More improved peanut (*Arachis hypogaea* L.) protocol for direct shoot organogenesis in mature dry-cotyledonary and root tissues

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Peanut is a legume of economic importance, whose improvement could greatly benefit from the integration of both classical and modern techniques. Although biotechnology techniques such as tissue culture and gene transformation have been reported in peanut, it has mostly focused on the application of standard organs, such as, leaf, stem, and embryo axis. Little has been achieved using seed cotyledon, primarily because the latter tissue has been limitedly successful *in vitro* culture for adventitious plant formation; and no protocol has been successfully developed for peanut root organogenesis. The *main purpose* of this study was to develop a tissue culture protocol that could induce direct shoot formation in dry peanut cotyledon more efficiently and root tissue. This goal was achieved by preparing mature dry seeds in four explant types, including, the whole cotyledon, half cotyledon, diced cotyledon and two-side-cut cotyledon, and root segments from germinating embryos pre-cultured for 0, 1, 2, 3, 4, 5, 6, or 7 days on hormone-free culture medium. The culturing of those explants on nutrient media containing kinetin, 6-benzylamino purine, 2, 4-dichlorophenoxyacetic acid, or thidiazuron (TDZ) used alone or in combinations of cytokinin with auxin resulted in greatest direct multiple shoots per explant. Greater TDZ concentrations (5-30 mg/l) are recommended for greater number of shoots per mono/side-cut cotyledon (78-93). The proximal region formed more shoots than the distal region. However, overlapping multiple shoot formation in both regions was greater than current protocols'. Thidiazuron-based treatments induced greater shoot formation, in cotyledon, than other growth-regulator treatments. No root tissue explants that were pre-cultured more than one day formed shoots. All newly formed shoots were transferred onto control medium without growth regulators for rooting and subsequently grew normally in the greenhouse.

Keywords: Peanut seed shoot organogenesis; peanut tissue culture; shoot organogenesis; thidiazuron; plant micropropagation.

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Introduction

Mature dry-seed is the most prevalent peanut biological material year-round. In addition to its economic and biological importance, peanut seeds are readily available and cost- and space-effective for storing genetic material across seasonal cycles. Cotyledon, particularly, is

inherently the most abundant tissue in peanut seed, which makes it potentially the most beneficial seed tissue for maximizing adventitious plant formation for micro-propagation. However, in addition to root, it is noticeably one of the least exploited tissues in peanut tissue culture and genetic engineering, primarily, because of its reticence and lower responses for adventitious plant formation *in vitro* compared to other tissues, such as, leaf, stem, and embryonic axis [1-6].

The present study exploits dry cotyledonary and root tissues' potential to form reliable shoots *in vitro*. It reports the most efficient protocol that applies different treatments to achieve greater results for abundant multiple-shoot formation that outperforms all existing improved peanut protocols for shoot induction in seed cotyledon [3, 7-13]. This protocol also induced, for the first time, adventitious shoots in seed root tissue. The present success could increase the usage of these tissues in tissue culture for *in vitro* commercial and scientific micro-propagation as well as genetic engineering for crop improvement purposes.

Materials and Methods

1. Plant Material and explant preparation:
Peanut mature-dry seeds of cultivar '*Valencia*' (Valencia botanical type) obtained from a New Mexico seed company, Sunland (New Mexico, USA), were surface-sterilized by soaking into 100% ethanol for 3 minutes, then, transferring to 25% commercial Clorox for 8 minutes followed by four rinses with sterile distilled water. Cotyledon explants were excised from sterile seeds and related explants were prepared in (1) whole cotyledon, (2) diced cotyledon, (3) mono-side-cut cotyledon (half cotyledon), and (4) di-side-cut cotyledon (two-side cuts). Root tissue explants were excised from sterile embryos that were pre-cultured on Murashige and Skoog (MS) basal medium and vitamins (Sigma-Aldrich, St. Louis, MO, USA) without growth regulator treatment for 0, 1, 2, 3, 4, 5, 6, or 7 days prior to transferring to treatment media.

2. Explant culture and cultural conditions:
Thirteen explants were randomly cultured on individual Petri dishes containing MS medium, sucrose (30 g/l), and phytagel (5 g/l) medium. Except for the control, the medium also contained different shoot-inducing treatments that included 0, 5, 10, 20, or 30 mg/l kinetin, 6-benzyl-amino purine (BA) or thidiazuron (TDZ), and/or 3 mg/l 2, 4-dichlorophenoxyacetic acid (2,4-D). Five plates of the same treatment were randomly assigned to individual explant types for culture. Each plate contained five explants. The final pH of the medium was adjusted to 5.8 with 1N NaOH after the addition of Phytagel (2g/l), and autoclaved at 121°C for 20 minutes. All culture dishes were wrapped with Parafilm prior to incubation. Cultures were incubated at room temperature under a 16 h photoperiod (50 $\mu m^{-2}s^{-1}$) and transferred to the fresh media every four weeks. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

3. Observations and Data Collections:
Observations were made daily, and data were collected throughout the 55 day-study period, as needed. Most statistical analyses were performed using T-test for sample-pair-means comparisons of shoot formation differences.

