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# Thidiazuron induced somatic embryogenesis in *Coffea arabica* L. and *Coffea canephora* P ex Fr.

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The rapid direct and repetitive somatic embryogenesis in *Coffea arabica* and *C. canephora* genotypes was tested on Murashige and Skoog medium (MS) containing thidiazuron (TDZ) (1-phenyl-3-(1,2,3,-thiadiazol-5-yl)urea) in concentrations of 2.27–11.35s μM. Segments taken from cotyledon leaf, first leaf and stalk of regenerated plantlets produced clusters of somatic embryos directly from cut portions of explants on TDZ (9.08 μM) containing medium within a period of two months. Subculturing of these embryo clusters produced more secondary embryos on reduced TDZ (0.045–0.91 μM) containing medium and these subsequently developed into plantlets (80–85%) on development medium followed by rooting on MS basal medium. This direct somatic embryogenesis from leaf and hypocotyl explants in *Coffea* sp. is a strong evidence of cell totipotency. The rapid somatic embryo induction protocol would be useful for the mass propagation, direct regeneration and genetic transformation of selected elite lines.

**Key words:** *Coffea arabica, Coffea canephora*, cotyledons, hypocotyls, leaf, somatic embryos, thidiazuron

## Introduction

*Coffea* is an extremely important perennial agricultural crop in tropical areas with more than 6.5 million tons of green beans being produced every year on about 11 million hectares (ANONYMOUS 1997). The genus *Coffea* (Rubiaceae) consists of about 80 species in which only *Coffea arabica* (arabica) and *Coffea canephora* (robusta) are important for the production of *Coffea* beans (BERTHOUD and CHARIER 1988). *C. arabica* contributes nearly 70% of the coffee consumed world-wide due to its superior quality and *C. canephora* accounts for the rest 30%. In India, both arabica and robusta coffee are cultivated on almost equal areas (RADHAKRISHNAN 2002). Conventional breeding of coffee is difficult because of the long duration of cultivation before the seeds are set. Plant regeneration via various tissue culture methods could be very effective for propagation and improvement of coffee plants (SONDAHL and SHARP 1977).

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Somatic embryogenesis is a highly useful method for the large-scale propagation of species of economic interest. Somatic embryos are widely considered to be of single cell origin; hence this is advantageous for transformation studies. Moreover, the process of somatic embryogenesis offers a mean to propagate large numbers of transgenic plants over a short period of time. In *Coffea,* somatic embryogenesis was reported nearly 3 decades ago by STARITSKY (1970) and constitutes a model system for perennial species (SONDAHL and LOH 1988). The origin of somatic embryos in *Coffea* may be either direct, from explants (YASUDA et al. 1985; HATANAKA et al. 1991, 1995), or indirect, via embryogenic callus formation (SONDAHL and SHARP 1977, DUBLIN 1981, VAN BOXTEL and BERTHOULY 1996, PIERSON et al. 1983, NISHIBATA et al 1995, SANDRA et al. 2000). In spite of many reports on the somatic embryogenesis of *Coffea*, the induction of somatic embryogenesis and plantlet formation is very sensitive to culture conditions such as nutrient and hormonal composition of the medium (STARISTSKY and VAN HASSEL 1980). Thidiazuron has received considerable attention as a potent regulator of *in vitro* propagation systems and as an effective means of stimulating the development of adventitious shoots and somatic embryos in a wide variety of plants (HUETTEMAN and PREECE 1993, Lu 1993). In the present report the effect of TDZ on the somatic embryogenesis of *Coffea* sp. is discussed.

