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Full Length Research Paper

The relationship between bitterness and drought resistance of almond (*Prunus dulcis* Mill.)

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Cynogenesis has been suggested to be an adaptive response to drought stress in some plants. To determine the possibility of cyanogenic compounds role in almond resistance to drought stress, three different irrigation levels including moderate and severe stress (soil water potential, Ψ_{soil} = -1.2 and -1.8 MPa, respectively) and a control treatment (Ψ_{soil} = -0.33 MPa) were applied for five weeks to six different almond seedling genotypes including Bitter (homozygote bitter), Butte (heterozygote sweet), `Shahrood'$_{12}$ (SH12), `Shahrood'$_{18}$ (SH18), `Shahrood'$_{21}$ (SH21) and White (all heterozygote sweet). Cyanogenic compounds including amygdalin and prunasin and the nitrogen content in the roots and shoots were measured throughout the study. Results showed that nitrogen content tend to be lower in leaves and higher in roots however; differences were not significantly different between genotypes. Severe stressed plants generally showed higher root N content although this was only significantly different for SH18, SH12 and White. There was no amygdalin in vegetative parts of plants. Bitter genotype had the highest prunasin content in its roots compared to the other genotypes. Water stress had no effect on the content of cyanogenic compounds in the leaves of all genotypes, while in roots of all genotypes except Butte and White; water stress caused a decrease in prunasin content. Hence bitterness does not play any role in drought tolerance mechanisms in almond.

Key words: Prunasin, *Prunus dulcis*, almond, amygdalin, bitterness, compatible solute, drought resistance.

INTRODUCTION

The breeding of fruit tree rootstocks for environmental stress tolerance is difficult and time-consuming. The quantitative nature of stress tolerance and the problems associated with developing appropriate and replicatable testing environments make it difficult to distinguish stress-tolerant cultivars from sensitive ones. One approach to a better understanding of plant stress tolerance is to isolate those characteristics correlated to contribute to stress tolerance and determine their relative importance. Only then can focused breeding approaches be developed. Almond (*P. dulcis* Mill, syn: *P. amygdalus* Batsch) is a drought resistant species and highly adapted to a wide range of soil water availability (Alarcon et al., 2002; De Herralde et al., 2003; Isaakidis et al., 2004). The tolerance of almond trees to water stress is related to adaptive mechanisms present in their leaves or roots. Previous studies have indicated that almond drought resistance streams from both mechanisms involving avoidance as well as some degree of osmotic adjustment when plants are subjected to short-term water stress periods. Short-term drought (several weeks) in juvenile plants permits observation of changes in some physiological processes that are typical for progressive stages of drought. Almond genotypes use as rootstocks are commonly propagated from seeds. In plantations in Iran, the genotype Bitter, a selected cultivar, is traditionally used as rootstock (Rahemi and Yadollahi, 2005) due to its possible, but unproved tolerance to environmental stresses such as drought (Yadollahi and Rahemi, 2005), nematode and capnodis resistance (Dicenta et al., 1999; Mulas, 1994; Usai and Dhallewin, 1990). Bitterness in almond is related to amygdalin accumulation in the kernels (Vetter, 2000).

Accumulation of solutes, either actively or passively, is an important adaptation mechanism for plants in

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response to osmotic stress (Yang et al., 2003; Hokmabadi et al., 2005). Cellular dehydration is a general consequence of osmotic stresses, including water deficit and high salinity levels. In response to these conditions, many plants synthesize solutes that either help retain water within cells, or protect cellular components from injury caused by dehydration. One such type of solute, cyanogenic compounds have been shown to act in both capacities. Water availability is perhaps the best-studied environmental factor of cyanogenesis, but despite the focus of research on just a few species, the results have been sometimes contradictory (Pederson et al., 1996). For instance, Caradus et al. (1990) found that the frequency of cyanogenic compounds in *Trifolium repens* was higher in areas of low rainfall in New Zealand, while Foulds and Grime (1972a), in a study of *T. repens* in England, found the converse to be true. The results of surveys of cyanogenesis in *Lotus corniculatus* are, however, more consistent. For instance, on Mainland, Orkney, a clear negative correlation between soil moisture and the frequency of the cyanogenic compound was found (Abbott 1977). Similar results have been recorded for *L. corniculatus* in numerous other European populations (Foulds and Grime, 1972b; Blaise et al., 1991). Almond is usually planted in semi-dry areas, so it is important to select the right rootstock for any orchard establishment.

