Identification of Organic Compounds in Wood and Bark of Persian Oak Having Different Levels of Crown Dieback Using Gas Chromatography-Mass Spectrometry

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The influence of environmental factors on variations in organic compound content of sapwood and inner bark of mature Persian oaks were investigated in the Zagros forests, western Iran. Trees with four levels of defoliation were selected: healthy, light, moderate, and severely defoliated. First, wood and bark flours were prepared based on standard TAPPI test methods. The extractives of the tissues were obtained using Soxhlet and acetone solvents. Subsequently, the extractives compounds were derivatized using a silane-based compound and then analyzed by gas chromatography-mass spectrometry. Six dominant compounds were detected in the wood and bark of healthy oaks, with 1,6-anhydro-β-D-glucopyranose (levoglucosan) and 1,2-benzenedicarboxylic acid (phthalic acid) being the most abundant components. In trees with light defoliation, five dominant compounds were identified, among which phthalic acid and salicylic acid were the most abundant components. In trees with moderate dieback, 6-aza-5,7,12,14-tetrathiapentacene (heteropentacene) and salicylic acid were found in the greatest quantities, while in trees with severe dieback, salicylic acid and phthalic acid were the dominant components in the wood and bark of Persian oak trees. Salicylic acid and gibberellin A3 were common components in all bark and wood samples, while other compounds differed between different classes of dieback.

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INTRODUCTION

It has long been known that forest dieback is a major consequence of climate change, often exacerbating forest trees' mortality. In the last two centuries, most forests worldwide have faced this major challenge, which has now become an acute problem, particularly affecting oak-dominated forests (Kabrick *et al.* 2008). Zagros oak forests constitute the largest forested region in Iran (Mohammadzadeh *et al*. 2021). Recently these oak forests have been affected by severe drought, resulting in widespread dieback of the dominant Persian oak (*Quercus brantii*) (Ghanbary *et al.* 2017).

Evaluation of the dieback in the Zagros forests is still in a preliminary stage and no comprehensive studies have been conducted on the precise causes, although a number of publications are available focused on this phenomenon in Iran and around the globe (Mohammad Zadeh *et al.* 2021; Soheili *et al.* 2021; Tongo *et al.* 2021).

Plants have several adaptive strategies to reduce the harmful effects of drought stress (Palva *et al.* 2001), including the ability to synthesize many types of protective organic compounds. In the extracts of wood and bark of trees, a wide variety of organic compounds are found that have different chemical structures (Balaban and Uçar 2001). Extractives are non-structural organic compounds with low molecular weight that are not directly involved in growth and development and/or reproduction of an organism. These compounds may play a role in the defense system of plants against adverse biotic and abiotic conditions and aid in survival during interactions with the environment (Vaysi 2017a). Although the defensive role of secondary metabolites is recognized, the understanding of the mechanisms by which these compounds interact with environmental stress remains unclear.

Unlike primary metabolites, such as carbohydrates, proteins, and nucleic acids, the absence of secondary metabolites does not immediately lead to cell death, although in the long term it may lead to disorders that affect the survival of the organism, reduce fertility or its obvious properties, or cause no visible changes (Sumner *et al.* 2015). Given the wide diversity of extractives in wood and bark, and their effects on the physical properties of tree species and the natural durability of wood, it is likely that extract chemistry plays an important role in plants in terms of resistance to stress (Vaysi 2017b). Therefore, there is a possibility that the types and proportions of organic compounds in Persian oak tissues are related to different levels of dieback, which may increase resistance to early mortality. The work described here can be considered as one of the first studies in this regard.

One of the methods used to identify compounds in plant extracts is gas chromatography-mass spectrometry (GC-MS) (Tajik *et al.* 2013). Different types of chromatography are good methods for detecting components at different concentrations based on the molecular size and polarity of the compounds (Hosseini Hashemi *et al.* 2015).