## Materials and Methods

### Plant material

Seeds of *Coffea arabica* L. variety Hemavathy and *C. canephora* P. ex Fr. variety 274 and CXR variety (collected from CCRI, Balehonnur, Karnataka State, India) were sown in garden soil. The seedlings emerged after 30 days. The cotyledons and hypocotyls were used as *in vivo* derived explants. Similarly, the leaf and stalk portions of *in vitro* regenerated plantlets were used as explants for repetitive embryogenesis experiments. These *in vitro* sterile plantlets were obtained through inducing embryogenic callus, subsequently somatic embryos and plantlets by using a reported protocol (VAN BOXTEL and BERTHOULY 1996). Surface sterilization for *in vivo* explants was performed with 1% sodium hypochlorite (10 min). Subsequently the explants were sterilized with 0.1% mercuric chloride for 5 minutes followed by washing with sterile water five times. Fifty mm square explants were cut from the leaf blade with a scalpel, excluding the basal and apical portions, mid vein and margins. Hypocotyl and stalk explants were cut into ten mm segments and collected in petri dishes containing 0.025% cysteine HCl. The explants were cultured in 100 × 20 mm disposable petri dishes containing approximately 25 ml of the medium. Leaf explants were cultured with their adaxial side in contact with the medium.

### Media

Medium A comprises MS basal medium (MURASHIGE and SKOOG 1962), 2% sucrose (w/v) supplemented with 2.27 – 11.35 μM thidiazuron (TDZ, i.e. 1-phenyl-3-(1,2,3,--thiadiazol-5-yl)urea). Medium B comprises MS basal + 3% sucrose (w/v) + 2.27 – 11.35 μM TDZ, medium C is MS basal + 10% (v/v) coconut water (CW) + 2% (w/v) sucrose + 2.27 – 11.35 μM TDZ and medium D contains 2% (w/v) sucrose+ 10% (v/v) CW. Controls were maintained on medium E comprising MS basal and 2% sucrose. MS salts full or half strength concentration along with B5 vitamins (GAMBORG et al.1968), 2–3% (w/v) sucrose,

supplemented with TDZ 2.27–11.35 μM and CW 10% (v/v) (optional), were also used in the experiments. The clumps of the leaf-derived primary embryogenic nodules were further cultured on medium comprising half strength MS salts and B5 vitamins supplemented with 0.91 μM TDZ. The maturation of the somatic embryos was carried out in half strength MS salts, 10 mg $L^{-1}$ thiamine HCl, 3.2 mg $L^{-1}$ pyridoxine HCl, 2.85 μM indole-3-acetic acid (IAA), 2% (w/v) sucrose and cultured for 60 days. The regenerated plantlets were rooted on half strength MS basal medium containing 2% (w/v) sucrose. All the media were gelled with 0.8% agar (w/v). The pH of all the media was adjusted to 5.7 and was autoclaved at 120 °C and a pressure of 1.06 kg/$cm^2$ for 15 minutes. All the cultures were incubated in the dark at 25 ± 1 °C for 45 days. Rooting was carried out under a 16:8 photoperiod.

### Statistical analysis

Fifty leaf (from each leaf 10 replicates), ten hypocotyl (from each hypocotyl 5 replicates) and ten stalk explants (from each stalk 5 replicates) each were used for the experiment. The experiment was repeated thrice and the mean ± SE values of the results were tested using the one-way ANOVA test and the results were analyzed using Tukey Multiple Comparison (software Prism 3.0). In the results, only the optimal TDZ concentration treated data are provided (Tabs. 1–5).

## Results

Initially 80–90 % of the leaf and hypocotyl explants remained green when cultured in the dark on MS medium with or without TDZ. No deterioration was observed within 20 days. Initially the embryos appeared as small white globular masses, which germinated and passed through successive developmental stages. The effects of TDZ on the frequency of embryo and callus formation per explant is described in tables 1–5.