White and Bitter almond have been provisionally nominated as suitable rootstocks for water stress and irrigation conditions in Iran respectively (Yadollahi et al., 2008). In this research work, we studied the effect of water availability on cyanogenic compound production capacity of nominated almond genotypes originating from seeds as well as to improve our understanding of physiological mechanisms involved in the response of young almond plants to drought stress. Such information is valuable in order to improve drought resistance, which in turn, requires study of cyanogenesis in which water stress is quantified and all of the other important environmental variables are either constant or controlled.

MATERIALS AND METHODS

Plant material and experimental design

The experiment was conducted during the 2005 growing season at the research green house in the Department of Horticultural Sciences, Tarbiat Modares University, Iran. Seeds of *Prunus dulcis* from six different almond genotypes were selected after controlled hand pollination with sweet pollen source (Butte cultivar) and Bitter genotype (only for bitter genotype) during the 2004 growing season. Genotypes were 1) homozygous sweet (cultivar `Butte' from Davies University, California, USA), 2) homozygous bitter (cultivar `Bitter' from cross pollinated seeds between 2 bitter genotypes, University of Tehran), 3) heterozygous sweet genotypes (commercial Iranian almond cultivars named: `Shahrood'$_{12}$ (SH12), `Shahrood'$_{18}$ (SH18), `Shahrood'$_{21}$ (SH21) and `White' (local almond cultivar with pale brown kernels from Agricultural Research Centre of Shahrekord, Iran,.. Seeds of similar shape and size were selected, then scarified and treated with fungicide and stratified at 4°C for 30 days until rootlets appeared.

Germinated seeds were transplanted on July 5, 2005 into the 7 L containers (1 seedling per container) in the experimental glass-house. Each container was 0.33 m in diameter and 0.33 m deep and a hole at the bottom for drainage. Day/night temperatures were 25 - 35/15 - 25°C respectively and relative humidity was 55% of the greenhouse. The soil consisted of humus, soil and sand (1:1:1). The soil comprised of silt (5 - 8%), clay (20 - 40%) and sand (50 - 75%), pH 7.3 - 7.5, Mg 360.9 mg^{-L}, Na 1150 mg^{-L}, Ca 495.9 mg^{-L}, K 540 mg^{-L}, Fe 200 □g^{-L}, Zn 174 □g^{-L}. Plants were supplied with a soluble 20:8:12 N: P: K fertilizer and well watered before beginning of measurements, until plants reached 20 cm in height. A factorial experiment was conducted with a randomized complete block design which included 3 irrigations factors, six genotype factors and 3 replications. On September 5, 2005 treatments were applied based on Ψ_{soil} from soil moisture content curve based on results obtained from the soil samples (Soil and Water Research Institute, Tehran, Iran) and the control pots which were weighed every day. Plants were kept in the nominated Ψ_{soil} (soil water potential) for 5 weeks. Treatments were: T1 = control pots were watered regularly to field capacity (well irrigated, Ψ_{soil} = -0.33 MPa). T2 = seedlings kept in Ψ_{soil} = -1.2 MPa as moderate drought stress, T3 = seedlings kept in Ψ_{soil} = -1.8 MPa as severe drought stress. The experiment ended on 7th of October 2005, a total of 5 weeks. Then plants were harvested, washed with tap and distilled water and divided into leaves and roots and immediately frozen in liquid nitrogen. Plant materials were then lyophilized by freeze drying (CHRIST, Germany) for 24 h at -50°C and -0.05 mbar atmospheric pressure. Dried materials were sent to the University of Adelaide, Australia for further analysis.

Extraction of metabolites from almond kernels

For quantification of amygdalin and prunasin, extracts were made from freeze dried materials, which were pre-weighed. Leaves and roots frozen in liquid nitrogen were ground to a fine powder using mortar and pestle. Frozen, ground tissue (0.2 g DW) was resuspended in 10 ml of methanol and 1 mg Polyvinylpyrolidone (PVP) by vortexing, and then heated in a water bath at 80°C for 10 min (Dicenta et al., 2002). The solvent was separated from particulate material by centrifugation (405 x g, 2 min) then stored at -20°C until HPLC analysis. Reference compounds (amygdalin and prunasin) were purchased from Sigma Company, USA.

