ABSCISIC ACID IN SEED DEVELOPMENT AND GERMINATION OF CARROT[1]

CLAUDINEI ANDREOLI and JAMES D. MAGUIRE[2]

ABSTRACT - The levels of abscisic acid (ABA) in primary and secondary umbel seeds of carrots (*Daucus carota* L.) during development and maturation were determined by gas liquid chromatography and related to seed growth and germination. The level of ABA was high during development, reaching a maximum at 21-28 DAF, then declined sharply as seeds matured. The high content of endogenous ABA during development did not impair seed growth and germination; however, the germinability of both seed sources was completely blocked by exogenous 10^{-4}M ABA. This indicates that ABA might activate two specific mechanisms of action: one promotive during embryogenesis and another one inhibitory during remobilization. Nonetheless, the data demonstrated, for the first time in an umbelliferous crop, the endogenous level of ABA in developing seeds, and the effect of exogenously applied ABA on seed germination.

Index terms: *Daucus carota*, chromatography, flowering, ABA.

ÁCIDO ABSCÍSICO NO DESENVOLVIMENTO E GERMINAÇÃO DE SEMENTE DE CENOURA

RESUMO - Os níveis de ácido abscísico (ABA) em sementes de cenoura (*Daucus carota* L.) oriundas de umbelas primárias e secundárias durante o desenvolvimento e a maturação foram determinados pela cromatografia líquida gasosa e relacionados com o crescimento e a germinação das sementes. O nível de ABA determinado foi alto durante o desenvolvimento da semente, alcançando o máximo aos 21-28 DAF, decrescendo rapidamente na maturação. O alto teor de ABA endógeno durante o desenvolvimento não afetou o crescimento da semente e a germinação; entretanto, a viabilidade da semente de ambas as posições foi completamente bloqueada na presença de 10^{-4} M ABA exógeno. Isto indica que o ABA pode apresentar dois mecanismos específicos de ação: um estimulador durante a embriogenese e o outro inibidor durante a remobilização. Os dados demonstraram, pela primeira vez em plantas umbelíferas, o nível de ABA endógeno na semente durante o desenvolvimento e o seu efeito na germinação.

Termos para indexação: *Daucus carota*, cromatografia, florescimento, ABA.

INTRODUCTION

Various naturally occurring inhibitors, among coumarin and phenolic acids, were thought to be important in preventing seed germination, but after the discovery of abscisic acid (ABA), research began its possible role in regulating seed germination and dormancy. ABA is also thought to play an important part in the phase of seed development, in controlling embryogenesis and in transition from this phase to seed maturation and embryo germination. Recent reviews on the role of ABA are those by Walton (1980, 1981), King (1982) and Black (1983).

Considerable evidence from several species has accumulated over the recent years showing that ABA content changes during seed development and maturation. Most show a similar pattern - a fairly steep increase during early development succeeded by a

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[2] Agronomist, Ph.D., EMBRAPA/Serviço de Produção de Sementes Básicas (SPSB), Ed. Palácio do Desenvolvimento, 9º andar, SBN, Caixa Postal 040305, CEP 70057 Brasília, DF, Brazil.

sharp drop as the seeds mature. This has been reported in wheat (*Triticum aestivum* L.) (McWha 1975, King 1976, Radley 1976, 1979), cotton (*Gossypium hirsutum* L.) (Davis & Addicot 1972), peas (*Pisum sativum* L.) (Eeuwens & Schwabe, 1975), triticale (*Triticosecale*) (King 1979) soybean (*Glycine max* Merr.) (Quebedeaux et al. 1976, Ciha et al. 1978, Ackerson 1984a) and bean (*Phaseolus vulgaris* L.) (Hsu, 1979, Van Onckelen et al. 1980). The ABA increase is more or less parallel to the increase in dry fresh weight but falls after reaching its maximum. In peas, however, ABA content increases at a rather late stage after the dry weight has levelled off and when the growth rate has diminished, i.e., during the maturation of the seed. In bean, Hsu (1979) found two peaks of free ABA, one at 22 days and another at 28 days after anthesis.