The antioxidative effects of secondary metabolites, such as polyamines and phenolic compounds, and their roles in plants under stress have been demonstrated in several studies. In comparison to susceptible plants, stress-resistant rice plants have a higher potential to increase the concentrations of polyamines (Yang *et al.* 2007). Sivasamy *et al.* (2022) showed that in onions (*Allium cepa*), secondary metabolites including phenol 2,4-bis (1,1-dimethylethyl) hexadecane, 7,9-di-*tert*-butyl-1-oxaspiro-(4,5)-deca-6,9-diene-2,8-dione, butyl-octyl ester, phthalic acid, and di-iso-octyl ester increased under stress. The *A. cepa* increases its defense capability by producing additional secondary metabolites that it employs as preventive defensive counter measures during stress. Moradi (2018) reported a 242% increase of salicylic acid and a 35% decrease in gibberellic acid occurred in a kind of thyme plants (*Thymus vulgaris*) under drought stress, respectively.

Quantification of metabolites by GC-MS in different tissues (bark and wood) of Persian oaks subjected to different levels of crown dieback could help to better understand the competitive ability of trees under stress and identify intrinsic responses of trees in forest stands. This study follows up on Mohammadzadeh *et al.* (2021), who described the variations of different nutrients from leaves, bark, and wood of Persian oaks with different severity of dieback. To the best of the authors' knowledge, this is the first report that investigates extractives in Persian oaks affected by drought stress. In the current study, it was hypothesized that Persian oak develops a proactive defense against drought stress by producing defensive metabolites. Therefore, the main objective of the study was to identify secondary metabolites in Persian oak trees with different levels of dieback, using GC-MS analysis.

EXPERIMENTAL

Study Area

Trees in the Gachan forest area, where oak mortality is widespread, were selected for this study. The Gachan forest covers an area of 6.6 hectares and is located about 7 km northeast of the city of Ilam (Iran). Basic information about the forest area is presented in Table 1 and Fig. 1. The main tree species in the stand were *Quercus brantii* (Persian oak), *Pistachia atlantica*, *Amygdalus scoparia*, *Acer monspessulanum*, and *Crataegus aronica*. The occurrence of drought, including crown dieback and tree mortality, was first reported in 2008 (Mirabolfathy 2013). The climate in the study area is classified as Mediterranean based on the De Martonne Aridity index, with dry and warm summers, and wet and mild winters.

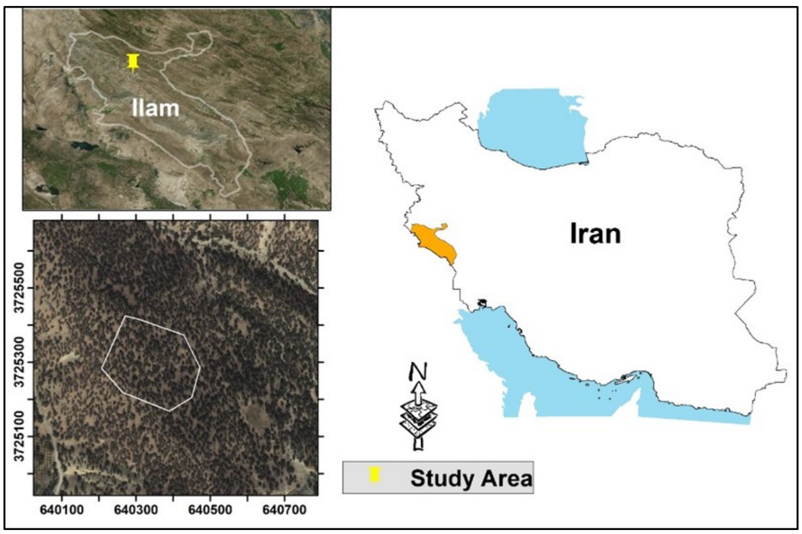


Fig. 1. The location of Gachan forest area in Ilam province and the Iran map (adopted from Mohammadzadeh *et al.* 2021)

Table 1. Basic Information of the Sampled Forest Stands in Gachan Area, Ilam Province, Iran

Sampling Site	Y UTM	X UTM	Alt (m)	Slope (%)	AAP (mm)	AAT (°C)	R.H (%)
Gachan	3726625	0639242	1821	35	551.8	16.19	56

Y: Longitude; X: Latitude; Alt: Altitude; APA: Average of Annual Precipitation (mm); AAT: Average of Annual Temperature; RH: Relative humidity. Climate data were based on the synoptic meteorological station of Ilam

