

Arbuscular mycorrhizal symbiosis regulates physiology and performance of *Digitaria eriantha* plants subjected to abiotic stresses by modulating antioxidant and jasmonate levels

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1 **Abstract:**

2 This study evaluates antioxidant responses and jasmonate regulation in *Digitaria eriantha* cv.
3 Sudafricana plants inoculated (AM) and non-inoculated (non-AM) with *Rhizophagus irregularis*
4 and subjected to drought, cold or salinity. Stomatal conductance, photosynthetic efficiency, biomass
5 production, hydrogen peroxide accumulation, lipid peroxidation, antioxidants enzymes activities
6 and jasmonate levels were determined. Stomatal conductance and photosynthetic efficiency
7 decreased in AM and nonAM plants under all stress conditions. However, AM plants subjected to
8 drought, salinity or non-stress conditions showed significantly higher stomatal conductance values.
9 AM plants subjected to drought or non-stress conditions increased their shoot/root biomass ratios,
10 whereas salinity and cold caused a decrease in these ratios. Hydrogen peroxide accumulation, which
11 was high in non-AM plant roots under all treatments, increased significantly in non-AM plant
12 shoots under cold stress and in AM plants under non-stress and drought conditions. Lipid
13 peroxidation increased in the roots of all plants under drought conditions. In shoots, although lipid
14 peroxidation decreased in AM plants under non-stress and cold conditions, it increased under
15 drought and salinity. AM plants consistently showed high CAT and APX activity under all
16 treatments. By contrast, the GR and SOD activity of AM roots was lower than that of non-AM
17 plants and increased in shoots. The endogenous levels of OPDA, JA and 12-OH-JA showed a
18 significant increase in AM plants as compared to nonAM plants. 11-OH-JA content only increased
19 in AM plants subjected to drought. Results show that *D. eriantha* is sensitive to drought, salinity
20 and cold stresses and that inoculation with AM fungi regulates its physiology and performance
21 under such conditions, with antioxidants and jasmonates being involved in this process.

22

1 **Key words**

2 Arbuscular mycorrhiza; cold stress; *Digitaria eriantha*; drought stress; jasmonates; salt stress.

3

4 **Abbreviations**

5 AM, arbuscular mycorrhiza (I); APX, ascorbate peroxidase; CAT, catalase; GR, glutathione
6 reductase; MDA, malondialdehyde; nonAM, noninoculated plants; SOD, superoxide dismutase

7

8

1 **Introduction**

2 Plants growing under natural conditions are exposed to a variety of biotic and abiotic stresses,
3 which limit their development and productivity due to inhibition of a number of physiological and
4 metabolic processes (Seki et al. 2003). Drought, salinity and cold are important plant growth
5 constraints in many parts of the world. The soils of central Argentina are semiarid with a variable
6 moisture regime and humidity restricted to part of the year. This region is generally characterised by
7 poorly developed soils, with little horizon differentiation which are highly drained, with low water-
8 holding capacity and organic matter content (Colazo et al. 2010). Due to its poor structure, this soil
9 is highly susceptible to wind and water erosion in vast areas of the region (Peña Zubiarte and
10 D'hiriart 2005; Colazo et al. 2010). Salinity affects approximately 2.5 million hectares in central
11 Argentina, 20% of which are permanent and semi-permanent saline lakes. This problem is
12 compounded by drainage practices and the expansion of irrigated agriculture in arid areas with high
13 evapotranspiration rates (Malpassi et al. 2004). In this region of Argentina, recent records show
14 about 60 days of frost per year (Collado 2003). Therefore, it is necessary to use species, especially
15 those producing forage, which can tolerate abiotic stress conditions such as drought, salinity and
16 cold.

17 The different plant responses produced to cope with environmental stresses are regulated by
18 crosstalk between hormones and signal molecules. The role of abscisic acid (ABA) in plant
19 responses to abiotic stresses is well known (Wilkinson and Davies 2002; Hirayama and Shinozaki
20 2007). In contrast, the role of other plant hormones such as jasmonic acid (JA) is less well known
21 (Sanchez-Romera et al. 2014). Jasmonic acid, a plant hormone belonging to the octadecanoid
22 family, is not only involved in the plant's response to biotic and abiotic stresses but also in the
23 regulation of plant development (Wasternack and Hause 2013). JA is synthesized by the

1 oxygenation of α -linolenic acid through a series of reactions within the chloroplast and the
2 peroxisome, with cis-12-oxophytodienoc acid (OPDA) being the main intermediate of JA
3 biosynthesis (Wasternack 2007). JA can be converted into numerous conjugates and derivatives,
4 some of which have a well-described biological activity, such as the JA-methyl ester (MeJA), cis-
5 jasmon and JA-isoleucine conjugate (JA-Ile, Wasternack and Hause 2013).