HPLC analysis of metabolite extracts

All HPLC analyses were made using Agilent 1100 series apparatus. For quantification of amygdalin and prunasin in kernel extracts, methanol extracts were filtered (0.45 µm Millex-HV unit, Millipore), diluted in methanol if necessary, then injected by auto-sampler (7.5 µl) and separated at 25°C through a Zorbax Eclipse XDB-C18 5 µm column (Agilent) with dimensions: 4.6 x 150 mm. Mobile phases were water (A) and acetonitrile (B) and conditions were: 20% B at 200 µl min^{-1} for 15 min; 20% B to 100% B and increasing from 200 µl min^{-1} to 400 µl min^{-1} over 12 min; 100% B at 400 µl min^{-1} for 2 min; 100% B to 20% B at 400 µl min^{-1} over 3 min; 20% B at 400 µl min^{-1} for 8 min. Wavelengths from 190 to 400 nm were monitored and R.T. for prunasin was 15.6 min based on the standard.

Root and leaf nitrogen analysis

Leaf and root samples from each replicate of each treatment were collected at the end of the experiment. Samples were washed once with tap water and twice with distilled water, dried in a forced-draft

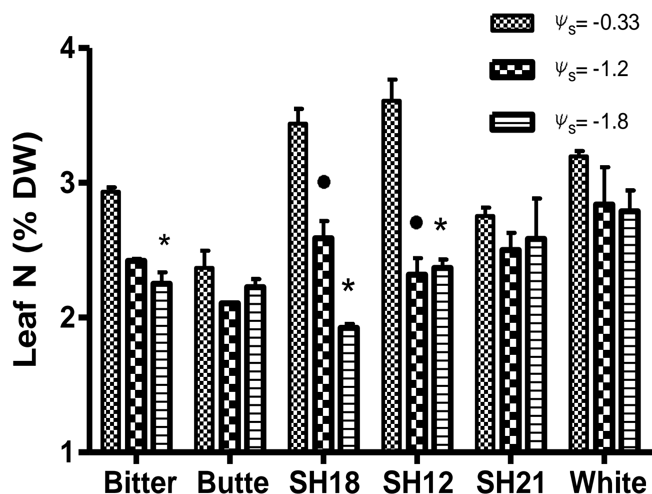

Figure 1. Leaf Nitrogen content (% DW) of 6 different genotypes of almond seedlings after 5 weeks exposure to water stress. Star (*) shows the significant differences ($P \leq 0.05$) between well irrigated treatment compared with severe water stress treatment. Each point is the average of three replications and vertical bars indicate ± SEM.

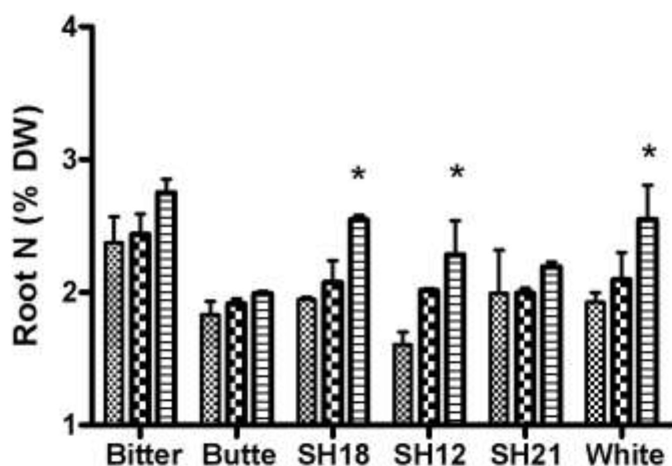

Figure 2. Root Nitrogen content (% DW) of 6 different genotypes of almond seedlings after 5 weeks exposure to water stress. Star (*) shows the significant differences ($P \leq 0.05$) between well irrigated treatment compared with severe water stress treatment. Each point is the average of three replications and vertical bars indicate ± SEM.

oven at 70°C and ground in a mill to pass a 40-mesh screen. Samples were digested using salicylic acid, and nitrogen (N) content was determined by the Kjeldahl procedure (Emami, 1995).

Statistics

All data were subjected to a two-way analysis of variance (ANOVA) with previous data transformation whenever required using SPSS statistical program. Significantly different means ($P \leq 0.05$) were separated using Duncan's multiple range test (DMRT). Graphs were designed using Graph Pad Prism 5 software.

RESULTS

Root and leaf nitrogen analysis

In control plants SH12 and Butte showed the greatest and lowest N content in their leaves, respectively (Figure 1). Butte, SH21 and White did not show any significant changes in leaf N content in response to water stress. Leaf N content reduced with moderate water stress in SH18 and SH12, while under severe stress, plants of Bitter genotype also showed a significant reduction (Figure 1). In control treatments Bitter had the greatest and SH12 had the least root N content. Moderate stress had no significant effect on root N content while severe stressed plants generally showed higher root N content, although this was only significantly different for SH18, SH12 and White (Figures 1 and 2).

