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Drought stress tolerance in vetch plants (*Vicia* sp.): agronomic evidence and physiological signatures

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Abbreviations:
APX: ascorbate peroxidase activity (units/mg protein); Cart: carotenoid (mg/gFW); CAT: catalase activity (units/mg protein); Chl*a*: chlorophyll *a* (mg/gFW); Chl*b*: chlorophyll *b* (mg/gFW); Chl*a*/Chl*b*: Chl*a*/Chl*b* ratio; DTF: days to flowering; DPPH: 2,2-diphenyl-1-picrylhydrazyl (mg/ml); DTM: days to maturity; Fv/Fm: maximum photochemical efficiency of photosystem II; HI: harvest index; H_2O_2: hydrogen peroxide content (µmol/g FW); LPC: leaf proline content (µmol/gFW); LSC: leaf soluble carbohydrate (µmol/ml); MDA: malondialdehyde content (nmol/gFW); MSI: membrane stability index (%); PN: pod number (No./plant); POX: peroxidase (units/mg protein); RWC: relative water content (%); SN: seed per plant (No./plant); SP: seeds in pot (No./pod); STI: stress tolerance index; TSW: 1000-seed weight (g); YSI: yield stability index

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Abstract

This study aimed to investigate vetch genotypes' responses to moderate and severe drought stress and identify stress tolerance markers in arid conditions. Ten vetch genotypes (*Vicia dassycarpa* Ten., *V. pannonica* Crantz., *V. michauxii* Spereng., *V. sativa*-Ardebil, *V. sativa*-Dashtyar, *V. sativa*-Fereydonshahr, *V. sativa*-Mashhad, *V. sativa*-Semirom, *V. sativa*-Shahrekord and *V. villosa* Roth.) were cultivated under three water-deficit conditions: control, moderate and severe drought stress. These conditions represented maximum allowable depletion levels of 30, 50 and 85% of soil available water, applied after the six-leaf stage in the 2019–20 and 2020–21 growing seasons. The findings highlight the vetch's response to drought stress is influenced by stress severity and genotype. The result indicated a wide range of genetic diversity in agro-physiological traits among the studied *vicia* germplasm. *Vicia dassycarpa* Ten. shows highest straw yield and shorter days to flowering and maturity. *Vicia michauxii* Spreng. demonstrates high grain yield and advantageous traits like increased water content, photochemical efficiency of photosystem II, chlorophyll *b*, carotenoids and membrane stability index. It has lower soluble carbohydrate, DPPH (2,2-diphenyl-1-picrylhydrazyl) and proline content. Additionally, *V. michauxii* Spreng. exhibits superior agronomic traits such as more seeds per pod, per plant and higher 1000 seeds weight, serving as reliable markers for drought tolerance. The results emphasize *V. dassycarpa* Ten. for fodder and *V. michauxii* Spreng. for grain production in water-limited regions. Further research on gene expression related to drought tolerance traits should enhance our understanding of vetch.

Introduction

The genus *Vicia*, commonly referred to as vetches, is a large and complex group with 210–240 species, primarily distributed in temperate regions of the northern hemisphere and originating from the Mediterranean and Irano-Turanian regions (Maxted, 1993; Leht, 2009). It is a diverse genus of leguminous plants that includes several prominent species. Common vetch (*Vicia sativa*) is the most widely cultivated species of vetch (Nguyen *et al.*, 2020) and is mainly used for animal feed as a cheap and rich source of protein and minerals of high digestibility and high energy content. A study on the genetic characterization of Spanish core collections of common vetch found that the species has high genetic diversity and is a valuable germplasm for resilient agriculture (De la Rosa *et al.*, 2021). Hairy vetch (*Vicia villosa* Roth.) is another species of vetch that is considered a cosmopolitan species due to its capacity to naturalize under different conditions (Renzi *et al.*, 2020). Documented studies and published information on the environmental stress tolerance of other cultivated *Vicia* species, including *Vicia pannonica* Crantz. and *V. michauxii* Spereng., are scarce.

Drought stress is a substantial abiotic factor affecting plant growth, yield and quality worldwide (Fahad *et al.*, 2017). Although arid regions suffer from insufficient and uneven rainfall, the amount of water allocated to agriculture has declined. This is mainly attributed to the increasing urban population, industrial development and the adverse effects of climate change (Gitz *et al.*, 2016; Tzanakakis *et al.*, 2020).

Consequently, crops are exposed to water scarcity stress for a significant duration of their growth cycle. Drought stress impacts plant growth and development in various ways, such as decreasing photosynthetic rate and stomatal conductance (Ahmad *et al.*, 2019), reducing chlorophyll and carotenoid levels (Nematpour *et al.*, 2019) and increasing reactive oxygen species (ROS), soluble protein and proline content to the detriment of the plant (Ahmad *et al.*, 2019; Nematpour *et al.*, 2019). According to Irani *et al.* (2015), water deficit had a significant impact on the physiological traits of sainfoin genotypes, including decreased dry matter yield and relative water content, and increased carotenoid content, free proline content and enzyme activities. Plants have developed various strategies to cope with water scarcity, including

physiological and biochemical responses that differ by species. These strategies include adjusting growth patterns, reducing transpiration loss, accumulating compatible solutes, enhancing transpiration efficiency, regulating osmotic and hormonal functions and delaying senescence (Kabbadj *et al.*, 2017). Thus, the development of crop varieties with enhanced drought tolerance is imperative to safeguard food security and maintain sustainable crop production in water-limited environments.

There is variation in drought tolerance among different species of plants, as well as among ecotypes and cultivars within each species (Khan *et al.*, 2016; Nematpour *et al.*, 2019; Zhang *et al.*, 2019). Haffani *et al.* (2014, 2017) compared three vetch species (*Vicia narbonensis* L., *V. sativa* L. and *V. villosa* Roth.) under water stress and found that *V. narbonensis* showed the highest drought tolerance, attributed to its larger leaf area, higher relative growth rate and better ability to maintain high values of water use efficiency and stress tolerance index under water-limited conditions. Abdelhaleim *et al.* (2022) observed significant differences among faba bean genotypes and irrigation treatments for most traits, with the exception of the number of pods in the first season. They found that yield traits decreased with increasing drought stress, while proline content increased. Irani *et al.* (2015) demonstrated significant differences among sainfoin genotypes, with ecotypes Baft, Najafabad and Sirjan identified as drought-tolerant or moderately drought-tolerant based on their stress tolerance index and proline content under water-deficit conditions. Saeidnia *et al.* (2017) observed high genotypic variation for all measured traits in their study on smooth bromegrass (*Bromus inermis* Leyss) genotypes. They also found that carotenoid content and water-soluble carbohydrates were correlated with drought tolerance, while leaf proline content did not show any correlation with drought tolerance. Kebede (2018) studied vetch species, finding differences in growth features, phenology, forage and seed productivity. *Vicia villosa* Roth., *V. dasycarpa* Ten. and *V. atropurpurea* Crantz. were identified as potential for integration with other crops, but further testing is required. Arteaga *et al.* (2020) studied the responses of 47 *Phaseolus vulgaris* genotypes to water-deficit and salt stress treatments. They proposed using proline as a biochemical marker for large-scale screenings of bean genotypes to exclude the most sensitive cultivars. Muktadir *et al.* (2020) proposed that supplementing selection in breeding programmes with metabolite-based biomarkers and bioindicators, and the sequencing of the faba bean genome for molecular breeding are necessary. They also suggested a combination of screening methods and breeding scale expansion to improve faba bean yield under drought conditions. Nunes *et al.* (2022) found that differences in leaf water potential, despite no differences in relative water content, improved yield in Portuguese cowpea landraces, highlighting the significance of small differences in stomatal responses or water-saving strategies and the need to preserve diverse genetic pools for exploring other drought adaptation traits. The vetch plant, often overlooked, holds potential for cultivation during autumn in water-limited areas (Haffani *et al.*, 2014; Nguyen *et al.*, 2020; Renzi *et al.*, 2020; De la Rosa *et al.*, 2021). Consequently, it becomes imperative to identify drought-tolerant genotypes that exhibit high performance and comprehend the factors associated with tolerance (Nematpour *et al.*, 2019).

In arid and semi-arid regions characterized by limited rainfall during cold seasons and high temperatures during warm seasons, vetch genotypes with high production and drought tolerance potential could alleviate pressure on limited underground water resources. These genotypes can provide valuable rich protein food for humans and fodder for livestock by mainly growing in the cold season. Hence, this study aimed to (i) investigate how different vetch genotypes respond to moderate and severe drought stress and (ii) identify physiological markers associated with stress tolerance in arid conditions. The findings can improve vetch growth and performance, paving the way for crop rotation with cereals under irrigated conditions.

Material and methods

Plant material

Ten vetch landraces, namely *V. dassycarpa* Ten., *V. pannonica* Crantz., *V. michauxii* Spereng., *V. sativa*-Ardebil, *V. sativa*-Dashtyar, *V. sativa*-Fereydonshahr, *V. sativa*-Mashhad, *V. sativa*-Semirom, *V. sativa*-Shahrekord and *V. villosa* Roth., were received from Dryland Agriculture Research Institute, Iran, and Pakan Bazr and Dashtyar Seed companies, Isfahan, Iran. The seeds of these species were collected from various regions across Iran (Table 1 and Fig. S1). At the start of the experiment, the seeds were sown in the field, and the entire plants were collected during the flowering stage. Subsequently, these plant samples were sent to the Botanical Laboratory of Isfahan Agricultural and Natural Resources Research and Educational Center (IANREC) for species identification purposes.

