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Research Article

Mitigation of chilling and freezing stresses through colonization with arbuscular mycorrhizal fungi in spring barley

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Abstract

Cold stress is an important limiting factor for cereal production. Barley is a host species for arbuscular mycorrhizal fungi (AMF) with a high genetic diversity in response to cold stress. In order to explore the mechanisms for the ameliorative effect of AMF under cold stress, an experiment was undertaken using completely randomized block design with three factors including temperature treatment, AMF inoculation and plant cultivar. Two spring barley cultivars with different cold tolerance ('Reyhan' as tolerant and 'Torkaman' as susceptible) were inoculated with two AMF species (*Diversispora versiformis* and *Rhizophagus irregularis*) and were grown under chilling (CH, 5 °C) and freezing (FR, −5 °C) temperatures for three weeks. Dry matter production, photosynthesis rate, and membrane integrity parameters decreased, while the antioxidant defense and the synthesis of phenolics were activated under CH stress. Inoculation of plants with AMF alleviated the adverse effects of CH stress on growth and membrane parameters, while exacerbated CH effect on the antioxidant system and phenolics accumulation. Plants could not survive FR stress when they were not cold acclimated through prior exposure to CH treatment, unless they were inoculated with AMF which resulted in survival rates almost similar to cold-acclimated plants. Our results suggested that AMF alleviated CH stress through reducing H_2O_2 and improved membrane integrity while the substituting effect of AMF for cold acclimation and increasing FR survival was mediated by the activation of antioxidant defense and phenolics synthesis and accumulation of proline.

Keywords: Antioxidant defense, Barley, Cold acclimation, Electrolyte leakage, Phenolics metabolism, Survival rate

Introduction

Cold stress is a prevalent factor limiting the growth and productivity of crop plants worldwide (Ruelland *et al*., 2009). Low temperature consists of chilling (CH, 0–15 °C) and freezing (FR, <0°C) temperatures, affects the fluidity, integrity and function of plasma membranes, and impairs metabolic processes and enzymatic activities (Theocharis *et al*., 2012). Chilling stress diminishes photosynthetic capacity of leaves through inhibition of chloroplast development and modification of the pigment composition and content, accompanied by photosystem damage (Miura and Furumoto, 2013). Plant water status is also disturbed in CH-stressed plants due to decreased hydraulic permeability of the plasma membranes and reduction in the nutrient uptake with osmotic functions (Ruelland *et al*., 2009; Theocharis *et al*., 2012; Miura and Furumoto, 2013).

Increased generation of reactive oxygen species (ROS), which attacks and damages macromolecules and membrane lipids, is a common response to stresses such as CH stress (Suzuki and Mittler, 2006). The level of damage depends on the balance between the generation of ROS and their scavenging by the antioxidant enzymes, particularly superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Plant membrane is the primary site of oxidative injury under CH stress, leading to lipid peroxidation accompanied by an increased leakage of electrolytes (Theocharis *et al*., 2012).

Despite the impairment of growth, CH temperatures can improve tolerance to FR stress and increase plant survival following a freeze-thaw cycle (Janska *et al*., 2010). Gradual exposure to CH temperatures, meaning 'cold acclimation' is accompanied by several biochemical and molecular alterations such as activation of antioxidant defense system, synthesis and accumulation of cryoprotectant molecules, and increase in the proportion of unsaturated fatty acids (Gusta and Wisniewski, 2013).

Phenylpropanoid pathway is involved in the synthesis of a diverse array of phenolic compounds such as phenolic acids, flavonoids and anthocyanins (Boudet, 2007). Phenylalanine ammonia-lyase (PAL) catalyzes the initial reaction in this pathway, while polyphenol oxidase (PPO) mediates the hydroxylation of monophenols and oxidation of polyphenols (Boudet, 2007). Modification of the phenolics metabolism has been reported under CH stress in certain plant species (Yang *et al*., 2018). However, the functional

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significance of phenolics in plants CH tolerance is still obscure and its role, as a component of cold acclimation process in improving frost tolerance, has not been explored yet.

The symbiosis of plant roots with arbuscular mycorrhizal fungi (AMF) alleviates the adverse effects of different environmental stresses such as salt and drought (Abdel Latef *et al*., 2016). However, it is only in the recent years that AMF application for increasing the tolerance of plants against CH and FR has attracted scientific attention. Available reports have shown that AMF colonization alleviates CH stress in plants through attenuating membrane lipid peroxidation, increasing osmolytes accumulation, improving leaf water status, and photosynthesis and activation of antioxidant enzymes in maize (Zhu *et al*., 2010), cucumber (Chen *et al*., 2013) and barley (Hajiboland *et al*., 2019).

Barley is the second most important staple food crop after wheat that is cultivated worldwide under different environmental conditions, and undergoes cold stress in the temperate and cold regions (Rizza *et al*., 2011). A wide range of CH and FR tolerances were found in barley cultivars (Rizza *et al*., 2011; Joudmand *et al*., 2019) suggesting a high genetic diversity in the physiological and biochemical responses under cold stress in this species. Barley is a mycorrhizal host species, however, the mechanisms for the ameliorative effect of AMF association under CH and FR stresses are not adequately understood in this crop. The aim of this study was to evaluate the effect of AMF on CH and FR stresses in two spring barley cultivars. Our working hypothesis is that AMF-mediated metabolic alterations responsible for alleviation of low temperature stress is different under CH and FR conditions in barley cultivars.

Material and methods

Two spring barley (*Hordeum vulgare* L.) cultivars that differed in terms of cold resistance, 'Reyhan' (tolerant) and 'Torkaman' (susceptible) were prepared by Seed and Plant Improvement Institute (SPII) (Karaj, Iran). Seeds were surface-sterilized with 10% sodium hypochlorite and germinated on perlite in the dark in 5 L plastic container filled with washed sterilized perlite. Twenty four seeds were sown in each container. Five-day-old young seedlings were exposed to light and irrigated with Hoagland nutrient solution at 300 mL $week^{-1}$ for three weeks. Plants were grown under growth chamber conditions with a day/night temperature regime of 28/17 °C, a relative humidity of 70/80% and a photoperiod of 17/7 h at a photon flux density of approximately 200 µmol $m^{-1}s^{-1}$ provided by fluorescent lamps.

