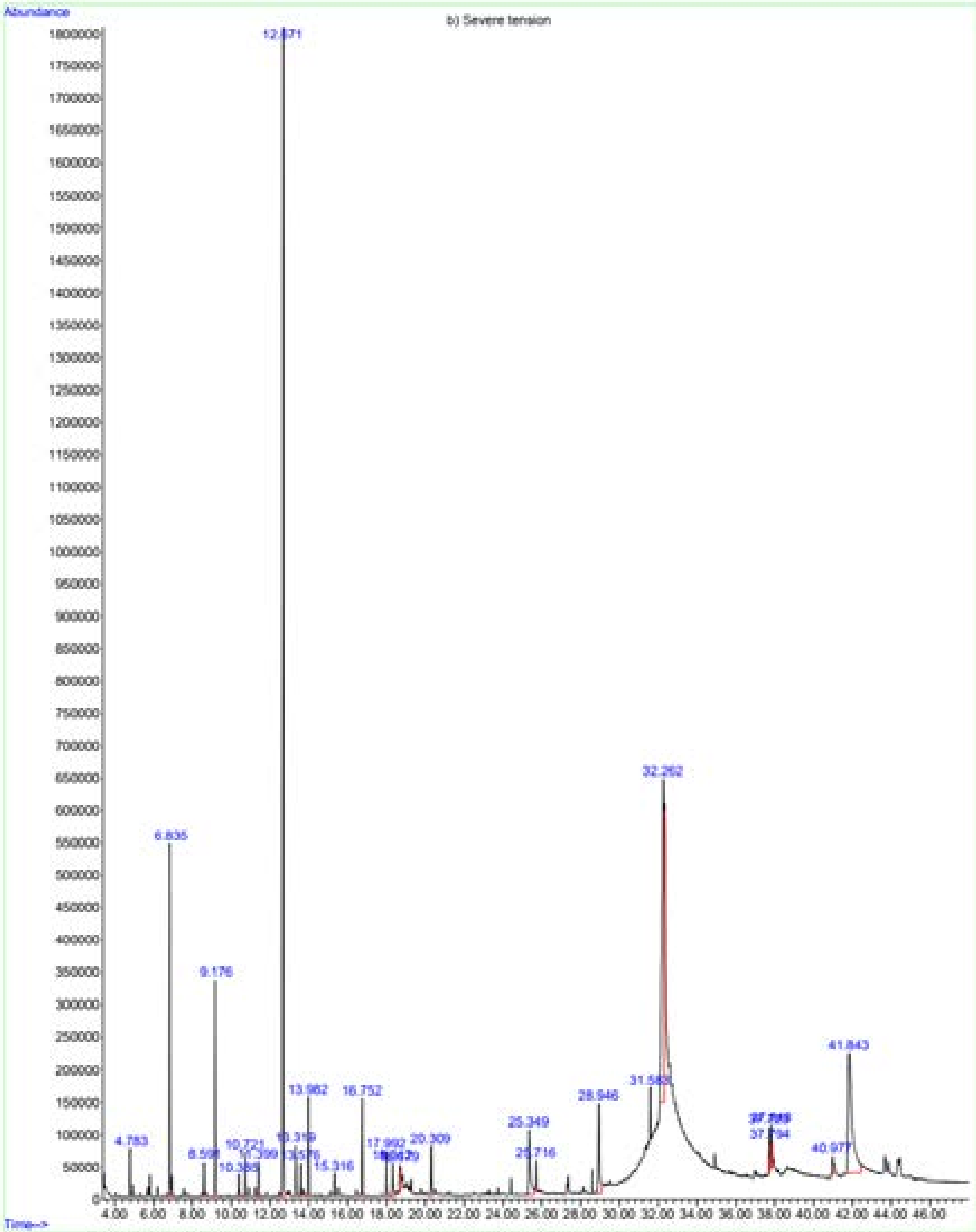
V: *Mean comparison of irrigation, nitrogen and methanol levels on oil weight (percent and amount) and oil volume (amount) in black cumin*

