South African Journal of Botany 95 (2014) 123–128

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Antioxidant enzyme activity, proline accumulation, leaf area and cell membrane stability in water stressed *Amaranthus* leaves

M.M. Slabbert [a], G.H.J. Krüger [b,*]

[a] Department of Horticulture, Tshwane University of Technology, Pretoria, South Africa
[b] School for Environmental Sciences and Development, North-West University, Potchefstroom, South Africa

ARTICLE INFO

Article history:
Received 18 June 2014
Received in revised form 13 August 2014
Accepted 27 August 2014
Available online 29 September 2014

Edited by J van Staden

Keywords:
A. hybridus
A. hypochondriacus
Antioxidative enzymes
A. tricolor
Cell membrane stability
Leaf area
Leaf water status
Metabolic response
Osmoregulation
Proline
Relative water content
Water stress

ABSTRACT

Traditional crops are extremely important for food production in low income, food-deficit countries (LIFDCs) where they continue to be maintained by socio-cultural preferences and traditional uses. Significant potential exists to improve these crops, one of which is to select for improved productivity during moisture stress conditions. Germplasm of *Amaranthus tricolor*, *Amaranthus hypochondriacus* and *Amaranthus hybridus* were subjected to various screening methods to measure metabolic and physiological changes during water stress. The activities of enzymes involved in the oxygen-scavenging system during abiotic stress conditions (superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR)), free proline production, leaf area (LA), cell membrane stability (CMS), leaf water potential (LWP) and relative water content (RWC) were measured in these three amaranth species during induced water stress. This study showed significant differences in metabolic responses during water deficit of the three species tested. Moisture stress and a decrease in RWC and LWP were first experienced in *A. hybridus* and *A. hypochondriacus*, followed by *A. tricolor*. There was an indirect correlation between leaf water status (RWC and LWP), enzyme activity, proline production and leaf area. The combined effect of GR, APX and SOD could ensure higher levels of regulation of the toxic effect of H_2O_2 which could be associated with drought tolerance in *Amaranthus*. Distinct differences in onset of proline accumulation and the amount of accumulated in leaves upon induced water stress was noticed for the three amaranth species tested. Proline accumulation during water stress conditions in amaranth seems to be indirect and could possibly have a protective role apart from osmoregulation during stress conditions. This contention is supported by the decrease in leaf area and high cell membrane stability for two of the species tested. This study forms part of a project aimed at the development of improved traditional crops to contribute to food production and quality for subsistence farmers in areas with low precipitation or variable rainfall patterns.

1. Introduction

Water deficit is one of the most common environmental limitations of crop productivity and economic losses, and it is a permanent constrain that farmers face daily (Fuglie, 2007; Hyman et al., 2008). Chronic or sporadic periods of water deficit stress leads to reduced growth and quality in plants, and high losses in yield of 50% and more are experienced (Wang et al., 2003). Exposure of plants to extreme stress conditions such as drought will initiate a diverse set of physiological, morphological and developmental changes in order to survive, as have been widely reported (Gomes et al., 2010; Ozkur et al., 2009). Physiological traits relevant or modified by the responses to water deficits span a wide range of vital processes and there is no single response pattern that is highly correlated with yield under all drought environments (Cattivelli et al., 2008).

Plants have evolved a number of antioxidant enzymes that ameliorate oxidative stress by scavenging toxic oxygen species (Liang et al., 2003; Sun et al., 2007). Superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate reductase (APX) co-operate to reduce the damaging effect of oxygen radicals through the Halliwell–Asada pathway. There are numerous reports on these enzymes protecting plants during oxidative stress initiated by drought (Ahmed et al., 2009; Boogar et al., 2014; Ozkur et al., 2009). Plants able to tolerate drought must therefore be able to increase their defense mechanisms under conditions of severe water deficit. Water stress causes oxidative injury, and the ability to increase the levels of antioxidative capacity or increased levels of antioxidants during water stress can limit membrane damage and enzyme activity can be an important measurement of drought tolerance (Dawood et al., 2014).