Site location and experimental setup

The experiment was conducted at Lavark Research Farm in Najaf-Abad, Iran (32°32′N, 51°23′E, 1630 m above mean sea level) on a Typic Haplargid, silty clay loam soil, with pH 7.5. It had a hot and dry climate (Fig. S2). Soil characteristics, including field capacity (31.2 cm^3/cm^3, %), permanent wilting point (21.8 cm^3/cm^3, %), available water content (9.4 cm^3/cm^3, %) and soil density (1.36 g/cm3), and some physical and chemical properties were recorded (Table 2).

The experiment followed a split plot design with three soil moisture levels (30, 50 and 85% maximum allowable depletion, MAD) and ten vetch genotypes. Planting occurred on 2 November 2019 and 5 November 2020. Each experimental unit had eight rows, 4 m in length, with 0.25 m row spacing and 0.05 m spacing between plants. Drip irrigation tapes with 16 mm drippers (flow rate: 1.3 litres/h) were placed alongside rows at 0.25 m intervals. Irrigation was managed using a ball valve and volumetric counter.

Single superphosphate (16% P_2O_5, Nahade Gostar Co., Khorasan Razavi, Iran) was applied at a rate of 100 kg/ha during land preparation and before planting. Potassium sulphate (52% K_2O, Jonoobgan Co, Kerman, Iran) at a total rate of 50 kg/ha and urea fertilizer (45% N, SPC Co, Shiraz, Iran) at a total rate of 100 kg/ha were applied in two stages: 50% at the two- to four-leaf seedling stage and 50% at the 50% flowering stage. All applications were administered through drip fertigation.

Soil moisture environment levels

Water-deficit levels were imposed on vetch plants after the six-leaf stage based on MAD of soil available water (TDR soil moisture meter, Spectrum Technologies, Inc. USA).

Vetch evapotranspiration was calculated using weather data and the FAO-Penman–Monteith equation (Allen *et al.*, 1998),

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Table 1. Landraces and geographical origin of the studied *Vicia* plant species

	Geographical location			
Landraces	Latitude	Longitude	Above mean sea level	Source
V. dasycarpa Ten.	37°23′31″	46°14′21″	1464	DARI, Iran
V. pannonica Crantz.	37°23′31″	46°14′21″	1464	DARI, Iran
V. michauxii Spereng.	32°59′19″	50°24′45″	2450	DARI, Iran
V. sativa Ardebil (A.)	38°15′0″	48°18′0″	1311	DARI, Iran
V. sativa Dashtyar (D.)	32°38′30″	51°39′40″	1548	Dashtyar, Com., Iran
V. sativa Fereydonshahr (F.)	32°56′19″	50°6′45″	2150	DARI, Iran
V. sativa Mashhad (M.)	36°32′57″	59°38′6″	1156	Pakan Bazr, Com, Iran
V. sativa Semirom (Se.)	31°24′24″	51°33′51″	2364	DARI, Iran
*V. sativa Shahrekord (*Sh.*)*	32°19′34″	50°50′36″	2049	DARI, Iran
V. villosa Roth.	32°38′30″	51°39′40″	1548	Dashtyar, Com., Iran

DARI, Dryland Agriculture Research Institute, Iran.

Table 2. Some physical and chemical properties of the tested soil at 0–30 cm depth

Year	Soil texture	pH	EC (dS/m)	P (mg/kg)	K (mg/kg)	Total N (%)	Organic carbon (%)
2019–20	Clay loam	7.7	2.16	48.7	325	0.03	0.54
2020–21	Clay loam	7.3	1.74	67.5	360	0.02	0.61

which is given by:

$$E_T = \frac{408\Delta(\mathrm{Rn} - G) + \gamma\frac{900}{T+273}u(es - ea)}{\Delta + \gamma\,(1 + 0.34\,u)} \quad (1)$$

where E_T = evapotranspiration (mm/day); Δ = slope of the saturation vapour pressure curve (kPa/°C); Rn = net radiation at the crop surface (MJ/m^2/day); G = soil heat flux density (MJ/m^2/day); γ = psychrometric constant (kPa/°C); T = mean air temperature (°C); u = wind speed at 2 m height (m/s); e_s = saturation vapour pressure (kPa); e_a = actual vapour pressure (kPa).

Three water-deficit conditions were implemented: control, moderate and severe drought stress, corresponding to depletion levels of 30, 50 and 85% of available water. MAD and irrigation depths were determined using Eqns (1) and (2) based on Allen *et al.* (1998) and Kiani *et al.* (2016). Applied irrigation was measured using a flow meter.

$$\theta_{\mathrm{irrig}} = \theta_{\mathrm{fc}} - (\theta_{\mathrm{fc}} - \theta_{\mathrm{pwp}}) \times \mathrm{MAD} \quad (2)$$

Equation (1) calculates θ_{irrig} (threshold soil water content at irrigation) using θ_{fc} (soil water content at field capacity) and θ_{pwp} (soil water content at wilting point) and multiplies by MAD.

$$D_{\mathrm{irrig}} = (\theta_{\mathrm{fc}} - \theta_{\mathrm{avg}}) \times Z_e \quad (3)$$

Equation (2) calculates D_{irrig} (irrigation depth) by multiplying the difference between θ_{fc} and θ_{avg} (average soil water content at root zone) by Z_e (root depth). Applied irrigation was measured using a flow meter during the experiment.

Measurement of traits

The drought tolerance of ten vetch genotypes was investigated by measuring various biochemical, agro-physiological and agronomic traits. For biochemical traits – including chlorophyll *a*, chlorophyll *b*, total carotenoids, leaf proline, soluble carbohydrates, ascorbate peroxidase activity, catalase activity, peroxidase activity, hydrogen peroxide, malondialdehyde and membrane stability index – in each experimental unit the newest fully developed leaf with all leaflets from four randomly selected plants during the 50% flowering growth stage was clipped, immediately wrapped in aluminium foil with the treatment number, and placed in liquid nitrogen. The samples were then transported to the laboratory and stored in an −80°C freezer until the traits were measured. For agro-physiological traits such as relative leaf water content and Fv/Fm ratio, two middle leaflets from the most recently developed leaf of randomly selected plants at the 50% flowering stage were used. Additionally, days to flowering and days to maturity were recorded when 50% of the plants reached these stages. Agronomic traits, including plant pod number, pod seed count, total seed count per plant, 1000-seed weight, straw yield, grain yield and harvest index, were measured at agronomic maturity stage. Stress tolerance index and yield stability index were also calculated to assess the genotypes' tolerance to stress and yield consistency, respectively.

Chlorophyll (Chl) and carotenoid (Cart) contents

The method outlined by Lichtenthaler and Wellburn (1983) was employed to measure the levels of chlorophyll and carotenoids.

$$\mathrm{Chl}a = 12.25 \times \mathrm{A663.2} - 2.798 \times \mathrm{A646.8} \quad (4)$$

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$$\text{Chl}b = 21.50 \times \text{A646.8} - 5.10 \times \text{A663.3} \quad (5)$$

$$\text{Cart} = (1000 \times \text{A470} - 1.82 \times \text{Chl}a - 85.02 \times \text{Chl}b)/198 \quad (6)$$

where A is light absorbance at 663, 646.8 and 470 nm using a U-1800 UV-VIS spectrophotometer (Hitachi, Tokyo, Japan).

Maximum quantum efficiency of photosystem II, Fv/Fm

Chlorophyll fluorescence was measured using a portable fluorimeter (Opti-Sciences Inc., Hudson, NH, USA) as described by Van Kooten and Snel (1990). Two middle leaflets, both together from the latest fully developed leaf on two randomly selected plants per experimental unit, were selected and measured between 10:00 a.m. and 12:00 p.m., following a 25 min dark adaptation period. Fv/Fm, representing the maximum quantum efficiency of photosystem II, was calculated as (Fm–Fo)/Fm to assess stress impact on photosynthetic light reactions (Fo: minimal fluorescence, Fm: maximal fluorescence, Fv: variable fluorescence).

Relative water content (RWC)

Early in the morning (6 to 8 a.m.), from the latest fully developed leaf on five randomly selected plants for each treatment, two middle leaflets were detached and placed inside a plastic bag within an ice container, then immediately transferred to the laboratory. Sampled leaves were weighed for fresh weight (FW), immersed in distilled water at room temperature to reach saturated weight (SW) after 16–18 h, and dried at 70°C for 72 h to obtain dry weight (DW). RWC was calculated using the equation by Manette *et al.* (1988).

$$\text{RWC}\ (\%) = ((\text{FW-DW})/\ (\text{SW-DW})) \times 100 \quad (7)$$

Leaf proline content (LPC)

LPC in leaves was analysed following Bates *et al.* (1973). Leaf samples (0.2 g) were homogenized in 10 ml 3% aqueous sulfosalicylic acid (w/v) using a Polytron homogenizer (PT2100 with a 7 mm probe; Brinkmann Instruments, Westbury, NY). The homogenate was then centrifuged at 9000 × *g* for 15 min at room temperature in a 15 ml polypropylene microcentrifuge tube. The 2 ml extract were mixed by vortexing with 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent (2.5% ninhydrin in 60% acetic acid) in a 15 ml polypropylene tube with a screw top. The mixture was heated at 100°C for 1 h in a water bath, then immediately placed in an ice bath for 10 min. Afterward, 4 ml of toluene was added to the mixture, which was vortexed to obtain two separate phases. The supernatant was measured at 520 nm against a toluene blank using a U-1800 UV-VIS spectrophotometer (Hitachi).