6 On the other hand, during abiotic stress, different metabolic pathways are uncoupled and
7 electrons are transferred to molecular oxygen to form reactive oxygen species (ROS) such as
8 superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) or hydroxyl radicals (OH^{\bullet}) (Noctor et al.
9 2014). These ROS are toxic molecules capable of causing oxidative damage to proteins, DNA and
10 lipids (Miller et al. 2010). It has been estimated that, under CO_2 scarcity conditions due to abiotic
11 stress-induced stomatal closure, up to 50% of the entire photosynthetic electron flow may end up as
12 $O_2^{\bullet-}$ (Biehler and Fock 1996). Antioxidant systems eliminate excess ROS produced under such
13 stress conditions (Gill and Tuteja 2010). The scavenging of ROS is achieved through the action of
14 different enzymatic and non-enzymatic compounds, including superoxide dismutase (SOD),
15 glutathione reductase (GR), catalase (CAT), ascorbate- or thiol-dependent peroxidases, and the
16 enzymes of the ascorbate-glutathione pathway. Non-enzymatic mechanisms include compounds,
17 such as ascorbic acid, glutathione and α -tocopherol, capable of directly scavenging several ROS
18 (Scheibe and Beck 2011).

19 Arbuscular mycorrhizal (AM) fungi have been used and studied for its influence on the
20 antioxidative responses in plants of agronomic importance, such as grape (Alarcon 2001), soybean
21 (Porcel and Ruiz-Lozano 2004), rice (Ruiz-Sánchez et al. 2010), wheat (Abdel Latef 2010), tomato
22 (Dell'Amico et al. 2002; Abdel Latef and Chaoxing 2011) or lettuce (Ruiz-Lozano et al. 1996;
23 Aroca et al. 2008; Baslam and Goicoechea 2012) subjected to abiotic stress. In tomato plants,

1 inoculation with AM fungi caused an increase in SOD, CAT, peroxidase (POD) and ascorbate
2 peroxidase (APX) activity in salt-affected leaves and reduced oxidative damage to lipids as
3 indicated by malondialdehyde (MDA) content (Abdel Latef and Chaoxing 2011). At the molecular
4 level, Aroca et al. (2007) found that AM symbiosis regulates root hydraulic properties and enhances
5 *Phaseolus vulgaris* tolerance to drought, cold and salt stress. Such regulation closely correlated with
6 the regulation of PIP2 protein levels and phosphorylation state. In addition, depending on the
7 presence of AM fungi, differential expression of PIP genes studied under each stress was observed.

8 AM also affect the above-ground part of plants, leading frequently to higher tolerance to
9 abiotic stresses than that of non-mycorrhizal (nonAM) plants. For instance, AM symbiosis
10 increased photosynthetic efficiency by over 40%, induced the accumulation of the antioxidant
11 molecule glutathione and reduced the accumulation of hydrogen peroxide and oxidative damage to
12 lipids in rice plants subjected to drought stress (Ruiz-Sánchez et al. 2010). Increases in
13 photosynthetic activity and water use efficiency have been reported in AM plants growing under
14 drought (Birhane et al. 2012; Liu et al. 2015) or under salt stress conditions (Sheng et al. 2008;
15 Hajiboland et al. 2010). The alleviation of metabolic inhibitions of photosynthesis by AM
16 symbiosis has been found to be related to the stimulation of carbohydrate transport and metabolism
17 between source and sink tissues (Kaschuk et al. 2009). AM fungi modulate source-sink relations
18 and can stimulate photosynthesis rates sufficiently to compensate for fungal carbon requirements
19 (Kaschuk et al. 2009; Dodd and Pérez-Alfocea 2012).

20 *Digitaria eriantha* Steudel, subspecies *eriantha*, is a native component of grasslands in
21 eastern and southern Africa. It has been adapted and grown in the semi-arid central region of
22 Argentina as a perennial grass, with biomass values of 3,200 kg dry matter ha⁻¹ (Veneciano 2006).
23 Nowadays, *D. eriantha*, a source of forage, is available throughout the year. Indeed, it is an

1 economically and nutritionally important resource for cattle, particularly cows in calf in winter
2 months, and for weaning calves in the summer (Frasinelli and Martínez Ferrer 1999). With regard
3 to its adaptation to specific climatic conditions, some studies have provided evidence that *D.*
4 *eriantha* plants respond to abiotic stress at different stages of their life cycle. For example, Di
5 Giambatista et al. (2010) found that, during germination at 25-30° C, although seeds tolerated an
6 osmotic potential of -1 MPa, the germination rate decreased significantly at lower levels of osmotic
7 potential. Additionally, Garbero et al. (2010; 2012) found that exposure of *D. eriantha* cv.
8 Sudafricana plants to cold caused an increase in MDA content, affected antioxidant defense and
9 chlorophyll biosynthesis and resulted in serious anatomical damage to leaves. This negative effect
10 of cold on growth, anatomy, ABA and JA levels and antioxidant defense was obvious only in *D.*
11 *eriantha* cv. Sudafricana plants but not in cv. Mejorada INTA, thus indicating that cv. Sudafricana
12 is more sensitive to cold stress.

13 In order to expand the use of *D. eriantha* in Argentina, it is necessary to improve its
14 tolerance to environmental stresses such as cold, drought and salinity. To achieve this, the use of
15 AM fungi could be a worthwhile strategy. Elucidation of a plant's biochemical and physiological
16 responses common to different abiotic stresses when associated with AM fungi will help us to
17 improve the characteristics of this species for its growth in semi-arid habitats.