*Coffea arabica* showed direct somatic embryogenesis wherein 70% of *in vitro* leaf explants (161.6 ± 2.5 embryos) (Fig. 1A) and 50% of *in vitro* stalk explants of regenerated plants produced embryos (39.9 ± 1.25 embryos) (Tabs. 1, 2), but only 10% of cotyledonary leaf explants (12.6 ± 0.81 embryos) produced embryos when they were cultured on medium A (Tab. 3). In *C. arabica* 60% of the *in vivo* coteledonary leaf explant produced callusing. In *C. canephora* 274 variety 60% of *in vitro* leaf explants (Tab. 1, Fig. 1B) and 72% *in vitro* stalk of regenerated plants (Tab. 2, Fig. 1D) produced direct somatic embryogenesis from the margins and surface respectively (180.1 ± 3.0 and 102.8 ± 6.8 embryos) on medium A. Medium A containing 9.08 μM TDZ supported direct somatic embyogenesis from leaf explants as compared to medium C where in 10% coconut water was incorporated (Tab. 1). Though coconut water was tested in order to establish its effect on somatic embryogenesis, it was found to be insignificant in this study as there was no response for embryogenesis. In *C. canephora* 274 variety only 22% of *in vivo* cotyledonary leaf explants produced embryos (16.5 ± 1.51) on medium A (Tab. 3). Callusing was not noticed in *in vitro* leaf and stalk explants in contrast to cotyledonary leaf explants where 50% of *in vitro* stalk explants of regenerated plants with marginal callusing was noticed (Tab. 3). The higher sucrose concentration in medium B (with 3% sucrose) reduced somatic embyogenesis in *C. canephora* from *in vitro* leaf and stalk explants, while only 20 and 60% explants produced 36.0 ± 0.66 and 40.5 ± 1.65 embryos per explant respectively (Tabs. 1, 2). Me-

dium C supported direct embryo formation from *in vitro* leaf explants of *C. arabica* only along with callusing but the number of embryos per explant was less (Tab. 1). Almost 100% of *C. canephora* 274 variety and 64% of *C.arabica in vitro* leaf explants produced

**Tab.1.** Effect of TDZ on *Coffea* somatic embryogenesis from *in vitro* leaf explants

| MS medium | Supplements | | | % of explants responded | | No. of embryos per explant | | % of explants producing callus | |
|---|---|---|---|---|---|---|---|---|---|
| | Sucrose %(w/v) | TDZ (μM) | CW (v/v) | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety |
| A | 2 | 9.08 | 0 | 70 | 60 | 161.6±2.5[b] | 180.1±3.0[a] | – | – |
| B | 3 | 9.08 | 0 | 20 | 20 | 24.3±1.6[a] | 36.0±0.6 | – | – |
| C | 2 | 9.08 | 10 | 16 | – | 13.3±0.5 | – | 64* | 100** |
| D | 2 | 0 | 10 | – | – | – | – | 10* | 10*** |
| E | 2 | – | – | – | – | – | – | – | – |

*Greenish-white non friable callus, ** light yellow friable callus, *** white non friable callus, incubation = 45 days
E = control. a = $p < 0.01$, b = $p < 0.05$, compared to number of embryos on medium 'C' for *C. arabica* and medium 'B' for *C. canephora*, CW = coconut water.

