Temporal and Spatial Distribution of Hormones in Peanut Ovules During Early Development

Q. L. Feng[1], S. C. Mohapatra[2], H. E. Pattee[3], and H. T. Stalker[1]*

ABSTRACT

Study of hormonal regulation of fruit development requires quantitative measurement of reproductive tissues at different developmental stages. This study reports the application of ELISA (enzyme-linked immunosorbent assay) for the measurement of free indole-3-acetic acid (IAA), dihydrozeatin riboside (DHZR) and abscisic acid (ABA) in ovules of peanut (*Arachis hypogaea* L., cv. NC 6). IAA levels in apical and basal ovules gradually increased with aerial peg elongation from 4 to 10 d after pollination (DAP), but decreased when peg elongation ceased by 14 DAP. Higher DHZR levels were observed in aerial ovules at 10 DAP, and the ABA level was highest at 1 DAP. Aerial apical ovules contained higher IAA and DHZR levels than basal ovules. At day 1 after soil penetration (DASP), IAA and ABA levels in both ovules decreased, whereas DHZR levels increased. Peg tip swelling and ovary enlargement at 5 DASP were accompanied by increased levels of all three hormones. The highest levels of the hormones were observed in both ovules at 7 DASP, i.e., when embryos were at the globular stage and ovaries were rapidly enlarging to form pods. Basal ovules also had higher hormonal levels than apical ovules after soil penetration. Progressive changes in hormone levels during peanut ovule and embryo development suggest a physiological relationship between the two events.

Key Words: Indole-3-acetic acid, dihydrozeatin riboside, abscisic acid, enzyme-linked immunosorbent assay, ovule, embryo, peanut, *Arachis hypogaea*.

Regulation of fruit development by different plant hormones has been firmly established, and has been implicated in peanut (*Arachis hypogaea* L.) fruit (pod) development (Ziv and Zamski, 1975; Zamski and Ziv, 1976). Studies on hormonal changes during progressive fruit development (temporal) and in different fruit parts (spatial) require quantitative measurement of the hormone(s) of interest. Because of the large quantities of tissues required for this purpose, most attempts to quantify endogenous hormones during peanut pod development have relied on bioassays alone (Jacobs, 1951; Lee *et al.*, 1972) or bioassays in combination with gas chromatography (Feng and Pan, 1989). Shlamovitz *et al.* (1995) used RIA (radioimmunoassay) to determine IAA and ABA levels and ELISA (enzyme-linked immunosorbent assay) to confirm IAA quantities and GC methodology to confirm ABA levels in peanut pegs and immature pods. They found small differences in IAA levels during pegging to pod initiation stages, but a significant decrease in ABA levels over the same period. The objectives of this study were to (a) apply ELISA to hormones of peanut ovules, (b) develop ABA and DHZR assays in addition to IAA, and (c) include analyses of early developmental stages of peanut reproductive tissues which have not been previously reported.

Materials and Methods

Plant Materials and Ovule Isolation

Plants of *A. hypogaea* cv. NC 6 were grown in 15 × 15-cm pots to prevent peg penetration to the soil and in soil beds for normal fruit production. Flowers at anthesis were identified daily by placing colored tags at the reproductive node. Aerial pegs were excised 1, 4, 7, 10 or 14 d after pollination (DAP). A second tag was attached to pegs on the day they reached the soil surface. Pegs that had just reached the soil surface at 9 DAP and ones at 1, 3, 5, or 7 d after soil penetration (DASP) were collected. Corresponding pegs also were collected from potted plants where peg penetration to the soil was prevented. Excised pegs were placed on dry ice (-60 C) in the greenhouse and carried to the laboratory. Apical and basal ovules were excised under a microscope at room temperature (24 C) and then stored at -80 C until use. Further, all samples were handled by the same methodology to equalize the temperature effects. Separate samples consisting of 80 to 120 ovules were used for each hormone. Sampling was repeated three times for each of the three hormones and for each of the developmental stages listed above.

