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Organogenesis from Immature Pecan Embryonic Axes in Vitro

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Abstract. **Immature embryos were excised during kernel development from fruits of the pecan [*Carya illinoensis* (Wangenh.) C. Koch] cultivars Desirable and Stuart. The cotyledons were removed and the main embryo axes were used as explants. Explants were cultured in vitro on media containing various levels of cytokinins and auxins. Morphogenesis in 'Stuart' preceded that of 'Desirable' by 1 to 2 weeks. In both cultivars, the percentage of embryo axes forming shoots only or both shoots and roots increased until ≈4 to 6 weeks before nut maturity, as judged by shuck dehiscence. After this time, developmental responses declined. Production of normal plants was highest on a medium containing IBA, BA, and kinetin at 0.5, 4.4, and 9.3 μM, respectively. Shoots only were obtained on a medium containing cytokinin without auxin and roots only on a medium containing auxin with no cytokinin. Axillary shoots elongated from embryo axes of both cultivars. This response was greatest on a medium containing cytokinin as the only hormone for 'Desirable', but with both auxin and cytokinin for 'Stuart'. Chemical names: indole-3-butyric acid (IBA); *N*-(phenylmethyl)-1*H* purin-6-amine (BA); *N*-(2-furanylmethyl)-1*H*-purin-6-amine (kinetin).**

Pecan is the most valuable horticultural nut tree crop native to North America (Thompson and Young, 1985). There are >1000 named scion cultivars (Thompson and Young, 1985); however, clonal rootstocks are yet to be developed due to the lack of practical clonal propagation techniques (Wood, 1987). Commercially, pecan trees are propagated by grafting or budding scions onto rootstocks derived from open-pollinated seeds. This practice results in great genetic diversity of rootstocks in the field. Clonal rootstocks would be of great potential value to the pecan industry by allowing incorporation into the matrix rootstocks desirable traits such as tree size control, enhanced nutrient uptake, control of alternate bearing, more uniform growth, and pest resistance. Micropropagation is a potentially efficient method for obtaining improved clonal rootstocks. This technique has been attempted with nodal stem segments taken from seedling pecans (Hansen and Lazarte, 1984; Wood, 1982) with low regeneration success, i.e., only 1.5 plantlets per explant were obtained (Hansen and Lazarte, 1984). In addition, nodal seedling tissue from in vivo-germinated embryos often results in profuse contamination of cultures by microorganisms (Wood, 1982). The effectiveness of current methodologies could be enhanced by using embryos germinated in vitro, thereby providing a source of axenic nodal stem segments. In vitro germination of embryos would also provide a controlled system for defining the hormonal requirements for organ development in pecan tissues.

Mature pecan nuts germinate readily under appropriate conditions, such as exposing the seeds to high moisture conditions before planting (Sparks et al., 1974) or germinating the seeds at 30 or 35C (van Staden et al., 1976; van Staden and Dimalla, 1976). Kinetin can effectively substitute for temperature or stratification requirements (Dimalla and van Staden, 1977). Nuts collected from the tree just before shuck dehiscence have already attained regenerative capacity (Tedders et al., 1970); however, the ontogenetic stage at which the pecan embryo develops the optimum capacity for potential regeneration and the levels of growth regulators stimulating normal growth and development are unknown. The objectives of this study were to determine: a) the stage in the development of the pecan fruit during which the excised embryo demonstrates the greatest capacity for germination under defined environmental and cultural conditions, and b) the interaction of various levels of the plant growth regulators cytokinin and auxin on root and shoot morphogenesis.

