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Micropropagation of *Eria dalzelli* (Dalz.) Lindl. through TCL *in Vitro* Culture

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ABSTRACT

Efficient shoot regeneration of *Eria dalzelli* (Dalz.) Lindl. was achieved for the first time using shoot tip transverse thin cell layers (tTCLs) and thidiazuron (TDZ). Protocorm-like bodies (PLBs) or proliferating shoot buds was observed when tTCLs of shoot tip sections were cultured on Mitra *et al.* (1976) basal medium supplemented with 9.08 μM TDZ. The highest percentage of PLB survival was 96%, producing healthy shoots with 2-3 leaves. Shoots rooted when cultured on the same basal medium supplemented with 11.42 μM IAA. Regenerated plantlets grew normally with a 90% survival rate. This simple protocol will be useful for large-scale propagation of *E. dalzelli*.

Keywords: orchid, TCL, TDZ

Abbreviations: IAA, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **NAA**, α-napthaleneacetic acid; **PLB**, protocorm-like body; **TDZ**, thidiazuron (*N*-phenyl-*N*-1,2,3-thidiazuron-5′-ylurea)

INTRODUCTION

Current interest in propagation of commercially important native orchid species has created a need to develop practical propagation methods (Sharma and Tandon 1990; Malabadi *et al.* 2004, 2005; Malabadi *et al.* 2008a, 2008b). With increased popularity a potential need exists for the introduction of new native orchids. Although orchid sales are quickly rising, production and sales of native orchids, at best, are slowly increasing. Production of native orchids has not been fully commercialized, but is centralized within hobby growers and small, specilized nurseries. These nurseries only offer a small selection of showy genera and species. The small market for native commercial orchids is influenced by a lack of knowledge from the consumer, lack of interest by the industry, difficulties in propagation methods, and the long time period required to obtain flowering plants. A major obstacle to propagation of native orchids is the difficulty in seed germination, while seedling development can be long process and flowering plants are often produced only after 3-5 yerars of growth.

Eria dalzelli (Dalz.) Lindl. is an important epiphytic native orchid species of the Western Ghat forests of Karnataka. Over-exploitation and slow growth rate of plants has reduced the number of native orchids in the Western Ghat forests of Karnataka. Furthermore, there are no reports of *in vitro* propagation of this orchid species in the literature. So it is essential to take measures such as plant tissue culture methods for propagating this native orchid species.

Plant tissue culture methods have played an important role in the micropropagation of several commercially important orchids to meet the demands of growing market throughout the world (Wimber 1963; Morel 1964; Rao 1977; Wang 1988; Sharma and Tandon 1990; Sharma *et al*. 1991; Lakshmanan *et al*. 1995; Ichihashi 1997; Chang and Chang 1998; Ichihashi 1998; Kanjilal *et al*. 1999; Malabadi *et al*. 2004, 2005; Malabadi and Nataraja 2007a, 2007b; Nayak *et al*. 1997, 2002; Huan *et al*. 2004; Teixeira da Silva *et al*. 2006; Malabadi *et al.* 2008a, 2008b). In orchids, plantlets are usually regenerated through protocorm-like bodies (PLBs) or by direct shoot organogenesis. Transverse thin cell layers (tTCLs) of plant tissues such as apical meristems, stem nodes, and PLBs have been successfully used as explants for plant regeneration in a few orchids as well as other plant species (Begum *et al.* 1994; Nayak *et al*. 2002; Malabadi *et al.* 2004, 2005; Teixeira da Silva *et al*. 2006; Malabadi and Nataraja 2007a, 2007b; Zhao *et al.* 2007; Malabadi *et al.* 2008a). This culture system was first developed by Tran Thanh Van for progrmming different patterns of morphogenesis in *Nicotiana tobacum* (Tran Thanh Van 1973a, 1973b, 1980). Therefore, in order to save time and plants in nature, an improved method such as tTCLs is used as an effecient propagation method for the fast multiplication of *E. dalzelli*. The application of tTCL technology is very efficient in terms of using a small number of plants as an explant source but resulting in the production of a great number of plantlets in a more controlled manner than a conventional explant. This is very cost effective for small-scale tissue culture industries and commercial production of plantlets, and also generates employment for rural people.