Leaf soluble carbohydrate (LSC)

LSC content was determined using a modified method of Yemm and Willis (1954). Fresh leaves (approximately 0.5 g) were ground with liquid nitrogen until a homogeneous powder is obtained. It was poured into a falcon containing 4 ml 80% ethanol (v/v), placed on ice and let to react for 5 min, then centrifuged at 10 000 rpm for 15 min. The supernatant was collected and filtered into a new falcon tube (funnel + filter paper). The extract was then mixed with 4 ml of anthrone reagent (0.2% anthrone in concentrated sulphuric acid), heated in a water bath for 10 min, cooled on ice and the absorbance was measured at 625 nm using a U-1800 UV-VIS spectrophotometer (Hitachi).

Antioxidant enzyme activities

Catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) activities were evaluated using an extract prepared following the protocol of Bradford (1976) with some modifications. In brief, fresh leaf samples (0.1 g) were homogenized in 1 ml of sodium phosphate buffer 50 mM (pH 7) containing 2 mM α-dithiothreitol, 0.2% Triton X-100, 2 mM EDTA, 50 mM Tris-HCl and 2% polyvinylpyrrolidone. The mixture was then stirred on a magnetic stirrer for 15 min. The homogenate was centrifuged at 14 000 rpm for 30 min at 4°C, and the resulting supernatant was used for the enzyme assays.

Catalase activity (CAT, µmol H_2O_2/min/mg protein) was measured in a 3 ml reaction mixture consisting of 450 µl sodium phosphate buffer 25 mM (pH 7), 50 µl of the enzyme extract and 250 µl of H_2O_2 20 mM. The activity was determined following Aebi (1974) by observing the reduction in absorbance at 240 nm for 180 s using a U-1800 UV-VIS spectrophotometer (Hitachi).

Ascorbate peroxidase activity (APX, units/mg protein/min) was measured according to Nakano and Asada (1981). The reaction mixture included 3 ml of sodium phosphate buffer (pH 7), 4.51 µl of 1 mM H_2O_2 solution, 100 µl of 5 mM ascorbate solution (prepared by dissolving 0.0264 g of ascorbate in 30 ml of 50 mM potassium phosphate buffer, pH 7) and 50 µl of the enzyme extract. The decrease in absorbance at 290 nm, indicating hydrogen peroxide-dependent oxidation of ascorbate, was monitored continuously over a period of 180 s.

Peroxidase activity (POX, units/mg protein/min) was assessed based on Herzog and Fahimi (1973). The reaction mixture, in a total volume, contained 3 ml of sodium phosphate buffer (pH 7), 4.51 µl of H_2O_2, 3.35 µl of guaiacol and 50 µl of the enzyme extract. The increase in absorbance due to guaiacol oxidation was measured at 470 nm for 180 s using a U-1800 UV-VIS spectrophotometer (Hitachi).

DPPH radical scavenging assay

The radical scavenging activity of the extracts was evaluated using a method adapted from Brand-Williams *et al.* (1995) with modifications. The reaction mixture consisted of 0.1 ml of the plant extract and 5 ml of 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol. After incubating for 30 min, the decrease in the purple colour was measured at 517 nm using a spectrophotometer, with 80% methanol serving as the blank. A control solution (AB Control) containing only methanol and DPPH was also used. Various concentrations of butylated hydroxytoluene were used as a positive control, mixed with 1 ml of 0.2 mM DPPH in methanol, and the absorbance was read at 517 nm against the blank, using a U-1800 UV-VIS spectrophotometer (Hitachi). The percentage of DPPH scavenging was calculated using the following equation:

$$\text{DPPH quenched} = [(\text{A sample} - \text{A blank})/\ (\text{A blank})] \times 100 \quad (8)$$

where A sample and A blank represent the absorption of the plant extract samples and blank, respectively. The inhibition percentage

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was plotted against sample concentration, and the IC50 value (the concentration required to inhibit 50% of the DPPH radicals) was determined through linear regression analysis.

H_2O_2 concentration

To determine the concentration of hydrogen peroxide (H_2O_2) generated in the samples, 0.2 g of leaf tissue was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was then centrifuged at 10 000 g for 4 min at 4°C. The supernatant, containing the soluble fraction of the tissue, was used to assess the H_2O_2 concentration. Specifically, 0.5 ml of the supernatant was combined with 0.5 ml of potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI solution. The reaction between H_2O_2 and KI forms iodine, which reacts with starch present in the solution to produce a coloured iodine–starch complex. This mixture was incubated in the dark for 1 h, after which the absorbance of the iodine–starch complex was measured at 390 nm using a U-1800 UV-VIS spectrophotometer (Hitachi). The concentration of H_2O_2 was determined using a standard curve. To establish the standard curve, known concentrations of H_2O_2 (ranging from 0 to 10 µM) were prepared and treated in the same manner as the samples. The absorbance of these standards was measured at 390 nm, and a plot of absorbance *v.* H_2O_2 concentration was created. The H_2O_2 concentration in the samples was then calculated based on this standard curve, as described by Velikova *et al.* (2000).

Malondialdehyde (MDA) concentration

MDA concentration, an indicator of lipid peroxidation, was assessed following the method described by Nematpour *et al.* (2019). Leaf tissue (0.2 g) was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid with a Polytron homogenizer (PT2100 with a 7 mm probe; Brinkmann Instruments), which was prepared using purified water. The homogenate was then centrifuged at 10 000 × g for 10 min at 4°C in a 1.5 ml polypropylene microcentrifuge tube. The supernatant was mixed with 0.5% thiobarbituric acid in 20% trichloroacetic acid and heated at 95°C for 30 min. After cooling, the mixture was centrifuged again at 10 000 × *g* for 10 min in the same type of microcentrifuge tube. The absorbance of the supernatant was measured at 532 and 600 nm using a U-1800 UV-VIS spectrophotometer (Hitachi).

The concentration of MDA was calculated using the equation:

$$\text{MDA} = \frac{\text{A532} - \text{A600}}{\epsilon} \times \frac{\text{volume of reaction mixture}}{\text{amount of leaf tissue}} \quad (9)$$

where ε is the molar extinction coefficient for MDA–TBA complex, equal to 155 mM/cm. The molar extinction coefficient is a constant used to convert absorbance into concentration, accounting for the specific interaction between MDA and TBA.

Membrane stability index (MSI)

MSI was determined following the method of Sairam *et al.* (1997). Leaf samples (0.4 g) were incubated in 20 ml of distilled water at 40°C for 30 min. The electrical conductivity (EC1) of the solution was measured. The samples were then boiled at 100°C for 10 min, and the electrical conductivity (EC2) was measured again. MSI was calculated using the following formula:

$$\text{MSI} = [1 - (\text{EC1}/\text{EC2})] \times 100 \quad (10)$$

Days to flowering stage (DTF) and days to maturity stage (DTM)

The time taken for flowering and maturity stages to occur in 50% of the plants within each experimental unit was recorded as the number of days.

Yield and yield components

During the harvest stage, the plant pod number, pod seed count and total seed count per plant were determined by averaging the values obtained from 20 randomly selected plants within each experimental unit. Additionally, in each treatment, the weight of five groups of 100 seeds was measured and recorded to calculate the weight of 1000 seeds. A 1 m^2 area from each plot was harvested, and the grain and straw yields were recorded after drying in an oven at 65°C for 48 h. Both the herbage biomass and the grain yields were reported based on a moisture content of 12.5%. The harvest index (HI) was also calculated using the following equation:

$$\text{HI} = (\text{GY} / \text{TB}) \times 100 \quad (11)$$

where HI is the harvest index, GY is the economic yield based on the grain yield (kg), and TB is the total biomass (kg).

Stress tolerance index (STI)

To determine the STI for the two water-limited levels, the following equation was employed (Nematpour *et al.*, 2019):

$$\text{STI} = (Y_s \times Y_p)/(\bar{Y}p)^2 \quad (12)$$

In the equation, Y_p refers to the total dry weight of the genotype under normal irrigation conditions, Y_s represents the total dry weight of the genotype under water-deficit stress levels, and $\bar{Y}p$ represents the average total dry weight under both water-deficit stress and normal irrigation conditions.

Yield stability index (YSI)

The identification of high-yielding genotypes under both normal and water stress conditions was based on the YSI, which is determined using the formula (Sabouri *et al.*, 2022):

$$\text{YSI} = Ys / Yp \quad (13)$$

Here, Y_s represents the total dry weight of an experimental genotype under water stress, and Y_p represents the total dry weight of the same genotype under normal conditions.

Statistical analyses

A combined analysis of variance was conducted using a split–split plot arrangement within a randomized complete block design, utilizing SAS statistical software (ver. 9.4). Treatment means were compared using the least significant difference (LSD) test

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at a significance level of $P < 0.05$. Histograms were created using Microsoft Excel 2016 software. Furthermore, principal component analysis (PCA) was carried out using Stat Graphics Centurion (ver. 16.1.15), and cluster analysis was performed using SAS JMP (Version 11.0).

Results

Analysis of variance

Significant interaction effects were observed among the three soil moisture environments and vetch genotypes for most measured traits (Table S1). These interactions underscore the differential responses of the vetch genotypes to varying levels of soil moisture, emphasizing the need for a nuanced understanding of each genotype's stress tolerance strategies.

Agronomic traits

The agronomic traits measured – straw yield, grain yield, HI, pod number, pod seed count, total seed count and 1000-seed weight – are crucial for assessing plant performance under stress conditions. Each trait provides insights into the plant's productivity and stress resilience.