Water stress induces numerous metabolic changes in plants, and many plants respond to a decrease in osmotic potential by intracellular

Abbreviations: APX, ascorbate peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; LA, leaf area; LWP, leaf water potential; RWC, relative water content; SOD, superoxide dismutase.

* Corresponding author.

http://dx.doi.org/10.1016/j.sajb.2014.08.008
0254-6299/

accumulation of organic solutes e.g. ions, alcohols, sucrose or amino acids. One of the most prominent metabolic changes is a tremendous free-proline accumulation (Aziz et al., 1998; Manivannan et al., 2007). The role of proline during stress includes functioning as a compatible osmolite/osmoregulation (Abdalla and El-Khoshiban, 2007; Huang et al., 2000), preventing hyperosmotic stress (Kishor et al., 1995) by balancing concentration differences between the cytoplasm and the central vacuole of the plant cells, is involved in stabilizing proteins/enzymes and enzyme activities (Gill and Tuteja, 2010), stabilizes subcellular structures (membranes and proteins) (Ashraf and Foolad, 2007; Efeoghu et al., 2009), and is involved in the conservation of nitrogen and energy for a post-stressed period (Hare et al., 1998). Proline is also known to induce expression stress responsive genes, such as drought and salt stress (Chinnusamy et al., 2005; Dawood et al., 2014; Zhu et al., 1998).

The measurement of solute leakage from plant tissue is a proven method for measuring membrane integrity in relation to environmental stresses (Blum, 1998; Blum and Ebercon, 1981; Farooq and Azam, 2006). This technique involves measurements of electrolyte leakage into an aqueous medium, where the degree of cell membrane stability is considered to be one of the best physiological indicators of water stress tolerance (Kocheva et al., 2004; Labuschagne et al., 2008).

Maintenance of turgor pressure during stress is important to preserve metabolic responses in crop species, and is a well recognized mechanism in breeding toward water stress tolerance. The ability of seed propagated plants to adapt to water stress involves either tolerance to cellular dehydration or minimisation of water loss and maintenance of turgor pressure (Morgan, 1992). Relative water content (RWC) allows for the comparison of metabolic changes in the plant, at the same cellular water status (Blum, 1998). RWC further allows the estimation of plant water status in terms of cellular hydration and is under the possible effect of both leaf water potential and osmotic adjustment (Blum, 1998). RWC can be used effectively to evaluate drought tolerance and selection of the most drought tolerant genotypes (Abdalla and El-Khoshiban, 2007).

A renewed interest in the traditional crop *Amaranthus* has developed not only because of its growth abilities, but also because of its good nutritional qualities. As a vegetable crop, *Amaranthus* is a very nutritious summer leafy vegetable. The leaves are rich in protein, iron, calcium and vitamins A, C & D (Brenner et al., 2001). In the present study, the effect of water stress on the activities of the enzymes of the antioxidative pathway (SOD, APX and GR), leaf area (LA), osmoprotection (free proline production), cell membrane stability (CMS), in comparison with changes in leaf turgor maintenance (RWC, LWP), was assessed to obtain an indication of the changes in metabolic traits which could indicate possible mechanisms of drought tolerance in different amaranth genotypes.

2. Materials and methods

2.1. Cultivation, water stress and sampling

Experiments were carried out at the Agricultural Research Council-Roodeplaat, Pretoria, at 25°59′S; 28°35′E and at an altitude of 1200 m above sea level. Three amaranth species, i.e. *Amaranthus hybridus*, *Amaranthus hypochondriacus* and *Amaranthus tricolor* were cultivated in pots in a temperature controlled greenhouse. Cultivation and leaf sampling was done as described by Slabbert and Krüger (2011).

2.2. Analytical procedures

2.2.1. Leaf water status

LWP and RWC measurements were made pre-dawn from control and stressed plants according to the methods described by Slabbert and Krüger (2011).

