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In vitro Rapid Clonal Propagation in Static Liquid Medium and Acclimatization of *Piper solmsianum* and *P. tuberculatum*

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ABSTRACT

An efficient and rapid tissue culture system is described for multiplication of Piper solmsianum and P. tuberculatum, two important American species, through shoot tip and nodal explants cultures. Stem segments with 2-3 nodes (3-4 cm long), taken from eight to ten-month-olds in vitro plantlets, were placed on 0.6% agar gelled (G) and liquid (L) MS mineral salts plus vitamins, 3% sucrose and supplemented with 0.02 mg/L IAA + 0.02 mg/L GA_3 (G1 or L1), 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP (G2 or L2) or 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP (G3 or L3).The best shoot elongation, shoot proliferation and rooting response of stem segments was observed in liquid culture medium (L3). An average of 9.1±0.9 cm shoot elongation, 2.4±0.5 shoot proliferation, and 5.5±0.8 nodes formation for P. solmsianum, and 8.6± 0.7 cm shoot elongation, 2.1±0.3 shoot proliferation, and 6.7±0.6 nodes formation for P. tuberculatum were observed after 120 days of culture. For acclimatization, elongated shoots were separated and rooted in MS medium supplemented with 0.02 mg/L IAA + 0.02 mg/L GA_3. Plantlets, thus developed were established in several potting mixtures with 30 to 100% of survival rate in both species.

Keywords*: Clonal propagation, liquid culture medium, nodal esplants, Piper solmsianum, P. tuberculatum.*

INTRODUCTION

Piper L. belong to the Piperaceae family, order Piperales. The order consists of the several families such as the Aristolochiaceae, Hydnoraceae, Lactoridaceae, Piperaceae and Saururaceae, and has been classified among the basal Angiosperm[2]. The Piperaceae familiy comprises 14 genera and ca. 1950 species, of which the genus *Piper* is the largest, with more than 600 species described worlwide[6]. *Piper solmsianum* is a shrub measuring 1-3 meters tall, commonly found in Southern Brazil and *P. tuberculatum* known as "matico" or "cordoncillo", is abundant in the West Indies and is widely distributed from Brazil to Mexico[37].

The most significant compound of *Piper* species is the piperamide piperine, which constitutes the active pungent principle of *Piper nigrum*[27]. A large number of reports have demonstrated the remarkable potential of such amides as insecticides[19], molluscicides[30] and antifungal agents[26]. Together with amides, various other classes of phytochemicals have been isolated from *Piper* species, principally the chromenes[14], phenylpropanoids, lignans and neolignans[4], alkaloids[28], flavokawains[30], and several compounds of mixed-biosynthetic origin[27]. Recently, it was reported that the knowledge of the chemistry, bioactivity, and ecology of Piperaceae species provides preliminary clues for an overall interpretation of the possible role and occurrence of major clases of compounds[12].

In *Piper solmsianum*, leaves and stem barks from two different specimens have previously been investigated and yielded one benzofuran lignan and one glycosylated flavonoid[21] as well as five phenylpropanoids and two tetrahydrofuran lignans[16]. From spikes, in addition to sitosterol, syringaldehyde, 3,4,5-trimethoxybenzoic acid, isoelemicin and grandisin, two new tetrahydrofuran lignans were isolated and characterized as *rel*-(*7R,8R,7´R,8´R*)-3´,4´-methylenedioxy-3,4,5,5´-tetramethoxy-7-7´-epoxylignan and *rel*-(*7R,8R,7´R,8´R*)-3,4,3´,4´-dimethylenedioxy-5,5´-dimethoxy-7,7´-epoxylignan on the basis of spectroscopic data, incluiding 2D NMR spectrometric techniques. Also, their *in vitro* activity was determined against the trypomastigote form of *Trypanosoma cruzi*[17].

With respect to *P. tuberculatum* there have been isolated and characterized amides from seeds: 8 (*Z*) - *N*-(12,13,14 - trimethoxycinnamoyl) - Δ^3 – pyridin – 2 - one, *N* - (12,13,14 - trimethoxydihydrocinnamoyl) - Δ^3-pyridin-2-one, piplartine, piperine, $\Delta^{\alpha,\beta}$-dihydropiperine, 5,6 - dihydropiperlonguminine and pellitorine[24]; and from seeds and leaves six amides: pellitorine, $\Delta^{\alpha,\beta}$-dihydropiperine, piplartine, dihydropiplartine, *cis*-piplartine and fagaramide) and two antifungal cinnamoyl derivatives, methyl 6,7,8-trimethoxy-dihydrocinnamate and methyl *trans*-6,7,8-trimethoxycinnamate[36]. These amides were active against the fungus *Cladosporium sphaerospermum* and *C. cladosporioides*[24,36].