Results and Discussion

Naturally, cotyledon is a nutrient-storage tissue for the seed embryo and is different, in this respect, from other standard organs, such as, leaf, stem, and root. Thus, the expectation for morphogenetic responses is generally low. The results of this study demonstrate that the peanut cotyledon is potentially as efficient as other standard organs for *in vitro* plant formation. The study also resulted in the first successful, direct adventitious shoot formation in peanut root tissue.

1. Callus formation

Table 1. Shoot organogenesis in whole-cotyledonary, diced-cotyledonary, mono/side-cut cotyledonary, and di/side-cut cotyledonary tissues.

1a. Shoot organogenesis in whole-cotyledonary tissue:

Treatment	Whole cotyledon				
	CG	SER (%)	SPA	ESA	SCR (%)
Control	-	0	0	0	0
3 mg/l 2, 4-D	+	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l Kinetin	+	0	0	0	0
5 mg/l Kinetin	-	10±1.3	10±0.8	5±0.2	50±1.5
10 mg/l Kinetin	-	11±1.1	11±0.4	9±1.2	82±2.6
20 mg/l Kinetin	-	11±1.1	15±1.0	13±0.8	87±1.2
30 mg/l Kinetin	-	14±1.0	15±1.3	14±0.8	93±1.7
3 mg/l 2, 4-D + 5 mg/l BA	++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l BA	++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l BA	++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l BA	+	0	0	0	0
5 mg/l BA	-	5±0.9	2±0.2	2±0.3	100±3.3
10 mg/l BA	-	7±1.0	3±1.1	3±1.1	100±3.8
20 mg/l BA	-	10±0.5	5±0.4	1±0.2	20±1.2
30 mg/l BA	-	12±0.4	4±1.1	1±0.8	25±1.5
3 mg/l 2, 4-D + 5 mg/l TDZ	-	68±1.4	15±0.4	3±0.2	20±0.7
3 mg/l 2, 4-D + 10 mg/l TDZ	-	73±1.2	16±1.0	4±0.9	25±1.1
3 mg/l 2, 4-D + 20 mg/l TDZ	-	78±1.3	17±2.2	4±1.1	24±1.7
3 mg/l 2, 4-D + 30 mg/l TDZ	-	78±1.2	18±1.1	6±0.8	33±1.4
5 mg/l TDZ	-	75±1.3	18±1.2	5±0.2	28±1.4
10 mg/l TDZ	-	78±1.3	21±1.2	7±0,6	33±1.2
20 mg/l TDZ	-	82±1.2	25±1.2	9±1.0	36±1.7
30 mg/l TDZ	-	95±1.9	28±1.3	9±1.1	32±1.7

1b. Shoot organogenesis in diced-cotyledonary tissue:

Treatment	Cotyledonary dice				
	CG	SER (%)	SPA	ESA	SCR (%)
Control	-	0	0	0	0
3 mg/l 2, 4-D	+	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l Kinetin	++	0	0	0	0
5 mg/l Kinetin	+	0	0	0	0
10 mg/l Kinetin	+	1±0.2	5±0.5	2±0.2	40±1.1
20 mg/l Kinetin	+	3±0.3	3±0.1	3±0.8	100±2.1
30 mg/l Kinetin	+	5±0.5	8±0.5	2±0.7	25±1.3
3 mg/l 2, 4-D + 5 mg/l BA	++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l BA	++	0	3±0.6	0	0
3 mg/l 2, 4-D + 20 mg/l BA	++	5±0.1	5±0.7	2±0.1	40±1.3
3 mg/l 2, 4-D + 30 mg/l BA	++	2±0.2	5±0.8	1±0.7	20±0.5
5 mg/l BA	+	1±0.1	4±0.6	1±0.1	25±0.7
10 mg/l BA	+	5±0.4	3±0.6	2±0.2	67±1.4
20 mg/l BA	+	8±0.4	4±0.6	1±0.2	25±1.3
30 mg/l BA	+	9±0.8	7±0.7	2±0.3	28±1.2
3 mg/l 2, 4-D + 5 mg/l TDZ	++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l TDZ	++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l TDZ	++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l TDZ	++	0	0	0	0
5 mg/l TDZ	+	1±0.1	4±0.4	1±0.4	25±1.0
10 mg/l TDZ	+	5±0.2	8±0.8	4±0.8	50±1.3
20 mg/l TDZ	+	8±0.7	10±1.0	4±0.9	40±1.5
30 mg/l TDZ	+	9±0.7	11±1.0	6±1.0	55±1.3