Sampling Methods

Sampling of sapwood and secondary phloem (inner bark) of Persian oak trees (*Quercus brantii*) showing different intensities of dieback were chosen. The selected trees were mature and seed-originated, with diameters at breast height (DBH) ranging between 30 and 40 cm. The forest sampling area had uniform conditions (habitat, altitude, topography) and all trees were in the same biosocial classes. Selected trees were classified based on severity of crown dieback (class of defoliation) according to Kabrick *et al.* (2008): healthy trees (up to 5%); slight (5% to 33%); moderate (34% to 66%); or severe (100% defoliation). Three trees were randomly selected from each level of dieback. Because the forest is protected, it was not possible to obtain permission to cut trees for sampling. Core samples were taken using a 5 mm increment borer (Haglöf Sweden®) at breast height on each selected tree. The borer was drilled into the side of the trunk facing the dominant slope. The sapwood was distinguished from heartwood based on their different colors. The phloem tissues were separated from the rhytidome and sapwood.

Measurement of Chemical Compounds

Phloem and sapwood specimens were cut into small chips and subsequently ground to flour and passed through an 80-mesh sieve, according to the TAPPI T257 om-85 standard (2009). The tissue remaining on the sieve was collected for analysis. Approximately, 5 g (± 0.005 g) of coarse flour was extracted in acetone using Soxhlet for 8 h (Holmbom 1977; Tunalier *et al.* 2005). Extractives were stored at 4 °C until required.

Subsequently, nitrogen gas was passed through the extractives to evaporate the solvent. The extractive compounds were derivatized using a silane-based compound before GC-MS analysis. The purpose of the derivatization was to replace -OH groups on the extracted compounds with $OSi(CH_3)_3$ groups. This a critical sample preparation technique that transforms hard-to-analyze polar compounds into stable, volatile derivatives that are well suited for separation and identification by gas chromatography (Knapp 1979). Approximately 0.003 g extractive residue was redissolved in 90 µL N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) reagent for carrying out the interaction between the extract and BSTFA and then incubated in a water bath at 70 °C for 1.0 h before analysis by GC-MS on an HP 6890 Gas Chromatograph (Agilent Technology, Santa Clara, CA, USA), equipped with a split/split less injector and a 5973 Mass Selective Detector (MSD). The GC conditions used were: HP-5MS capillary column (SGE, 30 m, 0.25 mm), carrier gas, helium, flow rate 1.0 mL/min; oven temperature initially 60 °C, ramping to 260 °C at 6 °C/min, with post run off at 280 °C (Ashrafi 2011). Constituent compounds were identified using retention times published in the literature (Conde *et al.* 1999) and calculation of Quatz index as well as Adams table. The Quatz index, I, was calculated using published methods (Meszaros 2006; Vaysi 2011),

$$I = \frac{100n + 100\,(t_{rx} - t_{rn})}{t_{rn+1} + t_{rn}} \tag{1}$$

where n denotes the numbers of C in normal alkane; t_{rx} is retention time of unidentified compound; and t_{rn} is the retention time of normal alkane.

Correspondingly, the Quatz indices were adjusted with the Adams table before arranging both alphabetically and by ascending-descending trend of Quartz index (Ebadi 2022;).

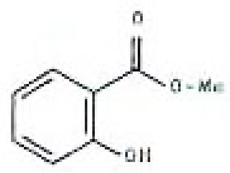
RESULTS

The GC-MS analysis of extractives from Persian oak sapwood and phloem detected approximately 100 different compounds, amongst which several more common compounds with varied contents were identified in trees with different levels of crown dieback. As shown in Table 2-1, six compounds were present in high quantities in the wood and bark of healthy oak trees.

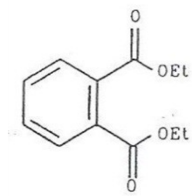
The compound in the highest concentrations in bark of Persian oak was levoglucosan (1,6-anhydro-β-D-glucopyranose) (6.36%), whereas 4-fluoro-3-methyl-anisole (0.42%) was in the lowest concentration (Table 2-1A). Various acids, glycosides, and aromatic compounds were found in the wood of healthy trees, with isomenthone and benzene carboxylic acid in the lowest and highest concentrations, respectively (Table 2, section 1 B).

Bark extractives of Persian oak in slight dieback category (5% to 33%), contained 72 identified compounds, five of which are shown in Table 2, section 2A. The highest and lowest levels of compounds were identified as 1,2-benzene dicarboxylic acid (phthalic acid) and hexadecamethyl octasiloxane (cetane), respectively.