The continued rise in ABA content during development and its rapid fall at maturity raises questions about the role of ABA in controlling development and embryogenesis. Attemps have been made to relate ABA level with three processes, namely, enzyme and protein synthesis, precocious germination, and seed growth.

During seed growth two aspects must be considered - growth and differentiation of the embryo (embryogenesis) and accumulation of reserves in storage tissue (cotyledons and/or the endosperm). The possible role of ABA in embryogenesis (zygotic and somatic) has been discussed in detail by several authors (Ammirato 1974, Choinski et al. 1981, Dure 1975, King 1982, Rajaseharan et al. 1982, Walbot 1978). Abscisic acid evidently does not inhibit embryogenesis or the accumulation of food reserves, since these reserve processes both occur when the ABA level in the seed is high. Walbot (1978) pointed out, for instance, that ABA does not affect the linear increase in fresh and dry weight of *Phaseolus vulgaris* embryos; and King (1976) has shown that in wheat the fast-growing grains contain the most ABA. Indeed, rather than inhibiting the accumulation of dry matter, ABA might possibly play a positive role in regulating the build-up of reserves (McWha 1975, Tietz et al. 1981, Ackerson 1984b). Two reported effects of ABA are the inhibition of specific RNA synthesis and translation (Ho 1983, Ho & Varner 1976). Obviously the transcription and translation processes involved in the synthesis of stored reserves are not inhibited. Abscisic acid might, however, not only suppress the synthesis of certain germination enzymes, but also induce another set of proteins involved in embryogenesis (Dure 1975, Dure et al. 1981, Walbot 1978, Ackerson 1984a). Therefore it is crucial to realize that ABA, as one of the growth regulators, serves to coordinate the overall physiology of plants. This could be accomplished either by suppressing or by promoting certain physiological processes. It is inappropriate to view ABA simply as an inhibitor in seed germination or other cellular processes. In cereal seed, attempts have been made to relate ABA action to the control of production of the hydrolytic enzyme, α -amylase. In part, this relationship is based on the well-known ABA inhibition of GA-induced α -amylase synthesis in the endosperm of mature germinating grain (Ho & Varner 1976, Ho 1983, Higgins et al. 1976). It would be interesting to determine whether this relationship extends back in time during seed development into the period of grain development. Evidence for this proposition is that at the time that ABA content declines at desiccation of the maturing cereal grain, GA-induced of α -amylase becomes possible (Evans et al. 1975, King 1976, Nicholls 1979, King & Gale 1980). Also, by artificially drying immature grain it has been possible to induce α -amylase in response to added GA (King 1976, Nicholls 1979, King & Gale 1980), but drying also enhances the degradation of applied ABA (King 1979).

In umbelliferous crop species, few reports have dealt with the hormonal regulation of seed growth, germination and dormancy. The presence of inhibitors in some umbelliferous fruits has been reported (Aki 1962, Aki & Watanabe 1961, Chatuverdi & Muralia 1975).

Aki (1962), working with carrot seed, found that the content of the inhibitor "carrotol" reached a maximum in the immature seed 31 days after flowering, and was found in the pericarp. Moreover, they determined that carrotol content of these seeds did not change during storage. However, the level of endogenous abscisic acid during seed development and post-maturation of carrot seed have not been determined.

The objectives of this study were: to determine the levels of ABA during development and post-maturation of carrot fruits on different umbel orders, and to relate the levels of ABA to seed growth and germination.

MATERIALS AND METHODS

Seed production

Carrot plants (*Daucus carota* L. hybrid cv. Spartan Bonus) were grown by the seed-to-seed method at the Othello Experiment Unit in the Columbia Basin, in Washington, during 1982 and 83. Seeds were planted in August, vernalized over winter, and plants produced seed the following August. Irrigation by furrow was applied twice weekly throughout post-anthesis and maturation. Because of the heavy application of fertilizer to the previous crop, no fertilizers were applied in 1982.