1c. Shoot organogenesis in mono/side-cut cotyledonary tissue:

Treatment	Mono/side-cut cotyledon				
	CG	SER (%)	SPA	ESA	SCR (%)
Control	-	0	0	0	0
3 mg/l 2, 4-D	-	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l Kinetin	+	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l Kinetin	+	5±0.2	7±0.4	5±0.4	71±2.0
3 mg/l 2, 4-D + 20 mg/l Kinetin	-	15±1.1	10±0.8	7±0.5	70±1.4
3 mg/l 2, 4-D + 30 mg/l Kinetin	-	25±1.1	10±0.6	6±0.5	60±0.7
5 mg/l Kinetin	-	5±0.9	10±1.1	4±0.7	40±0.9
10 mg/l Kinetin	-	8±0.8	11±1.1	10±0.9	91±2.1
20 mg/l Kinetin	-	20±1.0	11±0.9	11±0.9	100±2.2
30 mg/l Kinetin	-	22±1.1	18±1.0	15±1.3	83±2.0
3 mg/l 2, 4-D + 5 mg/l BA	+	4±0.6	5±0.8	3±0.5	60±1.3
3 mg/l 2, 4-D + 10 mg/l BA	+	10±0.8	16±0.9	16±1.3	100±2.2
3 mg/l 2, 4-D + 20 mg/l BA	-	35±2.5	30±1.2	21±0.9	70±1.6
3 mg/l 2, 4-D + 30 mg/l BA	-	35±1.3	30±1.2	20±1.2	67±1.1
5 mg/l BA	-	12±0.3	5±0.7	2±0.2	40±1.0
10 mg/l BA	-	21±1.1	25±1.0	18±07	72±1.7
20 mg/l BA	-	38±1.3	54±1.3	42±2.1	78±2.2
30 mg/l BA	-	58±1.4	71±2.0	53±1.3	75±2.2
3 mg/l 2, 4-D + 5 mg/l TDZ	+	44±1.4	55±1.8	23±0.7	42±1.2
3 mg/l 2, 4-D + 10 mg/l TDZ	+	58±1.6	56±1.3	26±0.5	46±1.1
3 mg/l 2, 4-D + 20 mg/l TDZ	-	65±1.3	60±1.9	31±1.1	52±1.4
3 mg/l 2, 4-D + 30 mg/l TDZ	-	65±1.3	67±1.7	30±0.9	45±1.1
5 mg/l TDZ	-	82±1.6	105±2.1	78±2.1	74±1.4
10 mg/l TDZ	-	88±1.9	110±2.2	85±2.3	77±1.8
20 mg/l TDZ	-	94±1.4	119±2.3	89±2.0	75±1.6
30 mg/l TDZ	-	98±1.7	127±2.5	93±2.4	73±1.4

1d. Shoot organogenesis in di/side-cut cotyledonary tissue:

Treatment	Di/side-cut cotyledon				
	CG	SER (%)	SPA	ESA	SCR (%)
Control	-	0	0	0	0
3 mg/l 2, 4-D	+	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l Kinetin	++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l Kinetin	+++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l Kinetin	+++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l Kinetin	++	0	0	0	0
5 mg/l Kinetin	-	0	0	0	0
10 mg/l Kinetin	-	0	0	0	0
20 mg/l Kinetin	-	0	0	0	0
30 mg/l Kinetin	-	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l BA	+++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l BA	+++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l BA	+++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l BA	++	0	0	0	0
5 mg/l BA	-	0	0	0	0
10 mg/l BA	-	0	0	0	0
20 mg/l BA	-	0	0	0	0
30 mg/l BA	-	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l TDZ	+++	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l TDZ	+++	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l TDZ	+++	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l TDZ	++	0	0	0	0
5 mg/l TDZ	-	0	0	0	0
10 mg/l TDZ	-	0	0	0	0
20 mg/l TDZ	-	0	0	0	0
30 mg/l TDZ	-	0	0	0	0

Notes: CG: callus growth ("-": no callus growth, "+": little callus growth, "++": median callus growth, "+++": large callus growth); **SER**: shooting explant rate (%) plus standard error; **SPA**: shoot primordia average plus standard error; **ESA**: explant shoots average plus standard error; **SCR**: Shoot conversion rate plus standard error.