Prior to germination, inoculums of *Diversispora versiformis* (formerly *Glomus versiforme*) and *Rhizophagus irregularis* (formerly *Glomus intrardices*), obtained from propagation on clover plants, and containing hyphal segments, spores, and root pieces, were placed approximately 1 cm below the seeds and covered with perlite. Mycorrhizal colonization of root fragments in the inoculums was 40% and 80% for *D. versiformis* and *R. irregularis*, respectively. In order to equalize the colonizing capacity of inoculums, 100 and 50 g inoculum L^{-1} perlite for *D. versiformis* and *R. irregularis* were applied to the container, respectively. Autoclaved AMF inoculums were applied to the –AMF treatments. In order to determine the colonization frequency at harvest, the fine roots were cleared in 10% KOH, acidified with excess 2% HCl and stained with 0.05% aniline blue. The root colonization rate (%) was determined by the gridline intersect method (Giovannetti and Mosse, 1980).

Following three weeks of growth under control conditions, the plants were randomly assigned to two temperature treatments including control (28/17 °C day/night) and chilling (CH) temperature (5/3 °C day/night). Chilling temperature was provided as a gradual temperature reduction by 5 °C day^{-1} from 25 °C to 5 °C under the same light conditions as control treatment. Plants were harvested three weeks after starting temperature treatments. Prior to the harvest, net photosynthesis rate was determined with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK). At harvest, the plants total fresh weight (FW) was determined, and subsamples were taken and stored in liquid nitrogen for further biochemical determinations. At harvest, plants dry weigh (DW) was determined in another group of plants after oven drying at 70 °C for 48 h. Oven-dried samples were mineralized in a muffle furnace at 550 ºC for 8 h. After resolving in 1% HCl, potassium was determined in the leaf samples by flame photometry (PFP7; Jenway, UK).

For determination of the survival rate, after the three-week CH treatment, plants were exposed to freezing (FR) treatment. This treatment was applied in dark by exposing the plants to 4 °C for 3 h; subsequently, the temperature was lowered by 1 °C h^{-1} to reach −5 °C. Following 48 h FR treatment, plants were allowed to thaw for 12 h through increasing the temperature up to 4 °C by 1 °C h^{-1}. The survival rate (%) was determined as the capacity of plants to recover from FR stress and start growing after three days under control (25 °C) conditions (Campos *et al*., 2003).

To determine electrolyte leakage, leaf disks (1×1 cm) were prepared, washed and floated in deionized water. Ten leaf disks were prepared from each replicate plant and placed in 10 mL vials. After 22 h incubation at room temperature, the electrical conductivity (EC) of the medium was determined (EC1) using an EC meter (HI 9812, Hanna instruments, Italy). Total conductivity was determined after killing the leaf tissues at 90 °C for 2 h (EC2). Electrolyte leakage was calculated as the ratio (%) of EC1 to EC2 according to Campos *et al*. (2003).

Leaf concentration of chlorophyll was determined after extracting the pigments in cold acetone and allowing the samples to stand for 24 h in the dark at 4

°C (Lichtenthaler and Wellburn, 1983). Proline was quantified using the method of Bates *et al*. (1973). The concentration of H_2O_2 was determined using potassium iodide (KI) at 390 nm (Junglee *et al.*, 2014). Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma) at 532 nm (Heath and Packer, 1968). Phenolics were extracted three times in 70% aqueous methanol at 4 ºC in the dark; following centrifugation, supernatant was employed to specify total soluble phenolics at 750 nm using Folin-Ciocalteu reagent (Swain and Hillis, 1959). Total soluble protein concentration was determined using Bradford reagent (Sigma, USA).

Osmotic potential was measured by an osmometer (Micro-Osmometer, Heman Roebling Messtechnik, Germany) following homogenization on ice and centrifugation at 4000 g for 10 min at 4 °C. Relative water content (RWC%) was calculated using the data pertaining to turgid weight (TW), FW and dry weight (DW) of the leaf disks according to the following equation: RWC = (FW–DW)/(TW–DW) × 100 (Babu *et al.*, 1999).

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined using mono-formazan formation test at 560 nm (Giannopolitis and Ries, 1977). Peroxidase (POD, EC 1.11.1.7) activity was assayed using the guaiacol as substrate at 470 nm (Chance and Maehly, 1962). Catalase (CAT, EC 1.11.1.6) activity was assayed through measuring H_2O_2 reduction at 240 nm (Luck, 1962). Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was assayed as the conversion rate of L-phenylalanine to trans-cinnamic acid (Dickerson *et al*., 1984); moreover, polyphenol oxidase (PPO, EC 1.14.18.1) activity was determined as the change in the absorbance (ΔAbs) of pyrogallol at 334 nm based on the protocols described by Singh *et al*. (1999).

A complete randomized block design was employed as experimental design using four independent containers as four replicates per treatment. A three-way analysis of variance (ANOVA) was performed for the main effects including temperature treatment (two levels: control and CH stress), AMF inoculation (three levels: –AMF, inoculation with *Diversispora versiformis* or *Rhizophagus irregularis*) and plant cultivar (two levels: 'Reyhan' and 'Torkaman) and their interactions. Pairwise multiple comparison procedure was carried out by the Tukey's test ($P<0.05$) using Sigma Stat. In order to find an interrelationship among different sets of physiological parameters, principle component analysis (PCA) was employed using Minitab 18.0.

Results

The ANOVA table showed that, all the analyzed parameters were significantly ($P<0.001$) affected by CH stress (Table 1). The AMF treatment influenced physiological parameters except for PAL activity and the phenolics concentration. Differences between cultivars in response to CH and AMF treatments were not significant for shoot DW, Chl concentration and photosynthesis rate, while the majority of cold stress indicators including membrane integrity parameters, activity of antioxidant enzymes and proline concentration were differently influenced by the applied treatments in both cultivars (Table 1).

Plants dry matter production was expectedly reduced by CH temperature; however, this effect was more pronouncedly observed in 'Torkaman' (CH-susceptible cultivar) (71%) compared with 'Reyhan' (CH-tolerant cultivar) (48%). Under CH temperature, inoculation of plants with AMF improved shoot DW without significant differences between the effect of the two AMF species. In plants grown under control temperature, *D. versiformis* was the only AMF species that improved shoot DW in both cultivars. A significant reduction in root AMF colonization was observed upon exposure to CH temperature (Table 2).

Leaf concentration of Chl *a*+*b* decreased under CH stress but increased with AMF inoculation in the stressed plants (Table 2). The leaf photosynthesis rate was more reduced by CH stress in susceptible cultivar (50%) compared with the tolerant one (37%). Inoculation of plants with *R. irregularis* resulted in higher photosynthesis rate under control temperature conditions in both cultivars. Under CH stress, AMF colonization significantly improved the photosynthesis rate of CH-susceptible cultivar inoculated with *D. versiformis* (Table 2).