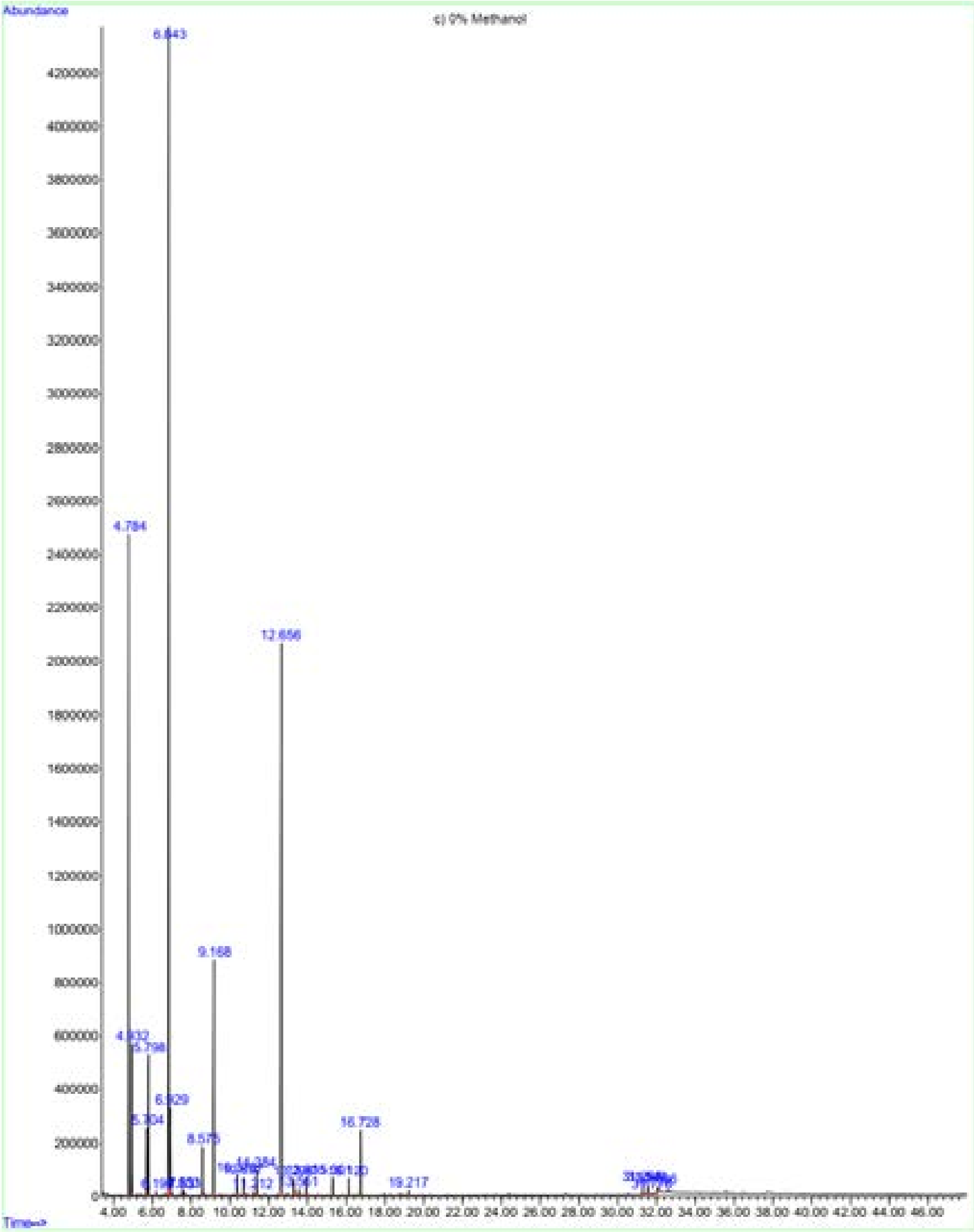
Treatment	Oil weight (%)	Oil Weight (g)	Oil volume (mL)
Control	83.3	10	12
Mild tension	86.7	10.4	10.4
Moderate stress	68.3	8.2	10
Severe tension	83.3	10	12.5
0 kg/ha Nitrogen	68.3	8.2	10
30 kg/ha Nitrogen	85.8	10.3	10.5
60 kg/ha Nitrogen	87.5	10.5	10.5
90 kg/ha Nitrogen	92.5	12.7	12.9
0 % Methanol	75.6	12.1	12.1
10 % Methanol	58.6	7.38	9.85
30 % Methanol	65.6	10.5	9.98