18

19 **Materials and methods**

20 *Experimental design*

21 The experiment consisted of a randomized complete block design with ten replicates per treatment.
22 The experiment had two factors: (1) inoculation treatment, with non-inoculated (non-AM) control

1 plants and plants inoculated with the AM fungus *Rhizophagus irregularis* (AM) and (2) abiotic
2 stress applications. This means that one group of plants was cultivated under optimal conditions
3 during the entire experiment and the other groups of plants were subjected to either drought, cold or
4 salt stress. Thus, there were eight treatments, each with ten replicates, giving a total of 80 pots.

6 *Soil and biological material*

7 A loamy soil was collected from the grounds at the Zaidin Experimental Station (Granada, Spain).
8 The soil had a pH of 8.1 (measured in water, 1:5 w/v); 1.5 % organic matter; nutrient concentrations
9 (g Kg^{-1}): total N, 1.9; total P, 1 (NaHCO_3 -extractable P); total K, 6.9. The available P in the soil was
10 27 mg Kg^{-1} . The soil was sieved (5 mm), diluted with quartz-sand (<2 mm) (1:1, soil:sand, v/v) and
11 sterilized by steaming (100 °C for 1 h on 3 consecutive days).

12 *Digitaria eriantha* Steudel cv. Sudafricana seeds were washed for 3 min in pure ethanol and
13 rinsed three times with distilled water. Ten seeds were then sown in 1000 ml pots containing a
14 sterilized mixture of soil/sand (1:1, v/v) and thinned to 5 seedlings per pot after emergence.
15 Mycorrhizal inoculum of *R. irregularis* (Schenck and Smith), strain EEZ 58 (Ri) was prepared as
16 described by Porcel et al. (2006) and 10 g of the inoculum were added to half of the pots at sowing
17 time, just below seeds. Non-inoculated pots received the same amount of autoclaved mycorrhizal
18 inoculum together with 2 ml of AM inoculum filtrate in order to provide a general microbial
19 population free of AM propagules.

21 *Growing conditions*

22 Inoculated (AM) and non-inoculated (nonAM) plants were cultivated in a greenhouse at 24:20 °C
23 (day: night), with 16:8 photoperiod, a relative humidity of 50-60% and an average photosynthetic

1 photon flux density of $800 \mu\text{mol m}^{-2} \text{s}^{-1}$, as measured with a light meter (LICOR, Lincoln, NE,
2 USA, model LI-188B). The plants were watered to field capacity and maintained under optimal
3 conditions for 6 weeks. After that period, the AM and nonAM plants were divided into four groups.
4 One group was kept as control at 24°C with soil at field capacity (under non-stress conditions). The
5 remaining three groups were subjected to the following treatments: 1) 24 °C and 60 % field capacity
6 for one week (drought stress); 2) 4 °C for 72 h (cold stress) and 3) 24 °C and soil irrigated with 200
7 mM NaCl for two weeks (salt stress). The duration and levels of stress imposed were based on
8 bibliographical references to these stresses (Pedranzani et al. 2005; Aroca et al. 2007; Di
9 Giambatista et al. 2010; Garbero et al. 2010, 2012). The plants were harvested after the stress
10 treatments.

11
12 *Parameters measured*
13 Mycorrhizal development and determination of plant biomass production

14 Mycorrhizal colonization was estimated by visual inspection of fungal structures after clearing the
15 roots in 10% KOH and staining with 0.05% (w/v) trypan blue in lactic acid as described by Philips
16 and Hayman (1970). The percentage of mycorrhizal colonization was calculated according to the
17 gridline intersect method (Giovannetti and Mosse 1980).

18 After treatments, AM and nonAM plants were harvested and the fresh weight (FW) of roots
19 and shoots was determined separately. Shoot and root dry weight (DW) was measured after after
20 being dried in a forced hot-air oven at 70 °C for 2 d. Shoot dry matter content was calculated as 1-
21 (FW-DW)/FW (Marulanda et al. 2007), and expressed as gram dry weight per gram FW.

22

1 Stomatal conductance and photosynthetic efficiency

2 Stomatal conductance was measured two hours after the onset of light using a porometer system
3 (Porometer AP4, Delta-T Devices Ltd., Cambridge, UK) following the user manual instructions.
4 Measurements were taken at the first true leaf from five different plants per treatment.

5 The efficiency of photosystem II (Qy) was measured using Fluor Pen FP100 (Photon
6 Systems Instruments, Brno, Czech Republic) which enables a non-invasive assessment of plant
7 photosynthetic performance to be carried out by measuring chlorophyll a fluorescence. Fluor Pen
8 quantifies the quantum yield of photosystem II as the ratio between actual fluorescence yield in the
9 light-adapted state (F'/V) and maximum fluorescence yield in the light-adapted state (FM),
10 according to the method described by Oxborough and Baker (1997). Measurements were taken in
11 the first true leaf of five different plants for each treatment.