Materials and Methods

Open-pollinated fruits were collected from 'Desirable' and 'Stuart' trees every 1 to 2 two weeks beginning 28 Aug. through 13 Nov. 1986 from an orchard in the vicinity of Madison, Ga. These cultivars were chosen for study because they are two of the most important ones commercially. Fruit were stored at 2–4C in plastic bags for 5 days. Before dissection, fruit were sterilized by immersion in 70% (v/v) ethanol for 20 min followed by three rinses in sterile distilled water. The fruits were opened and the embryos were excised aseptically. Embryos with cotyledons attached served as explant material for the 28 Aug. collection of 'Desirable' due to the small size of the embryos on this date and the risk of mechanical damage during cotyledon excision. After this date, for 'Desirable' and throughout the study for 'Stuart', only the main embryonic axes without cotyledon tissue were used as explants. The developmental response of the embryo with the cotyledons removed was monitored in the current study because previous in vivo studies have shown that growth of the seedling depends on the presence of the cotyledons (Wetzstein et al., 1983). Likewise, cotyledons may affect germination capacity in vitro so that their removal would permit a more accurate definition of hormonal effects on root and shoot production. Forty-eight fruits were dissected per cultivar for each collection date. These explants were placed in 60 × 15 mm petri dishes (four explants per dish) on a conditioning

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medium (Tulecke and McGranahan, 1985) solidified with 0.7% agar and with the plant growth regulators modified as in Table 1. Twelve explants were placed on each of the four hormone formulations. The hormones BA and IBA were used because they have been shown to be effective in supporting multiple shoot development in walnut (Driver and Kuniyuki, 1984), and kinetin was incorporated because of its morphogenetic effects on shoot induction (Zaerr and Mapes, 1985). After 4 weeks in the dark at 25C, the explants were transferred to a medium lacking hormones. Each explant was observed periodically and placed into one of three categories based on the organs developed in culture. The categories were roots only, shoots only, and normal plants with both shoots and roots.

Results

The gross morphological features serving as developmental markers for the fruits used in this study were determined at the time of collection (Table 2). In both cultivars, shell hardening was complete on the first sampling date. For the other characters observed, fruit development in 'Stuart' generally preceded that of 'Desirable' by at least 1 to 2 weeks.

The temporal pattern of development from the embryonic axes was similar for both cultivars (Fig. 1). Organ development was low at the early sampling dates, increasing for ≈6 to 7 weeks, followed by a declining capacity. However, the specifics for morphogenesis varied between cultivars in that the peak expression for 'Desirable' (Fig. 1A) occurred 2 to 3 weeks after that for 'Stuart' (Fig. 1B). In addition, 'Desirable' maintained a relative morphogenetic response of 0.8 or higher over 6 weeks, whereas 'Stuart' was at this level for only 3 weeks. The production of roots alone was consistently low for both cultivars for all sampling dates, with no obvious time optimum. However, there were periods that appeared to be optimum for shoots and normal plants. This period occurred over a span of 6 weeks (24 Sept. to 29 Oct.) for 'Desirable'. For 'Stuart', the interval was reduced by 2 weeks as an acute decline in shoot and plant production was observed by 15 Oct.

The seasonal cumulative potential for in vitro morphogenesis of the embryonic axes as a function of time followed a sigmoidal curve (Fig. 2). Again the pattern of both cultivars was similar, but with 'Desirable' demonstrating a slightly more pronounced lag period. By the end of the sampling period, the profile for 'Stuart' had reached a more obvious stationary phase than that for 'Desirable'.

Analyses of the cumulative response of each of the morphogenetic categories demonstrated that developmental capacity for shoot and intact plants followed a sigmoidal curve in both cultivars (Fig. 3). However, the relative response for the shoots only class was much higher for 'Desirable' (Fig. 3A) than 'Stuart' (Fig. 3B). In 'Desirable', the seasonal cumulative root growth was sigmoidal as well. But, for 'Stuart', there was no obvious lag period, perhaps reflecting the more advanced developmental state of the fruit from this cultivar (Table 2).

The plant growth regulator composition of the medium had a major influence on the response of the embryonic axis (Fig. 4). For 'Desirable', morphogenetic capacity was about equal on media 1, 3, and 4, representing a high cytokinin : auxin ratio, cytokinin alone, and auxin alone, respectively. The high cytokinin to auxin formulation was best for normal plant development, cytokinin alone yielded primarily shoots, while auxin alone resulted in the best root production. However, the majority of explants treated with auxin alone still produced normal plants. Medium 2, which contained a higher auxin to cytokinin ratio than medium 1, resulted in production of only shoots. Normal plant development in 'Stuart' was greatest on medium 1 followed by media 4, 3, and 2 in order of decreasing effectiveness. The primary response for 'Desirable' on media 2 and 3 was

Table 1. Plant growth regulators (in micromoles) in conditioning media to induce morphogenesis in pecan embryonic tissue in vitro.

