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Full Length Research Paper

Micropropagation of *Litsea glutinosa* (Lour) C.B

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***Litsea glutinosa* (Lour) C.B (Hindi: Maida lakri) is a medicinal plant of immense pharmaceutical value. The species is critically endangered due to its indiscriminate collection as raw material for pharmaceutical industry, where it is used for manufacturing drugs for pain, arousing sexual power and in treatment of diarrhea and dysentery etc. An attempt has been made for development of *in vitro* propagation procedure for the species, involving four steps, namely: culture establishment, shoot multiplication, rooting and hardening. Aseptic cultures were established on Murashige and Skoog (MS) medium supplemented 10.0 µM N^6-benzyladenine (BA) using nodal segments (1 cm). Four sets of simple randomized experiment were carried out on MS medium to study the effect of four doses of each BA, GA_3, IAA, (0, 2.5, 5.0 and 10 µM) and ascorbic acid (0, 284, 852 and 1136 µM) for *in vitro* shoot multiplication. MS medium supplemented with 5.0 µM BA with 852 µM ascorbic acid significantly proved optimum for *in vitro* shoot multiplication and resulted in 1.05 shoot number explant^{-1}, 1.72 node number shoot^{-1} and 1.79 node number explant^{-1} at one month after inoculation. The *in vitro* multiplied shoots were tested for *in vitro* root induction on MS culture media containing auxin IBA (Indole-3-butaric acid) treatments (0, 2.5, 5.0 and10.0 µM) in simple randomized designs experiment. MS media supplemented with 10.0 µM IBA, screened out to be significantly excellent for induction and growth of adventitious roots, resulting in 72.2% rooting and 0.72 root number explant^{-1} at 30 days after inoculation. The *in vitro* propagated plants exhibited excellent growth. Therefore, the present study recommends a four step micropropagation procedure for *in vitro* production of *L. glutinosa* plants on a commercial scale to meet the requirement of pharmaceutical industries and save the species from extinction.**

Key words: *Litsea glutinosa* (Lour) C.B, ascorbic acid, nodal segments.

INTRODUCTION

Litsea glutinosa (Family Lauraceae) is an evergreen tree of medium size, which grows to a height of about 25 m. Found in mixed primary and secondary forest and thickets throughout india and in the outer Himalayas' (Kirtikar and Basu, 1981). *L. glutinosa* contain photoconstituents like alkaloids, glycosides, flavonoids, saponins, tannins, phenolic compounds etc. The bark of *L. glutinosa*, "is one of the most popular of native drugs", is considered to be capable of relieving pain, arousing sexual power and good for stomach in treatment of diarrohea and dysentery. *L. glutinosa* is widely used as a demulcent and as an emollient. The phytochemical constituents of bark of *L. glutinosa* have been shown to possess effective antibacterial and antifungal activity (Hosamath, 2011). This species is critically endangered (Reddy and Reddy 2008). The conventional propagation is hampered due to low seed viability and no rooting of vegetative cuttings (Rabena, 2010).

Thus there is need for alternative *in vitro* propagation method for large scale multiplication, improvement and conservation of the species. The objective of the study was to develop a procedure for its micropropagation.