12 Hydrogen peroxide accumulation and determination of oxidative damage to lipids

13 The accumulation of hydrogen peroxide in leaves was determined using Patterson's method
14 (Patterson et al. 1984) with slight modifications as described by Aroca et al. (2003). Briefly, 250 mg
15 FW of shoots were homogenized with 5 mL 5% (w/v) TCA containing 0.1 g activated charcoal and
16 1% (w/v) PVPP. The homogenate was centrifuged at 18,000g for 10 min. The supernatant was
17 filtered through a Millipore filter (0.22 µm). A 1.2 mL volume of 100 mM potassium phosphate
18 buffer (pH 8.4) and 0.6 mL of colorimetric reagent were added to 130 µL of the supernatant. Fresh
19 colorimetric reagent was made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM
20 4-2 (2-pyridylazo) resorcinol (disodium salt). The samples were incubated at 45 °C for 1 h and
21 absorbance at 508 nm was recorded. Blanks were made by replacing leaf extract with 5% TCA.
22 Concentration values were expressed as nmol H₂O₂ g⁻¹ FW.

1 Lipid peroxides were extracted by grinding 500 mg of leaves and 6 mL of 100 mM
2 potassium phosphate buffer (pH 7) using an ice-cold mortar. Homogenates were filtered through
3 one layer of Miracloth and centrifuged at 15,000g for 20 min. The chromogen was formed by
4 mixing 200 μ L of supernatants with 1 mL of a reaction mixture containing 15% (w/v)
5 trichloroacetic acid (TCA), 0.375% (w/v) 2-thiobarbituric acid (TBA), 0.1% (w/v) butyl
6 hydroxytoluene, 0.25 N HCl and by incubating the mixture at 100 °C for 30 min (Minotti and Aust
7 1987). After cooling at room temperature, the tubes were centrifuged at 800g for 5 min, and the
8 supernatant was used for spectrophotometric measurement at 532 nm. Lipid peroxidation was
9 estimated as the content of 2-thiobarbituric acid-reactive substances (TBARS) and expressed as
10 equivalents of malondialdehyde (MDA) according to Halliwell and Gutteridge (1989). The
11 calibration curve was made using MDA in a range of 0.1-10 nmol. A blank was prepared by
12 replacing the sample with extraction medium, and controls for each sample were prepared by
13 replacing TBA with 0.25 N HCl. In all cases, 0.1% (w/v) butyl hydroxytoluene was included in the
14 reaction mixtures to prevent artifactual formation of TBARS during the acid-heating step of the
15 assay.

16 Determination of antioxidant enzymatic activities

17 Enzymes were extracted at 0-4 °C from 1 g FW of shoots with 50 mg polyvinylpyrrolidone
18 (PVPP) and 10 ml of 50 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA for superoxide
19 dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). For extraction of glutathione
20 reductase (GR), the buffer was supplied with 10 mM β -mercaptoethanol (Porcel et al. 2003). The
21 extracts were filtered through four layers of nylon cloth and centrifuged at 20,000 x g at 0-4 °C for
22 20 min. The supernatants were kept at -70 °C for subsequent enzymatic assays.

1 Total SOD activity (EC 1.15.1.1) was measured according to the method described by Beyer
2 and Fridovich (1987) based on the ability of SOD to inhibit the reduction of nitroblue tetrafolium
3 (NBT) by superoxide radicals generated photochemically. One unit of SOD was defined as the
4 amount of enzyme required to inhibit the reduction rate of NBT by 50 % at 25 °C. CAT activity (EC
5 1.16.1.6) was measured by the disappearance of H₂O₂ (Aebi 1984). The reaction mixture (3 ml)
6 contained 10.6 mM H₂O₂. The reaction was initiated by adding 25 µl of the extract and monitoring
7 the change in absorbance at 240 nm and 25 °C for 3 min. APX activity (EC 1.11.1.11) was
8 measured in a 1 ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1
9 mM hydrogen peroxide, and 0.5 mM ascorbate. The addition of the H₂O₂ initiated the reaction and
10 the decrease in absorbance at 290 nm was recorded for 1 min to determine the oxidation rate of
11 ascorbate (Amako et al. 1994). Finally, GR activity (EC 1.6.4.2) was determined by the procedure
12 described by Carlberg and Mannervik (1985). The reaction mixture (1 ml) contained 0.1 M HEPES
13 pH 7.8, 1 mM EDTA, 3 mM MgCl₂, 0.5 mM oxidized glutathione, 0.2 mM NADPH and 150 µl of
14 the enzyme extract. The rate of NADPH oxidation was monitored by the decrease in absorbance at
15 340 nm for 2 min. Two blanks, one without the enzyme extract and the other without oxidized
16 glutathione, were used as controls.

17 Extraction, purification and determination of jasmonates

18 12-*cis*-oxophytodienoic acid (OPDA), JA, 11-hydroxy-JA (11-OH-JA) and 12-OH-JA were
19 extracted, pre-purified and determined by GC-MS according to Miersch et al. (2008) using 0.5g
20 (FW) of leaf material. As internal standards, (²H₆)JA, (²H₅)OPDA, 11-(²H₃)OAc-JA, and 12-
21 (²H₃)OAc-JA were added in the appropriate amounts.

22

1 *Statistical analysis*

2 All data were subjected to two-way analysis of variance (ANOVA) with inoculation treatment and
3 abiotic stress as sources of variation. Post hoc comparisons with Duncan's Multiple Range Test
4 (Duncan 1955) were used to determine differences between the groups with the aid of the Statistical
5 Analytical Software (SAS) program, version 3.5 (1991).