Under control conditions, *V. pannonica* Crantz. had the highest straw yield (2048 kg/ha), suggesting its superior growth potential in optimal conditions. In contrast, *V. sativa* M. had the lowest straw yield (1079 kg/ha). Under moderate stress, *V. dassycarpa* Ten. performed best (1837 kg/ha), indicating its tolerance to moderate drought. Severe stress reduced straw yield significantly in all genotypes, with the greatest reduction in *V. pannonica* Crantz. (66%), but *V. dassycarpa* Ten. demonstrated minimal reduction (19%), highlighting its ability to maintain productivity under severe drought (Table 3). *Vicia michauxii* Spreng. consistently achieved the highest grain yield across all moisture levels, demonstrating superior stress tolerance and productivity. Conversely, *V. villosa* Roth. exhibited the lowest grain yield under control and moderate stress. Severe drought resulted in the largest reduction in grain yield for *V. pannonica* Crantz. (86%), whereas *V. michauxii* Spreng. experienced a lesser decline (66%), illustrating its robustness under extreme conditions (Table 3). *Vicia sativa* A. had the highest HI under control conditions (42.5), reflecting its efficient conversion of biomass to grain. Severe stress adversely affected the HI across all genotypes, with *V. dassycarpa* Ten. experiencing the largest decrease (75). However, *V. sativa* F. showed the smallest reduction (33), suggesting it retains a relatively high efficiency in converting biomass to grain under severe drought (Table 3).

Vicia dassycarpa Ten. had the highest pod number and demonstrated resilience with minimal reductions under drought conditions. *Vicia michauxii* Spreng. consistently had the highest pod seed count and total seed count across moisture levels, with significant reductions under severe stress, particularly in *V. villosa* Roth. These traits indicate that *V. michauxii* Spreng. and *V. dassycarpa* Ten. are more resilient in maintaining reproductive output under stress (Table S2). *Vicia michauxii* Spreng. maintained the highest 1000-seed weight across moisture levels, suggesting its seeds are less affected by drought stress compared to other genotypes. In contrast, *V. dassycarpa* Ten. and *V. pannonica* Crantz. had lower 1000-seed weights under stress conditions, which may indicate reduced seed weights under drought (Table S3).

Table 3. The mean comparison of the straw yield (kg ha^{-1}), grain yield (kg ha^{-1}) and harvest index of different vetch genotypes at the maturity growth stage under three levels of water-deficit stress: 30% (control), 50% (moderate stress) and 85% (severe stress) of the maximum allowable depletion levels of soil available water

Ecotype	Straw yield (kg ha^{-1})				Grain yield (kg ha^{-1})				Harvest index			
	Control	Moderate	Severe	Mean	Control	Moderate	Severe	Mean	Control	Moderate	Severe	Mean
V. dassycarpa Ten.	2000 ± 74	1837 ± 100	1625 ± 99	1821^{a}	742 ± 40	622 ± 23	119 ± 18	494^{cd}	27.1 ± 1.5	25.5 ± 1.4	6.7 ± 0.9	19.8^{d}
V. pannonica Crantz.	2048 ± 114	1762 ± 176	694 ± 91	1501^{bc}	495 ± 21	430 ± 18	70 ± 15	332^{e}	19.7 ± 1.3	20.3 ± 1.8	8.6 ± 1.0	16.2^{de}
V. michauxii Spereng.	1934 ± 91	1737 ± 57	1276 ± 111	1649^{b}	965 ± 90	945 ± 41	323 ± 37	745^{a}	33.3 ± 3.0	35.2 ± 1.3	20.9 ± 3.2	29.8^{abc}
V. sativa Ardebil	1237 ± 134	1131 ± 122	665 ± 33	1011de	876 ± 62	708 ± 58	139 ± 20	574^{b}	42.2 ± 4.3	39.3 ± 4.4	17.1 ± 2.0	32.9^{ab}
V. sativa Dashtyar	1462 ± 148	1239 ± 163	595 ± 56	1099^{d}	688 ± 43	666 ± 43	109 ± 13	487^{d}	32.6 ± 2.4	36.2 ± 3.9	15.4 ± 1.1	28.1^{c}
V. sativa Fereydonshahr	1548 ± 142	1025 ± 83	621 ± 81	1065de	800 ± 34	690 ± 54	172 ± 16	554^{bc}	34.6 ± 2.2	40.4 ± 3.0	23.2 ± 3.5	32.7^{ab}
V. sativa Mashhad	1079 ± 65	1142 ± 99	590 ± 41	937^{e}	798 ± 61	633 ± 57	170 ± 24	534^{bcd}	42.4 ± 1.3	35.8 ± 2.3	21.9 ± 1.5	33.4^{a}
V. sativa Semirom	1289 ± 142	1183 ± 66	726 ± 86	1066de	730 ± 37	544 ± 56	170 ± 27	481^{d}	37.0 ± 3.0	31.4 ± 2.7	18.8 ± 1.5	29.1^{bc}
V. sativa Shahrekord	1171 ± 70.5	1074 ± 81	682 ± 82	975de	790 ± 47	647 ± 69	153 ± 24	530^{bcd}	40.4 ± 2.5	37.6 ± 3.7	18.3 ± 1.7	32.1^{ab}
V. villosa Roth.	1852 ± 92	1489 ± 125	825 ± 54	1389^{c}	360 ± 12	318 ± 10	97 ± 16	258^{f}	16.4 ± 0.9	18.1 ± 1.6	10.6 ± 1.5	15.0^{c}
Mean	1562^{A}	1362^{B}	830^{C}		724^{A}	620^{B}	152^{C}		32.6^{A}	32.0^{A}	16.1^{B}	
LSD $_{5\%}$ (ME × E)	187.5				76.4				4.87			

This analysis was carried out over two growing seasons, with the water stress levels applied after the six-leaf stage.
Means followed by similar letter(s) are not significantly different at 5% probability level, using LSD's test.

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Agro-physiological traits

Agro-physiological traits – days to flowering, days to maturity, RWC, Fv/Fm ratios and Chl*a*/Chl*b* ratios – provide insights into how vetch genotypes adapt their physiological processes under stress. *Vicia pannonica* Crantz. consistently had the highest days to flowering and maturity, reflecting its extended development period. Severe stress reduced days to flowering in *V. sativa* M. and *V. sativa* D., with the most significant reductions in *V. sativa* F. and *V. pannonica* Crantz., indicating stress-induced acceleration of developmental processes in some genotypes (Table S3). The prolonged maturity observed in *V. pannonica* Crantz. under drought may be an adaptive strategy to optimize reproductive success under continued stress. *Vicia michauxii* Spreng. had the highest RWC under both control and severe stress conditions, suggesting its superior ability to retain water under drought (Table 4). In contrast, *V. sativa* M. had the lowest RWC under severe stress, highlighting its vulnerability to water deficiency. *Vicia pannonica* Crantz. maintained the highest Fv/Fm ratios, reflecting better photosynthetic efficiency under severe stress (Table 4). This genotype also had an increased Chl*a*/Chl*b* ratio under severe stress, suggesting enhanced chlorophyll activity relative to chlorophyll *b*, which could contribute to better stress adaptation.

Biochemical traits

Biochemical traits such as chlorophyll content, carotenoids, LPC, soluble carbohydrates, DPPH concentration and enzyme activities are critical for understanding the biochemical strategies underlying stress tolerance. *Vicia michauxii* Spreng. had the highest chlorophyll *a*, chlorophyll *b* and carotenoids content, indicating its robust photosynthetic capacity and higher antioxidant levels under drought (Table S4). Severe stress reduced these contents across genotypes, with *V. pannonica* Crantz. experiencing the highest reductions, potentially affecting its overall stress tolerance. *Vicia sativa* F. had the highest LPC under severe stress, suggesting its role in osmotic adjustment and stress mitigation. Similarly, *V. sativa* Se. accumulated the highest LSC, indicating a role in stress protection and energy storage (Table 5).

Vicia sativa Se. had the highest DPPH concentration under stress, reflecting its higher antioxidant capacity. Enzyme activities, including ascorbate peroxidase, catalase and peroxidase, varied among genotypes, with *V. sativa* M. showing the highest ascorbate peroxidase activity under control and moderate stress, and *V. villosa* Roth. maintaining high catalase activity across conditions (Table S5). Increased hydrogen peroxide and MDA levels under drought stress highlight oxidative stress. *Vicia michauxii* Spreng. and *V. sativa* Sh. had the highest hydrogen peroxide concentrations, while *V. sativa* M. maintained higher MSI values, suggesting better membrane integrity under severe stress (Table 6).