Relative water content (RWC) was influenced by CH temperature only in the CH-susceptible cultivar. Inoculation with AMF increased RWC in both cultivars and under both control and CH temperatures (Table 3). Osmotic potential decreased by CH temperature in both cultivars. Inoculation with AMF significantly decreased leaf osmotic potential under control temperature; under CH temperature, however, AMF inoculation was significantly effective only in the CH-susceptible cultivar inoculated with *D. versiformis* (Table 3).

Proline concentration increased by CH temperature in both species. Mycorrhization of CH-stressed plants further accumulated proline; the effect of both AMF species was significant in the CH-tolerant cultivar, and *R. irregularis* significantly influenced the CH-susceptible cultivar (Table 3). Under control temperature, leaf proline concentration increased in response to inoculation with *D. versiformis* in the CH-susceptible cultivar. In both cultivars, K concentration decreased under CH temperature, while increased upon mycorrhization under control and CH temperatures (Table 3).

Electrolyte leakage from the leaf tissues was significantly increased under CH temperature in both cultivars, while decreased by AMF inoculation in the CH-stressed plants without a difference between the two AMF species (Figure 1). The MDA concentration was increased by CH temperature. This effect was more prominent in the CH-susceptible (535%) than that in the

Table 1. Results of three-way ANOVA (mean squares) for the effect of chilling stress (CH, two levels), AMF colonization (there levels) and cultivar (CV, two levels) on the physiological parameters in barley plants.

Parameters	CV	CH	AMF	CV×CH	CV×AMF	CH×AMF	CV×CH×AMF
Shoot DW	315.188^{ns}	189631.021***	26841.063***	50.021^{ns}	2439.063***	3948.396***	1944.396***
Colonization	0.0464***	0.423***	0.00341***	0.00170^{ns}	0.00370^{ns}	0.00429^{ns}	0.0213*
Chl *a+b*	0.00662^{ns}	0.348***	0.0656***	0.000300^{ns}	0.00817**	0.0476***	0.000859^{ns}
Photosynthesis	0.975^{ns}	24.339***	9.473***	4.356***	4.230***	12.751***	1.784**
El leakage	68.547***	2060.319***	399.005***	65.924***	14.773**	458.235***	21.877***
MDA	54.312***	345.803***	60.408***	81.953***	11.075***	52.041***	25.045***
H_2O_2	0.0399^{ns}	10.863***	0.871***	0.383**	0.268**	3.683***	0.296**
SOD	6.354**	457.271***	14.856***	0.00611^{ns}	0.247ns	28.067***	8.730***
POD	0.165**	9.184***	0.275***	0.0667*	0.0802**	0.417***	0.150***
CAT	920.509**	38077.463***	1299.733***	156.688^{ns}	60.895^{ns}	119.449^{ns}	103.257^{ns}
PAL	114.040***	164.262***	3.542^{ns}	28.466***	15.200***	1.214^{ns}	9.310**
PPO	0.00126^{ns}	0.0782***	0.0172***	0.00124^{ns}	0.000284^{ns}	0.0224***	0.00106^{ns}
Phenolics	185.335**	10673.838***	18.899^{ns}	782.836***	2302.849***	112.530*	1012.782***
RWC	2.603^{ns}	17.350***	224.595***	3.151^{ns}	10.997***	3.014*	0.645^{ns}
O Potential	0.0726***	1.418***	0.278***	0.0393***	0.0155***	0.190***	0.0108***
Proline	250.024***	2023.691***	96.083***	5.026^{ns}	3.640^{ns}	31.762**	23.046**
Potassium	1.522^{ns}	93.023***	81.217***	2.534*	0.410^{ns}	3.134**	2.238*
Survival rate	22.578^{ns}	17095.127***	4406.959***	0.849^{ns}	48.891^{ns}	1369.215***	141.779ns

***** significant at P<0.001, ** P<0.01, * P<0.05, ns Non-significant. For analyzing colonization rate, two levels were considered for AMF**

Table 2. Shoot dry weight (DW), root colonization rate, leaf chlorophyll (Chl) *a+b* concentrations and photosynthesis rate in the tolerant ('Reyhan') and susceptible ('Torkaman') barley cultivars to chilling (CH) temperature grown without inoculation (–AMF) or inoculated with two AMF species and subjected either to control (28/17 °C) or CH stress (5/3 °C day/night) for three weeks.

Temperature treatment	AMF treatment	Shoot DW (mg plant^{-1})		Root colonization rate (%)	
		CH-tolerant	CH-susceptible	CH-tolerant	CH-susceptible
Control	–AMF	256±19^{b}	250±11^{b}	null	null
	D. versiformis	324±21^{a}	332±26^{a}	76±2.2^{a}	74±6.8^{a}
	R. irregularis	271±25^{b}	261±18^{b}	79±2.4^{a}	62±6.1^{a*}
CH stress	–AMF	132±8^{d}	72±17^{d}	null	null
	D. versiformis	182±8^{c}	200±15^{c}	54±5.8^{b}	45±2.1^{b*}
	R. irregularis	165±16^{cd}	186±6^{c}	51±9.8^{b}	48±7.6^{b}
		Chl *a+b* (mg g^{-1} FW)		Photosynthesis (µmol CO_2 m^{-2}s^{-1})	
		CH-tolerant	CH-susceptible	CH-tolerant	CH-susceptible
Control	–AMF	0.93±0.01^{a}	1.04±0.02^{a}	3.74±0.48^{b}	3.81±0.49^{b}
	D. versiformis	1.02±0.03^{a}	1.02±0.03^{a}	3.92±0.49^{b}	3.50±0.28^{b}
	R. irregularis	0.95±0.08^{a}	0.98±0.04^{ab}	6.70±0.20^{a}	5.08±0.14^{a}
CH stress	–AMF	0.52±0.02^{c}	0.66±0.03^{d}	2.34±0.78^{c}	1.89±0.65^{c}
	D. versiformis	0.93±0.03^{a}	0.90±0.02^{bc}	2.99±0.53^{bc}	5.16±0.74^{a}
	R. irregularis	0.82±0.01^{b}	0.82±0.05^{c}	3.03±0.70^{bc}	2.26±0.17^{c}

Data are means±SD (n=4). Data of each column indicated by the same letter are not significantly different (Tukey test, P≤0.05). * Significantly different from tolerant cultivar (t-test).