The objective of this study was to develop, for the first time ever, an *in vitro* propagation method using tTCL technology in *E. dalzelli*. This would help the conservation of this native orchid; moreover, an *in vitro* multiplication method can be used for the large-scale production to meet the growing demands of native orchids.

MATERIALS AND METHODS

Twenty plants of *Eria dalzelli* (Dalz.) Lindl., collected from the Western Ghat Forests of Karnataka state, India (14°5′ to 15°25′ N latitude and 74°45′ to 76°15′ E longitude with an average rainfall of 80 cm) were established in pots and grown under greenhouse conditions at the Department of Botany, Karnatak University, Dharwad, India. Shoot tips of *Eria dalzella* (0.5-0.8 cm) harvested from mother plants (approximately 4-6 years old) were carefully

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washed in double distilled water (DDW). They were surface decontaminated sequentially with 0.1% streptomycin (Sigma) (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma), and thoroughly rinsed with sterilized DDW. tTCLs 1-5 mm thick were cut from shoot tips and cultured on Mitra *et al.* (1976) basal medium with 3.0% sucrose (Sigma), 0.7% agar (Sigma), 0.5 gl^{-1} *myo*-inositol (Sigma), 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine (Sigma), 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin (Sigma). The medium was supplemented with a range of thidiazuron (TDZ) concentrations (0. 04, 0.22, 0.45, 2.27, 4.54, 9.08, 11.35, 13.62, 18.16, 22.71, 27.24, 31.78, 36.32, 40.86 and 45.41 µM) without any other plant growth regulators (PGRs) in 25 mm × 145 mm glass culture tubes (Borosil) containing 15 ml of the medium under cool white fluorescent light (100 µmol m^{-2} s^{-1}) at 25 ± 3°C with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. Media without TDZ served as the control. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. L-glutamine, biotin, *p*-aminobenzoic acid were filter sterilized (Whatman filter paper, pore size = 0.45 µm; diameter of paper = 25 mm) and added to the media after autoclaving when the medium had cooled to below 50°C.

The cultures were maintained for 6-10 weeks for the initiation of PLBs or proliferating shoot buds. The freshly initiated individual PLBs were transferred to Mitra *et al*. (1976) basal medium containing 9.08 µM TDZ. Healthy shoots with 2-3 leaves developed within 10-12 weeks. They were subcultured on the same medium for another 2 weeks for further shoot development. All experiments contained 30 cultures per replicate, with four replicates (120 cultures) per experimental treatment, and each treatment was repeated three times (120 × 3 = 360). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at the 5% level using SPSS (Microsoft Windows ver. 13.0.1.1 statistical software package).

Well-developed shoots (2-3 cm) 8 weeks old were further transferred to fresh Mitra *et al.* (1976) basal medium supplemented with various concentrations of auxins (IAA (indole-3-acetic acid), NAA (α-napthaleneacetic acid), IBA (indole-3-butyric acid) for rooting. All the shoot buds were cultured on Mitra *et al.* (1976) basal medium supplemented with IAA (0.57, 8.56, 11.42, 14.27, 17.13, 19.98, 22.84 µM), IBA (0.49, 2.45, 4.9, 7.35, 9.8, 12.25, 14.7, 19.6 µM) and NAA (0.53, 2.68, 5.37, 8.05, 10.74, 13.42, 16.11 µM) to test the rooting efficiency (**Table 2**). The shoots with well developed roots on basal medium supplemented with 11.42 µM IAA were washed thoroughly under running tap water and transplanted into 15 cm diameter plastic pots (2-3 plants) containing a potting mixture of charcoal chips, coconut husks and broken tiles (2: 2: 1) (**Fig. 1C**). Three to four plants were planted in each pot and the plants were watered daily and fertilized at weekly intervals with a foliar spray of a mixture of commercial DAP (di-ammonium phosphate) and NPK (nitrogen 20: phosphorous 10: potassium 10) (Malabadi *et al*. 2004, 2005; Malabadi and Nataraja 2007a; Malabadi *et al.* 2008a). 90% of plants survived.