6

7 **Results**

8 *Mycorrhizal development*

9 In this study no mycorrhizal colonization was observed in *D. eriantha* plants not provided with AM
10 inoculum. The inoculated plants showed between 68% and 72% of mycorrhizal root colonization
11 under the different treatments (data not shown). However, no significant differences were detected
12 following the application of abiotic stresses.

13 *Stomatal conductance, photosynthetic efficiency and plant biomass production*

14 AM symbiosis might result in altered rates of water movement into, through and out of host plants,
15 with consequent effects on tissue hydration and leaf physiology (Augé 2001). Therefore, parameters
16 reflecting water-related leaf physiology were determined in order to elucidate the impact of AM on
17 stress responses in *D. eriantha*.

18 Stomatal conductance showed a significant decrease under all stress conditions in AM as
19 well as in nonAM plants as compared to control conditions. Under non-stress, drought and salinity
20 conditions, however, the AM plants exhibited higher stomatal conductance as compared to nonAM
21 plants. The lowest values for stomatal conductance were observed under cold stress, with both AM

1 and nonAM plants showing similar values (Table 1). The efficiency of Photosystem II in non-AM
2 plants was similar under non-stress conditions and drought treatments but decreased under salinity
3 and cold stress conditions. In AM plants, photosynthetic efficiency decreased significantly under
4 drought conditions, while, under cold stress, AM plants exhibited increased photosynthetic
5 efficiency as compared to non-AM plants (Table 1).

6 The shoot/root biomass ratio was enhanced by AM symbiosis under non-stressful and
7 drought stress conditions, but was lower in AM plants than in non-AM plants under salinity and
8 cold stress conditions (Table 1). However, under all growing conditions, AM plants enhanced shoot
9 dry matter content except under salinity stress, when no significant differences between AM and
10 non-AM plants were found (Table 1).

11

12 *Hydrogen peroxide, oxidative damage to lipids and antioxidant enzyme activity*

13 Salt, drought and cold stresses are well known to induce oxidative stress in plants (Abdel Latef
14 2010; Aroca et al. 2003; Garbero et al. 2010). Plant cells contain an array of protection mechanisms
15 and repair systems that can minimize the occurrence of oxidative damage caused by reactive
16 oxygen species (ROS) (Abdel Latef 2010). Moreover, the antioxidant capacity of the host plant can
17 be activated by AM symbiosis (Abdel Latef and Chaoxing 2011). To elucidate these effects of AM,
18 ROS levels, oxidative damage to lipids and corresponding enzyme activity were determined in non-
19 AM and AM plants subjected to stress.

20 The level of hydrogen peroxide accumulated in roots was significantly higher in the nonAM
21 plants than in AM plants in all treatments, reaching maximum differences of up to 75% under
22 drought stress (Figure 1), while in AM plants no differences were observed among treatments. In
23 the shoots of AM plants, H₂O₂ increased by 95% under non stress and drought conditions, while, in

1 AM plants subjected to salinity or cold, it decreased by 40% and 68% respectively. H₂O₂
2 accumulation increased in nonAM plants by up to 40% under cold as compared to non-stress
3 conditions.

4 In roots, oxidative damage to lipids increased significantly by 115% only as consequence of
5 drought in both non-AM and AM plants as compared to non-stressed plants (Figure 2). Under
6 salinity, stress significantly decreased the levels of MDA in both AM and non-AM plants. In shoots,
7 MDA levels decreased in AM plants under non-stress and cold stress conditions. By contrast, under
8 drought and salinity treatments, the levels of MDA were significantly higher in AM plants as
9 compared to non-AM plants.

10 Among the enzymes known to be involved in ROS detoxification, SOD, CAT, APX and GR
11 were selected and their activity was determined in the shoots and roots of all plants (Table 2). In
12 roots, SOD activity was significantly lower in AM plants than in non-AM plants under non-stress,
13 drought and salinity conditions. Moreover, drought and salinity increased SOD activity in both non-
14 AM and AM plants. By contrast, cold stress drastically decreased SOD activity in non-AM plants
15 and increased this activity in AM plants to levels similar to those in non-stressed non-AM plants. In
16 shoots, SOD activity levels were lower under stress conditions as compared to non-stressed
17 treatments. However, AM plants showed significantly higher values than non-AM plants under the
18 different conditions assayed (Table 2).

19 CAT activity appeared to be consistently enhanced in both the roots and shoots of AM
20 plants under non-stressed and stressed conditions (Table 2). This increase was significantly higher
21 in roots subjected to cold stress and in shoots subjected to drought stress. In general, non-AM plants

1 did not show differences in CAT activity among the different stress treatments as compared to non-
2 stress conditions.

3 APX activity was always significantly higher in AM plants than in nonAM plants, in both
4 root and shoot tissues under all the treatments (Table 2), especially under drought and cold
5 conditions.

6 GR activity was always significantly higher in both the roots and shoots of non-AM plants
7 as compared to AM plants. GR activity in the shoots of non-AM plants decreased by about 43%
8 under drought and salinity conditions as compared to non-stress and cold conditions (Table 2).