Medium	IBA	BA	Kinetin	Molar ratio Auxin : cytokinin
1	0.5	4.4	9.3	1:27
2	5.0	52.8	0	1:11
3	0	52.8	0	0:53
4	5.0	0	0	5:0

Table 2. Gross morphological characteristics of fruits.

Collection dates (1986)	Cotyledon length (mm)		Embryo axis length (mm)		Endosperm condition[z]		Shuck[y]	
	Cultivar							
	Desirable	Stuart	Desirable	Stuart	Desirable	Stuart	Desirable	Stuart
August								
28	2.5	23	1	3	L	G	I	I
September								
10	15	25	2	3	L/G	M	I	I
17	35	30	2	3	G	M	I	I
24	36	30	4	3	M	M	I	I
October								
1	36	30	4	3	M	M	I	I
8	36	30	4	3	M	M	I	Sp
15	36	30	4	3	M	M	I	Sc
22	36	30	4	3	M	M	Sp	Sc
29	36	30	4	3	M	M	Sc	Sc
November								
13	36	30	4	3	M	M	Sc	Sc

[z]L = liquid, G = gelatinous, M = membranous.
[y]I = intact, Sp = partial dehiscence, Sc = complete dehiscence.

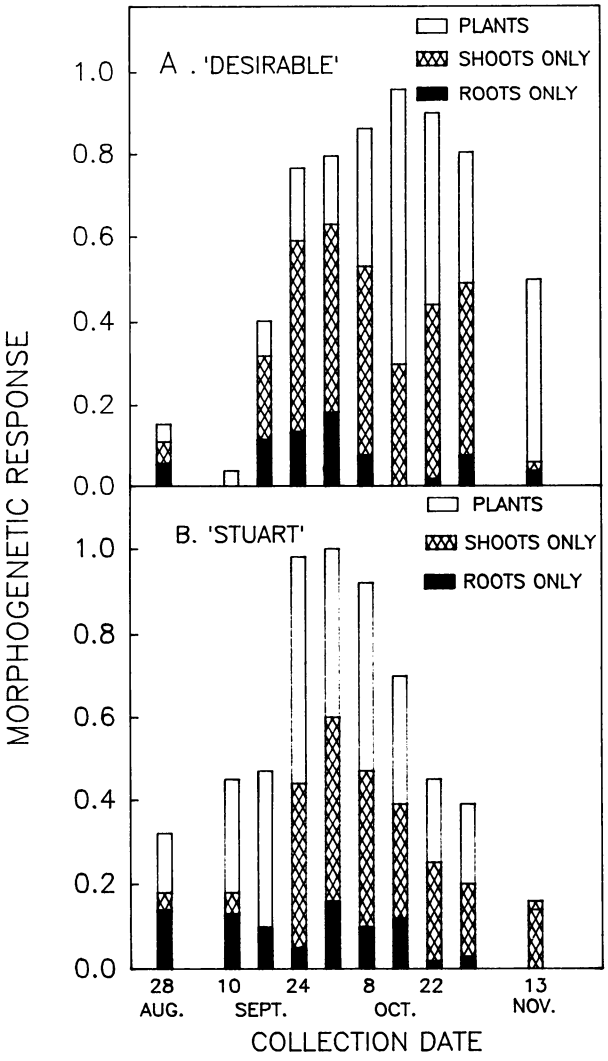

Fig. 1. Morphogenesis of pecan embryonic axes as a function of sampling date for 'Desirable' (**A**) and 'Stuart' (**B**) pecans. Each explant was categorized as producing a root only, shoot only, or a normal plant. The proportion of original explants exhibiting each of these growth patterns was calculated. A value of 1.0 was assigned to the sampling time with the highest number of explants producing roots, shoots, and normal plants. For any given sampling date, morphogenesis was plotted in an additive fashion; i.e., the portion of explants producing only roots was plotted first, then those explants producing only shoots were added, and finally the explants producing normal plants.

shoot elongation. The medium on which the most roots were formed was high in auxin (medium 4). All embryonic axes collected on 15 and 22 Oct. from 'Desirable' and on 8 and 15 Oct. from 'Stuart' and conditioned on medium 1 produced normal plants (data not shown).

