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Dynamics of abscisic acid and indole-3-acetic acid during the early-middle stage of seed development in *Rosa* x *hybrida*

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Abstract

Concentrations of endogenous abscisic acid (ABA) and indole-3-acetic acid (IAA) in *Rosa* x *hybrida* seed coat and embryos were determined at 28, 35, 42, and 49 days after pollination (DAP), a period encompassing the early-middle stages of seed development. No studies on rose have ever documented simultaneous change in ABA and IAA during these developmental phases in both seed coat and embryo. Plant growth regulators were extracted and then quantified by using high performance liquid chromatography (HPLC) based on solid phase extraction (SPE) purification. In both the seed coat and embryo, ABA content decreased from 28 DAP (4.39 pmol mg^{-1} and 1.36 pmol mg^{-1}, respectively) and onward. Endogenous IAA in seed coat followed the same trend. In contrast, IAA in embryo began to increase at 28 DAP (2.06 pmol mg^{-1}), peaked at 42 DAP (5.06 pmol mg^{-1}), and then declined dramatically at 49 DAP (1.17 pmol mg^{-1}). In embryo, the IAA/ABA ratio was always > 1.0 and showed a tendency to increase from 28 DAP to the maximum significant rate at 42 DAP (9.20). The ABA decrease associated with increased IAA levels in embryo could be a result of crosstalk between these two phytohormones. Such a change in the IAA/ABA ratio may signal the end of endodormancy caused by ABA at the pre-cotyledonary stage and the start of increased embryo cell division during the cotyledonary stage, which also results in increased hip weight.

Keywords: Diode array detection; Fluorescence detection; High-performance liquid chromatography; Hybrid tea rose; Plant growth regulators; Seed dormancy; Seed germination

Introduction

Seed development is a complex plant process during which a mature dry seed is formed following fertilisation. Basic embryonic pattern formation occurs during the early stage of seed development called morphogenesis (Meinke 1995; Raz et al. 2001). The latter stage, also called maturation, includes several physiological substages: embryo growth, seed filling, reserve accumulation, desiccation, and dormancy (Gutierrez et al. 2007). Regulation through these stages is a balancing act between plant growth regulators and the spatial and temporal expression of seed-specific gene networks (Ali-Rachedi et al. 2004). Seed structure (Bouchereau et al. 1999) and growth regulator mobilisation between the embryo and surrounding tissues (Brownfield et al. 2007) can also influence the physiologic aspects of seed development.

Dormancy is one of the physiological aspects that characterises a mature seed. It is generally defined as a block to completion of germination in an intact, viable seed under unfavourable conditions (Baskin and Baskin 2004; Finch-Savage and Leuber-Metzger 2006). In many plant species, endogenous abscisic acid (ABA) is known to be involved in the induction, and perhaps maintenance, of the dormant state, as well as in germination delay (Bais and Ravishankar 2002; Finch-Savage and Leuber-Metzger 2006). A decrease in ABA is usually detected at the start of the germination process due to synthesis suppression and catabolism (Balbuena et al. 2011). In *Rosa* spp. seeds, physiological dormancy is installed early in embryo development (Gudin 1994); bioassay (ELISA) (Pipino et al. 2013) techniques have detected high ABA levels during the late torpedo stage of embryos of floribunda roses 'Melglory' (1.20 pmol mg^{-1}) and 'Cassandra' (2.00 pmol mg^{-1}). Mature embryos are no longer dormant when their seeds are fully formed (Bo et al. 1995). At this stage, Pipino et al. (2013) found ABA decreased significantly in the two studied floribunda roses (0.40 and 0.20 pmol mg^{-1}, respectively).

Among other phytohormones, auxins seem to play a major role in embryogenesis and seedling growth. They provide positional information to embryos from the globular stage onward (Teale et al. 2006; Zhao 2010) and control cell division, elongation, and differentiation (Tromas and Perrot-Rechenmann 2010). The major auxin in developing seeds is indole-3-acetic acid (IAA) (Lee 1988). IAA has been shown to be involved in seed germination (Dewar et al. 1998; Guan and Scandalios 2002; Kucera et al. 2005) and can play a possible role in physiological dormancy (Ramaih et al. 2003). In *Arabidopsis thaliana*, dormant seeds contained less than half of the IAA in non-dormant seeds (Preston et al. 2009).