CH-tolerant (48%) cultivar. In the CH-stressed plants, AMF colonization reduced MDA concentration in both cultivars, while in the absence of CH treatment, AMF was only effective in the CH-tolerant cultivar inoculated with *D. versiformis* (Figure 1). Concentration of H_2O_2 increased by CH temperature, yet reduced by AMF colonization in both cultivars under CH stress. In the absence of CH treatment, however, AMF colonization augmented H_2O_2 concentration in the CH-susceptible cultivar (Figure 1).

Activity of SOD was higher in the plants exposed to CH stress, and was further increased upon mycorrhization in both cultivars. Nevertheless, under control temperature, the effect of mycorrhizal colonization depended on cultivar and AMF species. SOD activity decreased by inoculation with *D. versiformis* in the CH-tolerant cultivar, whereas it increased with *R. irregularis* in the CH-susceptible cultivar (Figure 2). POD activity was significantly increased by CH temperature in both cultivars, and AMF colonization significantly affected the tolerant cultivar inoculated with *D. versiformis* under CH stress (Figure 2). Similar to SOD and POD, the activity of CAT increased in response to CH temperature in both cultivars. Colonization with AMF did not impact CAT activity in the CH-susceptible cultivar, while in the CH-

Table 3. Leaf relative water content (RWC), osmotic potential, concentrations of proline and potassium (K) in the tolerant ('Reyhan') and susceptible ('Torkaman') barley cultivars to chilling (CH) temperature grown without inoculation (–AMF) or inoculated with two AMF species and subjected either to control (28/17 °C) or CH stress (5/3 °C day/night) for three weeks.

Temperature treatment	AMF treatment	RWC (%)		Osmotic potential (–MPa)	
		CH-tolerant	CH-susceptible	CH-tolerant	CH-susceptible
Control	–AMF	91.3±0.6 ^{d}	91.9±1.5 ^{b}	0.85±0.03 ^{d}	0.79±0.03 ^{d}
	D. versiformis	98.6±0.2 ^{a}	97.0±0.7 ^{a}	1.07±0.08 ^{c}	1.08±0.02 ^{c}
	R. irregularis	96.3±0.5 ^{bc}	97.5±0.7 ^{a}	1.30±0.03 ^{b}	1.29±0.04 ^{b}
CH stress	–AMF	90.0±1.4 ^{d}	88.9±1.4 ^{c}	1.44±0.03 ^{a}	1.30±0.03 ^{b}
	D. versiformis	98.1±1.1 ^{ab}	95.3±1.3 ^{a}	1.46±0.07 ^{a}	1.44±0.02 ^{a}
	R. irregularis	96.0±0.5 ^{c}	97.0±0.8 ^{a}	1.53±0.05 ^{a}	1.29±0.04 ^{b}
		Proline (µmol g^{-1} FW)		K concentration (mg g^{-1} DW)	
		CH-tolerant	CH-susceptible	CH-tolerant	CH-susceptible
Control	–AMF	61.09±6.92 ^{c}	26.30±4.97 ^{d}	14.27±0.96 ^{d}	15.45±0.73 ^{b}
	D. versiformis	57.03±4.82 ^{c}	50.33±3.59 ^{c}	19.69±0.29 ^{a}	18.70±0.65 ^{a}
	R. irregularis	69.58±4.73 ^{c}	38.86±6.43 ^{cd}	18.18±0.20 ^{ab}	18.35±0.90 ^{a}
CH stress	–AMF	99.71±6.27 ^{b}	88.95±9.68 ^{b}	11.39±0.56 ^{e}	10.22±0.34 ^{c}
	D. versiformis	120.9±16.3 ^{a}	95.41±12.83 ^{b}	15.73±1.51 ^{cd}	15.64±0.32 ^{b}
	R. irregularis	138.1±4.19 ^{a}	120.0±5.99 ^{a}	16.98±0.42 ^{bc}	15.41±0.42 ^{b}

Data are means±SD (n=4). Data of each column indicated by the same letter are not significantly different (Tukey test, P≤0.05).

tolerant cultivar, CAT activity increased through inoculation with *R. irregularis* and *D. versiformis* under control and CH temperatures, respectively (Figure 2).

Activity of PAL increased by CH temperature in both cultivars, but did not change following inoculation with AMF in the control plants. In the CH-stressed plants, AMF colonization increased PAL activity in the CH-tolerant cultivar, particularly regarding *D. versiformis*, while slightly decreased the activity in the CH-susceptible cultivar (Figure 3). Activity of PPO increased under CH temperature, yet decreased by inoculation with *R. irregularis* in the CH-stressed plants (Figure 3). Under control temperature, on the contrary, PPO activity was not affected by AMF (Figure 3). The concentration of phenolics was higher in the CH-stressed plants, but AMF effect was dependent on the cultivar. In the CH-tolerant cultivar, AMF colonization increased phenolics concentration, especially in *R. irregularis* under control and both AMF species under CH temperature. In the CH-susceptible cultivar, in contrast, phenolics concentration was significantly reduced by AMF inoculation in CH-stressed plants (Figure 3).

Plants grown in the absence of CH temperature died after exposure to freezing temperature, while surviving up to 40-80% when previously treated with CH temperature as acclimation treatment. Inoculation of unacclimated plants with AMF considerably increased their survival rate, though less effective compared with the acclimation treatment. The additive effect of both treatments was observed only in the CH-tolerant cultivar. In the CH-tolerant cultivar, the effect of *D. versiformis* was higher compared with *R. irregularis* concerning the increase in the survival rate of unacclimated plants (Figure 4).

The PCA of the various physiological data determined in this study divided all the parameters into two distinct subgroups. In one subgroup (subgroup I), growth, photosynthesis and water status parameters were clustered, while antioxidant defense, phenolics metabolism and membrane parameters, i.e. the biochemical indicators of cold stress, were shown to be interrelated, comprising the second subgroup (subgroup II) (Figure 5). The subsequent PCA of subgroup II indicated that the survival rate was distinctly clustered in a group and separated from the membrane integrity parameters (electrolyte leakage and MDA) and H_2O_2 (Figure 5).

Discussion

Two spring barley cultivars were different in terms of biomass production, photosynthesis rate and membrane integrity loss under CH stress, corroborating our previous results on different tolerances of these cultivars to low temperatures (Joudmand *et al*., 2019). Many researchers have observed a great variation in tolerance to low temperature among barley cultivars of various origins (Rizza *et al*., 2011), which is an excellent opportunity for breeders to select cultivars best suited to their agro-ecological zone.