Plant tissue cultures can be used for mass propagation of plants. Millons of plants are commercially propagated annually via micropropagation by shoot tips, nodal segments, suspension cultures and somatic embryogenesis, which are labor intensive and associated with developmental abnormalities as hyperhydricity[15,35]. In most studies on plant micropropagation traditionally agar (0.6-0.8%) is added to the culture medium to increase its viscosity[8]; however, recent reports have suggested that low concentration of agar provides a soft gelled medium which facilitates an adequate contact between the plant tissue and the medium, with better growth and their subsquent rooting[5]. In addition, liquid medium has also been attempted where agar was completely omitted from the medium[34]; in liquid medium propagation is accelerated, culture transfer frequencies may be decreased, labour is less intensive and cost of production is reduced[20]. For instance, reduced concentration of agar (1.0 to 0.2%) and its complete elimination (0.0%) favoured both shoot multiplication and rooting during micropropagation of *Boswellia serrata* (Burseraceae), an important tree species of Aravallis in Rajasthan, India, valued for its immense medicinal properties[34]. The assessment of genetic fidelity on micropropagated plants, and simultaneous quantitation of reserpine, ajmaline and ajmalicine were was observed in *in vitro* propagation of *Rauwolfia serpentina*, an important species because of its immense anti-hypertensive properties, using liquid medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA[10]. In another study which compared liquid and gelled media on the growth of plantlets from three Kenyan potato cultivars, the liquid medium gave more roots, nodes and leaves per plantlet than the solid medium[18]. Contrary to *Dracaena sanderiana*, an important indoor ornamental plant, distributed in tropical and subtropical open lands of India and Africa, whose rooting was high on MS solid compared to liquid medium when both media were supplemented with 1.5 mg/L IBA[3].

Cultures in liquid medium including the stirred tank reactor, bubble column reactor, balloon type bubble reactor, and air lift reactor[7],may be more efficient than agar culture medium. The major advantage of using bioreactor culture system for micropropagation of economically important plants is the potential for scaling-up in lesser time limit, reduction in the production cost as well as an automated control of physical and chemical environment during growth phase of the plant culture[20]; however, the lack of systematical and factorial experimental knowledge of the interaction between plant physiology and physical parameters with bioreactor designs affects the frequent use of this technology[25]. Besides, bioreactor culture systems often produce hyperhydric shoots due to submergence in liquid[38].

In temporary immersion systems (TIS) the immersion cycle, volumen of nutriente medium and the container are critical for efficient shoot proliferation[32], and compared to continuously submerged cultures, growth generally improved, but hyperhidricity was still a problem and was grater than 50% for some species as *Lessertia* (*Sutherlandia*) *frutescens* [31] and sugarcane[32].

In addition, in the micropropagation process of three Chilean native species of the genus *Rhodophiala* (*R. montana*, *R. splendens* and *R. ananuca*), a TIS, and conventional culture in static liquid, shaken liquid and gelled MS medium were compared. In gelled medium, hyperhydricity affected only 5% of explants, while in liquid medium was 16-40%, and survival to acclimatization reached 87-94% for explants whilst liquid medium only 38-69% [22]. Finally, for laboratories with limited financial resources, the bioreactor culture systems and TIS are expensive methods. Thus, the aim of this study was to establish a protocol for *in vitro* rapid clonal propagation of *Piper solmsianum* and *P. tuberculatum* using a conventional static liquid culture, for mass propagation and further acclimatization.

MATERIALS AND METHODS

Plant material

Mature inflorescences (spikes) of *P. solmsianum* C.DC., and *Piper tuberculatum* Jacq. were collected in Núcleo de Picinguaba, City of Ubatuba, São Paulo State, Brazil, in october 1998, and Cumbil river in Lambayeque, Peru, in december 2009, respectively. Botanical identification was performed by Dr. Guillermo E. Delgado-Paredes from Universidad Nacional Pedro Ruiz Gallo (UNPRG) based on taxonomic description realized by Yuncker[37]. The voucher specimens of *P. solmsianum* are deposited at Herbarium of Instituto de Biociências–USP, São Paulo, and voucher specimens of *P. tuberculatum* are deposited at the UNPRG Herbarium (HPR).