9 *Jasmonate levels*

10 The roots of AM plants are known to contain higher levels of jasmonates (Wasternack and Hause
11 2013). Alterations in the JA levels of shoots upon AM, combined with abiotic stresses are, however,
12 less understood. Therefore, jasmonate levels in the shoots of non-AM and AM plants subjected to
13 stress were determined. The levels of OPDA (JA precursor) were significantly higher in AM plants
14 than in non-AM plants for all treatments except under cold stress conditions, where the increase was
15 not significant (Figure 3A). In non-AM plants, only salt stress caused significant differences in
16 OPDA content with respect to non-stress conditions (Figure 3A). This is reminiscent of the role
17 played by OPDA in the salt-stress response of plants (Hazman et al. 2015). Drought and cold stress
18 did not show differences as compared to the non-AM treatment under non-stress conditions.

19 The level of JA in the shoots of non-AM plants did not differ among stress treatments as
20 compared to non-stress conditions (Figure 3B). However, the shoots of non-stressed AM plants
21 exhibited higher JA levels than the shoots of non-AM plants, with *D. eriantha* showing an increase
22 in JA upon mycorrhization. Under salt stress conditions, AM plants showed JA content similar to

1 that under non-stress conditions, while drought and cold stress decreased JA levels in AM plants to
2 those of non-stressed, non-AM plants.

3 The levels of 11-OH-JA did not differ among treatments in the nonAM plants, while, in AM
4 plants an increase was observed under drought stress conditions as compared to non-stressed AM
5 plants and to nonAM plants (Figure 3C). No differences in the 12-OH-JA levels of nonAM plants
6 were observed, except under cold condition, where there was a significant decrease with respect to
7 non-stress conditions. In AM plants, drought and salinity showed a significant increase in 12-OH-
8 JA levels as compared to non-stress conditions and nonAM plants (Figure 3D).

9

10 **Discussion**

11 AM fungi have been shown to improve plant tolerance to abiotic stresses (Ruiz-Lozano and Aroca
12 2010). Therefore, the effects of AM on the stress tolerance of *D. eriantha* were elucidated in terms
13 of mycorrhization, biomass production as well as, accumulation of ROS and jasmonates. To obtain
14 an overview of these processes, treatments were applied resulting in three different stresses:
15 drought, salt and cold stress.

16 For several species of agronomic interest, information concerning the link between abiotic
17 stress and mycorrhiza is abundant. To date, however, no studies have linked the regulation by the
18 AM symbiosis of plant physiology and performance under abiotic stresses with the alteration of
19 antioxidants and jasmonates levels in the host plant, and few studies have investigated common
20 mechanisms underlying AM-induced tolerance to different abiotic stresses (Aroca et al. 2007). To
21 gain an insight into these mechanisms, *D. eriantha* plants were inoculated with *R. intraradices* and
22 showed a colonisation rate of about 70 % colonisation in their roots. Given this finding, the values

1 for colonization observed in *Digitaria* are greater than those recorded for tomato plants (34%,
2 Dell'Amico et al. 2002 and 55% Abdel Latef and Chaoxing 2011), soybean (50%, Porcel and Ruiz-
3 Lozano 2004) and rice (45%, Ruiz-Sánchez et al. 2010) which were not subjected to stress and were
4 inoculated with the same fungus. In other studies using different species of *Rhizophagus*,
5 colonisation of grape roots did not exceed 41% (Alarcón et al. 2001). Colonization percentages in
6 the roots of *D. eriantha* were not affected by drought, salt or cold stress treatments as compared to
7 the non-stressed control group. This differs from other plant species, such as tomato, soybean, rice
8 and lettuce, which showed a significant decrease in colonisation rates when plants were subjected to
9 water stress (Dell'Amico et al. 2002; Porcel and Ruiz-Lozano 2004; Ruiz-Sánchez et al. 2010) or
10 salt stress (Abdel Latef and Chaoxing 2011; Aroca et al. 2013). These results indicate that *R.*
11 *irregularis* is highly efficient in terms of associating with *D. eriantha* cv. Sudafricana, even in the
12 abiotic stress situations studied.

13 In the present study, the shoot/root biomass ratios of non-stressed AM plants and those
14 subjected to drought increased. In addition, AM plants also enhanced shoot dry matter content as
15 compared to non-AM plants under all the conditions tested except for salinity. Nevertheless, salinity
16 and cold temperatures caused a decrease in the shoot/root biomass ratios of AM plants. This may be
17 due to the decrease in stomatal conductance and efficiency of Photosystem II (PSII), given the
18 inability of plants to counteract the toxic effect of NaCl (Evelyn 2009), representing a dehydration
19 that causes a common physiological disorder to water, salt and cold stresses (Solanke and Sharpe
20 2008). However, under saline conditions, AM symbiosis can stimulate root development in the host
21 plant as a strategy to cope with soil salinity. Indeed, in a previous study comparing the symbiotic
22 efficiency of two *Glomus* strains with differential adaptation to salinity, the mechanism used by
23 *Glomus* sp. to protect lettuce plants from the detrimental effects of salt was found to be based on the

1 stimulation of root development, while *G. deserticola* led to an improvement in plant nutrition
2 (Ruiz-Lozano and Azcón 2000). Thus, in this study of *D. eriantha* and *R. irregularis*, the fungus
3 also stimulated root development in the host plant under saline conditions (data not shown), which
4 resulted in a significant reduction in the shoot/root ratio of these plants.