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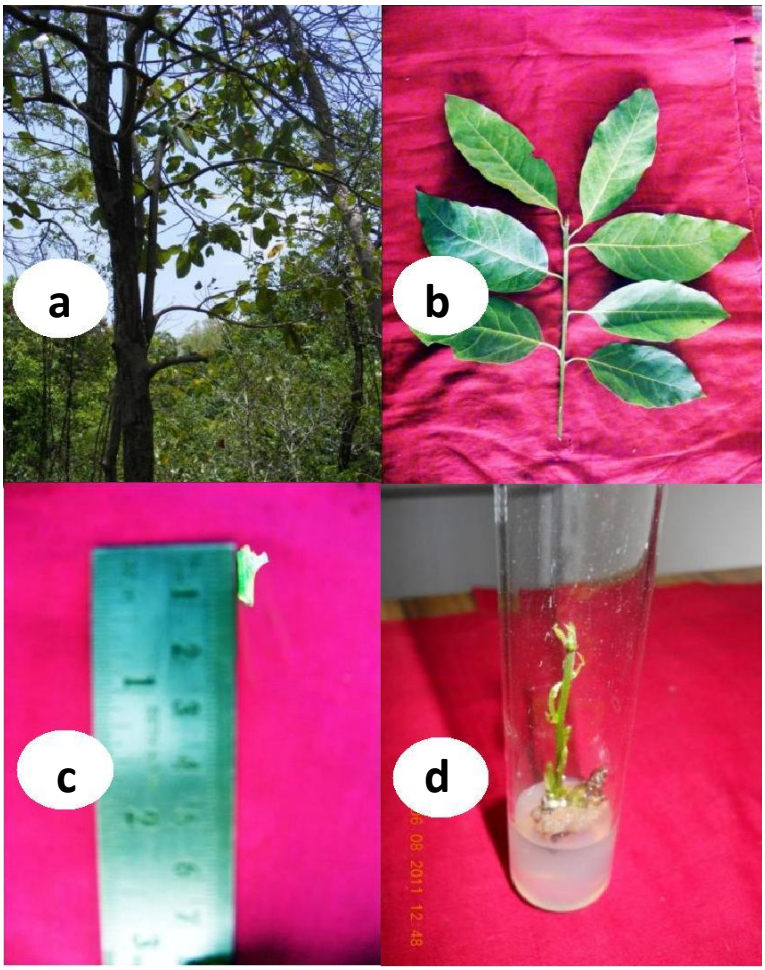

Figure 1. Explant collection, culture establishment and shoot multiplication in *Litsea glutinosa,* (a) mother plant, (b) a twig, (c) nodal explants, (d) *in vitro* shoot establishment on MS medium + 10 µM BA.

MATERIALS AND METHODS

The selected (mother) plants (Figure 1a) were used to collect twig (s) (Figure 1b), which were brought in laboratory and washed thoroughly for 15 min under running water for removing the debris from the surface. The washed twigs were defoliated and cut into nodal explants (approximately 1 to 1.5 cm long and 0.5 to 0.6 cm diameter) (Figure 1c). These explants were washed with 2% Cetrimide® and kept for 10 min with constant vigorous shaking (150 rpm) on an orbital shaker incubator. The explants were rewashed 4 to 5 times with distilled water to remove traces of Cetrimide®. The washed explants were sterilized for 5 min with a composite sterilization treatment comprising $HgCl_2$ (0.1%), Bavistin® (1.0%) and Streptomycin® (0.2%) in the laminar flow cabinet. Finally, the surface sterilized nodal explants were rinsed 4 to 5 times with sterile distilled water for removal of sterilizing agent under laminar flow cabinet. The nodal segments were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 10.0 µM BA for culture establishment (Figure 1d).

Shoot multiplication

Four sets of simple randomized experiment were carried out on MS medium to study the effect of four doses of each BA, GA_3, IAA, (0, 2.5, 5.0 and 10 µM) and ascorbic acid (0, 284, 852 and 1136 µM) for *in vitro* shoot multiplication. Shoot number explant^{-1}, node number shoot^{-1} and node number explant^{-1} were recorded (Figure 2a-c).

Root induction

A simple randomized experiment was carried out to study the effect of four doses of IBA (0, 2.5, 5.0 and 10 µM) on root induction at 30 days after inoculation.

Culture conditions and statistical analysis

The inorganic salts used for preparation of culture medium were obtained from Qualigens Pvt. Ltd., India and phytohormones and B vitamins from Sigma Chemicals Pvt. Ltd., MS medium was used in all the experiments with 5.0 µM BA. India. The medium contained 3% (w/v) sucrose, 0.65% (w/v) agar (Hi-media chemical Ltd., India). The pH of the medium was adjusted to 6.0 before autoclaving for 15 min at 1.06 kg cm^{-2} (121°C). Explants were cultured in 150 ml culture bottles containing 40 ml semi-solid medium. For *in vitro*