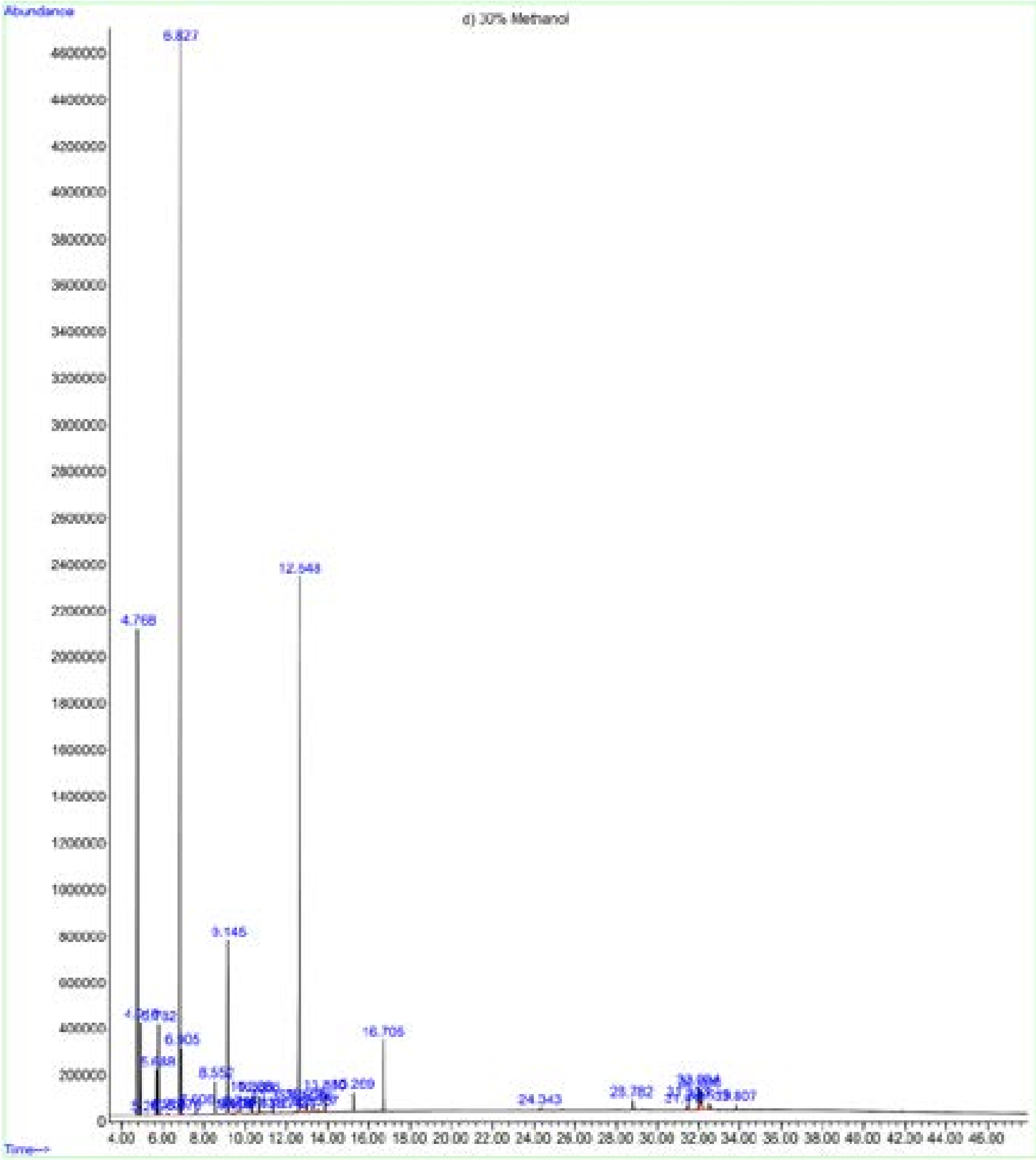
VI: *The four main phytochemical compounds of black cumin under nitrogen, methanol and drought stress conditions using GC-MS analysis*

