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Cyanogenic glucosides from dormancy to flowering time in early and late almonds

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Abstract. Cyanogenic glucosides are well-known defense compounds produced as a protection against herbivores. In a process called cyanogenesis, a toxic gas hydrogen cyanide (HCN) is released from cyanogenic glucosides upon tissue disruption. Our research, however, indicates that cyanogenic glucosides could develop new functions in other plant physiological processes, such as dormancy release in flower buds. In order to investigate this hypothesis, flower buds and individual parts of the fully-developed flower of five almond cultivars (early flowering time: 'Achaak' and 'Desmayo'; late flowering time: 'S3067' and 'Lauranne'; extra late flowering time: 'Penta') were collected in the experimental orchard of CEBAS-CSIC, in Santomera (Murcia, South-East Spain), to analyze the content of cyanogenic glucosides by LC-MS/MS. The two main cyanogenic glucosides, prunasin and amygdalin, were found in all the varieties, with the concentrations being highest in the bitter variety ('S3067'). Interestingly prunasin was observed in the flower buds from dormancy having its highest concentration right before flowering took place for all the five cultivars, suggesting that this compound could play an important role in flower development. Moreover, new derivatives of cyanogenic glucosides, namely prunasin amide, acid, anitrile, anitrile apioside and apioside, were also found in the tissues analysed, albeit in much lower concentration. The elevated levels of prunasin found in tissues as pollen or the presence of the new derivates in different parts of almond flower are also discussed.

Keywords. Prunasin – Amygdalin – Dormancy – Flowering time – LC-MS/MS.

Les glucosides cyanogènes, de la dormance à la floraison dans les amandes précoces et tardives

Résumé. *Les glucosides cyanogènes sont des composés de défense bien connus comme protection contre les herbivores. Dans un processus appelé cyanogenèse, un gaz toxique, le cyanure d'hydrogène (HCN) est libéré à partir de glucosides cyanogènes lors de la rupture du tissu. Notre recherche, cependant, indique que les glucosides cyanogènes pourraient développer de nouvelles fonctions dans d'autres processus physiologiques des plantes, comme la levée de dormance des bourgeons floraux. Afin de vérifier cette hypothèse, des bourgeons floraux et des parties individuelles de fleurs entièrement développées de cinq cultivars d'amandier (floraison précoce: 'Achaak' et 'Desmayo', floraison tardive: 'S3067' et 'Lauranne', extra tardive: 'Penta') ont été recueillis, auprès du verger expérimental de CEBAS-CSIC, à Santomera (Murcia, sud-est de l'Espagne), pour analyser la teneur en glucosides cyanogènes par LC-MS/MS. Les deux principaux glucosides cyanogènes, prunasine et amygdaline, ont été trouvés dans toutes les variétés, avec des concentrations plus élevées dans la variété amère ('S3067'). Il est intéressant de noter que pour les cinq cultivars, la prunasine a été observée dans les bourgeons floraux dès la dormance, la plus haute concentration étant trouvée juste avant la floraison pour les cinq cultivars, ce qui suggère que ce composé pourrait jouer un rôle important dans le développement des fleurs. En outre, de nouveaux dérivés de glucosides cyanogènes, tels que prunasine amide, prunasine acide, prunasine anitrile, prunasine anitrile apioside et prunasine apioside, ont également été trouvés dans les tissus analysés, mais avec une concentration beaucoup plus faible. Les niveaux élevés de prunasine trouvés dans des tissus comme le pollen ou la présence des nouveaux dérivés dans différentes parties de la fleur d'amandier sont également discutés.*

Mots-clés. *Prunasine – Amygdaline – Dormance – Temps de floraison – LC-MS / MS.*

I – Introduction

Cyanogenic glucosides are defense compounds of the plants presents in more than 3000 species including economically important crops such as almond (*Prunus dulcis* Miller D.A. Webb syn. *Prunus amygdalus* Batsch). Upon tissue disruption, in a process called cyanogenesis, the toxic gas hydrogen cyanide (HCN) is released from cyanogenic glucosides (Poulton, 1990). However, our research indicates that cyanogenic glucosides could develop new functions in other plant physiological processes, such as breaking dormancy or flower development. There are two cyanogenic glucosides in almond: prunasin and amygdalin, both derived from the amino acid phenlyalanine. Prunasin is the precursor of amygdalin. When both are degraded, glucose, benzaldehyde (bitter flavour) and hydrogen cyanide (toxic) are liberated (Sánchez-Pérez *et al*., 2008. When this last one is released, detoxification pathway is activated forming ammonia, aspartate and asparagines which could be a nitrogen supply for the plant (Swain and Poulton, 1994). Although the main function described of the cyanogenic glucosides is such as first chemical defense against pathogens and other predators, also they have other functions such as transport and storage of nitrogen and sugar for the kernel, being a precursor for protein synthesis or like metabolites source (Swain *et al*., 1992 and Sánchez-Pérez *et al*., 2008). On the other hand, breaking dormancy is the ability of the tree to start floral or vegetative budbreak. Consequently, flowering will only happen when the dormancy is broken. In almond, flowering time is one of the most important agronomic traits studied in breeding programs, because a late flowering variety avoids the lost of the yield because of the late or spring frosts. It is clear that cyanogenic glucosides have an important role in the plant and especially in the kernel as defense, for this the bitter taste of the kernel. Other functions as nitrogen supply and metabolites transport have been already detected but this is the first time that levels of cyanogenic glucosides have been measured from dormancy to flowering time, suggesting that these compounds could be involved in one of the two processes just mentioned. In relation with this, we try to answer the next questions: Could cyanogenic glucosides develop new functions in other plant physiological processes, such as dormancy release in flowerbuds and the flower development? How do cyanogenic glucosides evolve during the flower development? Is the content similar in all the tissues? Are there more cyanogenic glucosides derivates involve?