Principal components analysis (PCA) across all studied genotypes

Non-stressed environment

PCA of the non-stressed environment showed that PC1 (44% variation) had positive correlations with grain yield, PN, Chl *a*, SP, MSI, RWC, SPN, Fv/Fm and TSW, but negative correlations with HI, MDA, DPPH, LPC, LSC, POX and APX (Fig. 1(A)). PC2 (17% variation) had positive associations with Chl*a*/Chl*b*, grain yield and HI, and negative associations with DTF, DTM,

Table 4. The mean comparison of the relative water content (%), maximum photochemical efficiency of photosystem II (Fv/Fm) and Chla/Chlb ratio of different vetch genotypes at the 50% flowering growth stage under three levels of water-deficit stress: 30% (control), 50% (moderate stress) and 85% (severe stress) of the maximum allowable depletion levels of soil available water

Ecotype	RWC (%)				Fv/Fm				Chla/Chlb			
	Control	Moderate	Severe	Mean	Control	Moderate	Severe	Mean	Control	Moderate	Severe	Mean
V. dassycarpa Ten.	84.5 ± 0.41	82.7 ± 1.3	68.9 ± 2.2	78.7^{ab}	0.897 ± 0.01	0.813 ± 0.01	0.854^{abc}	0.853 ± 0.02	1.167 ± 0.02	1.150 ± 0.02	1.144 ± 0.05	1.154^{ab}
V. pannonica Crantz.	83.2 ± 0.83	78.4 ± 1.9	63.8 ± 4.2	75.1^{cd}	0.900 ± 0.01	0.828 ± 0.01	0.863^{ab}	0.860 ± 0.02	0.929 ± 0.04	0.899 ± 0.03	1.587 ± 0.08	1.138^{ab}
V. michauxii Spereng.	85.4 ± 0.92	82.5 ± 1.9	74.5 ± 2.58	80.8^{a}	0.893 ± 0.01	0.825 ± 0.01	0.866^{a}	0.878 ± 0.01	1.124 ± 0.03	1.133 ± 0.07	1.150 ± 0.04	1.136^{ab}
V. sativa Ardebil	84.0 ± 0.83	75.7 ± 0.3	63.5 ± 5.1	74.4^{cde}	0.875 ± 0.01	0.760 ± 0.01	0.829^{de}	0.852 ± 0.01	1.158 ± 0.03	1.078 ± 0.04	1.044 ± 0.09	1.094^{bc}
V. sativa Dashtyar	82.4 ± 1.4	74.4 ± 2.3	66.6 ± 4.7	74.5^{cde}	0.855 ± 0.01	0.787 ± 0.02	0.839^{cde}	0.875 ± 0.01	1.107 ± 0.02	1.090 ± 0.02	0.982 ± 0.05	1.060^{cd}
V. sativa Fereydonshahr	83.1 ± 0.8	80.3 ± 1.5	67.5 ± 3.8	77.0^{bc}	0.872 ± 0.01	0.810 ± 0.02	0.849^{bc}	0.865 ± 0.01	1.002 ± 0.03	1.061 ± 0.02	0.987 ± 0.04	1.017^{d}
V. sativa Mashhad	81.3 ± 0.41	78.6 ± 2.3	57.5 ± 2.6	72.5^{de}	0.872 ± 0.01	0.760 ± 0.01	0.826^{e}	0.845 ± 0.01	1.110 ± 0.03	1.038 ± 0.01	0.908 ± 0.05	1.019^{d}
V. sativa Semirom	81.7 ± 1.5	74.1 ± 0.65	59.6 ± 1.7	71.8^{e}	0.878 ± 0.01	0.772 ± 0.02	0.828^{de}	0.835 ± 0.01	1.119 ± 0.02	1.006 ± 0.01	1.300 ± 0.13	1.142^{ab}
V. sativa Shahrekord	82.9 ± 0.58	77.0 ± 0.84	59.9 ± 2.3	73.2^{de}	0.868 ± 0.01	0.800 ± 0.01	0.842^{cd}	0.858 ± 0.01	1.162 ± 0.03	1.037 ± 0.02	1.395 ± 0.05	1.198^{a}
V. villosa Roth.	85.2 ± 0.40	81.3 ± 1.7	69.2 ± 2.4	78.6^{ab}	0.852 ± 0.01	0.785 ± 0.02	0.830^{de}	0.853 ± 0.01	1.200 ± 0.03	1.116 ± 0.03	0.982 ± 0.04	1.099^{bc}
Mean	83.4^{A}	78.5^{B}	65.1^{C}		0.857^{A}	0.794^{C}	0.876^{B}		1.108^{B}	1.061^{C}	1.148^{A}	
LSD $_{5\%}$ (ME × E)	3.96				0.020				0.088			

This analysis was carried out over two growing seasons, with the water stress levels applied after the six-leaf stage.
Means followed by similar letter(s) are not significantly different at 5% probability level, using LSD's test.

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Table 5. The mean comparison of the leaf proline content (µmol/gFW), leaf soluble carbohydrate (µmol/ml) and DPPH (2,2-diphenyl-1-picrylhydrazyl) (mg/ml) of different vetch genotypes at the 50% flowering growth stage under three levels of water-deficit stress: 30% (control), 50% (moderate stress) and 85% (severe stress) of the maximum allowable depletion levels of soil available water

	LPC (µmol/gFW)				LSC (µmol/ml)				DPPH (mg/ml)			
Ecotype	Control	Moderate	Severe	Mean	Mean	Control	Moderate	Severe	Control	Moderate	Severe	Mean
V. dassycarpa Ten.	10.09 ± 0.39	11.65 ± 0.53	14.07 ± 0.19	11.94^{b}	141.3 ± 4.28	150.3 ± 4.06	168.5 ± 1.65	153.4^{c}	2736 ± 118	2664 ± 134	3331 ± 236	2911^{c}
V. pannonica Crantz.	11.41 ± 0.36	12.64 ± 0.42	14.80 ± 0.56	12.95^{a}	162.6 ± 6.70	160.2 ± 3.93	180.7 ± 3.70	167.8^{a}	3143 ± 112	3126 ± 139	4302 ± 94	3524^{a}
V. michauxii Spereng.	9.960 ± 0.16	10.28 ± 0.30	12.22 ± 0.37	10.82^{c}	150.8 ± 8.36	146.8 ± 8.38	165.3 ± 6.14	154.0^{bc}	2328 ± 122	2598 ± 133	2730 ± 124	2886^{c}
V. sativa Ardebil	10.52 ± 0.42	12.37 ± 0.59	14.39 ± 0.06	12.43^{ab}	147.2 ± 6.92	161.5 ± 7.70	171.7 ± 4.91	160.1^{abc}	2921 ± 100	2735 ± 121	3892 ± 178	3182^{b}
V. sativa Dashtyar	10.78 ± 0.37	13.02 ± 0.77	15.12 ± 0.44	12.97^{a}	161.7 ± 7.60	157.0 ± 8.12	177.5 ± 2.79	165.4^{a}	2875 ± 58	2919 ± 137	3938 ± 59	3244^{b}
V. sativa Fereydonshahr	11.84 ± 0.37	11.83 ± 0.55	14.26 ± 0.85	12.65^{ab}	150.7 ± 5.61	165.8 ± 5.28	187.7 ± 2.50	168.0^{a}	2868 ± 69	3055 ± 111	3853 ± 126	3259^{b}
V. sativa Mashhad	11.31 ± 0.40	13.26 ± 0.29	14.48 ± 0.08	13.02^{a}	160.3 ± 9.04	147.7 ± 7.25	189.0 ± 7.88	165.7^{a}	2979 ± 45	2783 ± 125	4019 ± 248	3260^{b}
V. sativa Semirom	11.40 ± 0.45	12.80 ± 0.50	15.33 ± 0.19	13.18^{a}	168.0 ± 3.79	150.7 ± 8.41	184.5 ± 3.73	167.7^{a}	2961 ± 74	3257 ± 188	4367 ± 61	3529^{a}
V. sativa Shahrekord	11.00 ± 0.51	13.17 ± 0.14	14.84 ± 0.28	13.00^{a}	165.3 ± 5.62	159.8 ± 6.64	173.8 ± 9.98	166.3^{a}	3111 ± 44	3063 ± 83	4253 ± 49	3476^{a}
V. villosa Roth.	11.367 ± 0.50	9.7781 ± 75	14.82 ± 0.42	11.99^{b}	155.3 ± 5.98	156.7 ± 8.22	179.2 ± 4.26	163.7^{ab}	2791 ± 201	2967 ± 151	4007 ± 296	3255^{b}
Mean	10.97^{C}	12.08^{B}	14.43^{A}		156.3^{B}	155.6^{B}	177.8^{A}		2871^{B}	2917^{B}	3969^{A}	
LSD $_{5\%}$ (ME × E)	1.00				11.62				191.6			

This analysis was carried out over two growing seasons, with the water stress levels applied after the six-leaf stage.
Means followed by similar letter(s) are not significantly different at 5% probability level, using LSD's test.