Root and leaf cyanogenic content

There was no amygdalin in the roots and leaves of plants (Figure 5). Among control plants, root prunasin content was the greatest for Bitter genotype. Root prunasin was higher than leaf prunasin in all genotypes, except for SH12, in which its leaf prunasin was the greatest in comparison with the others. Butte had the least prunasin in its roots among control plants (Figures 3 and 4).

DISCUSSION

In general the nitrogen concentrations were in the adequate range of 2.2 - 2.5 % DW for leaves (the growth rate reaches a maximum and remains unaffected by nutrient supply under irrigation). The ability to absorb and maintain adequate nutrients especially during stress periods could possibly be linked to drought stress resistance in almond (Issakadis et al., 2004). Seed chemical analysis before planting showed that Butte had the greatest amount of N in seeds rather than others. Bitter seeds germinated quickly and uniformly in comparison with others (data not shown) and the quicker the seed germination, the less risk of damping off. It seems that there is no relationship between seedling N content and seed N content. The majority of leaf nitrogen occurs in enzymes (especially Rubisco) which are related to photosynthesis. One would expect an increase in leaf nitrogen to cause an increase in net photosynthesis. It is true under conditions when carbon fixation is occurring at its maximal rate and photosynthesis is therefore limited by the amount of nitrogen-containing leaf constituents (Meziane and Shipley, 2001). Increase in root N content with severe water stress in just three of the genotypes might be related to their enzymatic activity in response to drought stress. Butte (heterozygote sweet genotype) had the least leaf N content in control plants. Nitrogen is usually the limiting element in plant growth especially in

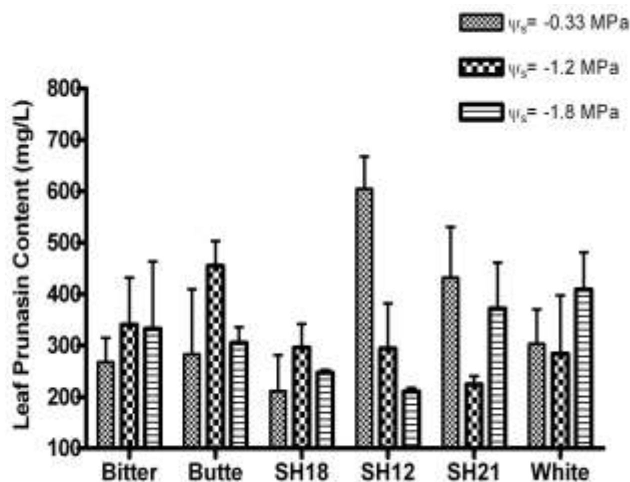

Figure 3. Leaf Prunasin content (mg/L) of 6 different genotypes of almond seedlings after 5 weeks exposure to water stress. Each point is the average of three replications and vertical bars indicate + SEM.

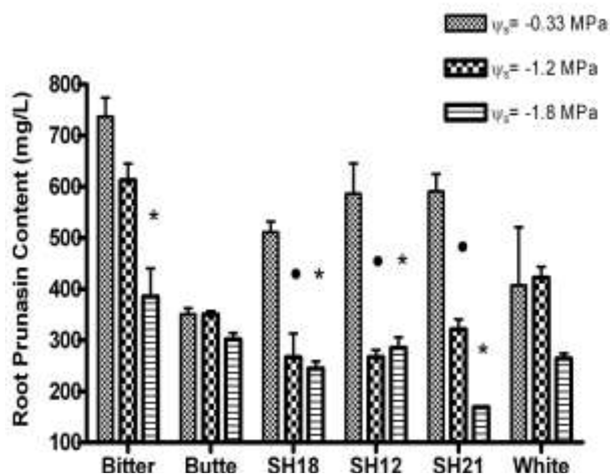

Figure 4. Root Prunasin content (mg/L) of 6 different genotypes of almond seedlings after 5 weeks exposure to water stress. Star (*) and dot (●) show the significant differences ($P \leq 0.05$) between well irrigated treatment compared with severe and moderate water stress treatments respectively. Each point is the average of three replications and vertical bars indicate + SEM.