Seed sterilization and germination

The culture was initiated from axenic seedlings explants. A total of 50 seeds per flask were thoroughly washed with distilled water for 10-20 min, decontaminated with 70% ethanol (v/v) for 60 s and 2.5% sodium hypochlorite (w/v) for 20 min and then washed three times with sterile water. Floating and submerged seeds were discarded; about 5-10 of them were transferred to glass test tubes containing 15 mL of MS medium[23] and 2% sucrose.

Micropropagation

Shoot-tips and nodal segmentsof 1 cm long containing an axillary bud, taken from six-month-olds *in vitro* seedlings, were used as explant source. MS medium, supplemented with 0.02 mg/L indolacetic acid (IAA), 0.02 mg/L gibberellic acid (GA_3) and 3% sucrose was used to initiate cultures and were maintained by annual subculturing on fresh medium containing the same formulation.

Experimental design

Stem segments with 2-3 nodes (3-4 cm long), taken from eight to ten-month-olds *in vitro* plantlets of *P. solmsianum* and *P. tuberculatum*, were placed on both gelled with 0.6% agar: G1, G2 and G3 treatments, and liquid: L1, L2 and L3 treatments, MS mineral salts plus vitamins, 3% sucrose and supplemented with 0.02 mg/L IAA + 0.02 mg/L GA_3 (G1 and L1 treatments), 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP (G2 and L2 treatments), and 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP (G3 and L3 treatments).Two excised stem segment were cultured in each flask (50x150 mm) with 20 mL of the culture medium. Each treatment was represented by 15 cultures by species and the experiments were repeated three times. Evaluations were performed after 120 days of culture. Additionally, for comparison, were evaluated plantlets of both species grown in G1 treatments with agar, after 12 months of culture.

Induction of rooting and acclimatization

Elongated shoots and nodal segments of 1-2 cm long were excised from the culture and transferred to semisolid MS medium supplemented with 0.02 mg/L IAA, 0.02 mg/L $GA_3$and 2% sucrose for vigorous root induction. One excised explant was cultured in each tube (18x150 mm) with 5 mL of the culture medium.

The rooted shoots (4-8-week-olds) were thoroughly washed with water to remove the adhering agar, transplanted to plastic pots containing a mixture of several substrates, and kept in the greenhouse for acclimatization.The substrates were sterilized by dry heat.The plants were watered at 3-day intervals and were supplied with 1/10 strenght MS inorganic solution twice a week before being transferred to open field. Polythene covers were completely withdrawn after 3-4 weeks of hardening. The survival rate was recorded 60 days after the transfer into pots.

Culture conditions

In all cultures, MS medium was supplemented with 1.0 mg/L thiamine.HCl and 100 mg/L *myo*-inositol. The pH of the medium was adjusted to 5.8±0.1, solidified with 0.6% agar-agar prior to autoclaving at 121 ^{o}C for 20 min, excluding the treatments in liquid culture medium. Cultures were maintained at 24-28 ^{o}C, 80±5% relative humidity, and 16-h photoperiod at a light intensity provided by cool white fluorescent tubes, with 10 µmol m^{-2} s^{-1}, for seed germination and 70 µmol m^{-2} s^{-1} for micropropagation and experimental treatments.

Statistical analysis

Results were processed and analyzed by analysis of variance (ANOVA) and the Tukey HSD multiple range test ($p \leq 0.05$) in order to compare treatment means. All the statistical analysis was carried out in the Stratgraphics Plus 5.0 software (StatPoint, Warrenton, Virginia, USA).

RESULTS AND DISCUSSION

Shoot elongation

In *P. solmsianum*, after 120 days of culture, the best response in shoot elongation (9.1±0.92), shoot proliferation (2.4±0.52), and nodes formation (5.5±0.85) was obtained in the presence of 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/LBAP, in liquid medium, and was found to be significantly higher ($P \leq 0.05$ level) than shoot elongation and nodes formation induced per stem segment explant in other treatments of the present study (Table 1). In *P. tuberculatum* similar results were observed. After 120 days of culture, the best response in shoot elongation (8.6±0.75) and nodes formation (6.7±0.67) was obtained in the presence of the same formulation in liquid medium, and also was found to be significantly higher ($P \leq 0.05$ level) than shoot elongation and nodes formation induced per stem segment explant in other treatments tested (Table 3).