Table 6. The mean comparison of the hydrogen peroxide content (µmol/g FW), malondialdehyde content (nmol/gFW) and membrane stability index (%) of different vetch genotypes at the 50% flowering growth stage under three levels of water-deficit stress: 30% (control), 50% (moderate stress) and 85% (severe stress) of the maximum allowable depletion levels of soil available water

	H_2O_2 (µmol/g FW)				MDA (nmol/gFW)			Mean	MSI (%)			
Ecotype	Control	Moderate	Severe	Mean	Control	Moderate	Severe		Control	Moderate	Severe	Mean
V. dassycarpa Ten.	0.834 ± 0.02	0.854 ± 0.01	0.873 ± 0.02	0.853^{ab}	7.082 ± 0.22	6.940 ± 0.33	8.937 ± 0.20	7.653^{cd}	89.15 ± 0.41	88.91 ± 0.27	84.16 ± 0.73	87.41^{a}
V. pannonica Crantz.	0.781 ± 0.02	0.881 ± 0.01	0.937 ± 0.01	0.866^{ab}	7.433 ± 0.18	7.837 ± 0.40	9.328 ± 0.13	8.199^{b}	86.25 ± 0.35	85.47 ± 0.46	80.80 ± 0.56	84.17^{d}
V. michauxii Spereng.	0.854 ± 0.01	0.828 ± 0.02	0.908 ± 0.02	0.863^{ab}	6.380 ± 0.22	6.695 ± 0.27	9.102 ± 0.25	7.392^{d}	89.55 ± 0.37	89.50 ± 0.39	85.10 ± 1.45	88.05^{a}
V. sativa Ardebil	0.818 ± 0.02	0.825 ± 0.01	0.899 ± 0.02	0.847^{b}	7.977 ± 0.16	8.043 ± 0.24	8.985 ± 0.18	8.335^{ab}	87.35 ± 0.35	86.43 ± 1.03	82.88 ± 0.61	85.55^{b}
V. sativa Dashtyar	0.809 ± 0.03	0.870 ± 0.02	0.916 ± 0.02	0.865^{ab}	6.818 ± 0.28	7.092 ± 0.42	8.900 ± 0.23	7.603^{cd}	88.14 ± 0.58	85.98 ± 0.77	82.15 ± 1.22	85.42^{bc}
V. sativa Fereydonshahr	0.789 ± 0.01	0.882 ± 0.02	0.924 ± 0.02	0.865^{ab}	7.548 ± 0.16	8.100 ± 0.21	8.915 ± 0.16	8.188^{b}	89.27 ± 0.22	85.24 ± 0.34	81.98 ± 0.91	85.50^{b}
V. sativa Mashhad	0.841 ± 0.01	0.871 ± 0.01	0.928 ± 0.02	0.880^{ab}	7.292 ± 0.13	8.295 ± 0.12	8.980 ± 0.21	8.189^{b}	87.35 ± 0.29	85.86 ± 1.14	81.32 ± 0.42	84.84^{bcd}
V. sativa Semirom	0.792 ± 0.02	0.876 ± 0.01	0.979 ± 0.06	0.882^{ab}	7.303 ± 0.22	7.909 ± 0.27	8.968 ± 0.28	8.060^{b}	87.81 ± 0.54	83.45 ± 0.84	82.13 ± 1.08	84.47^{bcd}
V. sativa Shahrekord	0.787 ± 0.02	0.891 ± 0.01	1.016 ± 0.13	0.898^{a}	7.712 ± 0.18	8.477 ± 0.08	9.405 ± 0.18	8.531^{a}	86.05 ± 0.48	84.20 ± 0.85	82.65 ± 0.36	84.30^{cd}
V. villosa Roth.	0.841 ± 0.01	0.843 ± 0.01	0.912 ± 0.02	0.865^{ab}	7.005 ± 0.35	7.238 ± 0.49	8.977 ± 0.27	7.740^{c}	89.52 ± 0.14	88.13 ± 0.56	83.18 ± 0.90	86.94^{a}
Mean	0.815^{C}	0.862^{B}	0.929^{A}		7.255^{C}	7.663^{B}	9.050^{A}		88.04^{A}	86.32^{B}	82.64^{C}	
LSD $_{5\%}$ (ME × E)	0.027				0.173				0.675			

This analysis was carried out over two growing seasons, with the water stress levels applied after the six-leaf stage.
Means followed by similar letter(s) are not significantly different at 5% probability level, using LSD's test.

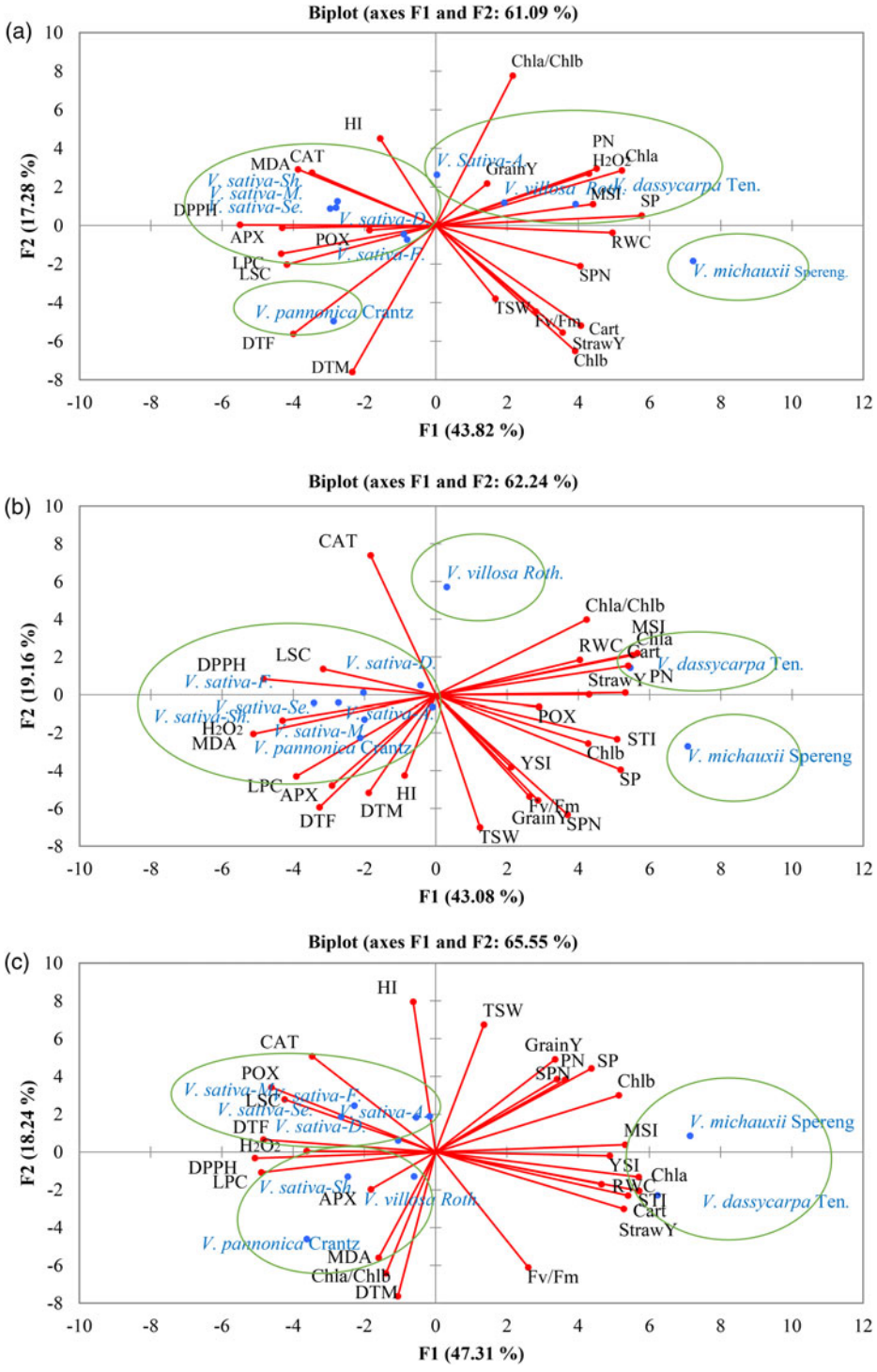

Figure 1. Distribution of the first two principal components of measured traits in ten vetch genotypes under three levels of water-deficit stress: (1A) 30% (control), (1B) 50% (moderate stress) and (1C) 85% (severe stress) of the maximum allowable depletion levels of soil available water. APX, ascorbate peroxidase activity (units/mg protein), Cart, carotenoid (mg/gFW); CAT, catalase activity (units/mg protein); Chl a, chlorophyll *a* (mg/gFW); Chl b, chlorophyll *b* (mg/gFW); Chla/Chlb, Chla/Chlb ratio; DTF, days to flowering; DPPH, 2,2-diphenyl-1-picrylhydrazyl (mg/ml); DTM, days to maturity; Fv/Fm, maximum photochemical efficiency of photosystem II; Grain-Y, grain yield (kg/ha); HI, harvest index; H_2O_2, hydrogen peroxide content (µmol/g FW); LPC, leaf proline content (µmol/gFW); LSC, leaf soluble carbohydrate (µmol/ml); MDA, malondialdehyde content (nmol/gFW); MSI, membrane stability index (%); PN, pod number (No./plant); POX, peroxidase (units/mg protein); RWC, relative water content (%); SN, seed per plant (No./plant); SP, seeds in pot (No./pod); STI, stress tolerance index; Straw-Y, straw yield (kg/ha); TSW, 1000-seed weight (g); YSI, yield stability index.

Chl*b*, straw yield and Cart. Increasing DTF and DTM decreased Chl*a*/Chl*b*, Chl*a*, H_2O_2, PN, MSI, SN and RWC. Grain yield had no significant correlation with TSW, but TSW was linked to straw yield, Fv/Fm, Chl *b* and Cart. Grain yield was associated with PN, MSI, H_2O_2 and Chl *a*. Genotypes were grouped: A (*V. sativa*-Sh, *V. sativa*-M, *V. sativa*-Se, *V. sativa*-D, *V. sativa*-F) by physiological traits and antioxidant enzymes, B (*V. pannonica* Crantz.) by late flowering and maturity, C (*V. michauxii* Spreng.) by grain yield-related traits, D (*V. dassycarpa* Ten., *V. villosa* Roth., *V. sativa*-A) based on MSI, grain yield and chlorophyll (Fig. 1(A)).