Effect of harvest

Just as flowering started, four replicated blocks of 50 carrot plants in each plot were selected for uniformity in plant size and maturity. When the stamens on the flowers of the primary of a plant were visible, the primary umbels were labeled with colored tags. Five plants of the same size and date of flowering were chosen at random and umbellets from the same radius of the primary and secondary umbels were harvested 7, 14, 21, 28, 35, 42 and 49 days after anthesis. The umbels were immediately placed on dry ice and brought to the laboratory. The developing and mature seed from each umbel position at each harvest were freeze-dried and stored at -20°C until extraction for ABA analysis. Changes in seed development were determined on samples of 400 fertilized ovules per lot at each harvest. These were weighed fresh and after oven-drying at 105°C for 24 hours. In addition, germination of freshly harvested seeds from each umbel was determined from each harvest stage. One hundred seeds were placed on filter moistened with either water or 10^{-4} M ABA solution in Petri dishes and incubated at 20°/30°C, with 12/12 h alternating light/dark (Association of Official Seed Analysis 1976). After 10 days the number of normal healthy seedlings was recorded.

Abscisic acid determination-Extraction

The overall scheme for the extraction purification from developing and mature seed for analysis of free ABA was the same as outlined by Quebedeaux et al. (1976), and Ciha et al. (1977). One to 4 g of carrot seeds were weighed, homogenized in 80% (v/v) cold methanol containing 100 mg of 2,6 ditert-butyl-4 -methyphenol (BHT) per liter transferred to 250 ml Erlenmeyer flasks and shaken for 24 hours at 40°C. A known aliquot of C + ABA was added to the extracts to determine the efficiency of ABA recovery from the seed extract. The homogenate was centrifuged and the supernatant was filtered through methanol-washed nº 1 Whatman filter paper, and then taken to dryness in vacuo at 35°C and stored at 20°C.

Preparative high performance liquid chromatography (HPLC)

The dried samples from the extraction were reconstituted in a known volume of 5% (v/v) ethanol in 0.2 N acetic and centrifuged at 1100xg for 10 min. A known volume of filtered supernatant was injected into a HPLC apparatus (Waters, Model 1600) controlled by a solvent programmer (Waters, Model 660) as described previously (Ciha et al. 1977). The sample was then eluted with a linear gradient of ethanol (5%-50% in 30 min.) in 0.2 N acetic acid at a flow rate of 5 ml/min. The eluate fraction containing ABA was collected based upon the retention time of an ABA standard with an UV detector at 254 mm. Aliquots were removed for counting in a scintillation counter as described by Ciha et al. (1977) and Quebedeaux et al. (1976). The recoveries of the ^{14}C-ABA were consistently between 40%-56%. The fraction was taken to dryness in vacuo at 35°C and stored at -20°C for gas-liquid chromatography (GLC) analysis.

Analytical quantification-gas-liquid chromatography (GLC)

All ABA samples for GLC electron capture analysis were methylated using diazomethane

(Schelenk & Gellermann 1960), dried and reconstituted in methanol for GLC. One µl sample was automatically injected into the gas-liquid chromatography apparatus (Model Varian 700) connected with an integrator (Model Varian CS 111). Operating conditions were: column temperature, 202°C; injector and detector temperatures, 234°C and 284°C, respectively; carrier gas (N) flow rate 30 ml/min. The methylated ABA sample peaks were integrated and the amounths of ABA calculated based on a calibration curve of known amounts of methylated ABA standards. ABA levels were corrected for C-ABA recovery in each sample as in Ciha et al. (1977).

RESULTS AND DISCUSSION

Seed development and maturation

Dry weight and moisture content changes of developing primary (Fig. 1) and secondary umbel seeds (Fig. 2) of carrot were determined from anthesis to maturation. Seeds from both umbel orders developed in typical fashion, growing rapidly between 7 and 28 days after anthesis. Seed dry weight reached a maximum at 35 and 42 DAF in the primary and secondary umbels, respectively. At that time, seed moisture content started to fall about 3% to 4% per day, but the rate decreased to 0.81% per day in the last 7 days. The changes observed in dry weight and seed moisture contents of developing carrot seeds are similar to those previously reported (Gray & Steckel 1982, Gray et al. 1984).