Chilling stress significantly reduced the colonization rate in both cultivars, which is consistent with the results observed in warm-climate cereals such as sorghum and maize (Lenoir *et al*., 2016) under cold stress, and in contrast with the extremely cold tolerant winter barley cultivars in our previous study (Hajiboland *et al*., 2019). Abiotic stresses impair several post-symbiotic steps related to the host plants including lower photosynthesis rate, hence the less carbon allocation to AMF under stress conditions, which has been documented using ^{13}C labeling (Lenoir *et al*., 2016).

The better performance of the AMF barley plants under CH stress was associated with improvement in the leaf photosynthesis rate and the content of pigments that are likely mediated by the modification in the internal

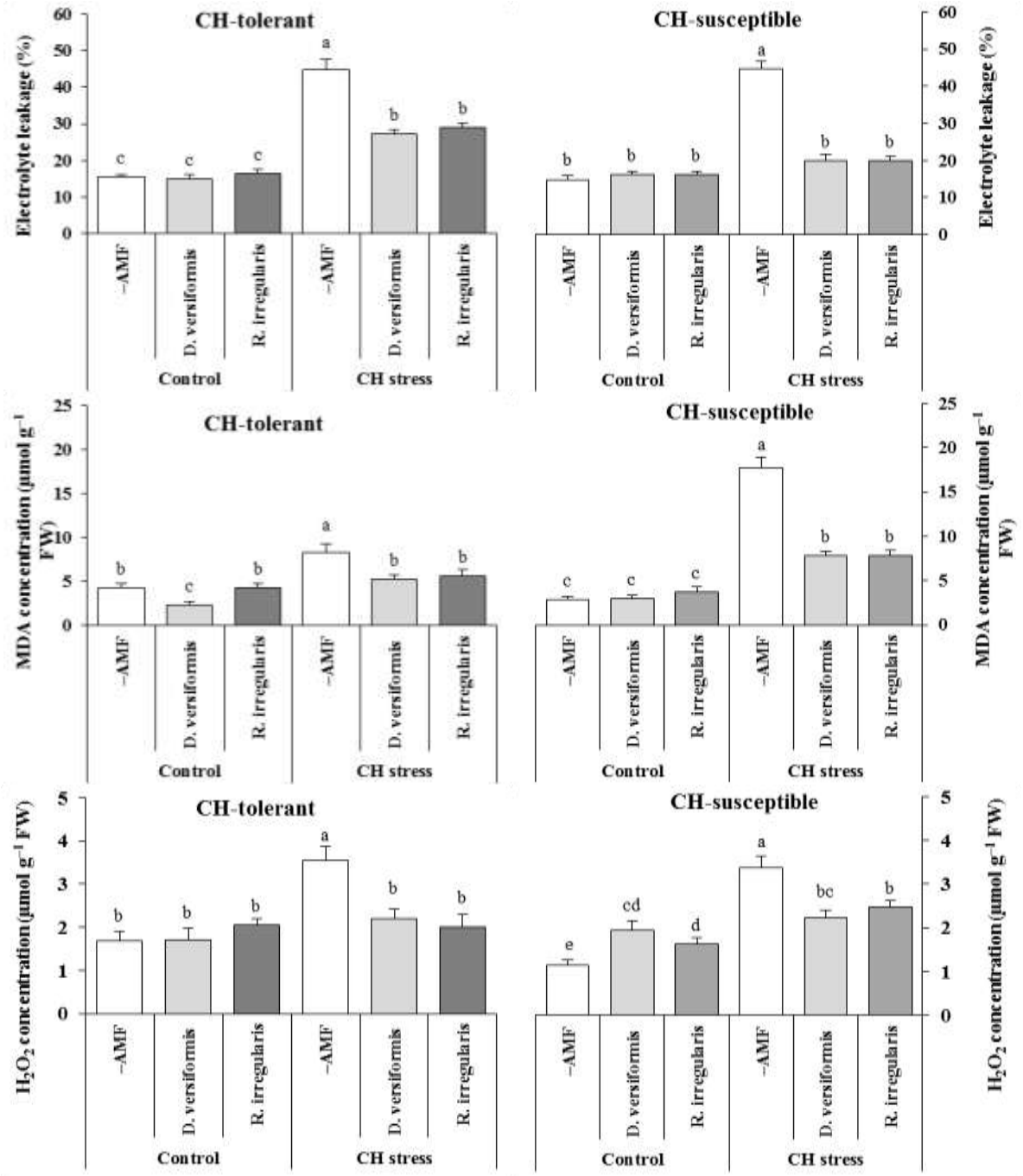

Figure 1. Electrolyte leakage, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations in the leaves of tolerant ('Reyhan') and susceptible ('Torkaman') barley cultivars to chilling (CH) temperature grown without inoculation (–AMF) or inoculated with two AMF species and subjected either to control (28/17 °C) or CH stress (5/3 °C day/night) for three weeks. Data are means±SD (n=4). Bars indicated by the same letter are not significantly different (Tukey test, P≤0.05).

hormone levels (Goicoechea *et al*., 1995). The role of AMF under CH stress was observed to be conspicuous regarding membrane integrity parameters, which is undoubtedly related to AMF-mediated reduction of ROS generation and/or accumulation under CH stress. Further corroborating this assumption is the significantly lower H_2O_2 accumulation in the AMF plants and its close interrelationship with the electrolyte leakage and MDA obtained in the PCA (Figure 5). Similar studies on salt, drought or heavy metal stresses, have further well documented the alleviating effect of AMF by reducing H_2O_2 and MDA and increasing the activity of antioxidant enzymes (Abdel Latef *et al*., 2016).