In wood of trees with slight dieback, four abundant organic acids, carbohydrates, and aromatic compounds were identified as most abundant amongst approximately 100 identified compounds (Table 2, section 2B). Salicylic acid was present in high concentrations (6.66%). Gibberellin A3 was present at a concentration of 2.05%.



(1)



(2)

Fig. 2. Structures of salicylic acid (1) and 1,2-dicarboxylic benzene (2)

Four compounds were present in high concentrations in the bark of trees with moderate dieback (34% to 66%) (Table 2-3A). Salicylic acid and 4-ethylisoquinoline (isoquinoline) were the most abundant compounds. In wood of oaks taken from trees with moderate crown dieback, six compounds with high amount were identified (Table 2, section 3B). 6-Aza-5,7,12,14-tetrathiamine tasene (tetracene) was present in the highest concentration, whereas hexadecamethylindole-2-1 (ester acid palmitic) was in the concentration. These compounds enhance systemic absorption and transport, primarily through the lipid-rich domain of the cell membrane and exhibit greater stability against enzymatic breakdown (*e.g*., by IAA oxidase). Acting as reservoirs or sustained-release forms of IAA, they undergo gradual hydrolysis by endogenous plant esterases, ensuring a controlled, time-extended release of bioactive free IAA.

Over 100 compounds were identified in bark and wood of sampled trees with severe dieback, but 66% of the total extractives were represented five compounds (Table 2, section 4). In bark, salicylic acid and gibberellin A3 showed the highest and lowest contents, respectively (Table 2, section 4A). In the extraction of dead oak wood with severe dieback, Phthalic acid, and salicylic acid were present in the highest and lowest amounts. Furthermore, 2,3-dihydroxy-6-quinoxaline, this compound is a chemical intermediate- building block- in the synthesis of biologically active compounds such as antibacterials, was present in low quantities (Table 2, section 4B).

DISCUSSION

Levoglucosan formed from the pyrolysis of carbohydrates, such as starch and cellulose (Lakshmanan *et al.* 1970). The research described in this paper examined the impact of dieback intensity on the extractives in phloem and sapwood tissues of Persian oak using GC-MS. The most abundant compounds identified were: trimethyls, propanoic acid, benzoic acid, and glucosides, such as levoglucosan and 3-fluoro-4-methylanisole another type of levoglucosan that is produced from cellulose pyrolysis. In general, the number and quantities of organic compounds in plants change remarkably under the influence of different types of stresses. The main roles of these compounds are not always clear, but it is likely that some increase resistance to degradation and to reactive oxygen species produced by environmental stress, as a component in adaptation to the stress (Holopainen 2011).

There were approximately 100 different compounds identified in phloem and sapwood of Persian oak. Among the identified compounds, salicylic acid has been commonly found. This compound is known to make the tissues more durable and resistant against induced stress, and has a key role in internal signaling in plants (Yuan and Lin 2008). Salicylic acid (*ortho*-hydroxybenzoic acid) is a plant growth regulator in the class of phenolic compounds, with a wide range of functions in various physiological processes such as ion exchange, stomatal opening and closing movements, cell membrane permeability, and photosynthesis rate (Bastam *et al.* 2013).

Production of salicylic acid may reduce the inhibitory effect and damage resulting from environmental stresses, such as drought and salinity (Yuan and Lin 2008), through non-enzymatic antioxidant effects resulting from an increase in the amount of protein and the activity of antioxidant enzymes (Ashraf *et al*. 2010). In general, the content of salicylic acid is increased in the drought stress. For example, a five-fold increase in salicylic acid concentrations occurred under water deficit in most of the shrubs tested by Munne-Bosch and Penuelas (2003).

As Tongo *et al*. (2021) suggested, Persian oak trees showing different dieback severities have varied physiological responses to environmental stresses such as drought, associated with defense and enabling them to tolerate drought stress whilst maintaining life processes. The current study supports the conclusions of Tongo *et al*. (2021) that trees with different levels of dieback show different responses to stress.