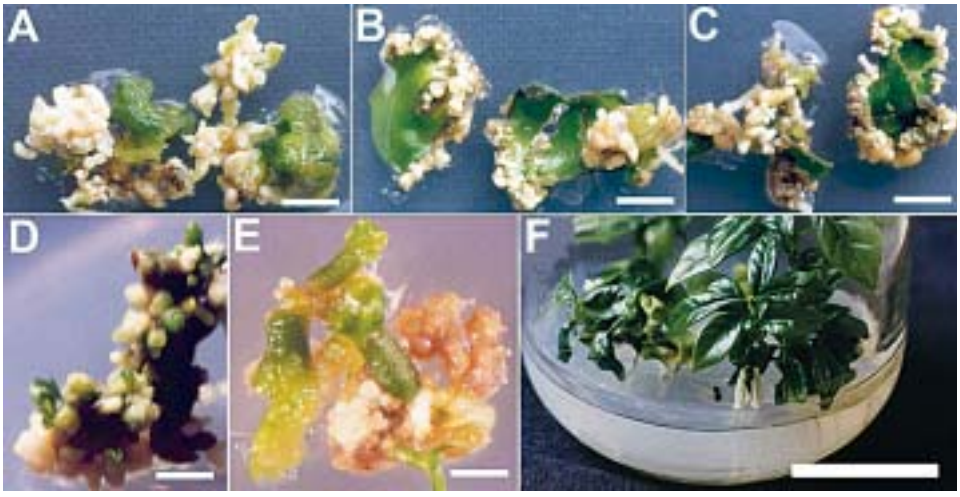


**Fig. 1.** **A)** Direct somatic embryogenesis from leaf explants of *Coffea arabica* on medium comprising MS basal medium supplemented with 2% Sucrose and TDZ 9.08 μM (bar 10mm); **B)** Direct somatic embryogenesis from leaf explants of *C.canephora* 274 variety on MS with 2% sucrose and TDZ 9.08 μM (bar 10 mm); **C)** Direct somatic embryogenesis from cotyledonary leaf explant of *C. canephora* CXR variety on MS medium containing B5 vitamins, 2% sucrose and supplemented with TDZ 9.08 μM (bar 10 mm); **D)** Direct somatic embryogenesis from *in vitro* stalk segments of *C. canephora* 274 variety on MS medium containing 2% sucrose and TDZ 9.08 μM (bar 4 mm); **E)** Development of somatic embryos originated from leaf explants of *C. canephora* 274 variety into plantlets. (bar 4 mm); **F)** Regenerated plantlet of *C. canephora* 274 variety (bar 10 mm)

**Tab. 2.** Effect of TDZ on *Coffea* somatic embryogenesis from *in vitro* stalk explants of regenerated plants

| MS medium | Supplements | | | % of explants responded | | No. of embryos per explant | | % of explants producing callus | |
|---|---|---|---|---|---|---|---|---|---|
| | Sucrose %(w/v) | TDZ (μM) | CW (v/v) | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety |
| A | 2 | 9.08 | 0 | 50 | 72 | 39.9±1.2[a] | 102.8±6.8[a] | – | – |
| B | 3 | 9.08 | 0 | 10 | 60 | 14.5±0.8 | 40.5±1.6 | – | – |
| C | 2 | 9.08 | 10 | – | – | – | – | 84*** | 100** |
| D | 2 | 0 | 10 | – | – | – | – | – | – |
| E | 2 | – | – | – | – | – | – | – | – |

* Greenish-white non friable callus, ** light yellow friable callus, *** white non friable callus, incubation = 45 days
E= control, a = $p < 0.01$, compared to those on medium 'B', CW = coconut water.

**Tab. 3.** Effect of TDZ on *Coffea* somatic embryogenesis from *in vivo* cotyledonary leaf explants

| MS medium | Supplements | | | % of explants responded | | No. of embryos per explant | | % of explants producing callus | |
|---|---|---|---|---|---|---|---|---|---|
| | Sucrose %(w/v) | TDZ (μM) | CW (v/v) | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety |
| A | 2 | 9.08 | 0 | 10 | 22 | 12.6±0.8a | 16.5±1.5a | 60 *** | 50 * |
| B | 3 | 9.08 | 0 | 12 | 20 | 12.0±0.2 | 20.2±0.6 | – | 20 * |
| C | 2 | 9.08 | 10 | – | – | – | – | 10 *** | 30 ** |
| D | 2 | 0 | 10 | – | – | – | – | 20 *** | 25 * |
| E | 2 | – | – | – | – | – | – | – | – |

* Greenish-white non friable callus, ** light yellow friable callus, *** white non friable callus, incubation = 45 days
E= control, a = $p < 0.01$, compared to those on medium 'B', CW = coconut water.

