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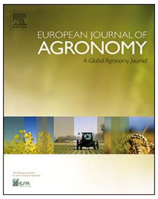

Cytokinins: A key player in determining differences in patterns of canopy senescence in Stay-Green and Fast Dry-Down sunflower (*Helianthus annuus* L.) hybrids

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ABSTRACT

Leaf senescence during grain filling can reduce crop yield. We studied, under field conditions and during grain-filling, the association between leaf cytokinin levels and the onset of leaf senescence in sunflower hybrids of contrasting canopy senescence patterns (Paraiso75, stay-green [SG] and Paraiso65, fast dry down [FDD]). At crop level, dynamics of live root length density (LRLD) and green leaf area index (GLAI) were followed, while at leaf level dynamics of total chlorophyll content, *trans*-Zeatin content, net photosynthesis and PSII quantum yield, were followed in leaf positions 17, 20, 22 and 24. Responses of these leaf variables to exogenous cytokinin applications to leaves at position 17 were also followed. SG exhibited greater ($p < 0.05$) LRLD and GLAI values at anthesis. In both hybrids, LRLD began to fall before GLAI. All variables decreased earlier ($p < 0.05$) in FDD. Initial leaf levels of *trans*-Zeatin were three times higher ($p < 0.05$) in SG. Exogenous cytokinin applications maintained leaf-level variables. These are the first results showing associations between LRLD dynamics with the dynamics of leaf cytokinin levels and changes in indicators of leaf functionality. Also, this is the first study in which estimates are made of cytokinin thresholds below which leaf senescence begins in two hybrids of contrasting canopy senescence patterns. These advances in the understanding, at both crop and leaf levels, of the controls and consequences of SG during grain filling, a trait known to improve crop water uptake under drought and increase biomass accumulation during grain filling, provide support for breeding efforts aimed at profiting from this trait to increase crop yields.

1. Introduction

Accelerated leaf senescence during grain filling can significantly reduce crop yield due to reductions in the levels of photoassimilate available for grain filling, as documented in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (Gregersen et al., 2008) and in maize (*Zea mays* L.) (Rajcan et al., 1999; Rajcan and Tollenaar, 1999). During leaf senescence the rupture of chloroplasts (Guiamét et al., 1999), the catabolism of chlorophyll and macromolecules, such as proteins (Lamattina et al., 1985; Martínez et al., 2008), nucleic acids and membrane lipids takes place, in what is referred to as the decommissioning process of the photosynthetic apparatus. This results in the re-mobilization of much of the N content of photosynthetic proteins to other growing organs (Lim et al., 2007).

In monocarpic species, leaf senescence is coordinated with senescence of whole plant and influenced by endogenous and exogenous factors. The regulation of this physiological process does not follow a simple signal transduction chain, but is influenced by a network of internal and external signals (He et al., 2001; Gan and Amasino, 1997; Buchanan-Wollaston et al., 2003 Gan and Amasino, 1997; Buchanan-Wollaston et al., 2003). Among the external signals, stress factors such as drought (Pic et al., 2002) and changes in the quality and intensity of light (Guiamét et al., 1989; Rousseaux et al., 1996, 2000) can play a part. Among the internal signals phytohormones (Noodén et al., 1997), root functionality (Lisanti et al., 2013) and demand for nitrogen by non-leaf organs, especially grain (Sinclair and De Wit, 1976; Sinclair and Horie, 1989; Van Oosterom et al., 2010) can have important roles in the induction of senescence. Among the hormones involved, cytokinins delay the process (Lim et al., 2007).

The predominant cytokinins in higher plants are isopentenyladenine, zeatin and dihydrozeatin (Sakakibara, 2006). Usually zeatin is the most abundant natural free cytokinin. Zeatin in higher plants is present in trans and cis forms, and these can be interconverted by zeatin isomerase. The *trans*-Zeatin form is more active in biological assays. The primary sites of synthesis of free cytokinins in whole plants are the apical meristems of roots (Akiyoshi et al., 1983; Akiyoshi et al., 1984;

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Barry et al., 1984; Beveridge et al., 1997; Samuelson et al., 1992; Takei et al., 2001; White, 1934). Cytokinins synthesized in roots move through the xylem together with water and minerals absorbed by the roots (Itai and Vaadia, 1971; Takei et al., 2001). In xylem exudate, cytokinins are mainly found in the form of zeatin riboside (Takei et al., 2001). Noodén et al. (1990) found, in the xylem exudate of soybean (*Glicine max* L.), that zeatin riboside, dihydrozeatin riboside, zeatin and dihydrozeatin were the most important forms of cytokinins present in the exudate, although further possible unknown forms could not be ruled out.

Gan and Amasino (1995), in tobacco (*Nicotiana tabacum* L.), found that the cytokinin content in senescent leaves decreases, and this has been proposed as a key signal involved in canopy senescence. The reduction of the cytokinin flow from the roots apparently fulfills an important role in soybean canopy senescence, and this reduction seems to be induced by messages from pods (Noodén et al., 1990). In maize (Ren et al., 2016), rice (*Oryza sativa* L.) (Liu et al., 2016), and tobacco (Singh et al., 1992) it has been shown that the application of cytokinins to leaves delays their senescence.

In many annual crops it has been observed that root biomass values reach a maximum at around flowering (Gregory et al., 1978; wheat; Mengel and Barber, 1974; maize; Sadras et al., 1989; sunflower). From this point onwards, root biomass begins to decline or, in some cases remains constant, and the same occurs with the functionality of the root system. Lisanti et al. (2013) found, in sunflower, a significant decrease of root biomass and of live root length density after flowering, effects that were associated with reductions in the volume of water absorbed by the crop. They also found that during the grain filling phase live root length density began to decrease before leaf senescence became evident. This phase is critical to crop performance because during it grain weight per plant is determined, therefore water and nutrient supply, especially of nitrogen, is of great importance for crop yield.