Only a few studies have been conducted to analyse IAA variation in rose seeds. Tillberg (1984) quantified IAA by reverse-phase, ion-pair high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) in mature *R. rugosa* var. *rubra* seeds during 14 weeks of stratification. He concluded that

endogenous IAA is low (0.74 pmol mg^{-1}) when germinability increases and germination is first visible. Kumar et al. (1995) used GC to analyse fully developed mature seeds of four *R. hybrida:* 'Priyadarshini', 'Queen Elizabeth', 'Delhi Princess', and 'Jantar Mantar.' They measured the mean content of IAA at 0.07 pmol mg^{-1}.

In order to understand better the physiological changes that take place during seed development and dormancy induction in hybrid tea roses (*Rosa* x *hybrida*), we systematically quantified ABA and IAA during the early-middle stage of seed development. Plant growth regulators were assessed at 28, 35, 42, and 49 days after pollination (DAP) in the seed coat (pericarp and *testa*) and embryo of *R.* x *hybrida* using high performance liquid chromatography (HPLC) based on solid phase extraction (SPE) purification.

Materials and methods

Plant material

Hybridisations were manually performed in the greenhouse of NIRP International (Bevera, Ventimiglia, Italy) between the end of May and the end of August 2011. One hundred hips from a cross (code 259) of tetraploid hybrid tea rose cultivars (female parent code 2364 x male parent code NC071101) were harvested at 28, 35, 42, and 49 days after pollination (DAP; 25 hips each developmental stage). They were then weighed, stored in polybags, immersed in liquid nitrogen (N_2), and maintained at -80°C until analysis. Later, hips were opened and their seeds were carefully excised using forceps and scalpel. Immediately, the seed coats (pericarp and *testa*) and embryos were collected separately and immersed in liquid nitrogen (N_2). To avoid analyte degradation, all analytical steps of the extraction procedures were performed at 4 °C, in the dark, and with amber glassware.

During hybridisation and hip development, monthly mean greenhouse temperatures were measured as 19.8 °C (May 2011), 21.7 °C (June 2011), 23.3 °C (July 2011), and 24.3 °C (August 2011).

Analysis of plant growth regulators by HPLC

ABA and IAA were assessed at 28, 35, 42, and 49 DAP in the seed coat and embryo of *R.* x *hybrida* by HPLC based on SPE purification, according to Bosco et al. (2013). Analytical reagent grade chemicals were used, unless otherwise indicated. Water (conductivity less than 0.05 μS/cm), methanol, and acetonitrile (Merck) were all of HPLC grade. ((±)-2-cis,4-trans-abscisic acid (ABA) and indole-3-acetic acid (IAA)**,** as well as SPE DSC-MCAX (bed wt. 300 mg, volume 6 mL) and SPE LC-NH2 (bed wt. 300 mg, volume 6 mL) cartridges were purchased from Sigma-Aldrich (Milano,

Italy). Stock standard solutions (1 mg mL^{-1}) of ABA and IAA were prepared using methanol as the solvent. All other standard solutions were prepared by dilution of the mother to obtain concentrations ranging between 0.0010 and 10 µg mL^{-1} ABA and 0.00010 and 10 µg mL^{-1} IAA.

The seed coat and embryo samples were weighed and ground in liquid N_2. Then, 0.3 g of each homogenized sample was suspended with 2 mL of 80% aqueous methanol containing 10-20 mg L^{-1} of butylated hydroxytoluene for 16 h at 4 °C in darkness under magnetic stirring. Extraction was performed as described by Bosco et al. (2013) using SPE DSC-MCAX and SPE LC-NH2 cartridges. A total of 20 µL of purified samples were injected into HPLC.

Chromatographic analysis was performed on an Agilent Model HPLC chromatographic system consisting of an HPLC series 1200 (Agilent Technologies, Böblingen, Germany) comprised of the following modular components: vacuum degassing unit, quaternary pump, auto injector, column oven, diode array detector (DAD G1315D), and florescence detector (FLD G1321A). The column used was a 150 mm x 4.6 mm i.d., 5 μm, Zorbax eclipse XDB-C18 (Agilent Technologies, Böblingen, Germany).