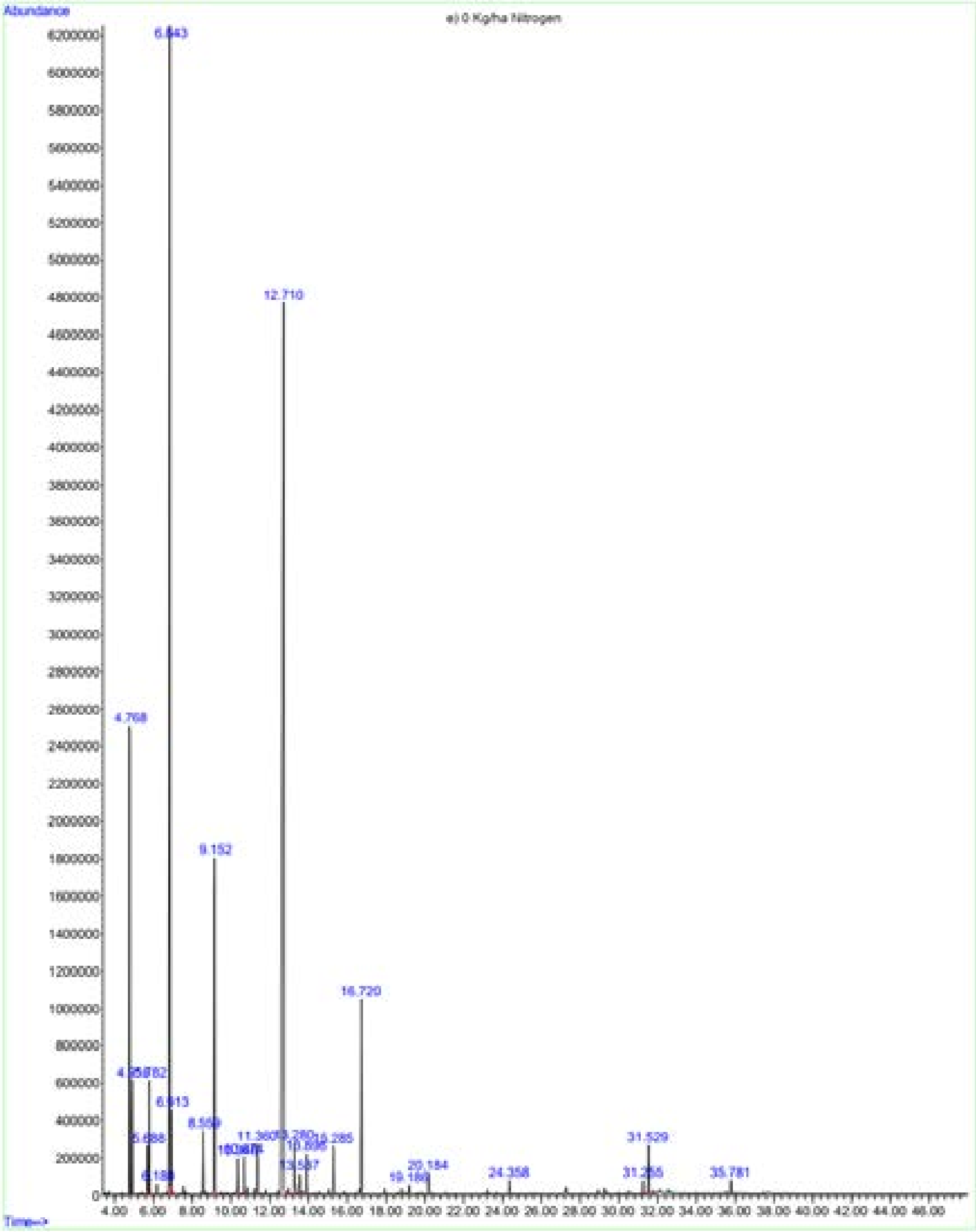
Treatment	Compound number	RT (min)	Hit Name	Mol Weight (amu)	Quality	% of total	corr. % max.
Control	28	12.89	Thymoquinon	164.08	90	56.06	100.00
		6.85	Cymol	134.11	97	11.13	19.84
		9.18	1-Pentene, 2-methoxy	154.14	25	7.26	12.94
		16.78	Junipene	204.19	99	7.18	12.81
Mild tension	26	6.84	Cymol	134.11	97	28.96	100.00
		12.66	Thymoquinon	164.08	98	23.52	81.22
		9.16	1-Pentene, 2-methoxy	100.09	30	9.52	32.88
		16.74	Kuromatsuene	204.19	99	7.78	26.87
Moderate stress	30	12.71	Thymoquinon	164.08	98	50.81	100.00
		6.83	Cymol	134.11	95	16.88	33.22
		9.16	1-Pentene, 2-methoxy	136.13	25	6.30	12.40
		16.74	Kuromatsuene	204.19	99	5.84	11.48
Severe tension	27	12.67	Thymoquinon	164.08	98	27.83	100.00
		32.26	9,12-Octadecadienoic acid	280.24	99	21.93	78.78
		41.84	9,17-Octadecadienal	264.25	97	14.97	53.81
		6.84	Cymol	134.11	97	5.82	20.92
0 % Methanol	27	6.84	Cymol	134.11	97	34.81	100.00
		12.66	Thymoquinon	164.08	98	22.94	65.91
		4.78	Alpha-Thujene	136.13	94	14.58	41.88
		9.17	Gamma-Terpinene	136.13	25	6.87	19.74
10 % Methanol	25	12.91	Thymoquinon	164.08	93	57.98	100.00