In sunflower variability exists in the patterns of canopy senescence during grain filling, ranging between the extremes of delayed senescence (stay-green, SG) and rapid senescence (fast dry down, FDD), and these differences are linked to variations in the accumulation of post-anthesis biomass (De la Vega et al., 2011). Furthermore, Lisanti et al. (2013) found, under both drought and irrigation, that a SG sunflower hybrid maintained greater soil water extraction rates for a longer time after flowering than a FDD hybrid, in association with a greater live root length density. This set of observations suggests that sunflower, in its SG and FDD forms, is an appropriate experimental model to examine the possible role of cytokinins in the control of leaf senescence and their role in the root/leaf communication during the grain filling phase.

The objectives of the present study were: 1) to track, in sunflower hybrids of contrasting canopy senescence patterns (SG and FDD), the dynamics of cytokinin levels in leaves during grain filling, and their association with root functionality and leaf senescence. Leaf cytokinin levels in the leaves were also manipulated by exogenous application of cytokinins to the leaves, something which aimed to 2) establish the threshold cytokinin level below which leaf senescence is triggered. In relation to the first objective, we postulated two hypotheses. The first that both timing of the onset and the rate of leaf senescence are strongly associated with levels of cytokinins in the leaves; variables which, in turn, would be linked to the observed differences in the dynamics of leaf senescence between SG and FDD hybrids. The second was that the cytokinin levels in the leaves are associated with the level of root functionality during grain filling. In consequence, the SG hybrid was expected to exhibit a more extended period of root functionality and maintain leaf cytokinin levels for longer periods than the FDD hybrid. In relation to the second objective, our hypothesis was that exogenous cytokinin applications would increase the level of this phytohormone in the leaves, reversing or delaying the onset of leaf senescence. There is no precedent in the literature, for sunflower or any other crop species, of studies that sought to document, in a simultaneous fashion, these variables in cultivars with different patterns of canopy senescence. Moreover, there are no precedents in sunflower, or in any other crop species, of attempts to determine the cytokinin levels below which the process of leaf senescence is triggered.

2. Materials and methods

2.1. Experimental design and crop growth conditions

The experiments were carried out at the Faculty of Agronomy, University of Buenos Aires (34° 35′S, 58° 29′W) during two growing seasons (2012/13 and 2013/14). Sowing dates were 22/11/12 and 30/11/13. Two sunflower hybrids (Nidera Semillas SA, Argentina), which do not differ in length of cycle or time to flowering but exhibit different canopy senescence patterns between flowering and physiological maturity, were used. Paraiso 75 belongs to the “SG” type and has greater green leaf area duration, while Paraiso 65 belongs to the “FDD” type and exhibits a greater canopy senescence rate during post-anthesis. The crops were grown under field conditions at a density of 7.5 plants m^{-2} (achieved by over-planting and thinning at 4-true-leaf stage), fertilized and protected from insects and diseases. The whole of the experimental area was protected from rain with a moveable rain-out shelter for the entire season, in order to avoid proliferation of foliar diseases. A split-plot experimental design with five replicates was used. Each of the five main plots contained two subplots, one for each hybrid (SG and FDD). Each main plot size contained 14 (seven for each sub-plot) 4.5 m-rows, spaced at 0.65 m between rows and each plot was fitted with an independent drip-irrigation system. The amount of water supplied differed between irrigations according to atmospheric demand, and aimed at maintaining soil water status close to field capacity.

Crop developmental status was recorded at weekly intervals using the Schneiter and Miller (1981) scale from the stage of twelve true leaves (V12) through to physiological maturity (R9, inflorescence bracts become yellow and brown). Three non-contiguous representative plants, in perfect competition and away from sub-plot borders and row extremes, were tagged in each sub-plot and used to record crop phenology.

During the grain filling period average daily incident PAR and average daily temperature were recorded using a 21X data-logger (Campbell Scientific Inc., Logan, UT), fitted with an HMP35C (Campbell Scientific, Inc) temperature sensor, and Li190SB PAR sensor (Li-Cor, Lincoln, NE) located at 40 m from the center of the experiment.

2.2. Response variable measurements

2.2.1. Live root length density (LRLD)

Every 7 or 8 days, commencing 8 days before the beginning of anthesis (Stage R5.1, 10% of the head area [disk flowers] in anthesis) and through to physiological maturity (Stage R9), samples were taken from the 0–20, 20–40 and 40–60 cm soil layers (more than 90% of the root system in sunflower is located in the 0–60 cm soil layer (Sadras et al., 1989; Angadi and Entz, 2002)) of each sub-plot. To do this a 5-cm diameter aluminum tube was inserted perpendicular to the soil surface at the midpoint between two representative neighboring plants in a row that were in perfect competition and away from sub-plot borders and row extremes. Three samples per position were taken in each of the five sub-plots. At each sampling position the tube was inserted three times in sequence to the required depths, and the resulting cylinders of soil released using a piston of a diameter equal to that of the internal one of the tube. Immediately after extracting each cylindrical sample, a 1.5-cm diameter soil core sampler was used to subsample that cylinder at three positions (one at each of the extremes and one at the cylinder mid-point, each aligned with the diameter of the sample core). Roots present in these three sub-samples were washed free of soil on a 590 μm mesh sieve and combined to form the composite sample using the Lisanti et al. (2013) technique, and this composite sample was used to