Statistical Analysis

Hip measurements, as well as the concentrations of ABA and IAA (pmol mg^{-1}) in seed coat and embryo were analysed at different DAP using one-way analysis of variance (ANOVA) and the Ryan-Einot-Gabriel-Welsch (F) post hoc test for multiple comparisons ($P < 0.05$). Prior to ANOVA, data distribution normality was tested by the Levene assumption of homoscedacity. Analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Hip Characterisation

The weights of the growing rose hips from 28 DAP to 49 DAP are shown in Table 1. Mean hip weight increased significantly over time and differed significantly at two instances. The first difference was observed at 35 DAP (5.03 g) when hips weighed was two times more than at 28 DAP (2.50 g). At 49 DAP, another significant increase in the weight of hips (7.55 g) was recorded. This value was three-fold that at 28 DAP. From 28 DAP onward, seed colour also changed slightly, from white-green to light brown, which is linked to the start of dehydration and pericarp hardening.

Endogenous ABA and IAA concentration

As shown in Figure 1, concentrations of endogenous ABA in seed coat and embryo generally decreased from 28 DAP forward. Specifically, seed coat ABA content peaked at 28 DAP (4.39 pmol mg^{-1}); a significant decrease was then observed at 35 DAP (0.45 pmol mg^{-1}; 0.10 times of initial content). Thereafter, during seed development, ABA concentration did not change significantly ($P<0.05$). A similar pattern was observed in embryo; in this case, ABA peaked at 28 DAP (1.36 pmol mg^{-1}) and troughed at 42 DAP (0.55 pmol mg^{-1}), which represented a relative reduction to 0.40 times of its initial concentration.

Figure 2 shows endogenous IAA concentration in seed coat decreased significantly between 28 and 35 DAP (0.66 pmol mg^{-1} and 0.12 pmol mg^{-1}, respectively); it reduced to 0.18 times of its starting value. Thereafter, it did not significantly differ. On the contrary, IAA content in embryo increased starting at 28 DAP (2.06 pmol mg^{-1}) and then peaked at 42 DAP (5.06 pmol mg^{-1}), a relative increase of 2.46 times its initial content. A further significant decline was detected at 49 DAP when the lowest IAA content was measured at 1.17 pmol mg^{-1}.

The ratio IAA/ABA in seed coat (Fig. 3a) was < 1.0 at all measured DAP and no statistical differences were observed. In embryo (Fig. 3b), the ratio was always > 1.0 and showed a tendency to increase from 28 DAP and forward to a maximum significant rate at 42 DAP (9.20). Later, a significant decrease was observed at 49 DAP as evidenced in the minimum measured rate of 1.10.

Discussion

Hip growth

This work focused on the early-middle seed development stage of *R.* x *hybrida*. During this period, we observed increased weight first at 35 DAP that equalled twice the weight at 28 DAP. Our results mirrored those reported by Guzicka et al. (2012) in *R. arvensis*, *R. spinosissima*, *R. virginiana*, *R. rugosa*, and *R. roxburghii* in which the increase was attributed mainly to enlargement of cells and supporting tissue, increased thickness of the hypanthium, and accumulation of seed reserves. A second increase, noted at 49 DAP, suggested the start of pericarp cell wall modification and consequent lignification. Pipino et al. (2013) described a similar pattern of relative increase in two floribunda roses ‘Melglory’ and ‘Cassandra’.

ABA and IAA concentration

158 Plant hormone study is not only hampered by the difficulty of measuring their extremely low concentrations in most
159 tissues, but also by interference in their determination from the presence of other substances. Accurate trace amount
160 quantification of these compounds demands robust methods. In this work, we used an analytical method that allowed
161 simultaneous detection of both ABA and IAA amounts. To clearly illustrate the dynamics of ABA and IAA in rose seed
162 coat and embryo during seed development, plant growth regulators were firstly separated by their hydrophobic and
163 acidic properties using anionic SPE, and then separated with HPLC (Bosco et al. 2013).

164 ABA is considered the likely primary cause of physiological dormancy in roses (Yambe et al. 1992; Bo et al.
165 1995; Zlesak 2006; Caser et al. 2011; Pipino et al. 2013). Using the HPLC method described above, we described ABA
166 dynamics from 28 DAP to 49 DAP. We found our results agreed with work by Finch-Savage and Leubner-Metzger
167 (2006) and findings of Taiz and Zeiger (2006) who all emphasized that ABA concentrations in embryo followed a
168 consistent trend: very low level during early embryogenesis, maximum level during mid- to late embryogenesis, and
169 decline to low levels when the seed reaches maturity. On the other hand, at the cotyledonary stage (28 DAP) in seed
170 coat, a high concentration of ABA (4.39 pmol mg^{-1}) was quantified, and then followed by a strong decay. The sizable
171 drop in ABA content to the basal level (0.45 pmol mg^{1}) after full embryo development might be related to termination
172 of the coat-imposed seed physiological dormancy, as proposed by Caser et al. (2011) and Pipino et al. (2013).