In addition to elongation of the primary shoot, axillary shoots were stimulated to grow from embryo axes collected after 1 Oct. (Table 3). Data compiled for the shoots only category in Fig. 4 included those axes in which the primary shoot alone elongated as well as those with axillary shoot elongation. Although the potential for axillary shoot elongation developed in 'Stuart' before it did in 'Desirable', the latter demonstrated a greater propensity for this growth response. Medium 2 was most effective for axillary shoot elongation in 'Stuart', whereas medium 3, containing only BA, was more efficient for 'Desirable'.

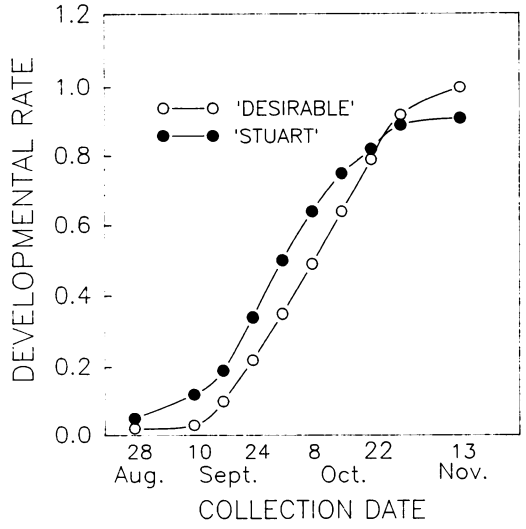

Fig. 2. Comparative developmental rate of the embryos from 'Desirable' and 'Stuart' pecans. Points on the graph represent the sum of all previous responses plus the response for that sampling date. The highest sum total was for 'Desirable'. This value was designated 1.0 and all others plotted relative to this value.

Establishment of axenic cultures with the main embryonic axes as the explant material was not a problem. Only 2% of the explants from 'Desirable' and 9% of the explants from 'Stuart' became contaminated during the time they were maintained.

Discussion

Under defined environmental and hormonal conditions, the main embryo axes of 'Stuart' and 'Desirable' have the same general pattern for morphogenic responses during seed maturation. The seasonal variation observed in both cultivars can be divided into three phases: 1) an early increase, 2) a middle maximum, and 3) a late decline. The onset of each stage occurs 2 to 3 weeks earlier in 'Stuart' than 'Desirable'. Within each cultivar, the timing of the onset of each stage is in parallel with the development of gross morphological features of the fruit. For example, in both cultivars, the decline of in vitro morphogenetic potential coincides with partial dehiscence of the shuck, which occurs 2 weeks earlier in 'Stuart' than in 'Desirable'. The in vitro analysis demonstrating a decline in development with shuck dehiscence correlates with the occurrence of vivipary, i.e., germination of the nuts on the tree. Factors preventing shuck opening, such as heavy late-season damage by hickory shuckworm or light damage by pecan weevil, will induce premature germination (Tedders et al., 1970).

There is variability within a cultivar for the type of growth response observed at any given sampling date. For example, some embryos produced only shoots or roots, whereas others produced both roots and shoots. This may be due to inherent genetic variability resulting from open-pollination.

Morphogenesis from the main embryonic axis of pecan is much greater than for apple (Korban and Skirvin, 1985). Callus was the only growth response obtained from 100% of the embryo axes of the 'Delicious' apple and 55% of 'Winesap'. From explants of the main embryonic axis of pecan, callus was formed only at the base of explants producing only shoots. In contrast, callus was observed at the apex of the embryonic axes of pecan explants producing only roots.