2.2.2. Antioxidative enzyme activity

Leaf material was sampled midday; freeze dried and stored at −80 °C until subsequent analyses. Samples of 0.04 g of freeze dried material were thoroughly macerated with cold enzyme extraction buffer [0.2% polyvinyl polypyrrolidone (PVP), 0.1 mM EDTA, and 50 mM potassium phosphate, pH 7.8]. The extracted sample was centrifuged (20 000 *g*, 10 min, 2–5 °C) and stored in ice.

Superoxide dismutase (SOD) activity was determined measuring nitrate formation from the oxidation of hydroxyl ammonium chloride at A_{530} as described by Malan et al. (1990) with slight modifications. A 425 μl reaction mixture containing 250 μl of a 65 mM potassium phosphate buffer (KH_2PO_4) (pH 7.8), 25 μl of 1.5 mM xanthine, 25 μl of 1 mM hydroxyl ammonium chloride ($NH_2OH \cdot HCl$) and 125 μl dH_2O, to which 75 μl of crude plant extract was added. After 20 min at 25 °C, 500 μl of reaction mixture was mixed with 500 μl of each 17 mM sulphanylic acid and 7 mM α-naphtylamine. After a further 20 min at 25 °C, the absorbancy of the reaction mixture was read at A_{530}. The SOD activity is expressed as units per gram dry weight (units g^{-1}, D_W).

The rate of NADPH oxidation over time at A_{340} was used as measure of glutathione reductase (GR) activity using a modified method of Tappel (1978) with slight modifications. The reaction mixture contained 500 μl of 0.1 M Tris–HCl buffer (pH 8.0), 375 μl of 0.5 mM EDTA, 50 μl of 0.25 mM glutathione (GSSG), 50 μl of 0.125 mM NADPH and 25 μl of crude plant extract. GR activity was expressed as units per gram dry weight (units g^{-1}, D_W).

The ascorbate peroxidase (APX) activity was assessed by measuring the oxidation of H_2O_2 by means of a decrease in absorbance at A_{265}, as described by Dalton et al. (1986) with slight modifications. The APX assay was performed in a 1.5-ml quartz cuvette containing 465 μl of 0.25 mM ascorbate, 830 μl of 50 mM KH_2PO_4 at pH 7.0, 25 μl of 1.0 mM H_2O_2, and 10 μl of crude plant extract. The APX activity was expressed as μgram per μl protein ($\mu g^{-1}/\mu l^{-1}$ protein). Protein was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard. All enzyme activities were expressed as percentage of controls (Srivastava et al., 1995).

2.2.3. Leaf free proline content

Proline was assayed from freeze dried leave material, using a 3% sulfosalicylic and ninhydrin extraction buffers (Bates et al., 1973). Samples of 0.04 g dry weight of leaves was homogenized with 3% (*w*/*v*) sulfosalicylic acid and centrifuged at 3000 *g* for 10 min. A 200 μl aliquot of the supernatant was mixed with 400 μl of the reagent mixture (30 ml glacial acetic acid, 20 ml phosphoric acid and 1.25 g ninhydrin) and heated in sealed test tubes at 100 °C for 1 h. After cooling down, 4 ml toluene was added to each sample. Proline content was read on a Titertek Multiskan ® Ascent (Titertek Instruments Inc., USA) at A_{520} and expressed as μmoles per gram dry weight ($\mu mol^{-1}/g^{-1}$, D_W).

2.2.4. Leaf area (LA)

LA was measured early morning by a leaf area meter (LI-3100, LI-COR Environmental, USA). Leaves were placed one at a time, with their apical side down, on the conveyer belt passing an interrupted light source and sensor. A digital reading was noted.

2.2.5. Cell membrane stability (CMS)

Leakage of electrolytes from tissue to an external solution was measured by the electron conductivity of the solution according to the method of Sullivan (1972). Each sample (five plants) consisted of 5 leaf disks ($n = 25$), cut with a number 6 cork bore, rinsed well with distilled water. Ten milliliters of deionized water was added to each tube, and the tubes were left at room temperature of approximately 20 °C for 24 h. Conductivity was measured for the water stressed plants (T1), samples were autoclaved for 15 min, and measured again (T2). Similar readings were obtained for the control (C1) and after