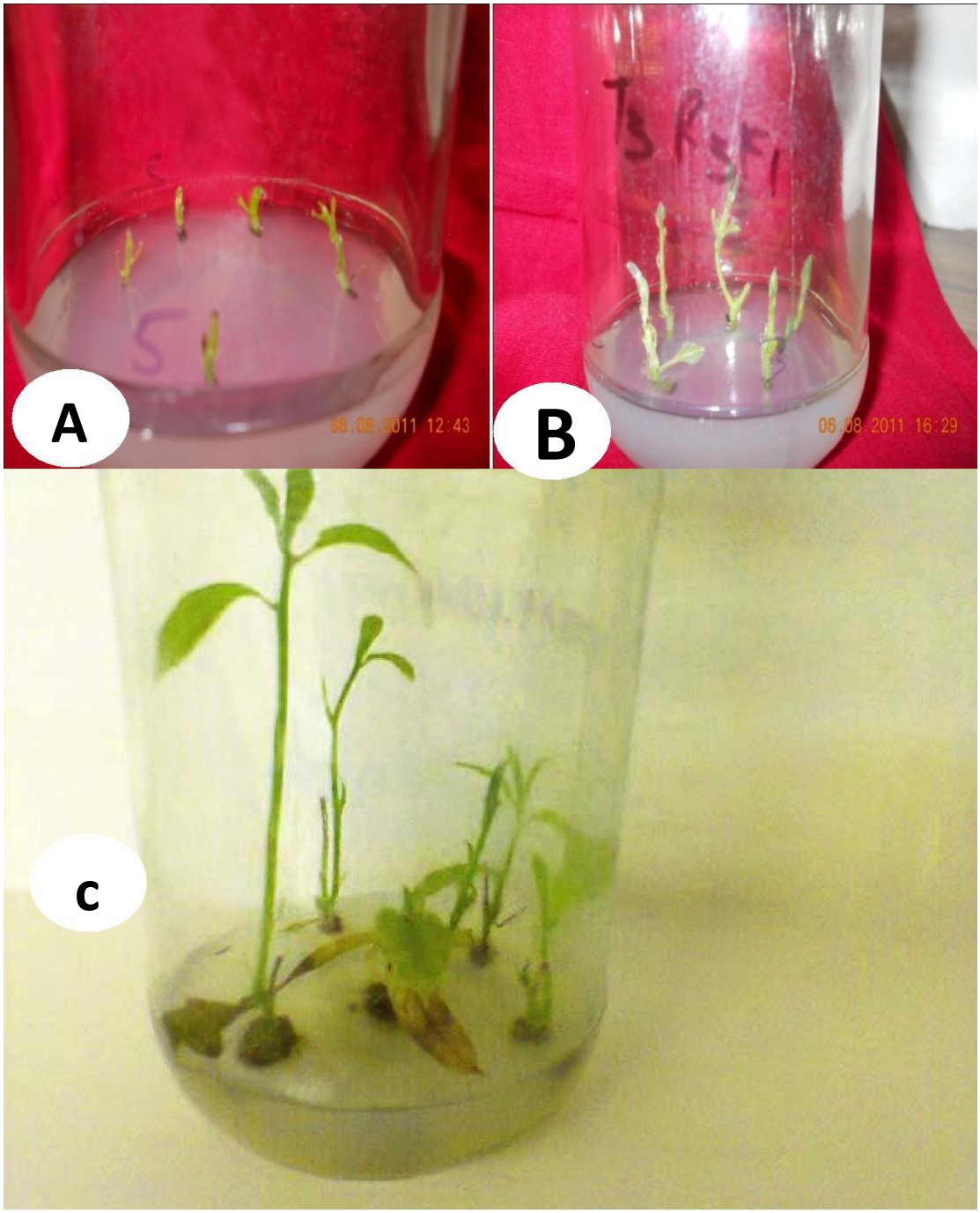

Figure 2. *In vitro* shoot multiplication in *Litsea glutinosa,* (a) MS medium supplemented 5 µM BA + 5 µM IAA, (b) MS medium 5 µM BA + 5 µM GA_3, (c) MS medium + 5 µM BA + 825 µM ascorbic acid at 30 days after inoculation.