Hormone Extraction

Frozen samples were homogenized in 1 mL of 80% cold (4 C) methanol containing 20 mg L^{-1} butylated hydroxytoluene and 10 mL L^{-1} acetic acid. Each sample was spiked with the respective standard hormone during homogenization to estimate percentage recovery. The slurry was stored overnight at 4 C and then filtered. Extraction was repeated two more times on the residue with the above solvent but for 8 hr each. The liquid phases were pooled and centrifuged at 4000 g for 5 min at 4 C. The supernatant volume was measured and diluted with 50% methanol. The diluted samples were passed through a C_{18} reverse phase cartridge (1 mL, Supelclean LC-18, SUPELCO) at the rate of 1 mL min^{-1} to remove pigments. The eluate was collected and evaporated to dryness with a stream of nitrogen. Samples used for ELISA of ABA or DHZR were dissolved in 200 mL of 25 mM Tris buffered-saline (TBS), pH 7.5, whereas samples used for IAA were first methylated with 250 mL of ethereal diazomethane for 20 min (Cohen, 1984) and then dried under a stream of nitrogen. The samples were then dissolved in 200 mL of 25 mM TBS and centrifuged at 4000 g for 5 min to remove insoluble material.

[1]Former Res. Assoc. and Prof., Dept. of Crop Science, North Carolina State Univ., Raleigh, NC 27695-7629.

[2]Prof., Dept. of Biol. and Agric. Eng., North Carolina State Univ., Raleigh, NC 27695.

[3]Prof., USDA-ARS, Dept. of Botany, North Carolina State Univ., Raleigh, NC 27695.

*Corresponding author.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed with assay kits obtained from IDTEK, Inc. (San Bruno, CA). Step-by-step procedures based on Weiler's (1982) approach were provided by the supplier(s) and are summarized below.

Precoated microplates were used for DHZR whereas microplates were coated in the laboratory for IAA and ABA. The first layer was polyclonal goat anti-mouse antibodies (0.25 mg mL^{-1} antibody in 50 mM $NaHCO_3$, pH 9.5, Jackson Laboratories, Inc., West Grove, PA) for the respective hormone. The second layer was the respective monoclonal antibodies (0.1 mg mL^{-1} $NaHCO_3$, pH 9.5) which were anchored to the polyclonals. The potential nonspecific binding sites were then blocked with rabbit serum albumin (200 mL, 10 mg mL^{-1} in 25 mM TBS) obtained from Sigma Chemical Co. (St. Louis, MO). The coated microplates were divided into two batches. One batch was used for standard curve development and the other batch was used for hormone quantification of the plant extract. For standard curve development, each well contained 100 mL of hormone-alkaline phosphatase conjugate and a known amount of standard hormone (varying between 0 to 10 pg in 100 mL TBS). For the plant extract assay, each well contained the same amount of hormone-enzyme conjugate and 100 ml of crude plant extract or fractions collected from the Supelclean LC-18 column. This was followed by the addition of 200 mL of the substrate *p*-nitrophenylphosphate (200 mL, 25 mg mL^{-1} DEAE buffer, 0.9 M, pH 9.8, Sigma Chemical Co). Color was developed by incubation for 1 hr at 37 C and absorbance was measured at 405 nm in a microplate reader (EL307C, BIO-TEK Instruments, Winooski, VT).

In the ELISA protocol used in this study, the hormone-enzyme conjugate had to compete with free hormones (in the standard or in the plant extract) for sites in the respective monoclonal antibodies. When the proportion of free hormone was increased in the same mixture, the binding by the hormone-enzyme conjugate decreased proportionately, which in turn decreased the color when the substrate was added after washing and removal of unbound hormones and hormone-enzyme conjugates. Thus, the standard curve showed an inverse relation between free hormone content and the absorbance at 405 nm.

High Performance Liquid Chromatography (HPLC)

ELISA data for each hormone were validated through HPLC with the earliest developmental stage (10-d old aerial peg, 1 cm long including the ovary and the meristems) where sufficient tissues could be obtained. Approximately 10 g of tissue were homogenized with 80% cold methanol. The slurry was maintained at 4 C for 8 hr followed by filtration. The residue was extracted two more times following the same procedure. The pooled extracts were evaporated *in vacuo* to remove methanol. The aqueous phase was adjusted to pH 7.0 with 1 N NaOH and partitioned three times with diethyl ether and then subdivided into two parts. For IAA and ABA, the aqueous phase from the above partition was adjusted to pH 2.5 with 1 N HCl and partitioned three times with diethyl ether. For DHZR, the aqueous phase was adjusted to pH 8.0 with 1 N NaOH and partitioned three times with *n*-butanol. The organic phases were reduced to dryness *in vacuo* and dissolved in 1 mL of 50% methanol. The 50% methanol solutions were passed through C_{18} reverse phase cartridges and the eluate was collected and dried *in vacuo*. These semipurified samples were dissolved in 200 mL of 15% methanol in 1 mM citrate buffer, pH 6.0.