Similar to the current research, leaf water content was affected under cold stress in cold-susceptible rather than cold-tolerant wheat cultivar (Liang *et al*., 2008). However, inoculation with AMF, increased RWC in both cultivars under both CH stress and control temperature. That is likely related to the formation of

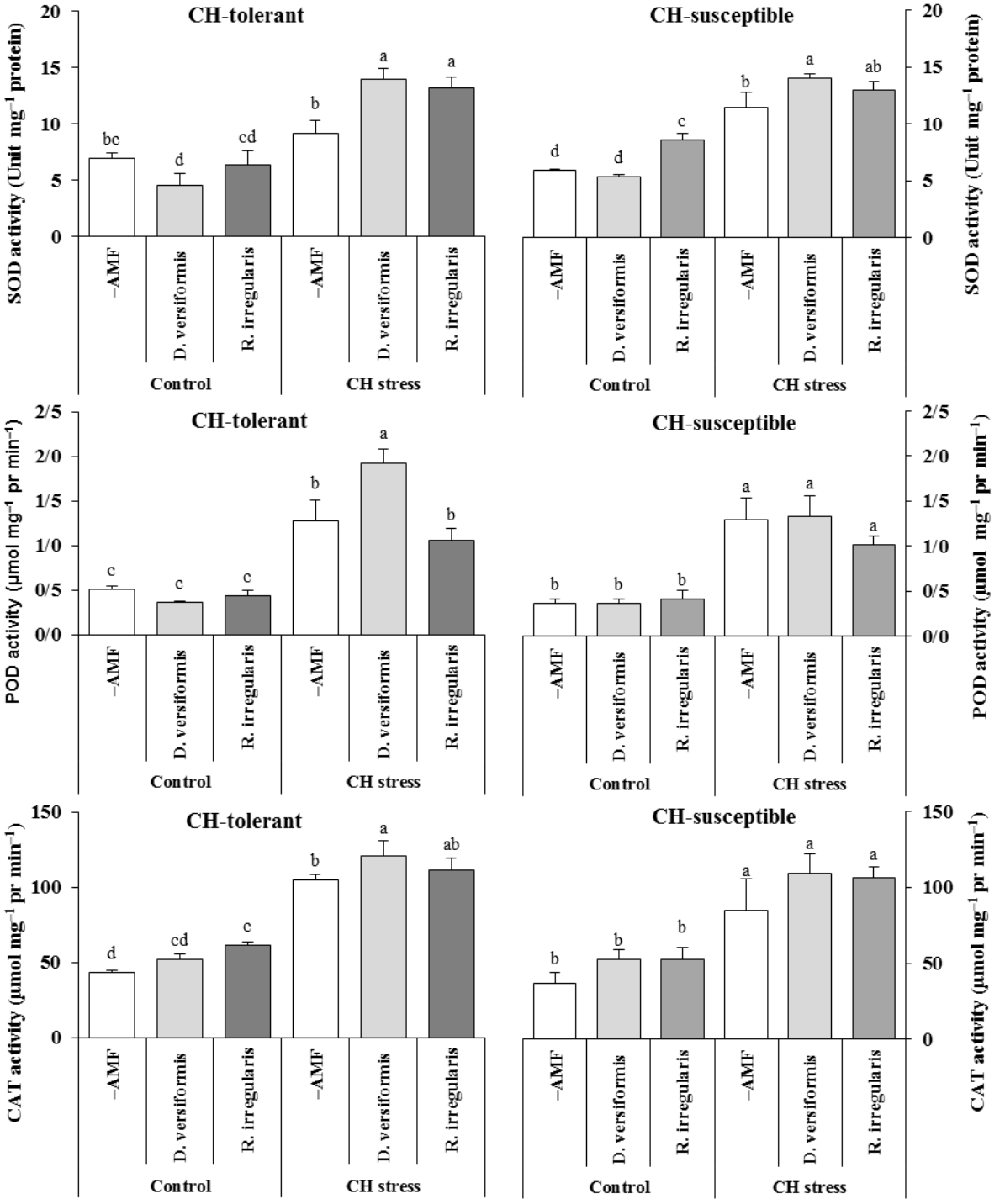

Figure 2. Leaf activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in the leaves in the tolerant ('Reyhan') and susceptible ('Torkaman') barley cultivars to chilling (CH) temperature grown without inoculation (–AMF) or inoculated with two AMF species and subjected either to control (28/17 °C) or CH stress (5/3 °C day/night) for three weeks. Data are means±SD (n=4). Bars indicated by the same letter are not significantly different (Tukey test, P≤0.05).

extended extra-radical hyphae, improved root hydraulic conductance (Abdel Latef *et al*., 2016) as well as higher K accumulation (Table 3).

The increased accumulation of proline under CH stress was further intensified via AMF colonization. Increase in the proline concentration induced by CH or AMF or a combination of both (up to 2.3-4.6 fold) significantly contributed to the osmotic potential of the leaves, which is comparable with that of K (32-44 µmol g^{-1} FW; 350-490 µmol g^{-1} DW) as the most important inorganic solute in the leaf. The contribution of proline to the leaf osmotic potential under CH stress does not exclude its protecting role against the damage induced by FR stress. The PCA pattern of cold stress indicators showed a close interrelationship between proline accumulation and survival rate (Figure 5), conferming