In both species, the three treatments in liquid medium were significantly better than those in gelled medium, and the combination of 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP did not influence shoot elongation.

The literature does not report studies on *in vitro* propagation of *Piper* species using liquid medium, and in agar medium the studies are limited. In *Piper barberi*, a critically endangered plant, a protocol was described for rapid multiplication through shoot tip and nodal explant cultures; nodal explants with a single axillary meristem showed three times better response with respect to shoot proliferation when compared to shoot tip explants with a cytokinin combination of 1.0 mg/L BAP and 0.5 mg/L KIN[1]. In *P. longum*, an important medicinal plant, an efficient and rapid tissue culture systems were developed through shootip tip multiplication and direct organogenesis, and maximum number of shoots were induced with 2.0 mg/L BA and 1.0 mg/L KIN[33]. Likewise, in this species, shoot tips explants regenerated shoots along with a few callus and embryoids on the MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA, and the NAA concentration of the same medium was lowered to 0.01 mg/L and added 10% coconut milk for subculturing[29].

There are other studies on the influence of agar concentration and liquid medium on *in vitro* plant propagation such as *Boswellia serrata*. In this species, reduced concentration of agar than normal and its complete elimination favoured both shoot multiplication and rooting during micropropagation, and there was an overall increase on dry and fresh weight and chlorophyll contents in such shoots; liquid medium devoid of agar proved to be best for micropropagation[34].Low agar levels and liquid culture medium have been reported to promote shoot proliferation in several culture systems on account of faster uptake of BAP and better absorption of water by plants implanted on softer gels, as observed on *in vitro* shoot production of almond (*Amygdalus communis*)[11], and in embryogenic cultures of *Pinus strobus*[13].

In another study, when comparing liquid and gelled MS medium, after 60 days of *in vitro* cultivation, similar shoot development was achieved in the three *Rhodophiala* species studied; however, the bulb growth index was significantly higher in liquid culture system for *R. montana* and *R. splendens*. In all cases higher browning in *R. ananuca* and hyperhydricity in *R. splendens* and *R. ananuca* were observed in explants developed in liquid media.

In the treatments (TIS, and conventional culture in static liquid, shaken liquid and gelled media), the response of explants was similar for all species tested[22].

Induction of rooting

In both species leaves, internodes and roots formation were induced in *in vitro* stem segments cultured in both gelled and liquid medium after 120 days of culture; however, among the three liquid treatments studied, the 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP combination was found to be slightly most effective, especially in *P. solmsianum* (Tables 2 and 4).

There are studies where better shoot growth and rooting in liquid medium have been observed. For instance, in the quest for cheap and suitable eco-friendly matrix with aseptic liquid culture media for rooting of ten plant species as *Nicotiana tabacum*, *Beta vulgaris*, *Musa* spp., *Tectona grandis*, and others, coir was selected due to its higher water retention capacity compared to paddy straw and jute[9].Additionally, in the propagation of three Kenyan potato cultivars liquid medium gave more roots, nodes and leaves per plantlet than the gelled medium, and no physiological differences among them were noted[18].

In *P. barberi* the *in vitro* shoots rooted on one-half and one-quarter MS basal medium, and the shoots rooted on one-quarter MS in the dark, produced eigth roots with an average root lenght of 3.36 cm and 98% survival[1]. In *P. longum* elongated shoots were separated and rooted in MS medium supplemented with 0.5 mg/l IBA[33].

Comparative study between the two species

Among the studies species, although *P. solmsianum* showed slight shoot elongation greater than *P. tuberculatum*, the result was not statistically significant; however, the nodes formation was significantly higher in *P. tuberculatum*, which would indicate an increased rate of multiplication (Table 5).

Acclimatization

Survival percentage under several potting mixtures on the acclimatization process of *in vitro* rooted plantlets of *P. solmsianum* and *P. tuberculatum* after 60 days is presented in table 6. *P. solmsianum* and *P. tuberculatum*, rooted plantlets survived 90 and 100% in the potting mixtures with sand-slim-peat moss, and soil-cropland-peat moss, respectively. The plants grew properly.