HPLC was performed with a Supelco LC-18 reverse phase column (3 mm, 150 mm × 4.6 mm I.D.). Solvent systems and programs used for IAA, ABA, and DHZR were adapted from Sweetser and Swartzfager (1978), Rose *et al.* (1987), and Eberle *et al.* (1986), respectively.

Results and Discussion

Hormone Assay

ELISA methodology for measurement of hormone concentrations in peanut was first validated using HPLC. First, peaks in the plant extract were compared with standard peaks with respect to retention time. Standard hormones for IAA, t-ABA, ABA, zeatin, zeatin riboside and DHZR eluted at 7, 5, 10, 22, 23 and 24 min, respectively. Peanut pod extracts gave peaks not only at these times but also at other times. Second, plant extract eluates corresponding to each peak were subjected to ELISA. This showed that peaks corresponding to the above retention times also gave maximum immunoreactivity for each hormone studied. With about 10 g of sample for each hormone, HPLC yielded values of 143, 119 and 1725 pg of IAA, DHZR and ABA mg^{-1} fresh tissue. For the same hormones, with 1 g of tissue, ELISA gave values of 164, 166 and 1708 pg mg^{-1} fresh tissue. Both the HPLC and ELISA values and variability are comparable to those published in the literature for other species (Weiler *et al.*, 1981; Weiler, 1982; Walker-Simmons, 1987). It was therefore concluded that ELISA can be used reliably to quantify endogenous hormones in peanut pods. Further examination showed that ELISA could detect as little as 0.05 pmol of IAA and 0.02 pmol of each ABA and DHZR. Because typical hormone concentrations in plant tissues range between 1 and 4 pmol mg^{-1} tissue (Brenner, 1981), the method used in this study had the requisite sensitivity. It was therefore possible to conduct ELISA with 1 g or less fresh tissue as opposed to the 10 g of tissue that would have been needed (but would not have been easily available) for HPLC.

Temporal and Spatial Changes

Although each peanut pod usually contains two seeds, the pods vary in size; and within a given time frame after fertilization, they also vary in orientation relative to the ground surface and to the peg axis to which they are attached. It is well known that hormonal distribution (especially auxins) within plant tissues is often influenced by the orientation of the plant organ of interest. While this consideration could not be ignored, collecting sufficient quantities of tissues with different orientations without depending on seasonal production of the field crop would have been nearly impossible. Therefore, the orientation question was addressed indirectly before the hormone study was undertaken. It was postulated that if hormone quantity was influenced by the orientation of the pod (i.e., whether the pod was in a vertical, horizontal or slanted position at an angle between horizontal and vertical), then there should be a consistent relationship between the size of the pod (and seeds therein) and the pod orientation. Examination of pods with different ori-

entations did not reveal relationships between pod orientation and either pod or seed size. It was therefore concluded that pod orientation would not be a major determinant of hormone distribution. However, because this could not be ruled out conclusively, care was taken to include pods of different orientation at the time of sample collection.

Temporal distribution relates to developmental stages representing time whereas spatial distribution relates to distribution in apical and basal ovules. Unlike other fruits, peanut pod development follows two clearly different but sequential time scales (Pattee and Mohapatra, 1987). The first scale refers to DAP whereas the second scale refers to DASP by the peg. Although DASP is a continuation of DAP, this time scale is not applicable to tissues which remain as aerial pegs and do not enter the soil.

IAA. Apical and basal ovules of aerial pegs contained approximately 8 pg IAA at 1 DAP, but the level decreased by 6 and 40% by 4 DAP, respectively (Fig. 1A). IAA levels started to increase again at 4 DAP and reached its maximum level by 10 DAP. During this period, the peg was elongating while embryo growth was suspended. Because IAA mediates cell extension (Evans, 1984) and is transported from the apex to the base of a peg (Jacobs, 1951), the increased IAA content in the ovules may facilitate its transport to the meristem where it stimulates cell division and elongation. IAA levels declined in aerial ovules by 14 DAP, which contributed to retardation of elongation in these pegs.