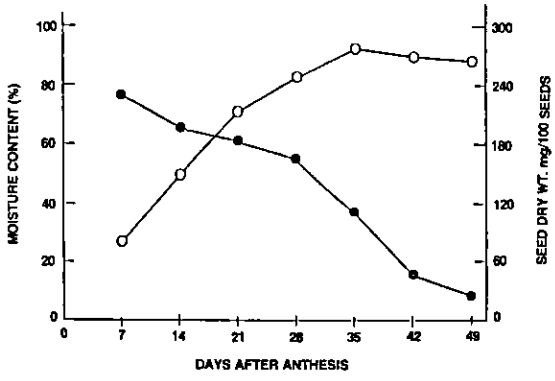

FIG. 1. Changes in dry weight (○——○) and moisture content (●——●) with time in primary umbel carrot seeds. Each point represents the mean of four replicates.

Seed Germination

Germinability of primary and secondary umbel seeds increased rapidly from zero, beginning about 14 days and 21 days after anthesis and reaching a maximum about 42 days and 49 days later, respectively (Fig. 3

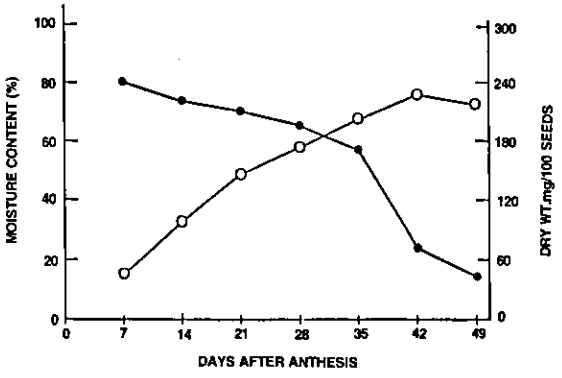

FIG. 2. Changes in dry weight (○——○) and moisture content (●——●) with time in secondary umbel carrot seeds. Each point represents the mean of four replicates.

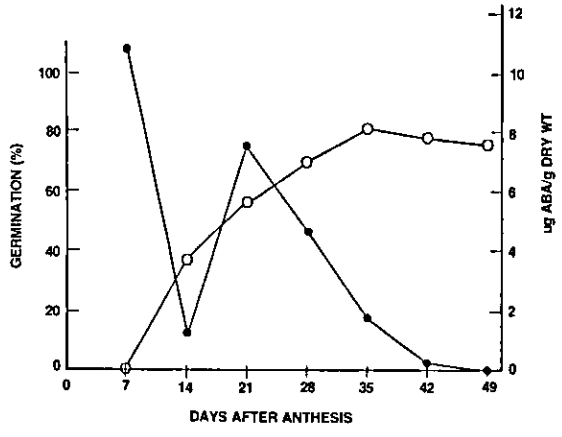

FIG. 3. Germination percentage (○——○) and endogenous ABA content (●——●) in primary umbel carrot seeds during development and maturation. Each point represents the mean of four replicates.

and 4). This increase in germination was completely blocked by exogenously applied 10^{-4} M ABA (Fig. 5A and B). Seeds taken from primary and secondary umbels of the parent plant were capable of germinating before the maximum seed dry weight stage, when seed moisture content was still high (Fig. 1 and 2). However, even after this stage, when the moisture content had dropped below 30%, seed germination continued to increase.

ABA content

A typical gas-liquid chromatogram of an ABA sample (methyl derivative) of carrot is shown in Fig. 6. This is the first time, as far as we are aware of, that ABA in developing seed of carrot has been identified and quantified by gas-liquid chromatography.