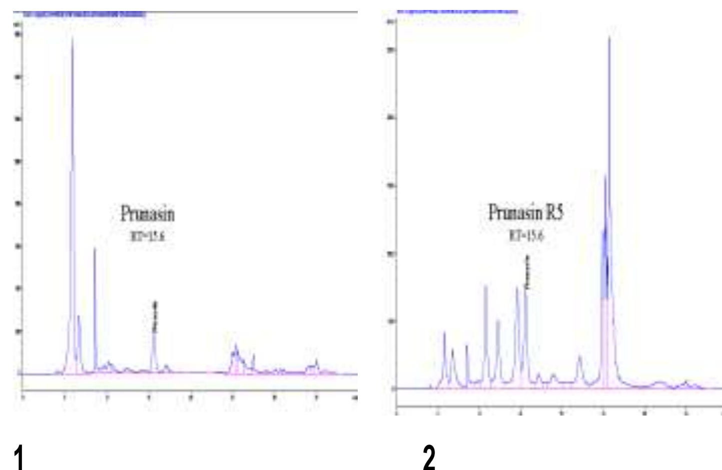

Figure 5. Spectrum of authentic of leaf (1) and root prunasin (2) at Retention Time = 15.6 minutes based on standard.

dry areas (Marschner, 1995). Plant N content and metabolism under some circumstances is highly correlated with photosynthesis capacity (Field and Mooney, 1986; Woodrow et al., 2002). However in this study a relationship between leaf N capacity and physiological indicators was not found (data not shown). Leaf N content tended to be reduced with severe stress but root N content tend to be increased. However the differences were not usually significant. In control plants, Bitter showed higher amounts of root N probably due to its ability to synthesize nitrogen compounds like cyanogenic glucosides (Dicenta et al., 2002). Simultaneously, the amount of prunasin in root material was greatest for Bitter genotype in comparison with others among control plants (Figures 2 and 4). This finding supports the idea that it may be better as a rootstock under irrigated conditions due to its higher resistance to nematodes and capnodis as mentioned before. A significant relationship between prunasin and nitrogen content was only observed between leaf N and root prunasin (Table I).

Water limitation was related to an increase in leaf nitrogen content of *Eucalyptus* seedlings planted under greenhouse conditions. Woodrow et al. (2002) reported that drought stress increased cyanogenic compounds in *Eucalyptus cladocalyx*. They also found that there was a significant relationship between cyanogenic compounds and leaf N content, therefore plants from dry land areas showed more tolerance due to their higher content of cyanogenic compounds. No amygdalin was found in roots and leaf tissues of almond. The same results have been reported previously (Vetter, 2000; Dicenta et al., 2002). It is known that diglycoside amygdalin is only found in reproductive tissues like seed parts and our finding supports that. Despite drought stress having no significant influence on leaf prunasin for all genotypes (Figure 3), root prunasin content for all genotypes tend to be reduced by drought stress; however this reduction was not significant for Butte and White (Figure 4). The response of almond genotypes used in the present research in addition with `GF677' rootstock to water stress was tested. Obtained results from the mentioned experiment showed a dramatic increase in proline accumulation in the leaves of all studied genotypes subjected to water stress (unpublished results). In Figure 3, it has been demonstrated clearly that prunasin had no compatible solute effect like proline or Glycine-betaine (the well known compatible solute in many plants) in

Table 1. Pearson's correlation coefficient for the relationships between the prunasin and total nitrogen contents in leaves and roots of the 6 almond seedling genotypes studied. Star (*) shows the significant correlation and ns shows non significant correlation.

	Leaf N	Root N	Leaf Prunasin	Root Prunasin
Leaf N	1			
Root N	-0.146^{ns}	1		
Leaf Prunasin	0.007^{ns}	0.0158^{ns}	1	
Root Prunasin	0.331*	0.012^{ns}	0.126^{ns}	1

drought resistant almond. However, there are several reports available in terms of increasing cyanogenic compounds in response to drought stress for instance in *Eucalyptus globulus* and *Trifolium* sp. (Bokanga et al., 1994; Schappert and Shore,1999). In the present research, there was considerable variability in the concentrations of cyanogenic compounds in the studied genotypes. Variability of prunasin was higher in roots than leaves which are in agreement with Dicenta et al. (2002) and Graham (2002). A specific distribution of both compounds (amygdalin and prunasin) was observed in the kernels. Prunasin (a monoglucoside) was found mainly in the vegetative parts, while amygdalin (a diglucoside) was localized only in bitter kernels. Prunasin thus appears to be the form of cyanogenic glycoside transported in the plant while amygdalin is utilized for storage, as previously suggested by Frehner et al. (1990). It is concluded that cyanogenic compounds in almond have a function in protecting the plant (roots and aerial parts) and the seed against plant eaters (insects, mammals, or birds) and no role as a compatible solute in drought stress, so bitterness doesn't play any role in drought tolerance mechanism in almond.

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