In another *Piper* study, the plants of *P. barbieri* were transferred to the field with 75% survival rate[1]. In *P. longum*, plantlets were established in soil, without specifying the survival percentage[33]; and in another study on rooting, the elongated shoots of *P. Longum* were transferred to non-sterile sand, after acclimatization, they grew roots within 15 to 20 days and were finnally transferred to polybags for further growth and field transfer[29].

Table 1: Effect of growth regulators on shoot elongation, shoot proliferation and number of nodes per culture from shoot apex and nodal explants of *P. solmsianum.* Data was recorded after 120 days

	Treatments	Shoot elongation (cm)	Shoot proliferation (No.)	Nodes (No.)
Agar-gelled MS medium	**G1**	6.7 ± 1.15 b	1.7 ± 0.42 a	4.1 ± 0.37 b
	G2	3.4 ± 0.53 c	2.1 ± 0.32 a	3.8 ± 0.63 b
	G3	3.8 ± 0.57 c	2.4 ± 0.52 a	4.0 ± 0.67 b
	G1 (after 12 months)	9.2 ± 0.32	2.3 ± 0.41	7.0 ± 0.45
Liquid MS medium	**L1**	7.1 ± 0.51 b	2.0 ± 0.00 a	4.3 ± 0.48 b
	L2	7.3 ± 0.99 b	2.4 ± 0.52 a	4.0 ± 0.47 b
	L3	9.1 ± 0.92 a	2.4 ± 0.52 a	5.5 ± 0.85 a

MS + vitamins (thiamine.HCl 1.0 mg/L and *myo*-inositol 100 mg/L) + 3% sucrose
G1 and L1, 0.02 mg/L IAA + 0.02 mg/L GA_3
G2 and L2, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP
G3 and L3, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP
Values with different letters in the same column are significantly different ($P \leq 0.05$)

Table 2: Effect of growth regulators on number of leaves, internodes and roots formed per culture from shoot apex and nodal explants of *P. solmsianum*. Data was recorded after 120 days

	Treatments	Leaves (No.)	Internodes (No.)	Roots (No.)
Agar-gelled MS medium	**G1**	4.1 ± 0.37 b	4.1 ± 0.37 b	4.2 ± 0.42 b
	G2	3.8 ± 0.63 b	3.8 ± 0.63 b	3.3 ± 0.67 c
	G3	4.0 ± 0.67 b	4.0 ± 0.67 b	4.1 ± 0.57 b
	G1 (after 12 months)	8.3 ± 0.21	6.6 ± 0.54	> 10
Liquid MS medium	**L1**	4.3 ± 0.48 b	4.1 ± 0.57 b	4.6 ± 0.70 b
	L2	4.0 ± 0.47 b	3.8 ± 0.42 b	4.2 ± 0.63 b
	L3	5.5 ± 0.85 a	5.3 ± 0.82 a	5.2 ± 0.92 a

MS + vitamins (thiamine.HCl 1.0 mg/L and *myo*-inositol 100 mg/L) + 3% sucrose
G1 and L1, 0.02 mg/L IAA + 0.02 mg/LGA_3
G2 and L2, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP
G3 and L3, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP
Values with different letters in the same column are significantly different ($P \leq 0.05$)

Table 3: Effect of growth regulators on shoot elongation, shoot proliferation and number of nodes per culture from shoot apex and nodal explants of *P. tuberculatum*. Data was recorded after 120 days

	Treatments	Shoot elongation (cm)	Shoot proliferation (No.)	Nodes (No.)
Agar-gelled MS medium	**G1**	6.1 ± 0.71 b	2.2 ± 0.42 a	4.5 ± 0.71 b
	G2	3.5 ± 0.44 d	2.0 ± 0.47 a	3.3 ± 0.48 c
	G3	3.5 ± 0.74 d	2.2 ± 0.42 a	3.4 ± 0.52 c
	G1 (after 12 months)	14.5 ± 0.43	2.1 ± 0.74	12.3 ± 0.26
Liquid MS medium	**L1**	5.3 ± 0.75 bc	1.7 ± 0.48 a	4.4 ± 0.70 b
	L2	4.9 ± 0.52 c	2.2 ± 0.42 a	4.0 ± 0.67 bc
	L3	8.6 ± 0.75 a	2.1 ± 0.32 a	6.7 ± 0.67 a