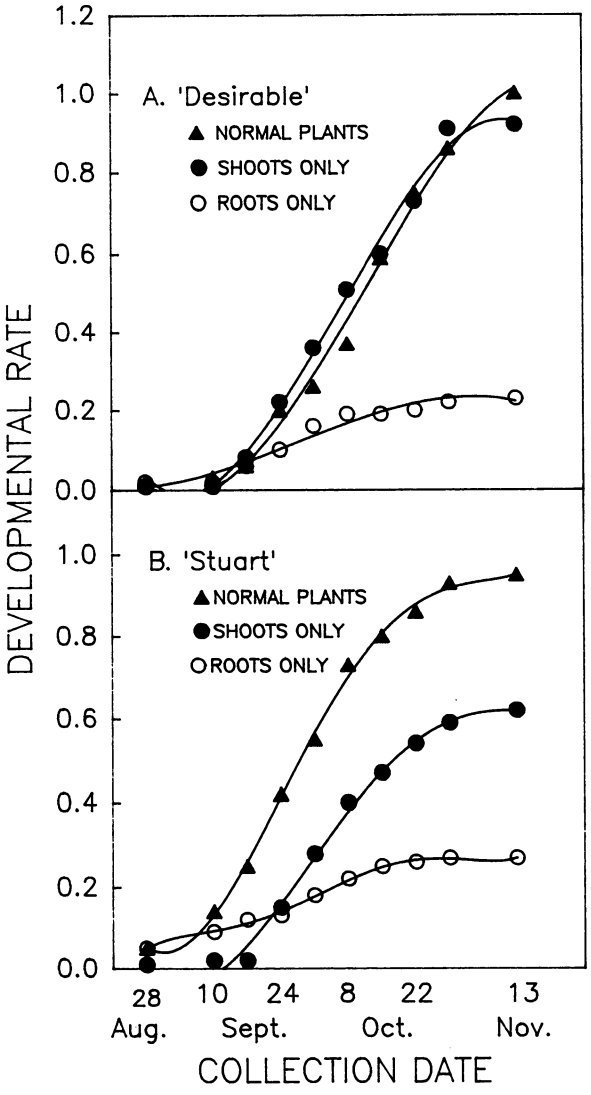

Fig. 3. Seasonal cumulative developmental rate for each of the three categories: roots only, shoots only and normal plants for 'Desirable' (**A**) and 'Stuart' (**B**) pecans. Points on the graph represent the sum of all previous responses plus the response for that sampling date. The highest sum total was for the plant category in 'Desirable'. This value was designated 1.0 and all others plotted relative to this value.

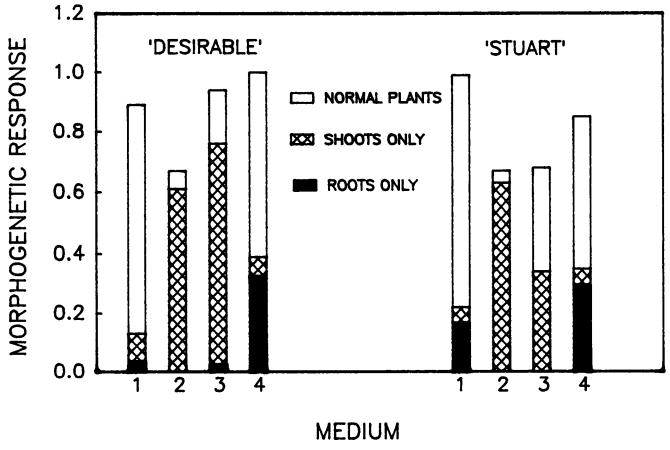

Fig. 4. Morphogenesis as a function of medium type. Data are based on the development from embryo axes collected throughout the sampling period and cultured as described in the Materials and Methods. The maximum value for organogenic response was obtained on medium 4 for 'Desirable'. This value was designated 1.0 and all other values plotted relative to this.

Table 3. Hormonal and temporal influence on axillary shoot elongation in pecan.

Collection date (1986)	Desirable				Stuart			
	Medium type[z]							
	1	2	3	4	1	2	3	4
October	*Embryo axis with axillary shoot elongation (%)*							
1	0	0	0	0	0	0	0	0
8	0	0	0	0	0	17	0	0
15	0	13	83	0	0	50	0	0
22	0	50	63	0	0	13	13	0
29	0	25	42	0	0	38	0	0
November								
13	0	0	0	0	0	0	0	0

[z]See Table 1.