In contrast to aerial pegs, IAA levels decreased in both ovules of the subterranean pegs at the equivalent developmental stage (for example, 1 DASP or 10 DAP). Thus, IAA levels in apical and basal ovules were 39 and 5% lower than those prior to soil penetration (9 DAP) (Fig. 1A). This contrasts with the results of Shlamovitz *et al.* (1995) who reported white pegs, which correspond to pegs in this study after soil penetration, increasing in IAA levels; and stage I pods returning to the initial IAA level of green pegs. The difference in the two studies is likely due to Shlamovitz *et al.* (1995) sampling entire peg or pods rather than using ovule tissues as done in this report. Decrease in IAA levels in ovules may result in a corresponding decline in IAA transport to the elongation zone, thus retarding peg elongation immediately after pegs penetrate the soil. However, IAA levels in apical ovules increased again between 1 and 3 DASP (10 and 12 DAP) whereas the IAA level in basal ovules decreased slightly during this period. IAA levels in both apical and basal ovules then sharply increased at 5 DASP (14 DAP), with the highest levels observed at 7 DASP (16 DAP) (Fig. 1A). IAA levels were four (for basal ovules) and six (for apical ovules) times higher than those at 1 DASP. At this time, the ovary was enlarging rapidly and the embryos reached the late globular or early heart stage. Thus, changes in the ovular auxin level appeared to be associated with ovary enlargement and cell division. High levels of IAA also have been found in the early stages of embryo development in tomato (Hocher *et al.*, 1992) and carrot (Michalczuk *et al.*, 1992). Liu *et al.* (1993) reported that synthesis and polar transport of auxin in globular embryos of *Brassica juncea* (L.) are essential for the establishment of bilateral symmetry, including cotyledon formation.

The IAA level was always higher in the basal ovule than the apical ovule in subterranean pegs, whereas the reverse was observed in aerial pegs of equivalent stages. In absence of experimental evidence for the physiological and developmental significance of this reverse relationship, we postulate that higher concentrations of the hormone in the apical ovule of an aerial peg may help establish a concentration gradient so that the hormone

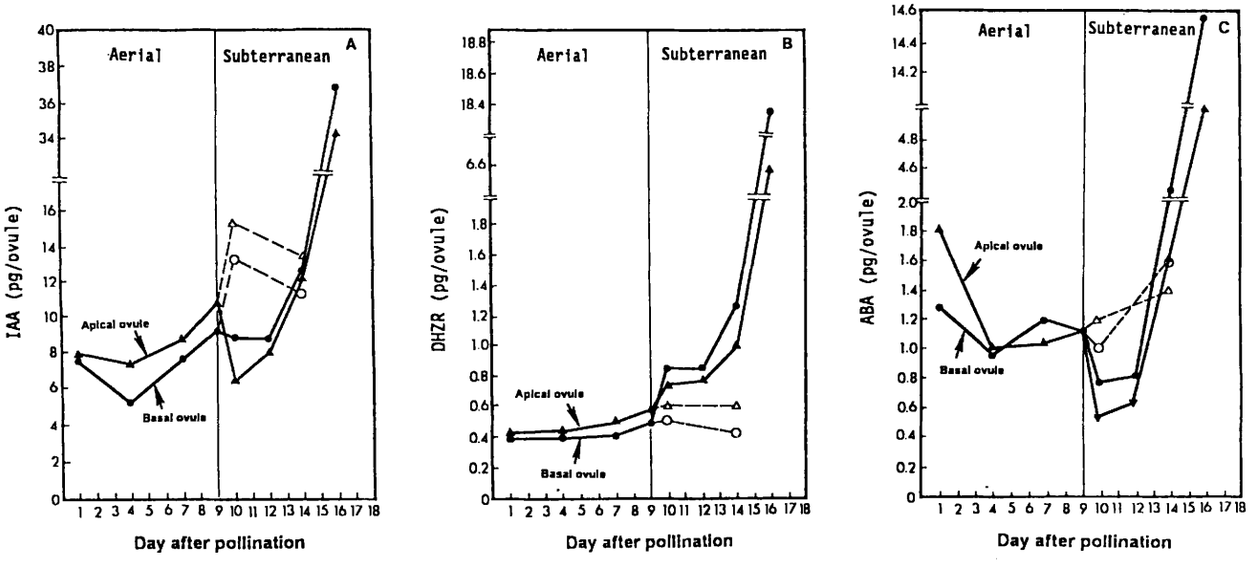

Fig. 1. Hormone levels in aerial and subterranean ovules of *A. hypogaea* cv. NC 6. Data are the means of three-replicate extractions of peanut ovules. The dotted lines show the hormonal changes in the ovules of pegs which were suspended in the air rather than penetrating into the soil. (A) IAA levels, (B) DHZR levels, and (C) ABA levels.

can be transported to the peg meristem. However, once the peg penetrates the soil and meristem activity is retarded, accumulation of hormone in the base of the ovary may occur and result in higher levels of the hormone in basal ovules. This may partially explain observations by Pattee and Mohapatra (1987) that basal ovules and embryos develop earlier and faster than apical ovules and embryos.