shoot multiplication experiments, the cultures were incubated at 25 ± 2°C under 16 h illuminations with fluorescent light ($50 \mu Em s^{-1} m^{-2}$). The experiments had thee replicates for *in vitro* shoot multiplication and three replicates for *in vitro* rooting (Figure 3). Each replicate had 10 propagules. In all five experiments the data were recorded at 30 days after inoculation. The data were subjected to one way (factor) analysis of variance for all the experiments with "F" test for ascertaining level of significance. If the data were found significant at $p \leq 0.05$, $LSD_{0.05}$ was computed for comparison of treatment means

FIGURE 2: *In vitro* shoot multiplication in *Litsea glutinosa*, (a) MS medium supplemented

Hardening and transplantation

The *in vitro* raised plantlets were removed from rooting medium, washed with distilled water and the plantlets were transferred to

Figure 3. The *in vitro* adventitious root induction *Litsea glutinosa,* root formation on semi-solid MS medium with 10 µM IBA at 30 days after inoculation.

sand beds in the mistchamber and the plantlets were covered with culture bottles to maintain humidity (Figure 4a).

Subsequently, they were transferred to perforated polythene bags and kept initially in mistchamber for 10 days and finally transferred to natural environmental conditions (Figure 4b).

RESULTS

In vitro shoot multiplication

Experiment 1

Shoot number explant^{-1}: Effect of different doses of BA at 0 to 10 µM produced statistically similar value for shoot number explant^{-1} at 30 days after inoculation. The effect of BA was found to be non significant on shoot number explant^{-1} at the stage of sampling (Table 1).

Node number shoot^{-1}: Use of BA significantly influenced node number shoot^{-1} at 30 days after inoculation. 5 µM BA enhanced node number shoot^{-1} by 25% in comparison to the control at the stage of sampling.

Node number explant^{-1}: Various doses of BA significantly influenced node number explant^{-1}at 30 days after inoculation. 5.0 BA µM significantly enhanced node number explant^{-1} by 27% in comparison to the control at the stage of sampling.

Experiment 2

Shoot number explant^{-1}: Effect of different doses of IAA significantly influenced shoot number explant^{-1} at 30 days after inoculation. 5 µM IAA significantly enhanced shoot number explant^{-1} by 22% in comparison to the control at the stage of sampling (Table 2).

Node number shoot^{-1}: Use of IAA significantly influenced node number shoot^{-1} at 30 days after inoculation. 5.0 µM IAA significantly enhanced node number shoot^{-1} by 33% in comparison to the control at the stage of sampling.

Node number explant^{-1}: Various doses of IAA significantly influenced node number explant^{-1}at 30 days after inoculation. 5.0 µM IAA significantly enhanced node number explant^{-1} by 55% in comparison to the control at the stage of sampling.

Experiment 3

Shoot number explant^{-1}: Effect of different doses of GA_3 significantly influenced shoot number explant^{-1} at 30 days after inoculation. Maximum shoot number explant^{-1} were obtained on 5.0 µM GA_3 which was significantly higher than shoot number explant^{-1} into other doses of GA_3. 5.0

Figure 4. Hardening and acclimatization of the *in vitro* raised plantlets of *Litsea glutinosa*, transferred into (a) sand bed covered with culture bottles in mistchamber, (b) growth of plantlets in the open environment.

Table 1. Effect of different doses of BA on shoot number explant^{-1}, node number shoot^{-1} and node number explant^{-1} at 30 days after inoculation. MS medium supplemented with uniform dose of 5.0 µM BA.

Doses BA (µM)	Shoot number explant^{-1}	Node number shoot^{-1}	Node number explant^{-1}
0	1.0	1.0	1.0
2.5	1.0	1.0	1.0
5.0	1.0	1.25	1.27
10	1.0	1.0	1.0
LSD$_{(0.05)}$	NS	0.09	0.01

Table 2. Effect of different doses of IAA on shoot number explant^{-1}, node number shoot^{-1} and node number explant^{-1} at 30 days after inoculation. MS medium supplemented with uniform dose of 5.0 µM BA.