		6.86	1-Methyl-2-isopropylbenzene	134.11	97	12.68	21.87
		9.18	Gamma-Terpinene	136.13	25	5.52	9.52
		16.77	Kuromatsuene	204.19	99	4.79	8.25
30 % Methanol	35	6.83	Cymol	134.11	97	35.55	100.00
		12.65	Thymoquinon	164.08	98	29.12	81.91
		4.77	Alpha-Thujene	136.13	94	10.64	29.93
		9.15	Delta.3-Carene	136.13	55	5.33	15.00
0 kg/ha Nitrogen	24	12.71	Alpha-Pinene	136.13	70	41.01	100.00
		6.84	Cymol	134.11	97	25.55	62.30
		4.77	Alpha-Thujene	136.13	95	6.77	16.50
		9.15	Gamma-Terpinene	136.13	40	6.41	15.62
30 kg/ha Nitrogen	30	6.86	1-Methyl-2-isopropylbenzene	134.11	97	29.73	100.00
		12.70	Thymoquinon	164.08	98	23.33	78.45
		4.79	Sabinen	136.13	94	9.79	32.91
		9.18	1-Pentene, 2-methoxy	100.09	30	8.47	28.50
60 kg/ha Nitrogen	24	6.81	1-Methyl-2-isopropylbenzene	134.11	97	36.79	100.00
		12.60	Thymoquinon	164.08	98	22.93	62.31
		4.77	Alpha-Thujene	136.13	94	16.30	44.32
		9.14	Delta.3-Carene	136.13	43	6.19	16.82
90 kg/ha Nitrogen	25	6.83	Cymol	134.11	97	34.62	100.00
		12.62	Thymoquinon	164.08	98	22.46	70.67
		4.78	Alpha-Thujene	136.13	94	15.22	43.97
		9.15	Beta-Phellandrene	136.13	25	6.34	18.31