RESULTS

In the present study, lower concentrations (0.04, 0.22, 0.45 µM) of TDZ could not effectively initiate PLBs or proliferating shoot buds (**Table 1**). All explants (shoot tip tTCLs) remained green for 4 weeks, eventually turned brown, and finally died (after 8-10 weeks). Incorporation of higher concentrations (22.71, 27.24, 31.78, 36.32, 40.86, 45.41 µM) of TDZ leads to the browning of explants, and ultimately in the death of explants (**Table 1**). Few explants remained green for 2-3 weeks and failed to induce organogenesis. All the explants cultured on Mitra *et al*. (1976) basal medium without TDZ (control) failed to induce PLBs. On the other hand, low initiation of PLBs or proliferating shoot buds was noticed when TDZ was incorporated at 2.27, 4.54, 11.35, 13.62 and 18.16 µM in the basal medium. The highest number of responsive explants (96.0 ± 3.0) showing maximum number of PLBs or proliferating shoot buds was observed at 9.08 µM TDZ (**Table 1; Fig. 1**). Therefore, 9.08 µM TDZ is the optimum concentration for inducing PLBs or proliferating shoot buds in *E. dalzelli* (**Table 1**). Shoot tip tTCLs remained green and developed small bud-like structures when cultured on 9.08 µM TDZ-supplemented basal medium after two weeks (**Fig. 1A**). The small bud-like structures were maintained for 4 weeks and then further subcultured on the same fresh medium for another 4 weeks.

Table 1 Effect of different concentrations of TDZ on the initiation of protocorm-like bodies (PLBs) or proliferating shoot buds in *Eria dalzelli* (Dalz.) Lindl.

TDZ (µM)	Responsive explants (%)	№ of PLBs or shoot buds per explant	№ of shoot buds produced per explant
Control*	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
0.04	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
0.22	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
0.45	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
2.27	12.0 ± 0.6 b	5.0 ± 0.2 b	2.0 ± 0.1 b
4.54	31.0 ± 1.8 b	19.0 ± 1.4 b	13.0 ± 1.0 b
9.08	96.0 ± 3.0 a	42.0 ± 2.7 b	30.0 ± 4.5 b
11.35	29.0 ± 2.5 b	11.0 ± 0.8 b	4.0 ± 2.1 b
13.62	16.0 ± 0.9 b	7.0 ± 0.5b	3.0 ± 0.2 b
18.16	5.0 ± 0.3 b	3.0 ± 0.0 b	2.0 ± 0.1 b
22.71	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
27.24	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
31.78	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
36.32	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
40.86	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
45.41	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c

*Control = Basal medium without TDZ
Data scored after 14 weeks represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different ($P<0.05$) according to DMRT.