II – Materials and methods

Flowerbuds and individual parts of the fully-developed flower (petals, sepals, pistils, and pollen) of five almond cultivars (early flowering time: 'Achaak' and 'Desmayo'; intermediate flowering time: 'S3067'; late flowering time: 'Lauranne'; and extra late flowering time: 'Penta') were collected from November 2013 to March 2014 every two weeks in the experimental orchard of CEBAS-CSIC, in Santomera (Murcia, South-East Spain). Three branches for each cultivar were collected every two weeks from the field in Santomera and placed in a growth chamber in controlled conditions (25ºC during the photoperiod of 16h and 20ºC during 8h of darkness, with a constant relative humidity of 60% during the night and 40% during the day). Almond branches were placed in a 5% saccharose and 1% Aluminum Sulfate solution. After five days the solution was changed. After 10 days the development state of the flower buds was measured. The date of dormancy breakage was established when 50% of the flower buds were in the b-c state of Fleckinger (Felipe, 1977). The flowering time date was determined when the 50% of the flowers in the tree were completely opened. To extract cyanogenic glucosides and their derivates, once the samples were collected and keep at -80ºC, they were grinded with a mortar and liquid nitrogen and weighed frozen. Then between 50 and 100 mg of the samples were added to 400 µL methanol 85% in a threaded tube of 1,5 ml. The samples with methanol were boiled 5 min in a bath and they were put in ice. After this were centrifuged 5 min 2000 g and the supernatant was collected and taken out to a HPLC tube and keep at -20ºC. 20 µL of this supernatant was filtered with 70 µL of water and 10 µL Linamarin (in-

ternal standard) 500 µM (final concentration 50 µM) in a ELISA filter (5x dilution). The order was lid + ELISA plate + ELISA filter. The mix from the filtering was centrifuged 5 min 3000 rpm and 60 µL were transferred to a HPLC vial in a HPLC tube and sent to analyze by LC-MS.

III – Results and discussion

The two main cyanogenic glucosides, prunasin and amygdalin, were detected in the flowerbuds of all the varieties (Figs. 1A and B), both early and late flowering cultivars, during the flower development. There were not significant differences among early and late flowering cultivars. The bitter cultivar (S3067) had the highest concentration of prunasin and amygdalin respectively. Whereas the prunasin peak was 1.727 µmoles/100 g, the amygdalin peak was 0.023 µmoles/100 g, what means that prunasin concentration was around 75 times more than amygdalin in the almond flowerbuds (Figs. 1A and B). According to Nahrstedt *et al*. (1972) only prunasin was detected in flowers of