Gibberellins are known to be present in high concentrations in terminal buds, cambium and young, growing cells, where these plant growth regulators may protect cell membranes and reduce ion leakage, thereby increasing resistance to stress (Gunes *et al*. 2007). Yadav *et al*. (2021) stated that phyto-hormones play crucial roles in drought stress tolerance in plants through regulation of cellular functions at the molecular level through cell signaling. Gibberellins are diterpenoid-based plant growth regulators with key roles including cell expansion in foliage, stems, and roots (Zhang *et al*. 2020). This group of growth regulators is essential in responses to environmental stress, often providing antagonism to the effects of abscisic acid (Yang *et al*. 2014). In general, the diterpenoid class of natural compounds is core to stress resilience and includes metabolites with crucial roles in plant defense against a/biotic stresses and adaptation with to ecological conditions (Pelot *et al*. 2018).

Analysis of phloem extracts from healthy trees suggested that the most common compound present was levoglucosan**,** which has an important role in the synthesis of polysaccharides in plants (Mousavi *et al*. 2018). Balaban and Uçar (2001) showed that similar carbohydrates are involved in energy production in *Quercus volcanica*. In addition, Balaban and Uçar (2001) found that cyclohexane extract was common compound in bark and wood of *Q. vulcanica* and this compound was ten time more abundant in bark compared with wood.

Table 2. More Common Chemical Compounds in the Extractives of Bark and Wood of Persian Oak Trees with different Levels of Dieback Detected by GC-MS

		Chemical Component	Retention Time (min)	Area (%)
1 Healthy Samples	(A) Bark	4-Fluoro-3-methylanisole	21.009	0.42
		1,6-anhydro-β-D-glucopyranose	22.85	6.36
		2-Phenylmethylene-Cyclohexanone	25.518	4.06
		2-Ethoxyethyl Phthalic acid	25.701	3.73
		Gibberellin A3	44.56	1.03
		Salicylic acid	47.09	1.63
	(B) Wood	Isomenthone	14.293	1.24
		1- Menthol	14.820	3.54
		2,6-Dimethoxyphenol	19.769	1.97
		2,1-Benzene dicarboxylic acid	25.726	16.83
		4-(t-Butyl)-3,3-diethyl-2(H3)-thiophenol	26.023	6.39
		Salicylic acid	55.726	5.13
2 Slight dieback	(A) Bark	1,3,5 -Triazine-2,4-diamine,1,6 -methoxy N-ethyl	26.033	4.2
		1,2-Benzene dicarboxylic acid	43.348	8.97
		Gibberellin A3	44.218	1.44
		1,1,3-Hexadecamethyloctasiloxane	49.592	0.49
	(B) Wood	Gibberellin A3	43.840	2.05
		1,1,3-Hexadecamethylcyclo octasiloxane	47.805	5.09
		1,1,3-Hexadecamethylindole	50.472	5.80
		Salicylic acid	53.47	6.66
3 Moderate dieback	(A) Bark	4- Ethyl accordine	44.189	1.04
		Salicylic acid	44.658	1.37
		Alpha-alpha-di(pyrropropylene dinitrilo)O-cresol	44.996	0.59
		Gibberellin A3	48.103	0.67
	(B) Wood	6-aza-5,7,12,14-tetrathiamine tasene	12.433	23.35
		Methyl 3-oxy-3-siglohexylpropanate	25.530	0.74
		Salicylic acid	44.950	1.31
		Hexa decamethyl hepta siloxane	46.718	2.24
		Hexa deca methyl octa siloxane	46.884	2.02
		Hexadecamethyl indole-2-one	48.790	0.62
4 Severe dieback	A) Bark	Gibberellin A3	45.431	0.62
		Alpha-alpha-di(pyrropropylene dinitrilo)O-cresol	46.203	0.83
		Salicylic acid	48.555	11.46
		Hexa deca methyl octa siloxane	45.208	1.25
		1-Nitro-10,9-dioxo-10,9-dihydro-Anthracene-2-carboxylic acid diethylamide	48.29	1.29
	(B) Wood	1,2-Benzene dicarboxylic acid	25.726	77.53
		Propanoic acid	26.378	0.3
		2,3-Dihydroxy-6-nitroquinoxylan	36.580	0.2
		Salicylic acid	42.943	3.23
		Heptamethyltrisiloxane	43.779	0.32

At slight crown dieback level, glucosides and aromatic compounds were present in both phloem and sapwood. 1,2-Benzene dicarboxylic acid was present in high quantities in the tissues. Vaysi (2017b) also reported phthalic acid, along with salicylic acids, in bark and wood of *Q. castaneifolia*. These compounds are thought to increase durability in oak wood.