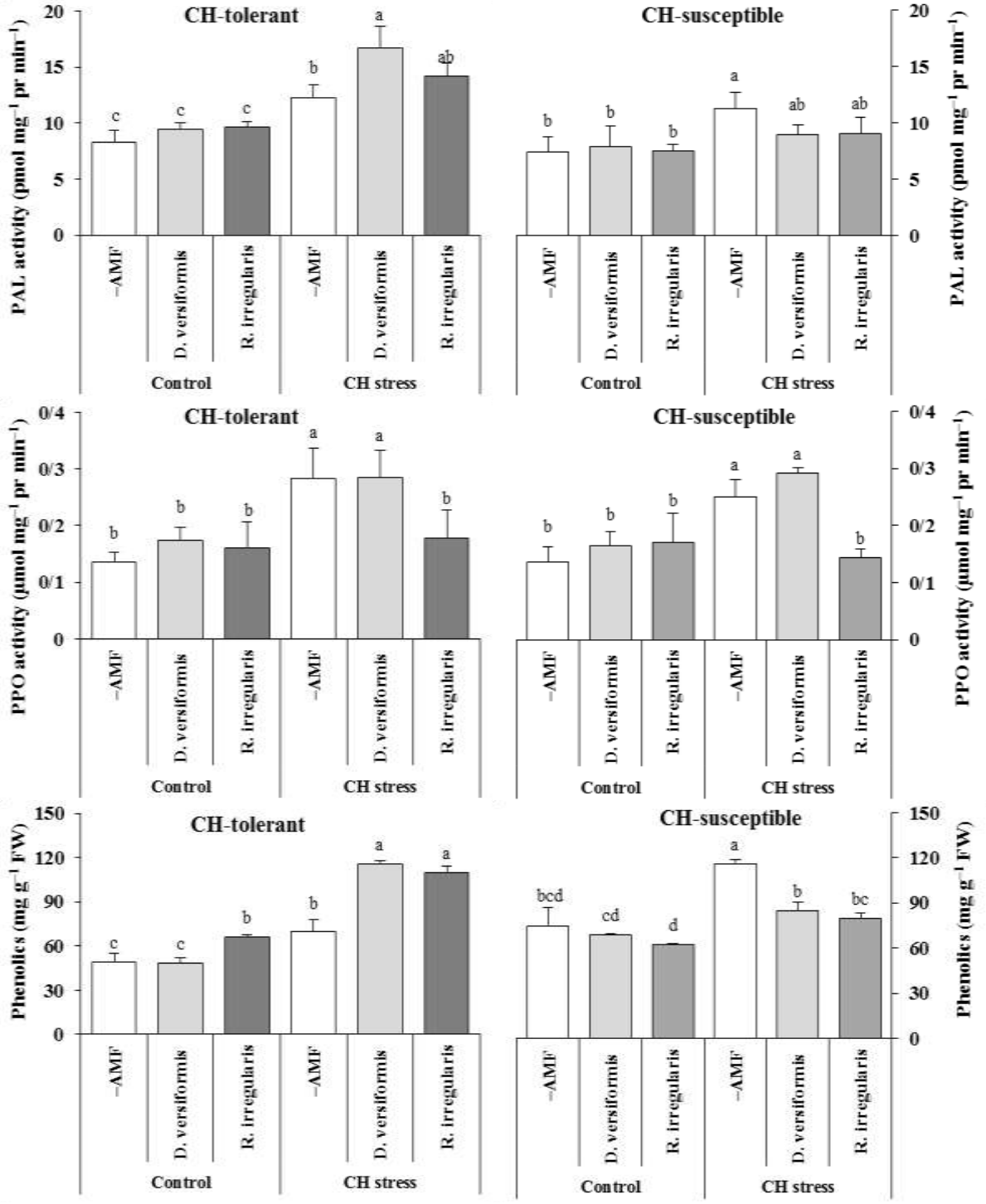

Figure 3. Leaf activity of phenylalanine ammonialyase (PAL), polyphenoloxidase (PPO) and concentration of phenolics in the tolerant ('Reyhan') and susceptible ('Torkaman') barley cultivars to chilling (CH) temperature grown without inoculation (–AMF) or inoculated with two AMF species and subjected either to control (28/17 °C) or CH stress (5/3 °C day/night) for three weeks. Data are means±SD (n=4). Bars indicated by the same letter are not significantly different (Tukey test, P≤0.05).

its crucial role in the FR resistance of cold-acclimated barley plants in this work. Accumulation of proline also seems to be a key event occurring upon mycorrhization, and enhancing the survival rate in the absence of acclimation treatment. Proline accumulating lines of barley plants showed higher winter survival (Tantau *et al*., 2004).

The activities of antioxidant defense enzymes were increased by CH stress. However, the two analyzed cultivars were not considerably different regarding the unstressed or stressed activity levels of antioxidant enzymes as opposed to the generalization provided by certain authors on a direct relationship between ROS scavenging capacity and plant tolerance to cold stress

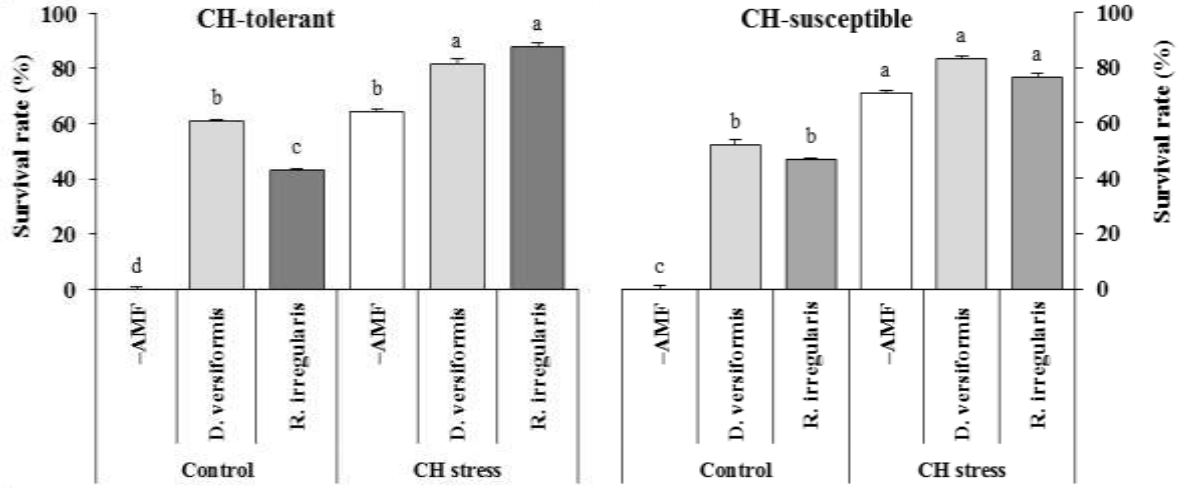

Figure 4. Survival rate in the tolerant ('Reyhan') and susceptible ('Torkaman') barley cultivars to chilling (CH) temperature grown without inoculation (–AMF) or inoculated with two AMF species and subjected either to control (28/17 °C) or CH stress (5/3 °C day/night) for three weeks then were exposed to freezing (–5 °C) temperature for two days in dark. Data are means±SD (n=4). Bars indicated by the same letter are not significantly different (Tukey test, P≤0.05).

(Suzuki and Mittler, 2006). Mycorrhization of CH-stressed plants exerted an additive effect on the activity of antioxidant enzymes which was more pronouncedly observed in plants inoculated with *D. versiformis*. The exacerbation effect of CH stress on the antioxidant capacity of plants by AMF was associated with the less H_2O_2 accumulation and lower electrolyte leakage and MDA concentration, indicating the effective ROS scavenging in the AMF plants compared with the –AMF counterparts. The PCA of the antioxidant defense indicators (SOD, POD and CAT) showed their determining role in the plants frost response (Figure 5). The elevated activity of ROS scavenging enzymes following cold acclimation is associated with increased freezing tolerance in pine trees (Tao *et al*., 1998).