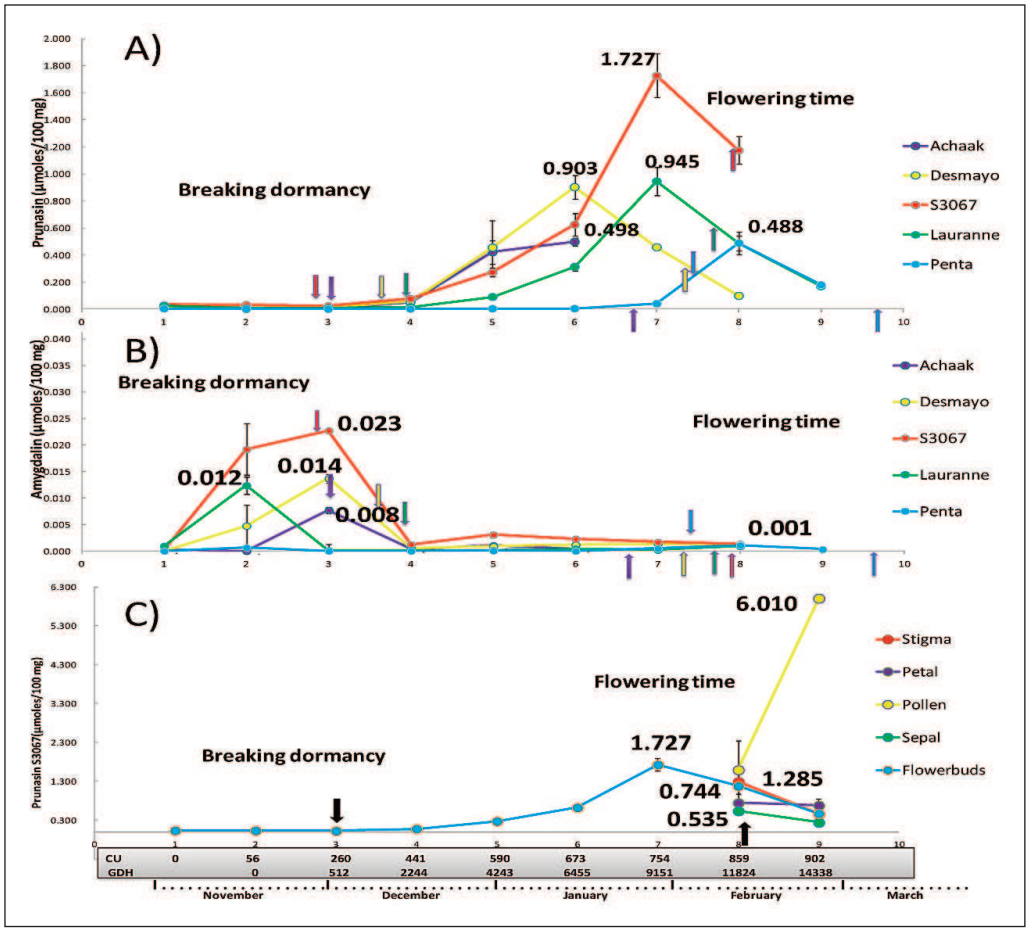

Fig. 1. Cyanogenic monoglucoside prunasin in flowerbuds during their development (A). Cyanogenic diglucoside amygdalin in flowerbuds during their development (B). Cyanogenic monoglucoside prunasin in flowerbuds, pollen, sepals, petals and pistils of S3067 variety during flower development (C). Down arrows indicate Breaking dormancy and up arrows indicate Flowering time.

Prunus avium, whereas amygdalin was not found. The highest concentration of prunasin was right before flowering took place for all the five cultivars (Fig. 1A), suggesting that this compound could play an important role in flower development. The highest concentration of amygdalin was when the break dormancy was taking place (Fig. 1B). So the tendency of the prunasin was to increase from break dormancy until flowering time whereas the amygdalin tended to decrease from break dormancy until flowering time. This could suggest that amygdalin could be used to break the flowerbud dormancy and prunasin to help the development of the the flowerbud to a flower. Lieberei *et al*. (1985), Selmar *et al*. (1988), Swain *et al*. (1992) and Sánchez-Pérez *et al*. (2008) suggested that cyanogenic compounds could have a nitrogen supply function. In relation with this, prunasin and amygdlain were detected in the almond kernel and during its development too (Swain *et al*., 1992 and Sánchez-Pérez *et al*., 2008) and in other parts of the almond tree like roots, stems and leaves (Dicenta *et al*., 2002). But not only flowerbuds had cyanogenic glucosides. Also other parts of the flower like petals, sepals, pistils and pollen contained prunasin and amygdalin and the derivates (Fig. 1C). In this sense, pollen was the tissue with the highest prunasin level compared with the other tissues, even with flowerbuds. London-Shafir *et al*. (2003) got the same results in almond pollen amygdalin, according to this author the amygdalin would inhibit inefficient pollinators allowing a more efficient pollinization by honeybees, which are able to tolerate the amygdalin toxicity up to a certain level. Regards to the others, petals, sepals and pistils had a tendency similar to the flowerbuds, so decreased after flowering until almost zero. Abarrategui (2010) detected prunasin by LC-MS in sepals, petals, pistils and pollen of bitter and sweet varieties of almond. Amygdalin level was almost zero in all the varieties except in the pollen of the bitter one. Moreover, new derivatives of cyanogenic glucosides namely prunasin anitrile, amide, acid, apioside and apioside anitrile were also found in the tissues analyzed (data not shown), although the concentration of these compounds was much more lower than prunasin. Only prunasin anitrile apioside had very high values. Pičmanová *et al*. (2015) suggested that these derivates could have a role in an alternative turnover pathway in which cyanogenic glucosides are converted to non-cyanogenic glucosides without any release of HCN.

IV – Conclusions

For the first time prunasin and amygdalin were detected in flowerbuds during the flower development in almonds. The bitter genotype was the highest concentrated. In the flowerbuds, prunasin concentration was around 75 times more than amygdalin. Prunasin had its highest concentration right before flowering took place, suggesting that this compound could play an important role in flower development. Amygdalin was the main compound detected during breaking dormancy while prunasin was the main compound during flowering time. Pollen was the tissue with the highest level of cyanogenic glucosides, even more than flowerbuds, especially in the bitter genotype. Other parts of the flower like petals, sepals and pistils contained prunasin and amygdalin in lower concentrations. Moreover, new derivatives of prunasin, namely prunasin amide, prunasin acid, prunasin anitrile, prunasin anitrile apioside and prunasin apioside, were also found in the tissues analyzed, although in much lower concentration.

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