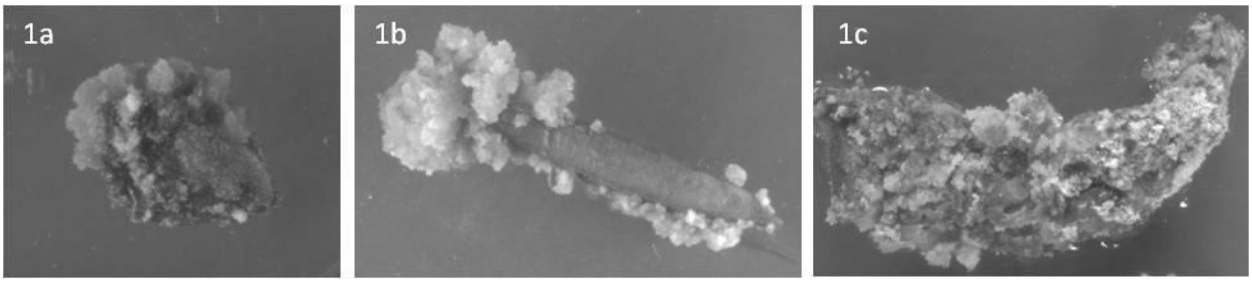

Figure 1. Cotyledon explants showing variable callus formation. **1a**: diced cotyledon friable callus; **1b**: di-sided-cut cotyledon showing mushy callus; **1c**: mono-sided-cut cotyledon showing disintegrating tissue without callus formation.

Overall, callus formation varied based upon explant type, preparation, and treatment (Table 1a-d; Figure 1a-c; Figure 2a-b). The general observations indicated that both cotyledon and root might not be the tissues of choice for quality callus formation. Good friable callus was only observed in diced cotyledon explant (Figure 1a). However, it was inefficiently small for consideration for shoot organogenesis during the experimental period. Limited callus formed in whole cotyledon, primarily, when 2, 4-D was combined with either kinetin or BA (Table 1a). Di-side-cut cotyledon developed more callus than any other explants tested only mushy-fluffy callus (non-friable) formed in the treated tissue during the study (Figure 2a). The control explants formed mostly fluffy-fibrous callus in root tissue (Figure 2b).

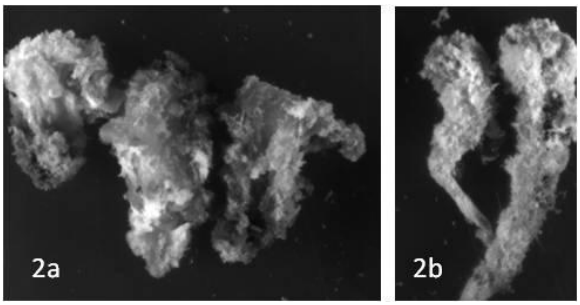

Figure 2. Root explant showing general poor callus formation, with *fluffy-mushy* callus formation in treated explants (2a) and *fibrous-Fluffy* callus formation in control explants (2b).

Although cytokinin or auxin can singly induce callus formation in plant tissue culture [14-17], the study showed that callus formation was the result of the combinations of 2, 4-D with either cytokinin used, across cotyledonary explant (Figure 1b, Table 1d). However, this callus was mushy and not friable for further successful multiplication, when separated from the original explant. For less understood reasons, the mono-side-cut cotyledon was the least responsive explant type for callus formation, because it seldom showed basic callus initiation (Table 1c). Related tissue generally disintegrated without further callus development or cell divisions (Figure 1c). This observation was initially expected from all cotyledon explants, considering its unusual structural genesis. No viable callus was observed from root explants, considering that types (Table 1a-d). The applications of 2, 4-D, BA, kinetin, or TDZ alone resulted mostly in little or no cotyledon callus initiation (Table 1a-d).

2. Shoot organogenesis in cotyledonary explants

Overall, the study showed that the dry-mature cotyledon is potentially a reliable tissue for *in vitro* micro-propagation, when proper preparation and conditioning are applied. However, shoot organogenetic responses varied with explant and treatment (Tables 1a-d). Similar observations have previously been reported in peanut [2, 18, 19,] and other plant species [15, 20]. Except di-side-cut cotyledon (Table 1d), all explants tested formed shoots *in vitro* (Table 1a-c). Mono-side-cut cotyledon (Figures 3a, b) was overall the most responsive explant for direct shoot formation, considering that 92% of the treatments applied to this explant, excluding the control, caused shoot formation (Table 1c). This was followed by the whole