DHZR. DHZR level in aerial ovules increased slightly between 1 and 10 DAP (Fig. 1B). Because cytokinin usually is not polarly transported in plant tissues (Wareing and Phillips, 1978), this increase probably does not contribute to peg elongation due to activity of intercalary meristems. DHZR decreased in aerial pegs after 10 d. However, after pegs penetrated the soil, DHZR levels in both ovules at 1 DASP (10 DAP) were 22 and 75%, respectively, higher than on the day prior to soil penetration (9 DAP). The levels were maintained until 3 DASP (12 DAP) (Fig. 1B), after which the DHZR level increased significantly. By 7 DASP (16 DAP), DHZR levels were 20 times higher in basal ovules and eight times higher in apical ovules than those at 1 DASP. The possible function of cytokinins in the early stages of plant embryogenesis is to stimulate cell division of the embryo (Danin *et al.*, 1993) and endosperm (Tollennar, 1977). Apical ovules appeared to have higher DHZR levels than basal ovules at aerial stages, whereas the situation was reversed after soil penetration.

ABA. Apical ovules at 1 DAP had a higher ABA level than at other aerial stages (4-9 DAP) (Fig. 1C). However, a gradual increase in ABA level was observed from 4-7 DAP. ABA content in basal ovules at 1 DAP was higher than that at later stages (4-10 DAP), but the highest level was found at 14 DAP. ABA levels in the apical and basal ovules at 1 DASP decreased by 56 and 38%, respectively, below levels in the aerial ovules at 9 DAP (Fig. 1C). There were only slight changes in ABA level between 1 and 3 DASP. This trend was also observed by Shlamovitz *et al.* (1995), who also reported a continued decrease in ABA during initial pod formation. However, after 3 DASP in our study, ABA content in both ovules increased dramatically (Fig. 1C). The contrast in ABA levels between Shlamovitz *et al.* (1995) and our results can easily be reconciled by noting that they used entire pods in their study versus only ovules being analyzed here, thus the large amount of tissue from pods would dilute the hormone concentrations when only specific tissues within the pod were observed to increase in hormone levels. Increase in ABA level during rapid growth of embryos also has been observed in other species, such as soybean (Ackerson, 1984), peach (Piaggesi *et al.*, 1991), tomato (Hocher *et al.*, 1991), and wheat (Walker-Simmons, 1987). ABA accumulation during early development of embryos has been suggested to be related to water uptake and phloem unloading in the seed coat in pea (Schussler *et al.*, 1991).

The observed changes in hormone content could have resulted from a combination of processes such as synthesis, degradation, changes in configuration, or transport. Questions about causes for hormone changes were not addressed because this study was designed primarily to compare hormone contents in intact ovules during early development. Investigations on metabolism and localization of the hormones in the embryo, ovule, or peg tissues and effect of exogenously applied hormones and hormone inhibitors will be needed to elucidate the exact mechanism of hormonal regulation of peanut fruit development. Such physiological investigations should be facilitated in the future because ELISA can now be used to quantify hormones for small samples of peanut reproductive tissues.

Acknowledgments

This research was partially supported by the North Carolina Agric. Res. Serv., Raleigh, NC 27695-7643 and the Peanut CRSP, USAID grant DAN-4048-G-SS-2065-00. The authors thank Mr. John A. Singleton for his technical assistance with HPLC.

Literature Cited

Ackerson, R.C. 1984. Abscisic acid and precocious germination in soybean. J. Exp. Bot. 35:414-421.

Brenner, M.L. 1981. Modern methods for plant growth substance analysis. Ann. Rev. Plant Phys. 32:511-538.

Cohen, J.D. 1984. Convenient apparatus for the generation of small amounts of diazomethane. J. Chromatog. 302:193-196.

Danin, M., S.J. Upfold, N. Levin, B.L. Nadel, A. Altman, and J. Van-Staden. 1993. Polyamines and cytokinins in celery embryogenic cell cultures. Plant Growth Reg. 12:245-254.