In oak sapwood and phloem from trees with moderate to severe crown dieback, the most abundant compound detected was salicylic acid. The measured quantity of salicylic acid increased with increasing crown dieback, possibly contributing to tolerance of stress factors and in this instance in improving drought tolerance. Salicylic acid has an important role in the physiological processes of plants, including the induction of defense responses against biotic (pests and pathogens) and abiotic stresses. Prithiviraj *et al.* (2005) showed that in the *Arabidopsis thaliana*, quantities of salicylic acid increased in different stages of growth, along with increasing stress. In the analysis of healthy sapwood samples in this present work, six compounds with acidic, glucosideic, and aromatic structures were obtained. Benzene dicarboxylic acid, an organic acid, was the most abundant extractive found in this stress level; this compound contributes to wood durability (Table 2). The high durability of oak wood is attributed to the content of phenolated compounds. Tannin, as a phenolic compound (polyphenol), is one of the main components in all vegetative parts of oaks, and it contributes natural durability; tannins are also considered to be defensive factors against herbivorous insects (Scalbert *et al.* 1988). Furthermore, phenolic compounds are produced in plants under drought conditions as part of the adaptation strategies against adverse impacts of extreme environments, helping to reduce oxidative damage (Celeste Varela *et al.* 2016).

In the analysis of sapwood from trees with slight crown dieback, salicylic acid was again the most abundant compound found; the plant growth regulator gibberellin A3 was present in lowest concentrations compared to other identified components in the extractives. Salicylic acid concentrations increased in trees tissues with increasing dieback, suggesting a role in the response to stress. Reductions in the concentrations of gibberellins are associated with decreasing drought tolerance in plants (Iqbal *et al.* 2022). During drought stress, gibberellin function as a growth promoter ceases, reducing the utilization of water (and nutrients) (Sharma and Kumar 2021).

Of the six most abundant compounds identified in sapwood samples of *Q. brantii* with 70% dieback, tetracene had the highest concentration, compared to the other five compounds. Polyamines are highly bioactive, low-molecular-weight aliphatic amines that occur ubiquitously as secondary metabolites in plants (Aktar *et al.* 2021). Polyamines have been shown to perform important roles in alleviating plant stress (Shi *et al.* 2022), through regulating the accumulation of sugar, proline, and other osmotically active compounds (Li *et al.* 2018). Polyamines concentrations may also increase the abscisic acid production under drought stress (Bitrián *et al.* 2012). Abscisic acid is also an inhibitor of plant growth, which during stress causes stomata to close and abscission, thereby greatly reducing transpiration.

In the analysis of oak sapwood from trees with severe crown dieback, salicylic acid and phthalic acid were present in the highest and lowest concentrations, respectively. Phthalic acid is well-known for driving plant physiological responses toward a new equilibrium; for example, it induced antioxidant defenses, improved enzymatic activities, and augmented the nutritional quality of tomatoes (Ahmad *et al.* 2019).

CONCLUSIONS

Gas chromatography-mass spectrometry (GC-MS) analysis of sapwood and phloem in Persian oak (*Quercus brantii*) across crown dieback severity revealed a clear metabolic shift linked to drought stress. Healthy trees showed high levoglucosan, indicative of normal carbohydrate turnover, while declining trees exhibited marked increases in salicylic acid (up to 11.5% in bark), highlighting its key role in defense signaling. Gibberellin A3, a growth-promoting hormone, decreased with stress severity, reflecting growth suppression. Moderately dieback trees displayed unique compounds (*e.g.*, 4-ethylisoquinoline, 6-aza-tetrathiapentacene, palmitate esters), suggesting induced synthesis of alkaloid- and lipid-based protectants, possibly acting as slow-release auxin reservoirs. Severely dieback trees showed metabolic simplification, dominated by phthalic acid (77.5% in wood) and salicylic acid, likely signaling cell wall breakdown. Low-level detection of 2,3-dihydroxy-6-quinoxaline hints at antimicrobial defense attempts. Overall, Persian oak appears to employ a tiered biochemical strategy: shifting from constitutive metabolites to inducible phenolic, hormonal, and heterocyclic defenses, thereby providing a metabolic signature of drought resilience and dieback progression.

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