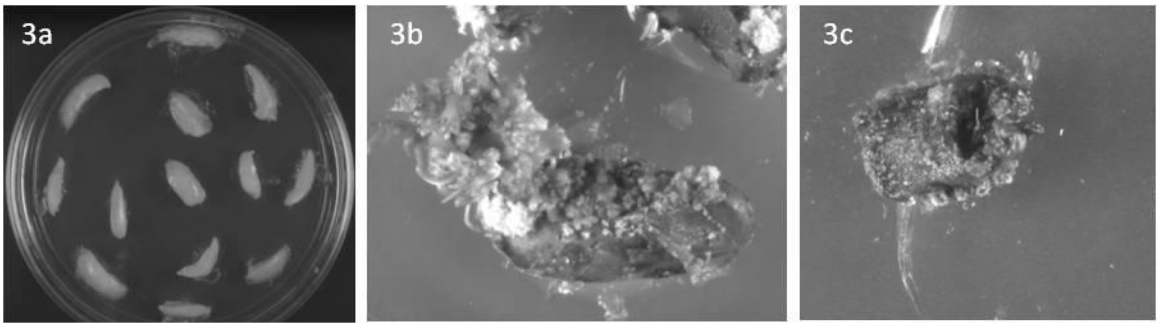

Figure 3. Shoot organogenesis in cotyledon explants. **3a**: fresh mono-sided-cut cotyledon on culture medium; **3b**: mono-sided-cut cotyledon explants showing developmental multiple shoot buds formation along cut edges spanning from *proximal* to *distal* regions; **3c**: diced cotyledon explants showing developmental shoot formation, with initial shoot bud formation on cut edges.

(Table 1a) and diced (Table 1b, Figure 3c) cotyledons, which formed shoots when treated, in 64% and 52% of explants, respectively. Mono-side-cut cotyledon also formed overall the greatest explant shoot primordia (127), shooting explant rate (98%), shoot conversion rate (100%), and explant shoots average (93) (Table 1c). Despite that this explant's shoot conversion rate spanned on a wider range (40-100%) among cultures treated with kinetin or BA alone or in combination with 2, 4-D compared to TDZ's (42-77%), the latter caused greater shoot averages per explant (23-93) (Table 1) than the formers combined (2-53). This could partly be justified by the fact that, when TDZ-treated, this explant generally formed greater shoot primordia (55-127) than when treated with kinetin (7-18) or BA (5-71) (Table 1c). As previously reported [2, 21], repetitive multiple shoots could form indefinitely from a single explant *via* repetitive shoot budding of subcultured shoot buds. However, only primary multiple shoot buds, which were observed during the experiment, were scored for this report. Further, early severing of shoot primordia or buds, into clusters of two to 5 units from the original explants, improved shoot conversion rate of up to 100%, irrespective of the treatment (data not shown).

In addition to more improved results than previously reported [2-4, 9-12, 22], the present investigation has demonstrated the potential of forming more abundant multiple shoots in both proximal and distal regions of the cotyledon (Figure 3b). Same evidence also underpins that the proximal region is relatively more prolific for shoot formation than the distal region. Similar gradient of shoot formation in cotyledon has also previously been reported in peanut [4, 11] and other species, including, winged bean [23] and *Pinus pinea L.* [24]. Being contiguous to the cotyledonary node region known to have preexisting meristem, the proximal region of the cotyledon might benefit from the persistence of cell rejuvenation with the potential of enhanced shoot formation. Achieving such vast multiple shoot formation was significant to ensure reliability of clonal propagation and transgenic plant development in cultivated peanut. Further, observed maximal cotyledon shoot primordia, number of shoots per explant, and shoot formation and conversion rates are greater than those that have been reported previously in peanut [1, 21, 25] and recent studies [2, 4, 9-12, 22]. Most of all those reported studies applied only one preparation type of cotyledon tissue, such as, the whole cotyledon [11, 12] or half-cotyledon [8, 10, 13]. General comparisons, based on explant type's results, showed that the present protocol induced greater shoot rates in whole and half-cotyledons, and greater shoot rates and explant shoot averages than all previous fully-reported studies [2, 3, 9, 10, 11, 12, 22].

Unlike callus induction, shoot formation was mostly observed from treatments that applied cytokinin alone, in both cotyledon and root

Table 2. Five percent probability T-test results of comparative influence of growth regulator treatments or explant type on shoot organogenesis.