173 Several studies have been conducted to better describe the role of ABA in rose dormancy. Yambe et al. (1992)
174 put forth that a mature embryo is not dormant, but that ABA is instead localised in the inner surface of the pericarp to
175 inhibit germination. In fact, in germinating mature seeds of hybrid tea rose 'Inspiration', the authors detected ABA at
176 levels of 5.33 pmol mg^{-1} in seed coat and 1.48 pmol mg^{-1} in embryo. Even if ABA were primarily located in the
177 pericarp and the *testa* tissues of mature seeds, the studies required to say this have not been conducted on endogenous
178 ABA seed coat changes during the early-middle seed development stage in rose. Bo et al (1995) did indeed show later
179 that the ABA content in mature seeds of hybrid tea rose 'Crimson Glory' was highest in the *testa* (5.22 pmol mg^{-1}),
180 moderate in the pericarp (3.22 pmol mg^{-1}), and lowest in the embryo (0.68 pmol mg^{-1}).

181 Recently, Pipino et al. (2013) studied ABA concentrations in developing embryos of two floribunda roses,
182 'Melglory' and 'Cassandra'. The highest ABA levels were found during the early developmental torpedo stage (9
183 DAP). At 30 DAP, the same hips contained fully formed seeds andembryo ABA concentrations were significantly
184 reduced (0.40 and 0.20 pmol mg^{-1}, respectively). From these results, Pipino and co-authors concluded that initial high
185 ABA concentration is involved in the inhibition of precocious germination. In the present study, a similar ABA content
186 decrease was also observed from 28 to 49 DAP in *Rosa* x *hybrida* embryos. Differences among rose species could be
187 explained to the fact that during embryo development ABA levels are under maternal control (Raz et al. 2001).

In model plant *A. thaliana* accession 'Columbia', Kanno et al. (2010) quantified ABA levels during the various developmental stages after flowering. The work found that endogenous ABA levels in whole siliques reached a maximum in the middle of development at 9–10 days after flowering (DAF), and then peaked a second time during ABA accumulation during late development at 15–16 DAF. Next, to analyse part-specific ABA levels, the siliques were separated into seeds and other component parts (silique envelopes including the pedicles, receptacles, valves, replums, septa, and funiculi). Results from the component part analysis suggested that the first peak in ABA levels (9 DAF) is attributed to accumulation in seeds while the second increase in ABA levels near the end of development should be attributed to its accumulation in envelopes (Kanno et al. 2010).

Well documented is the crucial role that IAA plays in embryonic pattern formation (Moller and Weijers 2009). Activation of IAA biosynthesis has been reported (Stone et al. 2008); however, its physiological role during seed maturation is not clear and its study has been limited. Free IAA decreases during the imbibitions of *Sorghum* grains (Dewar et al. 1998), and auxin regulates catalase expression in the scutellum of germinating maize kernels (Guan and Scandalios 2002). A peak in free IAA coinciding with initial seed swelling during imbibitions in *A. thaliana* (Kucera et al. 2005) suggests it is important in regulating seed germination.

This work represents the first quantification of IAA in seed coat and embryo of *R. hybrida* during the early-middle seed development stage. In seed coat, IAA was always at basal values, suggesting that during these stages *testa* and pericarp are already well structured and that intensive cell activity is no longer on. Indeed, IAA was not found in *A. thaliana* seed envelope during seed development (Kanno et al. 2010). However, in rose embryo, IAA content increased until a maximum was achieved at 42 DAP, suggesting it has a role in embryo growth. In fact, during this period, cotyledons are growing within the seed and this peak could be indicative of strong cell activity in rose embryo. As described by Pipino et al. (2013), at 30 DAP embryos are at the end of late torpedo and at the beginning of cotyledonary when seeds are already well formed. During the process of seed development, the trend observed in this work was consistent with that reported in other plant species studies: soybean by Hein et al. (1984), almond by Koukourikou-Petridou and Porlingis (2001), *Prunus persica* by Wan et al. (2010), *A.s thaliana* seeds by Kanno et al. (2010), and wheat by Fischer-Iglesias et al. (2001). Silveira et al. (2004) reported that during seed development in *Pinus taeda*, IAA content grew continually from the globular stage until it reached the maximum at the cotyledonary stage. Thereafter, it declined dramatically in the mature seed, which seems to coincide with the demonstration by Kanno et al (2010) that most IAA accumulation in seeds occurred during the early stage of development.