Doses IAA (µM)	Shoot number explant^{-1}	Node number shoot^{-1}	Node number explant^{-1}
0	1.0	1.0	1.0
2.5	1.0	1.0	1.05
5.0	1.22	1.31	1.55
10	1.0	1.0	1.0
LSD$_{(0.05)}$	0.10	0.03	0.25

µM GA_3 enhanced shoot number explant^{-1} by 44% in comparison to the control at the stage of sampling (Table 3).

Node number shoot^{-1}: Effect of different doses of GA_3 was found to be non significant at 30 days after inoculation. 5.0 to 10.0 µM GA_3 produced statistically

Table 3. Effect of different doses of GA_3 on shoot number explant^{-1}, node number shoot^{-1} and node number explant $^{-1}$ at 30 days after inoculation. MS medium supplemented with uniform dose of 5.0 µM BA.

Doses GA_3 (µM)	Shoot number explant^{-1}	Node number shoot^{-1}	Node number explant^{-1}
0	1.0	1.0	1.0
2.5	1.0	1.0	1.11
5.0	1.44	1.11	1.41
10	1.0	1.16	1.11
$LSD_{(0.05)}$	0.19	NS	0.25

Table 4. Effect of different doses of ascorbic acid on shoot number explant^{-1}, node number shoot^{-1} and node number explant^{-1} at 30 days after inoculation. MS medium supplemented with uniform dose of 5.0 µM BA.

Doses Ascorbic acid (µM)	Shoot number explant^{-1}	Node number shoot^{-1}	Node number explant^{-1}
0	0.38	1.0	0.38
282	0.50	1.16	0.58
852	1.05	1.72	1.79
1136	0.55	0.94	0.53
$LSD_{(0.05)}$	0.44	0.73	0.83

equal value for node number shoot^{-1} at the stage of sampling.

Node number explant^{-1}: Various doses of GA_3 significantly influenced node number explant^{-1} at 30 days after inoculation. 5.0 µM GA_3 significantly enhanced node number explant^{-1} by 41% in comparison to the control at the stage of sampling.

Experiment 4

Shoot number explant^{-1}: Effect of different doses of ascorbic acid significantly influenced shoot number explant^{-1} at 30 days after inoculation. 852 µM ascorbic acid significantly enhanced shoot number explant^{-1} by 176% in comparison to the control at the stage of sampling (Table 4).

Node number shoot^{-1}: Use of ascorbic acid significantly influenced node number shoot^{-1} at 30 days after inoculation. 852 µM ascorbic acid significantly enhanced node number shoot^{-1} by 72% in comparison to the control at the stage of sampling.

Node number explant^{-1}: Various doses of ascorbic acid significantly influenced node number explant^{-1}at 30 days after inoculation. Ascorbic acid at 100 µM significantly enhanced node number explant^{-1} by 371% in comparison to the control at the stage of sampling.

Experiment 5

***In vitro* adventitious rooting:** Various doses of IBA (0, 2.5, 5.0 and 10.0 µM) induced significant rooting and root number explant^{-1} at 30 days after inoculation (Table 5).

A**dventitious rooting (%):** MS medium supplemented with various doses of IBA significantly enhanced rooting (%) at 30 days after inoculation. 10.0 µM IBA enhanced rooting (%) by 767% in comparison to the control and maximum rooting of 72.22% was obtained on 10.0 µM IBA supplemented medium.

Root number explant^{-1}: Various doses of IBA significantly influenced root number explant^{-1} at 30 days after inoculation. 10.0 µM IBA significantly enhanced root number explant^{-1} by 134% in comparison to the control and maximum root number explant^{-1} (0.72) was obtained on 10.0 µM IBA supplemented medium.

DISCUSSION

The micropropagation of *L. glutinosa* comprises four steps namely: establishment of culture from nodal explants, shoot multiplication, root induction and harden-

Table 5. Effect of different doses of IBA on MS medium for *in vitro* rooting % and root number explant^{-1} at 30 days after inoculation. Values in the parentheses are arc sine transformation.