Eberle, J., A. Arnscheidt, D. Klix, and E.W. Weiler. 1986. Monoclonal antibodies to plant growth regulators. III. Zeatinriboside and dihydrozeatinriboside. Plant Phys. 81:516-521.

Evans, M.L. 1984. Functions of hormones at the cellular level of organization, pp. 23-79. *In* T.K. Scott (ed.) Hormonal Regulation of Development II. The Functions of Hormones from the Level of the Cell to the Whole Plant. Encyclopedia of Plant Physiology. Vol. 10. Springer-Verlag, New York.

Feng, Q.L., and R.C. Pan. 1989. Changes of auxin, gibberellin and ethylene levels during pod development in peanut. Plant Phys. Commun. 2:25-28.

Hocher, V., B. Sotta, R. Maldiney, and E. Miginiac. 1991. Changes in abscisic acid and its beta-D-glucopryanosyl ester levels during tomato (*Lycoperisicon esculentum* Mill.) seed development. Plant Cell Rept. 10:444-447.

Hocher, V., B. Sotta, R. Maldiney, and E. Miginiac. 1992. Changes in indole-3-acetic acid levels during tomato (*Lycoperisicon esculentum* Mill.) seed development. Plant Cell Rept. 11:253-256.

Jacobs, W.P. 1951. Auxin relationships in an intercalary meristem: Further studies on the gynophore of *Arachis hypogaea* L. Amer. J. Bot. 38:307-310.

Lee, T.A., D.L. Ketring, and R.D. Powell. 1972. Flowering and growth response of peanut plants (*Arachis hypogaea* L. var. Starr) at two levels of relative humidity. Plant Phys. 49:190-193.

Liu, C., Z. Xu Z., and N.H. Chua. 1993. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell 5:621-630.

Michalezuk, L., T.J. Cooke, and J.D. Cohen. 1992. Auxin levels at different stages of carrot embryogenesis. Phytochem. 31:1097-1103.

Pattee, H.E., and S.C. Mohapatra. 1987. Anatomical changes during ontogeny of the peanut (*Arachis hypogaea* L.) fruit: Mature megagametophyte through heart-shaped embryo. Bot. Gaz. 148:156-164.

Piaggesi, A., P. Perata, C. Vitagliano, and A. Alpi. 1991. Level of abscisic acid in integuments, nucellus, endosperm, and embryo of peach seeds (*Prunus persica* L. cv. Springcrest) during development. Plant Phys. 97:793-797.

Rose, G.S., P.A. Elder, J.A. McWha, D. Pearce, D., and R.P. Pharis. 1987. The development of an indirect enzyme linked immunoassay for abscisic acid. Plant Phys. 85:46-50.

Schussler, J.R., M.L. Brenner, and W.A. Brun. 1991. Relationship of endogenous abscisic acid to sucrose level and seed growth rate of soybean. Plant Phys. 96:1308-1313.

Shlamovitz, N., M. Ziv, and E. Zamski. 1995. Light, dark and growth regulator involvement in groundnut (*Arachis hypogaea* L.) pod development. Plant Growth Regulation 16:37-42.

Sweetser, P.B., and D.G. Swartzfager. 1978. Indole-3-acetic acid levels of plant tissues as determined by a new high performance liquid chromatographic method. Plant Phys. 61:254-258.

Tollennar, M. 1977. Sink-source relationship during reproductive development in maize. A review. Maydica 22:49-75.

Walker-Simmons, M. 1987. ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Phys. 84:61-66.

Wareing, P.F., and I.D.J. Phillips. 1978. Hormonal control in whole plant, pp. 97-140. *In* P.F. Wareing and I.D.J. Phillips (eds.) Control of Growth and Differentiation in Plants. Pergamon Press, New York.

Weiler, E.W. 1982. Plant hormone immunoassay. Phys. Plant. 54:230-234.

Weiler, E.W., P.S. Jourdan, and W. Conrad. 1981. Levels of indole-3-acetic acid in intact and decapitated coleoptiles determined by a specific and highly sensitive solid-phase enzyme immunoassay. Planta 153:561-571.

Zamski, E., and M. Ziv. 1976. Pod formation and its geotropic orientation in the peanut, *Arachis hypogaea* L., in relation to light and mechanical stimulus. Ann. Bot. 40:631-636.

Ziv, M., and E. Zamski. 1975. Geotropic responses and pod development in gynophore explant of peanut (*Arachis hypogaea* L.) cultured *in vitro*. Ann. Bot. 39:579-583.

Accepted 20 Jan. 1998