A consistent increase has been observed in both barley cultivars in the concentration of free phenolics and the activity of its metabolizing enzymes, PAL and PPO upon exposure to CH stress. Activation of phenolics metabolism by low temperature has been observed in certain plants species (Ramakrishna and Ravishankar, 2011) including tomato and watermelon (Rivero *et al*., 2001). Since phenolic compounds are powerful antioxidants that act as ROS scavengers and reducing agents (Rice-Evans *et al*., 1996), they are important for a successful growth under CH stress. Association of plants with AMF influenced the phenolics concentration and the activity of metabolizing enzymes mainly under CH stress. Activity of PPO was differently affected by the two AMF species in the CH-stressed plants, which is unlikely to be crucial for CH and FR responses of plants as this difference was not reflected in the membrane integrity parameters or survival rate, both similarly improved by the two AMF species. In the AMF plants, activity of PAL and concentrations of phenolics, further increased upon exposure to CH stress in the CH-tolerant cultivar while decreased in the CH-susceptible one. Application of PCA showed that PAL is clustered with survival rate, while PPO seemed to be less interrelated with this parameter (Figure 5). In agreement with the present results on the effect of PAL, inhibition of PAL activity by a chemical inhibitor has been shown to reduce the tolerance to the extracellular formation of ice in oilseed rape (Solecka and Kacperska, 2003). However, further in-depth studies are required for a detailed explanation regarding the mechanism of phenolics effect on plants FR resistance.

Survival of the barley plants was expectedly increased by a three-week exposure to CH stress. Modulation of carbon metabolism, enhanced ROS scavenging capacity, osmotic adjustment, alterations in the membrane lipids, and synthesis of dehydrins and anti-freeze proteins are triggered during the pre-exposure of plants to non-freezing temperatures (Ruelland *et al*., 2009; Janska *et al*., 2010). The several biochemical analyses performed in this work further revealed the considerable modifications in plant metabolism, in accordance with the above-mentioned evidence on the effect of cold acclimation in plants. Cold acclimation increases freezing tolerance through activating cold-responsive genes responsible for further downstream events contributing to the plants frost tolerance (Suzuki and Mittler, 2006).

AMF colonization significantly increased the frost tolerance of the barley plants in the absence of cold acclimation, and showed an additive effect with the acclimation treatment, which was significant in the CH-tolerant cultivar. Based on the results of the current research, the priming effect of AMF can be attributed to the same metabolic adjustments induced by CH temperature, most importantly the activation of antioxidant enzymes and PAL and accumulation of proline. The role of other biochemical players such as antifreeze proteins was not determined in this study and requires further elucidation.

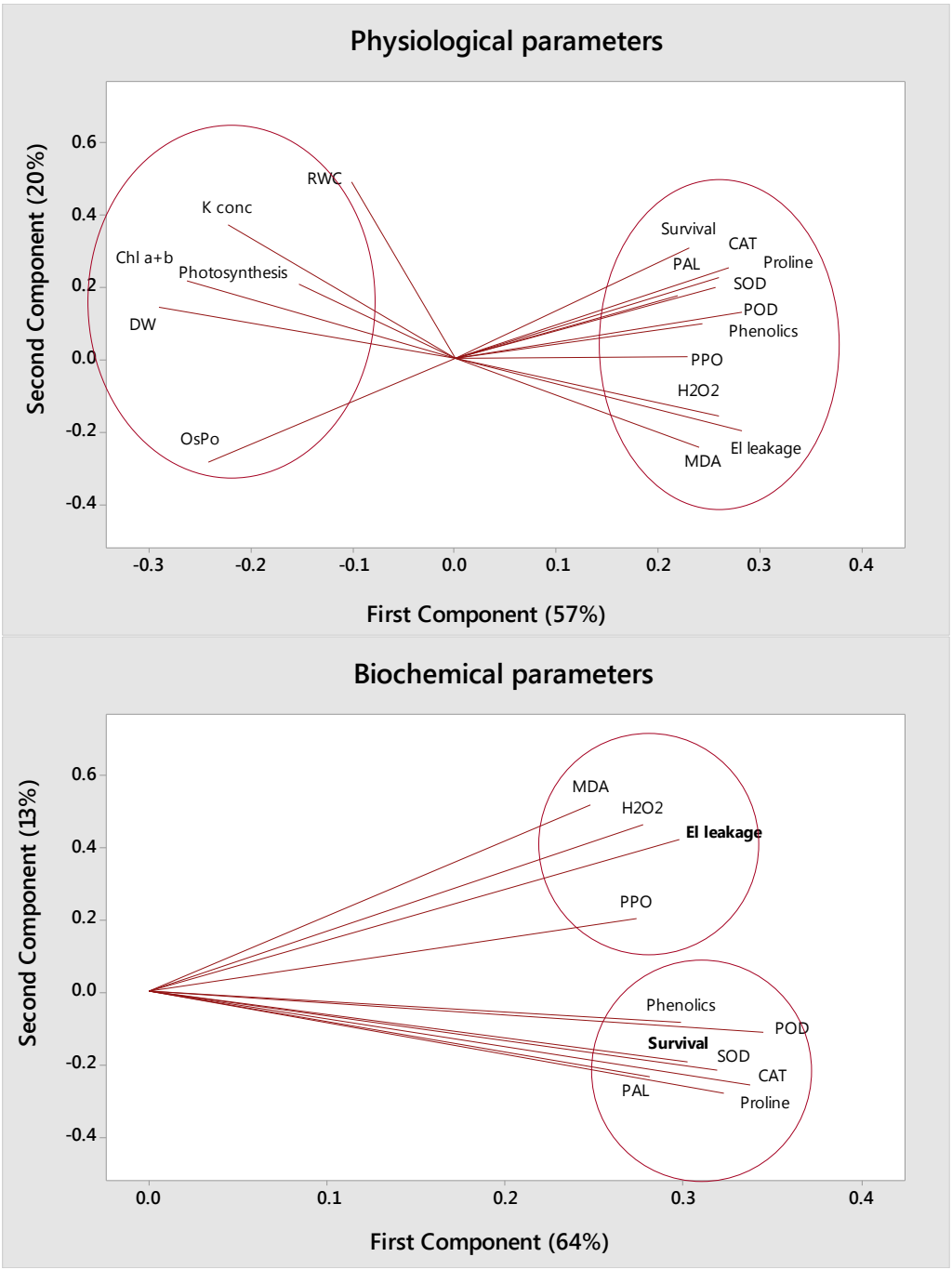

Figure 5. The PCA graph of physiological parameters and some of the biochemical indicators of cold stress in two spring barley cultivars ('Reyhan' as tolerant and 'Torkaman' as susceptible) subjected to chilling (5/3 °C day/night) and freezing (–5 °C) temperatures and grown without or with inoculation with AMF.

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