In accordance with the most studied relationship between ABA and gibberellins during seed maturation 'crosstalk' between ABA and IAA during seed development, and in particular during germination, has been reported during periods of seed development in *A. thaliana* (Liu et al. 2007). This suggests that the decrease in ABA associated

with increased IAA levels in embryo observed in this work could be the result of crosstalk between these two plant growth regulators. In species such as *Leymus chinensis* (Ma et al. 2010), when the ratio of IAA/ABA > 1.0, it signals promoting activity during embryo development, as well as promotion of a high germination percentage later. Here, the ratio might suggest a stop to endodormancy caused by pre-cotyledonary ABA, and increased cell division in embryo during the cotyledonary stage that leads to increased hip weight as previously noted.

In conclusion, the presented data confirmed that ABA metabolites accumulate differentially in the seed coat and seed tissues during development, and that ABA, both in seed coat and embryo, decreases when seeds are still mature and embryos are completely developed. These effects mark the termination of physiological dormancy and the beginning of physical dormancy due to a thickening of the pericarp. Moreover, for the first time, this work shows that IAA is an important factor to rose embryo and seed development, and that crosstalk between ABA and IAA during seed development in *R.* x *hybrida* may exist. Further analysis, to measure IAA levels during early seed development (< 28 DAP), would be the logical next step to a better understanding of its role in rose embryo formation.

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316 **Table**

317

318 **Table 1.** Mean weight and relative increase of *R.* x *hybrida* hips noted at different days after pollination (DAP). The
319 developmental stage of embryos is based on Pipino et al. (2013).

Time	Weight		Developmental stage of the embryo
(DAP)	(g)	Relative increase	
28	2.50 c	1.00	Late torpedo
35	5.03 b	2.01	Cotyledonary (mature seed)
42	5.61 b	2.24	Cotyledonary (mature seed)
49	7.55 a[§]	3.02	Cotyledonary (mature seed)

320 [§]Different letter indicates significant differences at the 0.05 level, Ryan-Einot-Gabriel-Welsch (F) post hoc test.

321

Figure captions

Fig. 1 Abscisic acid (ABA) contents in rose seed coat (black) and embryo (grey) collected at 28, 35, 42, and 49 days after pollination (DAP). One-way ANOVA was performed separately for seed coat and embryo; results are expressed as the mean of three determinations. [§]Different letters represent significant differences ($p < 0.05$) at REGW-F post hoc test. Seed coat (upper case); embryo (lower case).

Fig. 2 Indole-3-acetic acid (IAA) contents in rose seed coat (black) and embryo (grey) collected at 28, 35, 42, and 49 days after pollination (DAP). One-way ANOVA was performed separately for seed coat and embryo; results are expressed as the mean of three determinations. [§]Different letters represent significant differences ($p < 0.05$) at REGW-F post hoc test. Seed coat (upper case); embryo (lower case).

Fig. 3 Changes in the IAA/ABA ratio in *R.* x *hybrida* seed coat (a) and in embryo (b) at 28, 35, 42, and 49 DAP. One - way ANOVA was performed. [§]Different letters represent significant differences ($p < 0.05$) at REGW-F post hoc test.