Moderate stress environment

In moderate stress, the first and second principal components explained 62% of total variation. The first component (40% of variation) correlated traits like RWC, MSI, Cart, Chl*a*, Chl*a*/Chl*b*, PN, straw yield, POX and tolerance indices (Fig. 2). The second component correlated with CAT, TSW, MTD, DTF and HI. STI was linked to POX, Chlb and SP, indicating increased drought tolerance. Catalase activity, soluble carbohydrates, DPPH, H_2O_2 and MDA decreased. Days to flowering and maturity correlated negatively with MSI, carotenoids, RWC, Chl*a*, Chl*a*/Chl*b* and straw yield. 1000-seed weight

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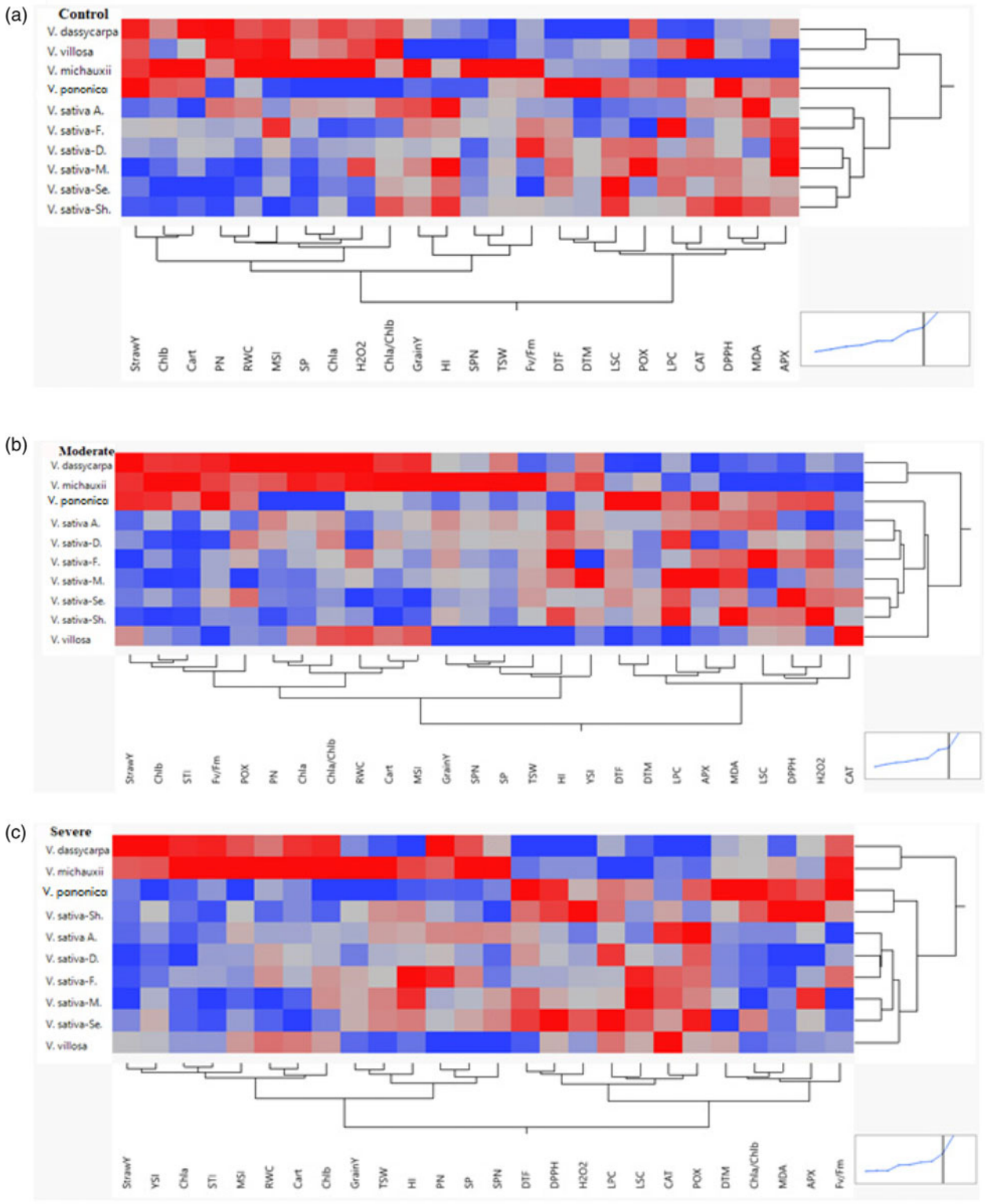

Figure 2. Cluster heat map of ten vetch genotypes under three levels of water-deficit stress: 30% (control), 50% (moderate stress) and 85% (severe stress) of the maximum allowable depletion levels of soil available water. Hierarchical clustering analysis groups the genotypes based on similarity and their correlation with different traits. The heat map displays a colour pattern where darker colours represent higher values, and lighter colours represent lower values. The colour scale ranges from dark red (high values) to dark blue (low values), with intermediate colours indicating varying degrees of trait expression. APX, ascorbate peroxidase activity (units/mg protein); Cart, carotenoid (mg/gFW); CAT, catalase activity (units/mg protein); Chl a, chlorophyll *a* (mg/gFW); Chl b, chlorophyll *b* (mg/gFW); Chla/Chlb, Chla/Chlb ratio; DTF, days to flowering; DPPH, 2,2-diphenyl-1-picrylhydrazyl (mg/ml); DTM, days to maturity; Fv/Fm, maximum photochemical efficiency of photosystem II; Grain-Y, grain yield (kg/ha); HI, harvest index; H_2O_2, hydrogen peroxide content (µmol/g FW); LPC, leaf proline content (µmol/gFW); LSC, leaf soluble carbohydrate (µmol/ml); MDA, malondialdehyde content (nmol/gFW); MSI, membrane stability index (%); PN, pod number (No./plant); POX, peroxidase (units/mg protein); RWC, relative water content (%); SN, seed per plant (No./plant); SP, seeds in pot (No./pod); STI, stress tolerance index; Straw-Y, straw yield (kg/ha); TSW, 1000-seed weight (g); YSI, yield stability index.

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correlated positively with grain yield, pod number, Fv/Fm and YSI (Fig. 1(B)).

Comparing control and moderate stress, 1000-seed weight and straw yield relationship decreased in stress. In stress, 1000-seed weight correlated positively with seed number, maturity, CAT, DPPH and Chl*a*/Chl*b*. Days to flowering and grain yield showed positive correlation in stress. Antioxidant enzyme relationships changed in stress, with negative or no correlation. Fv/Fm had different correlations with chlorophyll *b* and carotenoids but negative correlation with H_2O_2 in both environments (Fig. 1(B)).

Under stress, increased days to flowering and maturity correlated with higher free radicals, MDA, proline, ascorbate and DPPH. STI, straw yield and seed number decreased, while HI and 1000-seed weight increased. In control, increased maturity linked to reduced HI and H_2O_2, while grain yield, MDA and straw yield remained relatively unchanged. Improvement programmes should consider purpose and environmental conditions. Genotypes were classified into groups based on traits, showing suitability for different environments (Fig. 1(B)).

Severe stress environment

In severe stress, the first two components explained 65% of the variation. The first component correlated with stress tolerance traits, while the second correlated with grain yield. Genotypes high in both components showed high grain yield and drought tolerance (Fig. 1(C)). *Vicia dassycarpa* Ten. displayed potential as a forage genotype. Antioxidant enzymes and H_2O_2 had a strong positive correlation under severe stress. Chlorophylls, carotenoids and membrane stability were negatively correlated with H_2O_2. Increasing stress disrupted the balance between H_2O_2, chlorophylls and antioxidants (Fig. 1(C)).

Genotypes were classified into three groups based on severe stress performance (Fig. 1(C)). In severe stress, grain yield, TSW, pod and seed numbers and YSI increased. Antioxidant enzymes, proline and soluble carbohydrates correlated positively with H_2O_2. Days to flowering and maturity had no correlation. Comparing environments, the positive correlation between antioxidant enzymes and H_2O_2 increased with stress intensity. Under severe stress, H_2O_2 negatively correlated with chlorophylls, carotenoids and membrane stability. In the control environment, H_2O_2 had negative correlations with antioxidant enzymes but positive correlations with chlorophyll-a and membrane stability. Increasing stress disrupted the H_2O_2–chlorophylls–antioxidants balance in the control environment.

Clustering analysis

Under non-stressed conditions, genotypes clustered into distinct groups based on their characteristics. *Vicia dassycarpa* Ten. and *V. villosa* Roth. exhibited high straw yield, pod number, leaf water content, membrane stability and chlorophyll levels (Fig. 2). *Vicia michauxii* Spreng. showed high chlorophyll content, carotenoids, pod number, grain yield, seed number, seed weight and membrane stability. *Vicia pannonica* Crantz. displayed high DPPH activity and flowering days but low membrane stability and chlorophyll levels. *Vicia sativa* species had high HI, leaf carbohydrates, peroxidase, MDA and flowering days (Fig. 2).

Under moderate stress conditions, genotypes clustered into groups based on their traits. *Vicia dassycarpa* Ten. had high straw yield, peroxidase activity, pod number, chlorophyll a and leaf water content. *Vicia michauxii* Spreng. exhibited high chlorophyll content, STI, carotenoids, pod number, seed yield, seed number and seed weight. *Vicia pannonica* Crantz. showed high Fv/Fm, flowering and maturity days and ascorbate peroxidase activity but low pod number and chlorophyll levels. *Vicia sativa* species displayed high hydrogen peroxide, DPPH activity, MDA and leaf carbohydrates. *Vicia villosa* Roth. had high catalase activity but low Fv/Fm, grain yield, seed number, pod number, seed weight, flowering days and leaf peroxidase levels (Fig. 2).