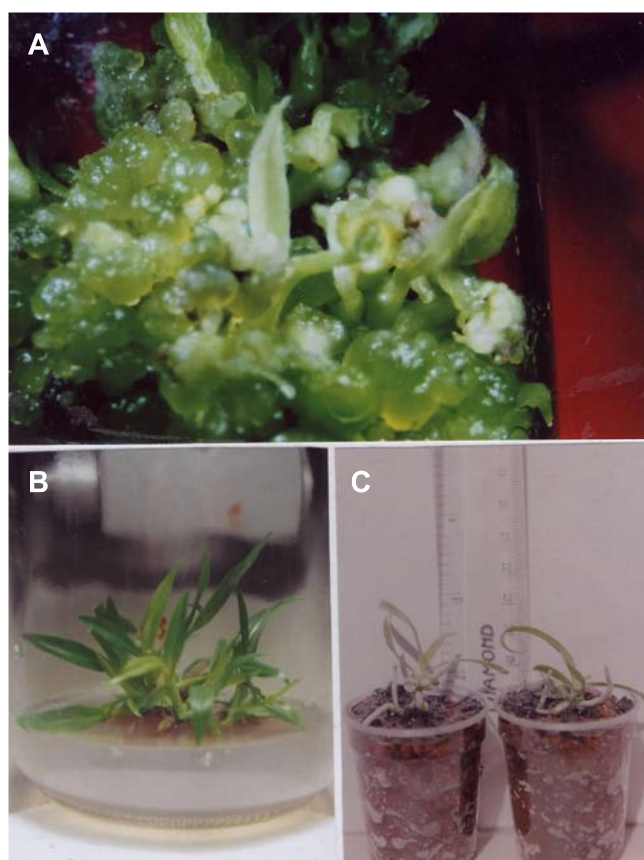

Fig. 1 ***In vitro*** **multiplication of** ***Eria dalzelli*** **(Dalz.) Lindl.** (**A**) Initiation of protocorm-like bodies or proliferating shoot buds in tTCL of shoot tip after 3-4 weeks of culture on Mitra *et al*. (1976) basal medium supplemented with 9.08 µM TDZ. (**B**) Healthy shoots formed rooting on Mitra *et al*. (1976) basal medium supplemented with 11.42 µM TDZ. (**C**) Well developed hardened plants ready for transfer to field conditions.

Table 2 Effect of different concentrations of auxin (IAA, IBA, NAA) on rooting of shoots regenerated with 9.08 µM TDZ treatment.

IAA (µM)	Rooting (%)	IBA (µM)	Rooting (%)	NAA (µM)	Rooting (%)
Control*	0.0 ± 0.0 c	0	0.0 ± 0.0 c	0	0.0 ± 0.0 c
0.28	0.0 ± 0.0 c	0.24	0.0 ± 0.0 c	0.26	0.0 ± 0.0 c
0.57	0.0 ± 0.0 c	0.49	0.0 ± 0.0 c	0.53	0.0 ± 0.0 c
2.85	0.0 ± 0.0 c	2.45	0.0 ± 0.0 c	2.68	0.0 ± 0.0 c
5.71	0.0 ± 0.0 c	4.90	0.0 ± 0.0 c	5.37	0.0 ± 0.0 c
8.56	18.0 ± 0.2 b	7.35	0.0 ± 0.0 c	8.05	0.0 ± 0.0 c
11.42	86.0 ± 5.2 a	9.80	6.0 ± 0.1 b	10.74	4.0 ± 0.3 b
14.27	59.0 ± 3.0 a	12.25	2.0 ± 0.1 b	13.42	2.0 ± 0.1 b
17.13	7.0 ± 0.3 b	14.70	0.0 ± 0.0 c	16.11	0.0 ± 0.0 c
19.98	2.0 ± 0.1 b	17.15	0.0 ± 0.0 c	18.79	0.0 ± 0.0 c
22.84	0.0 ± 0.0 c	19.60	0.0 ± 0.0 c	21.48	0.0 ± 0.0 c
25.69	0.0 ± 0.0 c	22.05	0.0 ± 0.0 c	24.16	0.0 ± 0.0 c
28.55	0.0 ± 0.0 c	24.50	0.0 ± 0.0 c	26.85	0.0 ± 0.0 c

*Control = Basal medium without IAA, IBA or NAA

Data scored after 14 weeks represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different ($P<0.05$) according to DMRT.