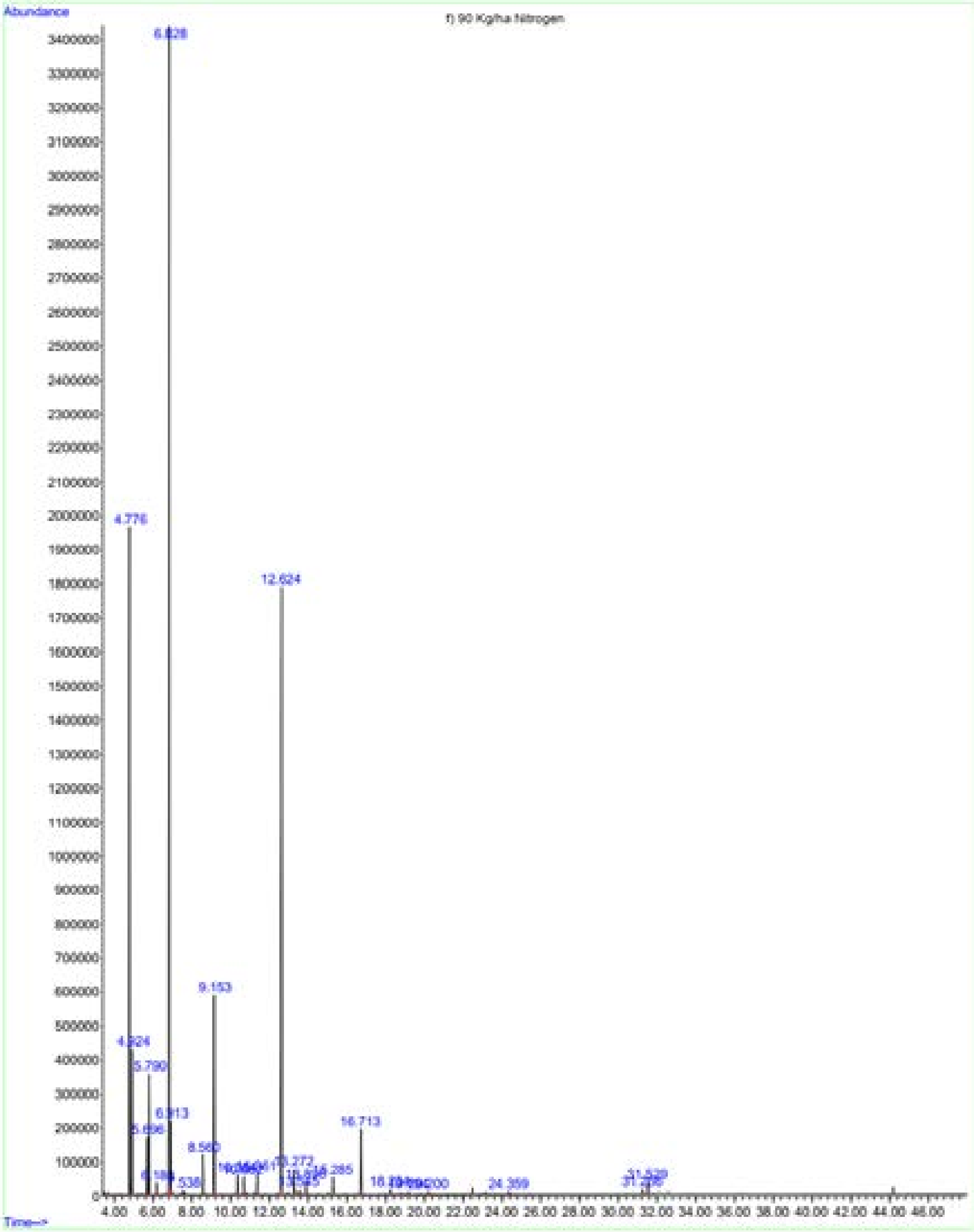

Abundance
a) Control
9000000
8500000
8000000
7500000
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
12.890
6.851
9.184
16.783
15.316
4.798
8.575
10.697
20.247
27.316
31.544
28.860
Time-->
4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00 26.00 28.00 30.00 32.00 34.00 36.00 38.00 40.00 42.00 44.00 46.00