Doses (µM) IBA	Rooting (%)	Root number explant^{-1}
0	8.33(10.97)	0.05
2.5	12.49(17.41)	0.11
5.0	33.33(35.24)	0.33
10	72.22(66.55)	0.72
$LSD_{(0.05)}$	14.59	0.18

ing and acclimatization. The present investigations pertain to standardization of BA, GA_3, IAA and ascorbic acid doses for shoot multiplications and different concentrations of IBA for root induction. The best combination for *in vitro* shoot multiplication in four experiments emerged to be MS medium supplemented with 5.0 µM BA + 852 µM ascorbic acid. There is no published report on the *in vitro* shoot multiplication in the species. BA exhibiting superiority over other sources of cytokinins for differentiation and growth of new shoots is well documented in other species. The possible reason could be that BA is much closely related to natural cytokinins as far as the structures of the latter is concerned.

Amendment of culture medium with ascorbic acid significantly influenced shoot number explant^{-1}, node number shoot^{-1} and node number explant $^{-1}$ at the stage of sampling. The increase in shoot number in the presence of ascorbic acid has also been reported in tobacco callus culture (Richard et al., 1988). According to Sharma and Chandel (1992) addition of ascorbic acid to the hormone supplemented medium was essential for bud break and further shoots multiplication. Mechanism of action of ascorbic acid, a common antioxidant/ antibrowning agent, is presently not known. Ascorbic acid or some product of its oxidation may possibly be increasing shoot number through ascorbate protection of endogenous phyto-hormones as implicated for tobacco, *Pinus* and *Picea* (Berlyn and Beck, 1980; Rumary and Thorpe, 1984; Richard et al., 1988). The auxins stimulate root deve-lopment by inducing root initials that differentiate cells of the young secondary phloem, cambium and pith tissue (Gianfagna, 1995). Roots formed *de novo* from diffe-rentiated cells other than radical are defined as adventitious roots (Casson and Lindsey, 2003). A key stage in adventitious rooting is the *de novo* formation of root meristem. The *in vitro* rooting is complex process and is controlled by several factors, major being hor-monal and nutritional status of media (Jarvis and Booth, 1981). A successful rooting procedure with high rooting percentage is essential for a competent micropropagation protocol.

In the present study 10 µM IBA stimulated adventitious root formation and was found to have significant effect on rooting percentage and root number. IBA is preferred over NAA as it produces strong fibrous root system and is less toxic than NAA (Ahuja, 1991). There are earlier reports by many workers supporting our finding of rooting response with IBA. In medicinal plant species like *Gentiana lutea*, (Petrova et al., 2011) 1 mg L^{-1} IBA was found effective for rooting. The best rooting was obtained on MS medium containing 0.5 mg/l IBA in *Solanum nigrum* (Kolar et al., 2008). On 3 mg L^{-1} IBA, rooting was obtained in *Chlorophytum borivilianum* (Bathoju and Giri, 2012). Maximum number of roots was obtained on 1.5 IBA mg L^{-1} IBA in *Centella asiatica* (Karthtikeyan et al., 2008). 100% *in vitro* rooting was obtained when shoot clusters were cultured on MS medium supplemented with 0.15 mg/L IBA in *Bacopa monnieri* (Sharma et al., 2010). IBA has been found effective for rooting of *in vitro* raised shoots of many tree species also. In *Dalbergia sissoo,* 1.0 mg/L of indole butyric acid (IBA) was reported to be the best treatment for rooting (Ali et al., 2012).

Conclusion

To the best of our knowledge this is the first report on micropropagation of *L. glutinosa*, a critically endangered medicinal tree species. As cultures have been established using nodal segments of mature trees from field, this method will be very useful for cloning of mature trees of this species. The high rooting frequency (72.2 %) obtained in the present study will help in its propagation in large numbers. The study will also be helpful for *ex situ* conservation of this endangered species in the form of *in vitro* cultures.

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