After 4-8 weeks, small bud-like structures formed healthy shoots with 2-3 leaves. The shoots that regenerated on 9.08 µM TDZ-supplemented basal medium were tested for rooting efficiency with different concentrations of auxins (IAA, IBA and NAA) (**Table 2**). The shoots failed to produce roots with lower concentrations of IAA (from 0.28 to 5.71 µM). Rooting efficiency was satisfactory by increasing the concentration of IAA from 8.56 µM to 19.98 µM (**Table 2**). Highest rooting percentage (86%) was with 11.42 µM IAA-supplemented basal medium (**Table 2; Fig. 1B, 1C**). The shoots cultured on basal medium supplemented with various concentrations of IBA (0.24-7.35 µM) and NAA (0.26-8.05 µM) failed to produce roots (**Table 2**). The lowest percentage of rooting (2.0-6.0%) was observed with IBA (9.8-12.25 µM) whereas 10.74 and 13.42 µM NAA showed 4.0 and 2.0% rooting, respectively (**Table 2**).

DISCUSSION

TDZ is a substituted phenyl urea with cytokinin-like activity (Mok *et al.* 1982) and therefore, stimulates rapid shoot differentiation. TDZ aids in rapid plant regeneration of a number of plant species through organogenesis (Malik and Saxena 1992). The potential of TDZ to stimulate shoot formation is very common in dicotyledonous plants as *Pisum sativum* (Massimo *et al.* 1996), *Cajanus cajan* (Eapen *et al.* 1998), *Arachis hypogaea* (Kanyand *et al.* 1994) as well as in monocotylednous plants viz. *Dendrocalamus strictus* Nees (Singh *et al.* 2001). In orchids, an efficient shoot regeneration of *Vanda coerulea* was achieved using thin shoot tip sections and TDZ (Malabadi *et al.* 2004a). PLBs or proliferating shoot buds was observed when thin shoot tip sections were cultured on Vacin and Went (VW) (1949) basal medium supplemented with 11.35 µM TDZ. The highest percentage of PLBs (95%) survived and ultimately produced healthy shoots with 2-3 leaves when subjected to a 4-week TDZ treatment. A culture period longer than 8 weeks with TDZ resulted in the formation of fasciated or distorted shoots. Shoots produced roots when cultured on half strength VW medium supplemented with 11.42 µM IAA in *Vanda coerulea* (Malabadi *et al.* 2004a). A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* 'Chiengmai Pink' was developed using TDZ (Chung *et al.* 2007). 5-25% of leaf tip segments of *in vitro* grown plants directly formed somatic embryos on half-strength MS medium supplemented with 0.3, 1 and 3.0mg/l TDZ (Chung *et al.* 2007). TDZ is effective in the induction of *in vitro* morphogenesis in shoot regeneration and multiplication (Earnst 1994; Chen and Piluek 1995; Nayak *et al.* 1997; Chen and Chang 2000) and direct somatic embryogenesis (Chen *et al.* 1999; Chen and Chang 2000) of several orchids (*Phalaenopsis*, *Doritaenopsis*, *Cymbidium aloifolium*, *Dendrobium aphyllum*, *Dendrobium moschatum*, *Oncidium*). Moreover, TDZ combined with 2,4-D are required for callus induction in *Cymbidium ensifolium* var. *misericors* (Chang and Chang 1998), *Oncidium* (Chen and Chang 2000a, 2000b), *Phalaenopsis* (Chen *et al.* 2000) and *Paphiopedilum* (Lin *et al.* 2000). However, Huang *et al.* (2001) reported that TDZ inhibits shoot proliferation and rooting in *Paphiopedilum*. In addition, Chen *et al.* (2002) reported that the best treatment of 4.54 µM TDZ induced only 20% of leaf explants to form shoots in *Paphiopedilum*. Very recently Hong and coworkers (2008) reported induction of totipotent callus on half-strength MS medium supplemented with 22.60 µM 2,4-D and 4.54 µM TDZ in darkness in a maudiae type slipper orchid, *Paphiopedilum* 'Alma Gavaert' (Hong *et al.* 2008). The callus proliferated more and was maintained without any morphogenesis on the same medium with a 2-month interval (i.e., subcultured every 4 weeks). When callus was transferred to half-strength MS medium supplemented with 26.85 µM NAA, an average of 4.7 PLBs/shoot bud formed from each explant after 120 days of culture in *Paphiopedilum* Alma Gavaert (Hong *et al.* 2008). Nayak *et al.* (1997) developed high frequency shoot proliferation in *Dendrobium moschatum*, *Cymbidium aloifolium*, and *Dendrobium apyllum* induced by 4.0 µM TDZ. Stem nodal explants of *Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60) directly formed shoots when cultured on modified half-strength MS basal medium supplemented with a combination of 4.52 µM 2,4-D and 0.45 µM TDZ (Chen *et al.* 2002). On PGR-free basal medium, the percentage explants with shoots were 33.3 and 0% while the number of shoots per explant were 1 and 0 in hybrid PH59 and PH60, respectively. In hybrid PH59, 4.52 µM 2,4-D plus 0.45 µM TDZ induced a higher percentage of explants with shoots and number of shoots per explant than did the PGR-free treatment. In hybrid PH60, although 4.52 µM 2, 4-D and 0.45 µM TDZ promoted shoot formation, the highest shoot number was found with 4.52 µM 2,4-D alone (Chen *et al.* 2002).