Under severe stress conditions, genotypes clustered based on their characteristics. *Vicia dassycarpa* Ten. exhibited high straw yield, YSI, chlorophyll levels, STI, carotenoids and pod number. *Vicia michauxii* Spreng. showed high chlorophyll levels, STI, membrane stability, leaf water content, carotenoids, seed yield, seed weight and pod number. *Vicia pannonica* Crantz. and *V. sativa*-Sh. displayed high Fv/Fm, ascorbate peroxidase, MDA, chlorophyll a/b ratio, flowering and maturity days, DPPH activity and hydrogen peroxide. *Vicia villosa* Roth. and *V. sativa* species had high catalase, peroxidase, leaf carbohydrates, leaf peroxidase, DPPH activity and HI (Fig. 2).

Discussion

This study investigated the growth and related agronomic, physiological and biochemical characteristics of ten vetch genotypes under moderate and severe water-deficit stress levels. The results provide valuable insights into the response of vetch plants to water-deficit stress under field conditions. On average, water-deficit stress led to several physiological and biochemical changes in the vetch plants (Tables 3–6 and S2–S5). Firstly, there was a decrease in relative leaf water content and Fv/Fm (Table 4), indicating reduced water availability and impaired photosynthetic efficiency. This reduction suggests a decline in the maximum quantum yield of photosystem II, indicating compromised photosynthetic capacity (Lawlor and Tezara, 2009; Soares-Cordeiro *et al*., 2009; Urban *et al*., 2017). Similar results were observed in *Vigna radiata* (Batra *et al*., 2014).

The decline in photosynthetic pigments, including chlorophyll *a*, *b* and carotenoids (Table S4), reflects the adverse impact of water-deficit stress on the plant's ability to capture light energy and carry out photosynthesis effectively (Ashraf and Harris, 2013). Additionally, the reduced MSI indicates membrane damage caused by water-deficit stress (Table 6), disrupting cell integrity and impairing various physiological processes (Wang *et al*., 2018; Schneider *et al*., 2019).

In response to water-deficit stress, there was an increase in LPC, concentration of LSC (Table 5), ROS scavenging enzymes (such as APX, CAT and POX) (Table S5) and oxidative stress markers such as DPPH, MDA and H_2O_2 (Tables 5 and 6). These changes suggest that the vetch plants activate defence responses to mitigate the harmful effects of water-deficit stress. The accumulation of proline and soluble carbohydrates helps in osmotic adjustment and maintaining cellular water potential (Swigonska and Weidner, 2013; Ozturk *et al*., 2021). The increase in antioxidant enzyme activity indicates the plant's attempt to counteract oxidative damage caused by the accumulation of ROS under stress conditions (Gill and Tuteja, 2010; Das and Roychoudhury, 2014).

The negative impact of water-deficit stress was evident in the decreased number of pods per plant, seeds per pod, seeds per plant, weight of 1000 seeds and overall grain yield (Tables S3 and S2). These reductions indicate that water-deficit stress directly impairs reproductive development and seed production in plants (Erice *et al*., 2010; Haffani *et al*., 2014).

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The study demonstrates that vetch plants' response to water scarcity is influenced by stress severity and plant genotype (Table S1). Abbasi *et al.* (2014) observed genotype-specific responses to drought stress in common vetch (*V. sativa* L.), impacting physiological and biochemical traits. Haffani *et al.* (2014) identified *V. narbonensis* as having high water use efficiency and stress tolerance.

Among the genotypes studied, *V. dassycarpa* Ten. stood out as the most resilient genotype under both moderate and severe drought stress conditions in terms of straw yield (Table 3 and Fig. 1(C)). The superior performance of *V. dassycarpa* Ten. indicates its ability to adapt to water scarcity through several traits: shorter time to flowering and maturity, enhanced photosynthetic efficiency with high Fv/Fm values, better osmotic adjustment shown by higher leaf proline and soluble carbohydrates, and a robust antioxidant response with increased activity of ROS scavenging enzymes like APX, CAT and POX. Hamidou *et al.* (2007) discovered that cowpea genotypes exhibiting increased net photosynthesis and solute accumulation demonstrated enhanced tolerance to water stress and improved recovery ability. Furthermore, *V. dassycarpa* Ten. demonstrated the shortest days to both flowering and maturity, which can be advantageous in limited water environments (Yordanov *et al.*, 2000; Farooq *et al.*, 2009).

Vicia michauxii Spreng. exhibited exceptional performance in terms of grain yield and various yield-related parameters (Figs 1–2). This genotype demonstrated the highest number of seeds per pod, seeds per plant and 1000 seeds weight, indicating its potential for high seed production and improved yield (Tables S2–S3). *Vicia michauxii* Spreng. also displayed favourable physiological characteristics associated with drought tolerance, such as high RWC, indicating efficient strategies for water retention and maintaining cellular hydration under drought stress. Haffani *et al.* (2014) found *V. narbonensis* had superior adaptation and water balance compared to *V. sativa* and *V. villosa* Roth., with higher water use efficiency.

The *V. sativa* genotypes, originating from regions with varying climates (ranging from very cold to moderate winters and moderate to hot summers) and rainfall (200–600 mm) (Table 1 and Fig. S1), were studied in dry and warm conditions in Najaf-Abad, Iran. This intraspecies variation highlights their adaptability to diverse conditions, suggesting that selecting genotypes within *V. sativa* is as critical as choosing among different species for improving drought tolerance and productivity in breeding programmes. Therefore, the significant differences observed in grain and straw yield can be attributed to the varied conditions of seed growth and production. A study on Spanish core collections of common vetch supports these findings, noting high genetic diversity and valuable germplasm for resilient agriculture (De la Rosa *et al.*, 2021).

Vicia michauxii Spreng. exhibited high values for Fv/Fm, chlorophyll *b*, carotenoids and MSI, reflecting its ability to maintain photosynthesis and cellular integrity under stress. In various studies conducted on chickpea (Pouresmael *et al.*, 2013), wheat (Sharma *et al.*, 2015), maize (Xu *et al.*, 2008) and cotton (Guo *et al.*, 2022), the use of Fv/Fm as a dependable indicator for assessing drought tolerance in different crop species has been supported.

The high levels of chlorophyll *b* and carotenoids further suggest that *V. michauxii* Spreng. retains its photosynthetic pigments, which are essential for capturing light energy and carrying out photosynthesis (Wang *et al.*, 2018; Schneider *et al.*, 2019). The elevated MSI reflects the ability of *V. michauxii* Spreng. to maintain membrane integrity and stability, which is crucial for various cellular processes under stressful conditions.

Furthermore, *V. michauxii* Spreng. displayed the lowest values for LSC, DPPH and LPC (Table 6). These parameters are indicators of oxidative stress and cellular damage caused by ROS. The low values observed in *V. michauxii* Spreng. suggest that this genotype has effective strategies for scavenging ROS and protecting cellular components from oxidative damage. Overall, the enhanced seed production exhibited by *V. michauxii* Spreng. results from high numbers of seeds per pod, seeds per plant and 1000 seeds weight. These findings highlight the potential of *V. michauxii* Spreng. as a valuable genotype for breeding programmes aimed at developing drought-tolerant vetch varieties. The study revealed contrasting responses among the vetch genotypes to moderate drought stress conditions. *Vicia sativa*-Se. demonstrated the highest sensitivity to moderate drought stress in terms of grain yield, with significant reductions compared to other genotypes (Table 3). This sensitivity was linked to the lowest MSI, compromised membrane stability and the lowest number of seeds per plant, indicating impaired reproductive development. Furthermore, *V. sativa*-Se. exhibited the highest DPPH activity, an indicator of oxidative stress. Conversely, *V. sativa*-F. displayed the highest sensitivity to moderate drought stress in terms of straw yield, accompanied by the highest HI and LSC, suggesting a reallocation of resources towards reproductive structures under stress.

Vicia pannonica Crantz. exhibited high straw yield under control and moderate drought stress conditions, indicating its potential for productive biomass production even with limited water availability. However, under severe drought stress, *V. pannonica* Crantz. showed the highest sensitivity, with significant reductions in both grain and straw yields. This genotype also exhibited the longest days to flowering and maturity, prolonging exposure to water deficit, and the lowest MSI values, indicating severe membrane damage and instability under severe drought conditions.

The result of PCA and heat map cluster indicated a wide range of genetic diversity in agro-physiological traits among the studied *vicia* germplasm, facilitating selection for genotypes for specific purposes such as grain and forage use (Majidi and Zadhoush, 2014). Agronomic and physiological assessments distinguished the studied species according to their systematic classification sections.

The diversity observed was predictable given the wide range of geographic origins and cultivation status of the germplasm, reflecting the impact of climate, landscape, agricultural use and history on the phenotypes of the species. The results also indicated that soil moisture levels can influence the relationships among traits, with drought stress altering gene expression for agronomic and physiological traits and changing their relationships. These findings indicate that RWC, Fv/Fm ratio, chlorophyll content, MSI and antioxidant enzyme activity are key physiological markers for assessing stress tolerance in vetch under varying drought conditions. The changes in these markers help to understand how different genotypes respond to stress and can guide breeding programmes for improved drought resistance.

Conclusions

The study highlights the importance of genotype-specific responses to drought stress in vetch. *Vicia dassycarpa* Ten. is recommended for fodder production due to its early flowering and maturity, while *V. michauxii* Spreng. is identified as ideal for grain production because of its superior agronomic and physiological traits under drought conditions. These findings provide valuable markers for breeding drought-tolerant vetch

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varieties, though further research on gene expression is needed to deepen our understanding.

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Data. The data utilized to substantiate the findings of this study are available in the Supplementary Material.

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