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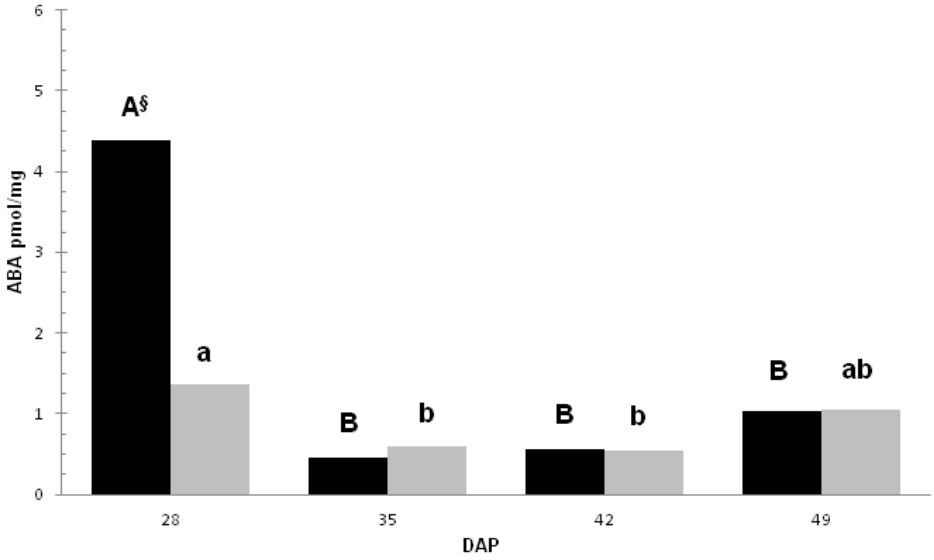


Figure 1

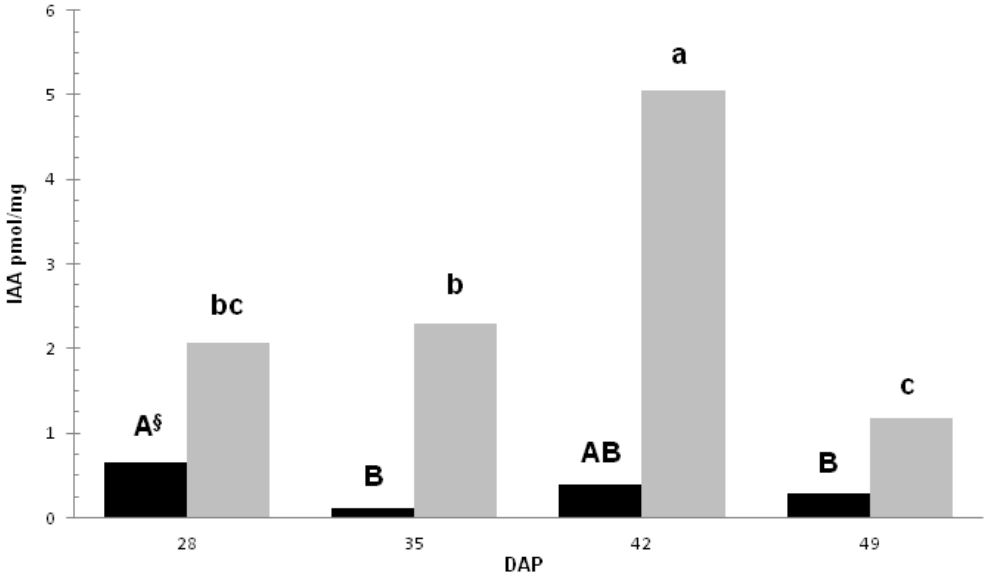
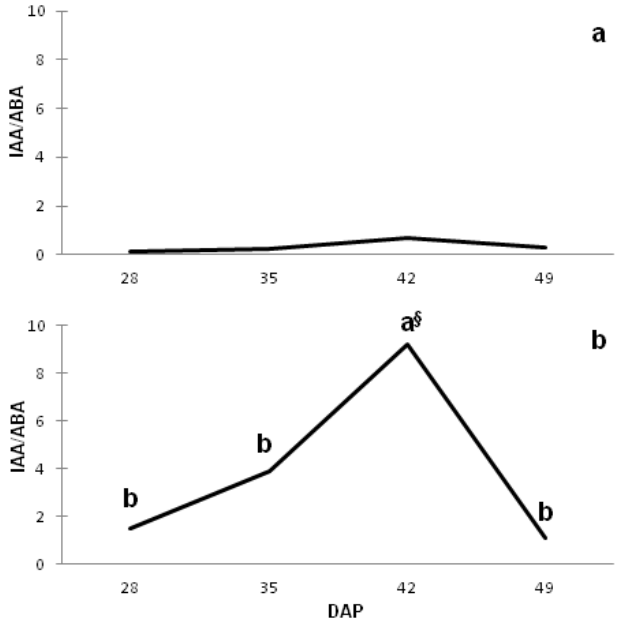


Figure 2


10
8
6
4
2
0
IAA/ABA
a
28
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42
49
10
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6
4
2
0
IAA/ABA
b
a§
b
b
b
28
35
42
49
DAP

Figure 3