MS + vitamins (thiamine.HCl 1.0 mg/L and *myo*-inositol 100 mg/L) + 3% sucrose
G1 and L1, 0.02 mg/L IAA + 0.02 mg/LGA_3
G2 and L2, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP
G3 and L3, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP
Values with different letters in the same column are significantly different ($P \leq 0.05$)

Table 4. Effect of growth regulators on number of leaves, internodes and roots formed per culture from shoot apex and nodal explants of *P. tuberculatum*. Data was recorded after 120 days

	Treatments	Leaves (No.)	Internodes (No.)	Roots (No.)
Agar-gelled MS medium	**G1**	4.5 ± 0.71 b	4.1 ± 0.74 bc	3.6 ± 0.52 b
	G2	3.3 ± 0.48 c	3.3 ± 0.48 c	3.6 ± 0.52 b
	G3	3.4 ± 0.52 c	3.4 ± 0.52 c	4.0 ± 0.82 a
	G1 (after 12 months)	13.1 ± 0.46	11.6 ± 0.54	6.2 ± 0.45
Liquid MS medium	**L1**	4.4 ± 0.70 b	4.5 ± 0.71 b	4.5 ± 0.71 a
	L2	4.0 ± 0.67 bc	4.0 ± 0.07 bc	3.9 ± 0.57 b
	L3	6.7 ± 0.67 a	6.7 ± 0.67 a	4.6 ± 0.52 a

MS + vitamins (thiamine.HCl 1.0 mg/L and *myo*-inositol 100 mg/L) + 3% sucrose
G1 and L1, 0.02 mg/L IAA + 0.02 mg/LGA_3
G2 and L2, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.02 mg/L BAP
G3 and L3, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP
Values with different letters in the same column are significantly different ($P \leq 0.05$)

Table 5. Comparative growth between *P. solmsianum* and *P. tuberculatum* per culture from shoot apex and nodal explants in L3 treatment. Data was recorded after 120 days

Species	Shoot elongation (cm)	Shoot proliferation (No.)	Nodes (No.)	Leaves (No.)	Internodes (No.)	Roots (N.)
P. solm.	9.1 ± 0.92 a	2.4 ± 0.52 a	5.5 ± 0.85 b	5.5 ± 0.85 b	5.3 ± 0.82 b	5.2 ± 0.92 a
P. tuberc.	8.6 ± 0.75 a	2.1 ± 0.32 a	6.7 ± 0.67 a	6.7 ± 0.67 a	6.7 ± 0.67 a	4.6 ± 0.52 a

MS + vitamins (thiamine.HCl 1.0 mg/L and *myo*-inositol 100 mg/L) + 3% sucrose
L3, 0.02 mg/L IAA + 0.02 mg/L GA_3+ 0.05 mg/L BAP
Values with different letters in the same column are significantly different ($P \leq 0.05$)

Table 6. Effect of several substrates on the acclimatization process of *in vitro* plantlets of *P. solmsianum* and *P. tuberculatum* after 60 days

Treatments/Potting mixtures	*P. solmsianum*		*P. tuberculatum*	
	High plantlets (cm)	Survival (%)	High plantlets (cm)	Survival (%)
Sand –cropland – garden soil	4.1	60	4.2	30
Cropland – gardensoil – peat moss	4.2	40	4.2	40
Sand – slim – peat moss	4.5	90	4.0	50
Cropland – slim – peat moss	4.2	50	4.5	20
Sand – garden soil – humus	4.2	50	4.2	40
Soil – cropland –peat moss	3.8	40	4.6	100

Fig. 1: *In vitro* clonal propagation of *Piper solmsianum* and *P. tuberculatum*

a. Propagation of *P. solmsianum* in static liquid medium, **b.** Hardened and soil acclimatized plantlets of *P. solmsianum*, **c.** Re-growth of *P. tuberculatum* in agar-gelled medium, and **d.** Hardened and soil acclimatized plantlets of *P. tuberculatum*

CONCLUSION

The present study describes a well-documented and reliable micropropagation protocol of *P. solmsianum* and *P. tuberculatum* from stem segments with 2-3 nodes, taken from eight to ten-months olds *in vitro* plantlets, in static liquid medium, with much higher rate of multiplication and lower costs. This protocol can be used as a basic tool for commercial cultivation of these medicinal plants.

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