light yellow friable callus and greenish white non friable callus respectively on medium C (Tab. 1) containing 2% sucrose. In *C. canephora* 274 variety *in vitro* stalk explant and cotyledonary leaf explants produced light yellow friable callus (Tab. 2, 3), which becomes embryogenic after culturing on Heller's (Heller 1953) medium for 3 months in the dark (data not shown). The callus obtained from *in vivo* coteledonary leaf explants of *C. arabica* is white and non embryogenic (Tab. 3). In both *C. arabica* and *C. canephora* 274 varieties the response of hypocotyl explants was almost similar to cotyledonary explants and a not very significant difference was found for somatic embryogenesis (Tab. 4). *C. canephora* CXR variety explants showed good response for direct somatic embryogenesis (Tab. 5) on MS medium supplemented with B5 vitamins and 9.08 μM TDZ (Fig. 1C). A maximum of 76.4 ±2.8 embryos and 91.6 ± 12.3 embryos were produced from *in vitro* leaf and cotyledonary leaf explants (Tab. 5). But some of the cotyledonary leaf explants showed greenish yellow colour callusing from the margins.

**Tab. 4.** Effect of TDZ on *Coffea* somatic embryogenesis from *in vivo* hypocotyl explants

| MS medium | Supplements | | | % of explants responded | | No. of embryos per explant | | % of explants producing callus | |
|---|---|---|---|---|---|---|---|---|---|
| | Sucrose %(w/v) | TDZ (μM) | CW (v/v) | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety | *C. arabica* | *C. canephora* 274 variety |
| A | 2 | 9.08 | 0 | 12 | 20 | 21.4±0.5[a] | 36.5±1.4[a] | 58 *** | 40 * |
| B | 3 | 9.08 | 0 | 14 | 18 | 24.2±0.2 | 26.0±0.4 | - | 18 * |
| C | 2 | 9.08 | 10 | - | - | - | - | 20 *** | 28 ** |
| D | 2 | 0 | 10 | - | - | - | - | 25 *** | 26 * |
| E | 2 | – | – | - | - | - | - | - | - |

* Greenish-white non friable callus, ** light yellow friable callus, *** white non friable callus, incubation = 45 days
E = control, a = $p < 0.05$, compared to those on medium 'B', CW = coconut water.

**Tab. 5.** Effect of TDZ on somatic embryogenesis of different explants of *Coffea canephora* CXR variety

| Medium with supplements | | | % of explants responded | | | No. of embryos per explant | | | % of explants producing callus | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Salts | TDZ (μM) | CW (v/v) | coty-ledon | *hypo-cotyl* | *In vitro* leaf | Coty-ledon | hypo-cotyl | *In vitro* leaf | coty-ledon | Hypo-cotyls | *In vitro* leaf |
| MS | 9.08 | 0 | 70 | 20 | 30 | 91.6±12.3[a] | 27.9±0.7[b] | 76.4±2.8[a] | 45* | 5*** | 52* |
| ½MS | 9.08 | 0 | 76 | 36 | 50 | 88.4±13.2[b] | 42.5±0.8[b] | 60.0±4.2[b] | 48* | 18* | 58* |
| MS | 9.08 | 10 | 24 | 28 | 20 | 26.5±1.0 | 33.5±0.9 | 44.2±0.8 | 60** | 66** | 75** |
| ½MS | 0 | 10 | – | – | – | – | – | – | 10* | 10*** | 12* |
| MS | – | – | – | – | – | – | – | – | – | – | |

* Greenish-white non friable callus, ** light yellow friable callus, *** white non friable callus, incubation= 45 days,
a = $p < 0.05$, b = $p < 0.001$ compared to those on MS with TDZ and CW. In all the media 2% sucrose and B5 vitamins used, CW = coconut water.

Matured embryos (greenish globular and tubular stage) physically removed from the explants and placed on developing medium formed individual plantlets (80–85%) (Fig. 1E). They produced shoots after 30–45 days. All the regenerated plantlets appeared to be morphologically normal. The regenerated plantlets were rooted on half strength MS basal medium within 45 days (Fig. 1F).