Abscisic acid could be detected in all seed extracts sampled throughout the course of seed development. In developing primary umbel seeds (Fig. 3), the ABA level was unexpectedly high, with maximum levels up to 10.85 µg/g dry weight. These values, found 7 days after anthesis, are the highest reported in the literature for seeds using similar analytical procedures (King 1982, Black 1983, Hsu 1979, Quebedeaux et al. 1976). The latter authors have also reported high concentration of ABA in developing soybean seeds during the active growth stage of pod filling (15 days post-anthesis). Compared with the developing primary umbel seeds, the secondary umbel seeds in general contain smaller amounts of ABA (Fig. 5). The level of ABA from both seed sources on a µg/g dry weight basis was high at 7 days post-anthesis, declined shortly, reaching a second peak between 21 and 28 days, and then decreased steadily to less than 0.2 µg/g dry weight at maturity. The period of greatest ABA content in the seed coincided with rapid growth in seed filling and increasing germinability. It is also interesting to note that, at this stage, maximum

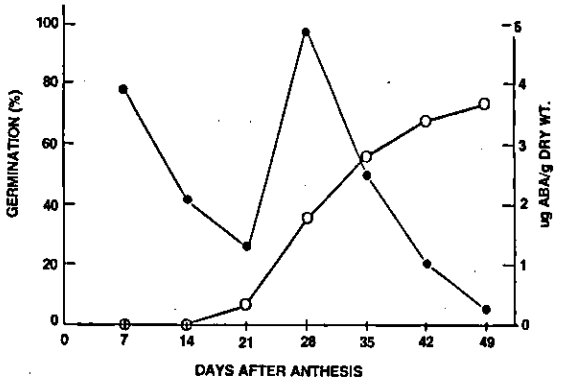

FIG. 4. Germination percentage (○——○) and endogenous ABA content (●——●) in secondary umbel carrot seeds during development maturation. Each point represents the mean of four replicates.

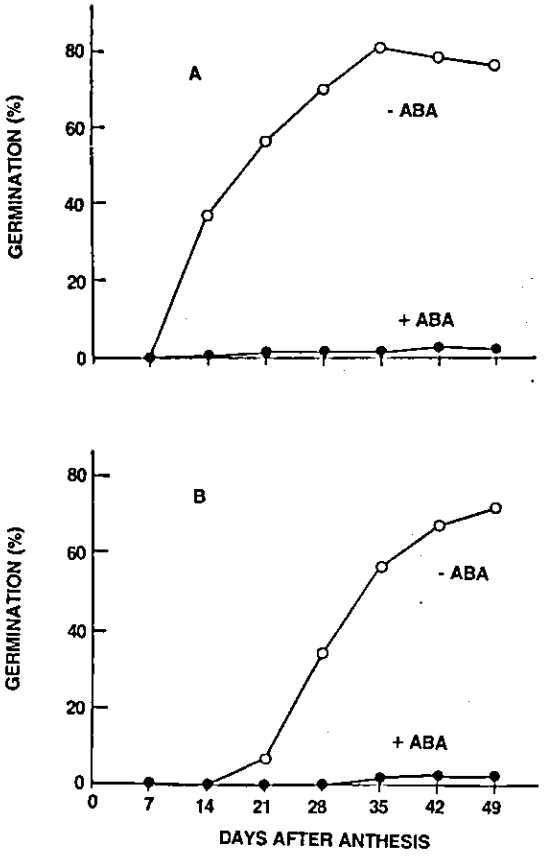

FIG. 5. In vitro germinability of primary (A) and secondary umbel (B) seeds of carrots during development an maturation. Seeds were incubated in Petri dishes in either water or 10^{-4}M ABA.

endosperm volume and active ambryo growth were observed (Gray et al. 1984).

The high level of ABA found in the young fruit from primary and secondary umbels (Fig. 3 and 4) was also observed in very young pea pods (Eeuwens & Schwabe 1975) and young bean fruits (Hsu 1979). The cause of this early high level of ABA was not investigated. It may represent high levels of ABA in those fruits that are not those which will abscise within the next few days. Early post-anthesis collections contain both fertilized and unfertilized fruits because it is not possible to distinguish them in the first few days after anthesis. Furthermore, Gray et al. (1984) showed that carrot embryos do not start to divide and grow until 21 days after anthesis.