Influence of individual growth regulator treatments on shoot formation	Calculated probability	Influence of 2, 4-D-based treatments on shoot formation	Calculated probability	Comparative explant type influence on shoot formation	Calculated probability
WC/SER/K* vs B	0.0231020^{s}	WC/SER/K* vs DK	0.00046146^{s}	SER-Ks/WC* vs CD	0.0001514^{s}
WC/SER/K* vs T	0.0001370^{s}	WC/SER/B* vs DB	0.0060122^{s}	SER-Ks/WC vs MC*	0.0289984^{s}
WC/SER/B* vs T	0.0000750^{s}	WC/SER/T* vs DT	0.0348990^{s}	SER-Ks/CD vs MC*	0.0184854^{s}
CD/SER/K vs B*	0.0136300^{s}	CD/SER/K vs DK	0.0677082^{ns}	SER-Bs/WC* vs CD	0.0052385^{s}
CD/SER/K vs T*	0.0136300^{s}	CD/SER/B* vs DB	0.0389971^{s}	SER-Bs/WC vs MC*	0.0350845^{s}
CD/SER/B vs T	0.1951290^{ns}	CD/SER/T* vs DT	0.0389971^{s}	SER-Bs/CD vs MC*	0.0263573^{s}
MC/SER/K vs B*	0.0297900^{s}	MC/SER/K* vs DK	0.0240111^{s}	SER-Ts/WC* vs CD	0.0000717^{s}
MC/SER/K vs T*	0.0000476^{s}	MC/SER/B* vs DB	0.0385939^{s}	SER-Ts/WC* vs MC	0.0132436^{s}
MC/SER/B vs T*	0.0016624^{s}	MC/SER/T* vs DT	0.0002613^{s}	SER-Ts/CD vs MC*	0.0000969^{s}
WC/SCR/K* vs B	0.0018879^{s}	WC/SCR/K* vs DK	0.0178420^{ns}	SCR-Ks/WC* vs CD	0.0139157^{s}
WC/SCR/K* vs T	0.0201474^{s}	WC/SCR/B* vs DB	0.0091887^{s}	SCR-Ks/WC vs MC*	0.0048501^{s}
WC/SCR/B* vs T	0.1217747^{ns}	WC/SCR/T* vs DT	0.0490459^{s}	SCR-Ks/CD vs MC*	0.0441714^{s}
CD/SCR/K vs B	0.4386886^{ns}	CD/SCR/K* vs DK	0.0139159^{s}	SCR-Bs/WC* vs CD	0.0173900^{s}
CD/SCR/K vs T	0.3377963^{ns}	CD/SCR/B* vs DB	0.0153597^{s}	SCR-Bs/WC* vs MC	0.0006656^{s}
CD/SCR/B vs T	0.2791103^{ns}	CD/SCR/T* vs DT	0.0038204^{s}	SCR-Bs/CD vs MC*	0.0029684^{s}
MC/SCR/K* vs B	0.0472945^{s}	MC/SCR/K* vs DK	0.0039326^{s}	SCR-Ts/WC* vs CD	0.1122825^{s}
MC/SCR/K vs T	0.3957082^{ns}	MC/SCR/B vs DB*	0.0278620^{s}	SCR-Ts/WC vs MC*	0.0025253^{s}
MC/SCR/B vs T*	0.0199934^{s}	MC/SCR/T* vs DT	0.0003860^{s}	SCR-Ts/CD vs MC*	0.0081109^{s}
WC/ESA/K* vs B	0.0021660^{s}	WC/ESA/K* vs DK	0.0157329^{s}	ESA-Ks/WC* vs CD	0.0470857^{s}
WC/ESA/K vs T	0.0908450^{ns}	WC/ESA/B* vs DB	0.0090845^{s}	ESA-Ks/WC vs MC*	0.0108855^{s}
WC/ESA/B vs T*	0.0004914^{s}	WC/ESA/T* vs DT	0.0070366^{s}	ESA-Ks/CD vs MC*	0.0094557^{s}
CD/ESA/K vs B	0.3943900^{ns}	CD/ESA/K* vs DK	0.0397085^{s}	ESA-Bs/WC vs CD*	0.0458606^{s}
CD/ESA/K vs T*	0.0315900^{s}	CD/ESA/B vs DB	0.1594658^{ns}	ESA-Bs/WC vs MC*	0.0434895^{s}
CD/ESA/B vs T*	0.0389972^{s}	CD/ESA/T* vs DT	0.0178955^{s}	ESA-Bs/CD vs MC*	0.0486634^{s}
MC/ESA/K vs B*	0.0704335^{s}	MC/ESA/K* vs DK	0.0095385^{s}	ESA-Ts/WC* vs CD	0.0021660^{s}
MC/ESA/K vs T*	0.0000272^{s}	MC/ESA/B* vs DB	0.0341951^{s}	ESA-Ts/WC vs MC*	0.0000269^{s}
MC/ESA/B vs T*	0.0032029^{s}	MC/ESA/T* vs DT	0.0000244^{s}	ESA-Ts/CD vs MC*	0.0000214^{s}

Notes: **MC**: mono/side-cut cotyledon; **SER**: shooting explant rate (%); **D**: 2, 4-dichlorophenoxyacetic acid (2,4-D); **K**: kinetin; **B**: 6-benzyl-amino purine; **T**: thiadizuron; **DT**, **DK**, or **DB**: are treatments combining 2, 4-D with either TDZ, kinetin, or 6-benzyl-amino purine; **CD**: cotyledon Dice; **WC**: whole cotyledon; **SCR**: shoot conversion rate (%); **ESA**: explant shoots average; **ns**: statistically not significant; **s**: statistically significant; *****: identifies statistically best treatment in a comparison; **WC, CD, or MC/SER**: shoot explant rate of WC, CD, or MC; **WC, CD, or MC/SCR**: shoot conversion rate of WC, CD, or MC; **WC, CD, or MC/ESA**: explant shoots average of WC, CD, or MC; **WC, CD, or MC/SER, SCR, or ESA / K vs B, K vs T, or B vs T**: individual treatments influence on the target explant shooting rate, shoot conversion, or shoot average.

tissues (Table 1 and Table 3). This supports the general, functional purpose of the cytokinin in tissue culture [26, 27]. Overall, statistical differences were observed among individual growth regulator treatments, for shoot formation, as 20 out 27 comparisons tested using T-test were significantly different in cotyledon (Table 2), amongst which 33.33%, 22.22%, and 18.51% were influenced by TDZ-, kinetin-, and BA-based treatments respectively.