5 The mycorrhizal colonisation of roots has a marked effect on the stomatal behaviour of the
6 host plant's leaves which also favours the exchange of CO₂ (Goicoechea et al. 1997; Augé et al.
7 2007; Ruiz-Lozano and Aroca 2010). When the stomatal conductance of AM plants differs from
8 that of non-AM plants, symbiosis in roots causes a fundamental change in the physiology of the
9 leaf, including alterations in its intrinsic biochemical and hydraulic properties (Augé 2000). Plants
10 colonised by *R. irregularis* presented higher stomatal conductance values than non-AM plants
11 (Augé 2001). In this study, non-stressed AM plants and AM plants subjected to drought and salinity
12 showed significantly higher stomatal conductance values (30% on average) than non-AM control
13 plants, a result similar to that reported by Augé et al. (2014) in a meta-analysis of the effect of
14 mycorrhization on stomatal conductance. By contrast, the values obtained under cold stress
15 conditions did not differ between treatments, indicating that mycorrhization did not mitigate the
16 effect of low temperatures.

17 *D. eriantha*'s Photosystem II efficiency was evaluated using chlorophyll *a* fluorescence. It
18 has previously been reported that mycorrhizal plants show higher values under drought (Ruiz-
19 Sánchez et al. 2010; Birhane et al. 2012) and salt stress (Ruiz-Lozano et al. 1996; Dell'Amico et al.
20 2002; Sheng et al. 2008) treatments. In the present study, the photosynthetic efficiency values for
21 AM and non-AM plants decreased significantly in relation to non-stressed plants when subjected to
22 stress. However, drought caused a reduction in the photosynthetic efficiency of AM plants as
23 compared to non-AM plants, while cold temperatures resulted in a significant increase in the

1 photosynthetic efficiency of AM plants as compared to non-AM plants. These results suggest that
2 the abiotic stresses tested affected the photosynthetic system by reducing the photosynthetic
3 efficiency of AM and non-AM plants and that damage to Photosystem II was more severe in plants
4 subjected to drought and salinity.

5 A positive correlation between tolerance to abiotic stresses in AM plants and maintenance of
6 Photosystem II efficiency has been demonstrated which, in turn, maintains (Porcel and Ruiz-Lozano
7 2004) or even increases (Ruiz-Sánchez et al. 2010; Evelyn 2009) the productivity of the plant.

8 Drought, salinity, extreme temperatures and oxidative stresses are accompanied by the
9 formation of ROS such as superoxide radicals ($O_2^{\bullet-}$) and H_2O_2 that damage membranes and
10 macromolecules (Miller et al. 2010; Noctor et al. 2014). Plants have developed various
11 antioxidative strategies to flush out these toxic components. The enhancement of antioxidant
12 defences increases tolerance to different abiotic factors (Wang et al. 2003). AM symbiosis
13 positively affects plants through nutrient acquisition or tolerance to environmental stresses
14 (Fuentelba 2014). In this study, AM plants reduced hydrogen peroxide levels under all stress
15 treatments, thus demonstrating their ability to counteract damage, which is in line with that
16 observed in mycorrhizal soybean and ryegrass plants subjected to drought (Porcel and Ruiz-Lozano
17 2004; Lee et al. 2012) and in two tomato cultivars under salt stress conditions (Hajiboland et al.
18 2010).

19 MDA levels in roots were similar in AM and non-AM plants for each stress treatment.
20 Drought increased MDA accumulation both in AM and non-AM plants, while salinity reduced its
21 accumulation in relation to non-stressed control plants. The reduction in H_2O_2 levels under the
22 different stress conditions could be explained by the significant increase in the CAT and APX
23 enzyme activity of AM plants as compared to non-AM plants with respect to all treatments, as
24 reported by Porcel and Ruiz-Lozano (2004). However, the values for SOD and GR activity were

1 lower in AM plants as compared to non-AM plants. Nevertheless, these two enzymes are not
2 directly involved in the removal of H₂O₂. SOD dismutates superoxide radicals into hydrogen
3 peroxide, which is then converted into water and molecular oxygen by CATs in peroxisomes, while
4 GR reduces dehydroascorbate to ascorbate in the ascorbate-glutathione cycle (Estrada et al. 2013).
5 H₂O₂ content in the leaves of non-AM plants was only increased by cold stress, while AM
6 symbiosis significantly decreased H₂O₂ under salinity and cold stress, similar to that observed in
7 tomato plants under saline conditions by Hajiboland et al. (2010). MDA content also decreased in
8 AM plants when subjected to cold temperatures, which is in line with that observed in tomato plants
9 subjected to salinity (Abdel Latef and Chaoxing 2011) and in soybean and rice plants under drought
10 stress conditions (Porcel and Ruíz-Lozano 2004; Ruiz Sánchez et al. 2010). However, under
11 drought and saline conditions, MDA levels remained high as compared to non-AM plants. The
12 erratic behaviour of oxidative damage to lipids observed in *D. eriantha* could be explained by the
13 findings of Porcel and Ruíz-Lozano (2004) who point out that H₂O₂ is involved in almost all areas
14 of the plant's aerobic biochemistry, such as electron transport in respiration and photosynthesis as
15 well as glucose oxidation, and is produced in large quantities by various enzymatic systems even
16 under optimal conditions. Moreover, under certain stress conditions, H₂O₂ can be used by plants as
17 a defence mechanism (Quan et al. 2008).