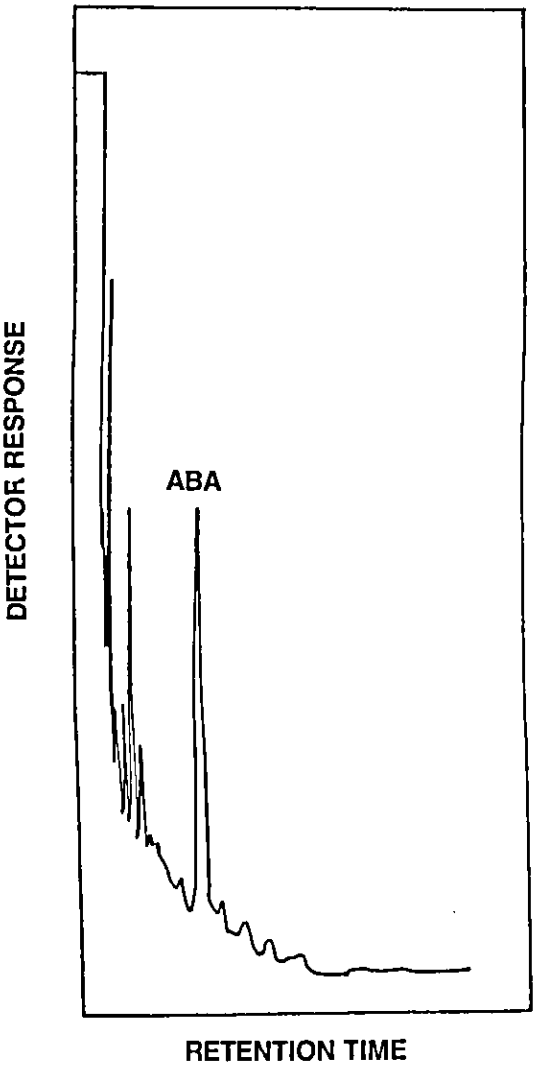

FIG. 6. Gas-liquid chromatograph of an ABA sample extracted from carrot seeds. The *retention time was based on standard* menthyl-ABA retention time.

Rapid increases in the rate of seed dry weight and the onset of germinability of primary and secondary umbel seeds from 21 days to 28 days post-anthesis (Fig. 1 and 2) appear to be correlated with a rapid rise in ABA levels in the developing seed (Fig. 3 and 4). It is clear that relatively high ABA concentrations do not interfere with development of seeds, but may be implicated in preventing precocious germination (Ackerson 1984b, King 1976, Van Onckelen et al. 1980, Hendrix & Radin 1984). However, in our study the germinability of both primary and secondary umbel seeds was completely blocked by 10^{-4} M exogenous ABA. Recent work on the influence of ABA on protein synthesis in cultured cotton embryos (Choinski et al. 1981) indicates that inclusion of ABA in the medium prevented precocious germination and allowed continued increases in catalase, malate dehydrogenase, citrate synthase, aspartate aminotransferase, and β -oxidation enzyme activities as well as *de novo* synthesis of malate synthase.

Several important questions related to ABA in carrot seeds need to be resolved: What is the site of origin for ABA which is eventually transported to the embryo? What is the role of maternal tissues in ABA metabolism and transport? It was observed that during maturation, when the seed moisture content declined sharply, there was a rapid decrease in the endogenous level of ABA (Fig. 3 and 4). It can therefore be speculated that seeds acquire the capacity to metabolize ABA and, as long a seeds remain by vascular connection to the mother plant, the ABA metabolism appears somehow inhibited, which results in a delayed but gradual decrease of endogenous

ABA. In summary, the ABA levels for developing carrot seeds are much higher than those reported for other crop species. For the first time, the endogenous ABA in developing seeds of an umbelliferous crop and the influence of exogenously applied ABA on seed germination have been measured.

CONCLUSIONS

1. Seed dry weight reached a maximum at 35 and 42 DAF in the primary and secondary umbel, respectively.

2. Germination starts at 14 and 21 DAF, and reaches a maximum at 42 and 49 DAF for primary and secondary umbel.

3. The level of ABA was high during development, reaching a maximum between 21-28 DAF, then declined sharply as seeds matured.

4. The high level of endogenous ABA during development did not impair seed growth and germination.

5. Germination of both seed sources was completely blocked by exogenous 10^{-4} M ABA.

6. ABA might activate two specific mechanisms of action: one promotive of enzymes during embryogenesis and another inhibitory during remobilization.

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