In many plants, high levels of abscisic acid (ABA) have been correlated with the inhibition of seed germination (Ho, 1982). Wood (1984) reported that both free and bound ABA was maximum in the pecan kernal at the initiation of rapid cotyledon growth and declined with seed maturation. The decreasing levels of ABA, as documented by Wood (1984), and the increasing competency of the embryonic axis up to the latter third of the sampling period, correlate with this concept; however, despite a continued decline in ABA, there is a concomitant decrease in morphogenesis—the opposite to what one would predict. These results parallel the observations of Bonamy and Dennis (1977) in peach seed. These investigators were unable to demonstrate a clear relationship between ABA content and metabolic competency in terms of germination potential of peach embryos.

The exogenous supply of plant growth regulators influences the developmental response of the embryo axes in pecan. The responses for most media were predictable in that shoots only were common on the media containing higher cytokinin concentration and the higher auxin concentration produced roots. The data in this report suggest that the main embryonic axes of both cultivars is high in cytokinin-like activity in terms of stimulating shoot elongation. This assumption is based on the response of the embryonic axes to the medium containing IBA only. On this medium, the major morphogenic response is the production of intact plants. In general, medium containing a high auxin : cytokinin ratio is expected to induce primarily a rooting response.

Axillary shoot elongation appears to be more readily stimulated in 'Desirable' than in 'Stuart'. Axillary shoot production in mature walnut was greater at a BA concentration of 2.0 mg·liter^{-1} than at either 5 or 10 mg·liter^{-1} (Heile-Sudholt et al., 1986). It is possible that lower levels of BA in the current experiments would have been more conducive for axillary shoot formation in 'Stuart'. To date, strategies for micropropagation of pecan have yielded limited success when based on axillary shoot proliferation from seedling stem segments (Hansen and Lazarte, 1984; Wood, 1982). The current results suggest that axillary shoot proliferation from cultured embryonic axis may be useful in establishing methods to generate clonal pecan plants to use as rootstocks. In vitro-germinated plants do not present

contamination problems, as observed in studies with in vivo-germinated seedlings (Wood, 1982).

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Analyzing Competition Between a Living Mulch and a Vegetable Crop in an Interplanting System

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Abstract. **Development of interplanting systems for vegetables has been impeded due to concerns about yield reductions and use of systematic experimental designs that limit analysis of fundamental competitive processes. This study employed an addition series and growth analysis combined with management strategies aimed at minimizing competition between the crop and the interplant. Pak choi [*Brassica rapa* L. (Chinensis Group)] was interplanted with strips of ryegrass (*Lolium perenne* L.) that covered 67% of the soil. Pak Choi was a weak competitor compared to perennial ryegrass. Mulch suppression using a sublethal rate of fluazifop provided the most promising management strategy to reduce competition from the ryegrass interplant. Timing of suppression and reduction of mulch root growth were critical elements of successful management. Chemical names used: (±)-2-[4-[[5-(trifluoromethyl)-2-pyrindinyl]oxy]phenoxy]propanoic acid (fluazifop).**

Vegetable growers often plant a late-maturing cabbage (*Brassica oleracea* L. var. *capitata*) crop for harvest during cool but inclement weather in the Pacific Northwest. Muddy field conditions prevail during crop growth or harvest, resulting in soil compaction that affects crop yields the following season. Living mulches planted between vegetable rows may decrease weed infestations, soil erosion, fertilizer and pesticide requirements, and soil compaction while enhancing organic matter, water infiltration, and moisture or nutrient retention (Akobundo, 1980; Hartwig, 1984; Horwith, 1985). However, concern about interference between the vegetable and living mulch has impeded development of viable production systems (Nicholson and Wien, 1983; Sweet, 1982).

Plants interfere with or reduce yields of a neighbor through environmental modification, alleopathy, or competition (Harper, 1977). Competition often is identified as the important mechanism of interference in most living mulch systems. Attempts to reduce competition in these systems have focused on mechanical or chemical suppression of mulch growth, screening for less competitive mulches, and variation of mulch planting dates (Akobundo and Okigbo, 1984; Cooper, 1985; Elkins et al., 1979; Hartwig, 1984; Loy, 1984; Nicholson and Wien, 1983; Peterman, 1985.)

Competition among plants is influenced by environmental

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