Table 3. Shoot organogenesis in root tissue.

Treatment	[1]SER	[1]ASE	[2]SER	[2]ASE	[3]SER	[3]ASE	[4]SER	[4]ASE	[5]SER	[5]ASE	[6]SER	[6]ASE
Control	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l Kinetin	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l Kinetin	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l Kinetin	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l Kinetin	0	0	0	0	0	0	0	0	0	0	0	0
5 mg/l Kinetin	0	0	9±1.1	2±0.1	0	0	0	0	0	0	0	0
10 mg/l Kinetin	5±0.1	2±0.1	15±1.3	3±0.5	0	0	0	0	0	0	0	0
20 mg/l Kinetin	10±1.3	5±0.8	23±2.1	5±0.5	0	0	0	0	0	0	0	0
30 mg/l Kinetin	18±1.6	6±0.9	25±2.5	6±0.9	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 5 mg/l BA	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 10 mg/l BA	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 20 mg/l BA	0	0	0	0	0	0	0	0	0	0	0	0
3 mg/l 2, 4-D + 30 mg/l BA	0	0	0	0	0	0	0	0	0	0	0	0
5 mg/l BA	2±0.3	3±0.3	6±0.7	3±0.3	0	0	0	0	0	0	0	0
10 mg/l BA	17±1.5	3±0.4	16±1.6	8±0.8	0	0	0	0	0	0	0	0
20 mg/l BA	18±1.7	65±4.4	23±2.5	15±1.2	0	0	0	0	0	0	0	0
30 mg/l BA	16±1.7	5±0.8	26±2.6	9±0.7	0	0	0	0	0	0	0	0

Notes: SER: Shooting explant rate (%) *plus* standard error; **ASE**: Average shoots /explant *plus* standard error. Embryo pre-culture period varied from 0 to 7 days and is presented in the table as follows: 1=0day, 2=1day, 3=2days, 4=3days, 5=5days, and 6=7days.

TDZ treatments induced the greatest shoot formation compared to any other treatments (Table 1a-c). Twenty out of twenty four TDZ-treatments, excluding those applied to di-side-cut explant, which did not respond at all (Table 1d), formed shoots. The second cotyledon best performing growth regulator treatments for shoot formation were BA-based, whose explant shoot averages ranged from 1 to 53. Eighteen out of 24 BA treatments formed shoots. Similar to the control, 2,4-D used alone did not induce shoots. Shoot conversion rate was generally independent of the treatment, but dependent of explant type (Table 2). The best performing treatment of the studies was the greatest TDZ concentration that formed the greatest number of shoots (93) and shoot primordia (127) per explant and rate of explants that formed shoots (98%) (Table 1c). Although high performances have generally been associated with low concentrations of TDZ in tissue culture across plant species [5, 20, 28-31], which justifies related common practices, we have consistently associated greatest responses with greatest concentrations of TDZ in peanut [2, 21].

Generally, the study showed that the combination of 2, 4-D with cytokinin treatments

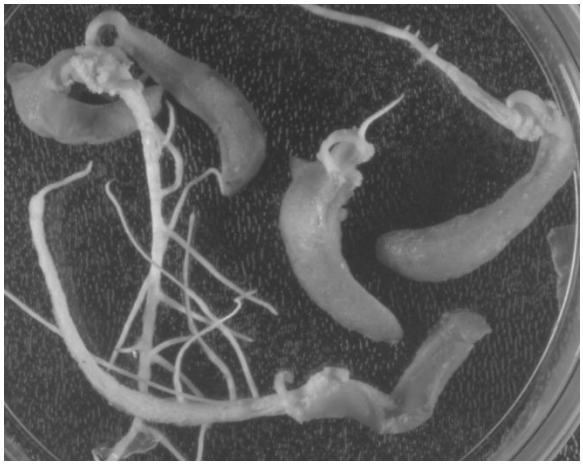

Figure 4. Cotyledon explants, cultured on hormone-free medium, forming roots and no shoots.