Abundance
b) Severe tension
Time-->
12.671
6.835
9.176
4.783
10.721
13.982
16.752
15.316
17.982
20.309
25.349
25.716
28.946
32.262
37.794
40.977
41.843

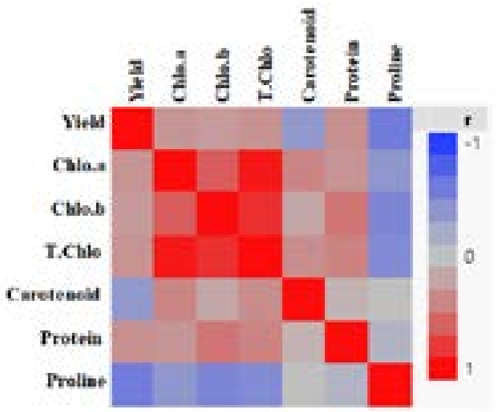

Abundance
c) 0% Methanol
6.843
4.784
12.656
9.168
4.832
5.798
6.929
5.704
8.575
16.728
19.217
Time-->


Abundance
d) 30% Methanol
6.827
12.548
4.768
9.145
6.905
8.552
16.705
24.343
28.782
Time-->


Abundance
e) 0 Kg/ha Nitrogen
6.843
12.710
4.768
9.152
16.720
31.529
24.358
35.781
Time-->



2: *Chromatograms of the phytochemicals compound under control (a), severe tension (b), 0% methanol (c), 30% methanol (d), 0 kg/ha nitrogen (e) and 90 kg/ha nitrogen (f) treatments using GC-MS analysis*



3: *Correlations among studied traits in black cumin. The strength and direction of the correlations among the different traits are indicated by the color (red indicates positive correlations while blue indicates negative correlations, and the shading represents the strength of the correlation).*

However, no significant decrease was observed in oil percentage with elevating water deficit stress (Aghaee and Ehsanzadeh, 2011). In our research, the oil percentage reduced by increasing methanol levels, which did not agree with the results of other research on some plants such as marigold (Aghaee and Ehsanzadeh, 2011), pumpkin and soybean (Mirakhori *et al*., 2009). With respect to the Greek oregano, the highest yield and oil percentage were obtained in 90 kg/ha N (Krol *et al*., 2020). Increase in the seed oil by applying nitrogen has also been reported in the study on soybean (Mirakhori *et al*., 2009). Increasing nitrogen levels reduced the percentage of pumpkin seed oil. In fact, with increasing nitrogen levels, the amount of protein increased in the seed (Aghaee and Ehsanzadeh, 2011). In black seed plant, the application of urea in the amount of 53.3 kg/ha significantly increased the oil percentage and oil yield (Moradzadeh *et al*., 2021) which was in agreement of our results.

The correlation among the measured traits was significant and positive at $P \leq 0.01$ (Fig. 3). Interestingly, chlorophyll a was highly correlated to Total chlorophyll (r = 0.992), while the lowest correlation was observed in chlorophyll b and yield (r = 0.508).

CONCLUSION

Overall, appropriate fertilizer and optimal environmental conditions are important to achieve high yield. In this study, methanol and nitrogen levels were applied under drought stress condition in black cumin. The results indicated that methanol and nitrogen application enhanced tolerance to the drought stress condition and higher yield in black cumin due to triggering the production of phytochemical compounds such as symol. It can be noticed that combined treatments of methanol and nitrogen were more effective than their individual applications. Our findings suggested that application of 10% methanol and 90 kg/ha nitrogen was the most effective treatments in black cumin. In view that all the growth indices and phytochemical compounds decreased in sequence of increasing drought stress levels, high drought stress (severe tension), could be concluded as the extreme drought level.

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Contact information

Daryush Talei: d.talei1348@gmail.com