18 With regard to enzymatic activity in leaves, CAT, APX and SOD showed significant
19 increases in AM plants as compared to non-AM plants, while GR activity decreased in AM plants
20 under all the stress conditions studied. Previous research has also reported increases in SOD, CAT
21 and APX activity in AM tomato plants subjected to saline stress (Abdel Latef and Chaoxing 2011).
22 Moreover, decreases in GR activity have been reported in the roots and stems of AM soybean plants
23 subjected to drought stress (Porcel and Ruiz-Lozano 2004). CAT and APX activities are both
24 involved in the scavenging of hydrogen peroxide, although APX has a much higher affinity for

1 H₂O₂ than CAT (Estrada et al. 2013). CAT activity is lower in the shoots of plants subjected to
2 salinity than in control plants not subjected to stress, which may indicate that, in *D. eriantha* plants
3 subjected to salinity, hydrogen peroxide could be preferentially scavenged by APX activity. Indeed,
4 in these plants, APX activity is significantly higher than in control plants not subjected to stress.

5 The involvement of jasmonates in the formation and development of mycorrhizal symbiosis
6 is widely accepted (Wasternack and Hause 2013; Bucher et al. 2014). Increases in JA have been
7 shown to correlate with the activation of genes for enzymes of the biosynthesis of this hormone
8 (Hause et al. 2007). Moreover, tomato plants defective in JA synthesis have been found to exhibit a
9 lower rate of AM root colonization than wild type plants (León-Morcillo et al. 2012). It was also
10 found that the application of JA either reduces (Ludwig-Muller et al. 2002; Herrera-Medina et al.
11 2008) or increases AM root colonisation (Landgraf et al. 2012). Thus, the role of JA in AM
12 symbiosis is still a matter of debate. In addition, there is no clear information on the precise role
13 played by this phytohormone and its family of compounds in abiotic stress responses in plants
14 which are associated with AM fungi. Our results show that JA levels in the shoots of non-AM
15 plants subjected to different abiotic stresses did not differ from those in unstressed control plants.
16 However, in AM plants, endogenous levels of JA increased significantly when subjected to drought
17 and salt stresses, while, under cold stress conditions, their behaviour was more irregular. Levels of
18 JA and its precursor OPDA in control plants were significantly higher in AM plants than in non-
19 AM plants, which is in line with the results obtained for the mycorrhizal roots of most plants by
20 Hause and Schaarschmidt (2009). The higher values for OPDA in relation to JA in all AM plant
21 treatments could be explained by the specific role played by OPDA in the expression of some genes
22 involved in stress responses (Wasternack and Hause 2013). Cold stress behaved differently from the
23 other stress treatments applied at all the jasmonate levels measured. Following cold treatment, the

1 levels of JA, OPDA and 11-OH-JA did not differ between non-AM and AM plants, while 12-OH-
2 JA levels slightly increased in AM plants as compared to non-AM plants. Whether the reduced level
3 of JA in cold-stressed plants contributes to increased or reduced stress responses remains to be
4 determined.

5 In summary, the results presented show that *D. eriantha* cv. Sudafricana is sensitive to
6 drought, salinity and cold stresses and that inoculation with AM fungi regulates its physiology and
7 performance under such stress conditions. The effects of AM symbiosis on antioxidant plant
8 responses and jasmonates accumulation depend on the intrinsic characteristics of the stress applied.
9 In general, the level of JA and its precursors was higher in AM plants under the different stress
10 conditions, which could help these plants to better cope with stressful conditions.

11

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16

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- 15

1 **Figure Legends**

2

3 Fig.1: Root and shoot hydrogen peroxide accumulation in *D. eriantha* plants non-inoculated
4 (nonAM) or inoculated (AM) with the AM fungus *R. irregularis* and cultivated under either
5 optimal, drought, salt or cold stress conditions. Bars represent means \pm standard error. Values
6 showing different letters are significantly different ($P \leq 0.05$), as determined by Duncan's
7 multiple range test (n=5).

8

9 Fig. 2: Oxidative damage of lipids in roots and shoots of *D. eriantha* plants non-inoculated
10 (nonAM) or inoculated (AM) with the AM fungus *R. irregularis* and cultivated under either
11 optimal, drought, salt or cold stress conditions. Bars represent means \pm standard error. Values
12 showing different letters are significantly different ($P \leq 0.05$), as determined by Duncan's
13 multiple range test (n=5).

14

15 Fig.3: Jasmonate content in *D. eriantha* plants non-inoculated (nonAM) or inoculated (AM) with
16 the AM fungus *R. irregularis* and cultivated under either optimal, drought, salt or cold stress
17 conditions. A) 12-*cis*-oxophytodienoic acid (OPDA); B) jasmonic acid (JA); C) 11-hydroxy-
18 jasmonic acid (11-OH-JA); D) 12-hydroxy-jasmonic acid (12-OH-JA). Bars represent means
19 \pm standard error. Values showing different letters are significantly different ($P \leq 0.05$), as
20 determined by Duncan's multiple range test (n=5).

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