## Discussion

This study provides a model for investigating the mechanisms underlying the process of somatic embryogenesis in *Coffea* genotypes. The most interesting aspect of our study on the somatic embryogenesis of *Coffea* genotypes is the rapid induction of direct somatic embryogenesis in both *C. arabica* as well as *C. canephora* on medium supplemented with TDZ irrespective of species and ploidy differences. This kind of response has not previ-

ously been reported. TDZ was first used for the mechanized harvesting of cotton bolls and more recently incorporated into tissue culture media as a means of inducing regeneration. It has been reported that the induction of somatic embryogenesis is commonly associated with modulations of auxins by TDZ (CAPELLE et al 1983, VISSER et al. 1992, HUTCHINSON et al. 1996). The utilization of TDZ in tissue culture of dicotyledonous plants for the direct differentiation of somatic embryos is gaining importance (GILL and SAXENA 1992, IANTCHEVA et al. 1999). In general TDZ at very low levels induces direct somatic embryogenesis in the geranium (QURESHI and SAXENA 1992), alfalfa (IANTCHEVA et al. 1999), water melon (COMPTON and GRAY 1992) and muskmelon (GRAY et al. 1992). The association between TDZ-induced responses and endogenous plant growth regulators of dicotyledonous plants has been documented by means of the quantification of their profiles. TDZ promotes the synthesis & accumulation of purines (CAPELLE et al. 1983) and also alters cytokinin metabolism (MOK et al. 1982). Since TDZ is involved in cytokinin metabolism, we decided to explore its effect on direct somatic embryogenesis in *Coffea*, and the results of present study proved this.

In all the treatments the initial appearance of small white globular embryos appears to be affected by the stage of maturity of the leaves; this was especially true with *in vivo* explants (data not shown). When 30 days old cotyledonary leaf explants of CXR and 274 varieties used the response was moderate to good compared to 2–3 month first and second leaves of seedlings (data not shown). Clumps of the leaf-derived primary embryogenic nodule mass proliferated to produce more secondary embryos when subcultured onto the medium containing half strength MS salts and B5 vitamins supplemented with 0.91 μM TDZ (data not shown). Trials were conducted to determine the optimal TDZ dosage for embryo formation by inoculating nodular clumps (5 × 5 mm) onto half strength MS basal medium containing different levels (0.045–4.54 μM) of TDZ. The use of TDZ at 0.91 μM most favored embryo proliferation.

The significance of this study is the appearance of rapid repetitive somatic embryogenesis from regenerated plantlets, which is applicable to both species of *C. arabica* and *C. canephora* by using TDZ. This is the first report of direct somatic embryogenesis from explants of *in vitro* regenerated plantlets derived through an indirect embryogenesis route. Use of other hormonal regimes results in callusing from cut portions (margins) and eventually used for indirect somatic embryogenesis to form plantlets (NISHIBATA et al. 1995). Various investigators have suggested the process of high frequency of somatic embryos in both *C. arabica* and *C. canephora* (HATANAKA et al. 1991, 1995; ETIENNE-BARRY et al. 1999; SANDRA et al. 2000). In all these cases, callus intermediates were involved and a long duration for embryo induction is involved. Callus derived somatic embryos known to possess somaclonal variations in regenerated lines. In the present report healthy regenerates were obtained from leaf, hypocotyls and stalk explants on MS salts containing medium supplemented with TDZ, through morphogenetic routes including direct somatic embryogenesis.

In conclusion, the present experiments have shown that leaf, hypocotyl and stalk explants of *C. arabica* and *C. canephora* are able to form somatic embryos directly in a defined medium containing optimal TDZ concentrations, which varies with genotypes and type of explant. Clumps of these embryo masses proliferated and germinated into plantlets on suitable media. This protocol is simple, reproducible, and easy to carry out and can provide good number of embryos and plants for mass propagation.

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