In *Costus speciosus*, rhizome thin sections cultured on B_5 basal medium without TDZ (control) or with low concentrations 0.45 and 4.54 µM TDZ completely failed to produce shoot buds. Higher concentrations of TDZ, particularly 36.32, 40.86 and 45.41 µM resulted in the browning of explants which finally necrosed (Malabadi *et al.* 2004b). On the other hand initiation of shoot buds was observed in the range 11.35 -27.34 µM TDZ with the highest percentage of rhizome thin sections (92%) producing shoot buds (12 ± 2.01) at 18.16 µM TDZ (Malabadi *et al.* 2004b). 87% of shoot buds elongated and gave rise to shoots. On this medium the explants remained green for 3 weeks and developed small bud-like structures from the central as well as peripheral regions during this period. After 2 weeks, the shoot buds elongated further and formed 2 to 3 leaves. A sharp decrease in the number of shoot buds formed was also noticed when the concentration of TDZ was increased from 18.16 to 31.78 µM (Malabadi *et al.* 2004b). Least and poor growth of shoot buds (1.8 ± 0.02) was noticed at 31.78 µM TDZ in *C. speciosus* (Malabadi *et al.* 2004b). Wilhelm (1999) reported successful micropropagation of juvenile sycamore maple (*Acer pseudoplatanus*) *via* adventitious shoot formation by the use of 0.04 µM TDZ. In case of pea 'Sugar Ann' and 'Patriot', an average of 20 shoots formed on MS basal medium supplemented with 0.5 or 1.0 µM TDZ (Massimo *et al.* 1996). TDZ either alone (4.54 or 9.08 µM) or in combination with IAA (5.71 µM) on MS medium induced a high frequency of shoot regeneration from primary leaf segments of 3 pigeonpea (*Cajanus cajan* L.) cultivars (Eapen *et al.* 1998). Singh *et al.* (2001) also reported that TDZ induced shoot multiplication in bamboo (*Dendrocalamus strictus*) and the maximum number of shoots (14.8 ± 1.0) were obtained from shoot explants cultured in 2.27 µM TDZ-supplemented half-strength MS basal medium. Treatment of soybean callus with TDZ, stimulated cytokinin accumulation (Thomas and Katterman 1986). These in-

vestigations all support the present findings.

CONCLUSION

A reliable protocol *via* PLBs or proliferating shoot bud formation from shoot tip tTCLs for *Eria dalzelli* propagation was achieved. The described protocol for micropropagation of *E. dalzelli* with TDZ is simple, efficient and can be applied for the conservation of this native orchid, and also for the commercial production as a low cost technology for generating jobs at the biotechnology sector for rural communities.

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