resulted in noticeable decrease of shoot formation, compared to cytokinin used alone (Tables 1a-c and Table 2). When a comparative influence on shoot organogenesis was statistically tested between auxin-based treatments and single cytokinin treatments, the results showed that individual growth regulator treatments best combination treatments (Table 2). Twenty four out of twenty seven comparisons were statistically significant in favor of single cytokinin treatments. Only 1 in 24 treatment comparisons showed a statistically significant difference in favor of 2, 4-D-based treatments. In select cases, the addition of 2, 4-D inhibited fully shoot formation (Table 1a-c). This occurred mostly when the whole cotyledon explant was treated with the combinations of 2, 4-D with either BA or kinetin (Table 1a). Similar observations were made when 2, 4-D was combined with kinetin, TDZ, or BA in treating diced cotyledon explant (Table 1b). Neither shoots nor shoot primordia formed in cultures treated with 2, 4-D alone (Table 1). The effect of combinations of a cytokinin and an auxin (NAA or 2, 4-D) on adventitious plant formation *in vitro* has generally been inconsistent. Some studies have reported consistent [22] and others inconsistent to no increase [14, 17, 24, 29, 32] of plant formation when treatments combined cytokinin and auxin, *in vitro*. It was also observed that about 2% of mono-side-cut cotyledons formed roots on control medium, but not shoots (Figure 4).

The studies also showed that shoot organogenesis response was generally dependent of explant type (Table 2). One hundred percent of differential comparisons aimed at determining the influence of explant on shoot formation showed statistical differences, using T-test (Table 2). Accordingly, the MC was the best responding explant across treatments for shoot organogenesis, considering that 6, 6, and 5 out of 6 comparative responses from TDZ-, kinetin-, and BA-based treatments, respectively, were statistically significant. The WC was the next best responding explant for shoot organogenesis, as 4, 3, and 3 out of 6 related comparative responses influenced by TDZ, kinetin, and BA, respectively, were statistically significant.

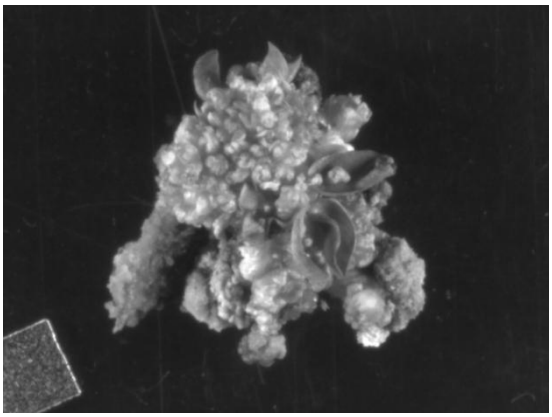

Figure 5. Root explants showing developmental shoot buds in the upper region subjacent to stem.

3. Shoot organogenesis in root explant

This is the first time peanut root tissue has been successfully used to induce direct adventitious plant formation *in vitro* (Table 3; Figure 5). Although multiple shoots were observed, the study showed that shoot-bud formation occurred only in the upper end of the root tissue that approximates the plant “crown region” (Figure 5). The greatest response observed (65 shoots/root explant) occurred in explants treated with 20 mg/l BA (Table 3).

Overall, shoot occurrence ranged from 2 to 65 shoots per explant. Amongst responding treatments, shoots formed from those that included kinetin or BA alone without auxin (2, 4-D) (Table 3). TDZ treatments' results are not included, because most related explants were contaminated and discarded. Generally, BA performed relatively better than Kinetin, considering that it induced not only the greatest average shoots per explant (65), but also the greatest shooting rate (26%) during one day of incubatory pre-culture period of explants (Table 3). The study also showed that extended pre-culture period of root explants on free-hormone MS medium had limited shoot organogenesis promoting effect, as only explants pre-cultured for zero and one day induced shoot organogenesis (Table 3). Similar to cotyledon, after shoot buds were split for further growth, extended repetitive-multiple shoots were observed in individual bud units. However, shoot-formation results that are reported in this study are limited to initial shoot bud formation in the original explants.

Figure 6. *In vitro*-induced plants are growing normally in the greenhouse.

4. Rooting and greenhouse acclimation

Induction of roots in shoots was merely achieved on basal medium. Shootlets that attained at least five millimeters long were severed from the cluster or explant and cultured on the freshly prepared medium where all units rooted within 7 to 15 days. Resulting plantlets were transferred into the greenhouse, where it all survived and grew normally (Figure 6).

Conclusion

This study showed that peanut cotyledon tissue might be more efficiently manipulated *in vitro* for increasing multiple shoot formation, similar to standard explants such as leaf, stem, or embryo axis. The mono-side-cut cotyledon responded the best for shoot formation, under the influence of TDZ, compared to the whole, diced, or di-side-cut cotyledonary explant. Shoots were induced for the first time in dry-peanut-seed root tissue. The study also suggests that both cotyledon and root might not be the tissues of choice for callus formation. Tissues shoot organogenesis depended upon explant preparation and conditioning. The study provides a model protocol, which can be reproduced across laboratory settings.

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