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Callus Induction and Plant Regeneration from Leaf Segments of Unique Tropical Woody Plant *Parasponia andersonii* Planch

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Abstract

The purpose of the present study was to develop effective methods for callus induction, shoot regeneration, and rooting for *Parasponia andersonii*. Leaf explants of *P. andersonii* were placed on Lloyd and McCown's (WPM) medium supplemented with various concentrations of TDZ and NAA for callus induction. Callus induction was observed on media containing 0.1 - 0.2 mg/l TDZ with 0.05 mg/l NAA. Maximum shoot regeneration was observed when the calluses were cultured on MS supplemented with TDZ and IBA. Shoots cultured on WPM medium supplemented with 0.5 mg/l IBA had the maximum rooting percentage (100) in 3 weeks. Rooted plants were transplanted to a potting mixture containing vermiculite (50%) and peat (50%) (v/v). After 2 months, more than 20% of plants survived and were transferred to the greenhouse. Thus, a new effective method has been developed for *P. andersonii* micropropagation that can be used in studies of plant-*Rhizobium* symbiosis and for the generation of transgenic *Parasponia* plants.

Introduction

Parasponia andersonii Planch belonging to Cannabinaceae (mentioned as Ulmaceae in J.C. Willis' Dictionary of the Flowering Plants and Ferns 4th Edition), it is the only known non-leguminous woody species that can form an effective nitrogen-fixing symbiotic relationship with *Rhizobium* and *Bradyrhizobium* (Trinick and Hadobas 1988, Becking 1992). In contrast, all other

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woody non-legume nitrogen-fixing symbioses are induced by the actinomycete *Frankia* (Benson and Silvester 1993). *Parasponia* plants are small to medium-sized trees, growing up to 15 meters tall. *Parasponia* species are typical pioneer plants forming a natural succession in covering bare, nitrogen-poor eroded soils (Becking 1983). Species of the *Parasponia* genus are found in South Sumatra, Java, the Philippines, New Guinea, Polynesia and Melanesia. Thus, *Parasponia* is generally distributed in South-East Asia and the Pacific Islands, along with other plants forming actinorhizal and/or rhizobial symbioses. *Parasponia* is a key species for studies on the accommodation of symbiotic bacteria in plant cells (Santi et al. 2013). In addition to its high agronomic potential, the nitrogen-fixing symbiosis offers scientists the unique opportunity both to study the differences between the partners at the molecular level, and to investigate the possibility of extension of the symbiosis to other non-legumes of agricultural importance (Sujatha and Kannaiyan 2002). Until now, this unique plant-bacterial association of *Parasponia*-rhizobium remains poorly studied. Effective experimental systems for studying of *Parasponia*-rhizobium symbiosis cannot be developed due to poor and extremely slow seed germination (up to 6 months) and high lethality of seedlings of this plant (Bender and Rolfe 1985). To study *Parasponia*-rhizobium symbiosis it is also necessary to develop effective methods of *Agrobacterium*-mediated transformation of *P. andersonii*. The most important step in *Agrobacterium*-mediated plant transformation is the regeneration of transgenic shoots from callus. The problem of *in vitro* reproduction of *Parasponia* was partially solved by two teams (Davey et al. 1993, Webster et al. 1995), who showed the possibility of micropropagation of *P. andersonii*. Also, Cao et al. (2012) generated composite plants of *P. andersonii*, whose roots contained the red fluorescent protein, DsRed1 reporter gene contributed by *Agrobacterium rhizogenes*-mediated transformation. Earlier, *Parasponia* stem segments were used for callus production (Davey et al. 1993, Webster et al. 1995). To generate transgenic plants, different types of tissues are used, but leaves are the most convenient source of explant due to their abundance and ease of manipulating the *Agrobacterium* infection. The leaf-disc method is most commonly used in *Agrobacterium tumefaciens*-mediated transformation of many plants.

The purpose of study was to develop effective methods for callus induction from leaf explants, shoot regeneration, rooting and acclimatization of shoot cultures of *P. andersonii*. These methods can later be used for both micropropagation and genetic transformation in this plant.

Materials and Methods

One-year-old *Parasponia andersonii* plant obtained from germinated seeds. Seeds were collected in Tahiti (Society Archipelago, French Polynesia) by Dr. Jean-Yves

Meyer and kindly provided by Dr. Priscille Frogier. The plants were grown in the greenhouse of the Institute of Biochemistry and Genetics (Ufa, Russia). Leaves excised from the mother plant were washed with running tap water, and surface-sterilized successively with 70% ethanol (2 min) and 0.5% sodium hypochlorite for 15 min. After washing 5 times with sterile distilled water, they were used in following experiments.

Sterile *Parasponia* leaves were cut into segments measuring 7 × 7 mm and placed in Petri dishes containing agarized (Bacto-agar "Helicon") basic medium of WPM (Lloyd and McCown 1981), MS and B5 (Gamborg et al. 1968), with the addition of half concentration of B5 medium's vitamins, 100 mg/l myo-inositol and 3% sucrose. Plant growth regulators were added to the media in different concentrations: BA (0.5 - 2 mg/l), TDZ (0.1 - 2 mg/l) and NAA (0.05 - 0.2 mg/l) for callus induction. The media were autoclaved at 121°C for 20 min., after adjusting the pH to 5.8. The treatments were maintained for 6 weeks at 28°C in the dark. Subcultures were carried out every 2 weeks. The percentage of callus-inducing explants was recorded for each treatment, after 6 weeks of cultivation.

To induce shoot regeneration, explants with calli were cut into pieces (about 5 × 5 mm) and then cultivated on WPM or MS basal medium containing 0.1 or 0.2 mg/l TDZ and 0.01 or 0.05 mg/l IBA, respectively. These explants were transferred to Binder climatic chamber (Germany) under a 16 hrs light/8 hrs dark photoperiod at 28 ± 1°C under a photon flux density of 50 µmol/m²/s, emitted from 'Fluora' fluorescent lamps. The number of regenerated shoots per explant in each treatment was recorded after 4 weeks of incubation. The explants were transferred to fresh media every 2 weeks.

To induce rooting, regenerated shoots (8 - 10 mm long) were excised and transferred to culture vessels (MK5-20PC, Caisson Labs. Inc.), containing 50 ml MS or WPM medium supplemented with different concentrations of NAA, IBA or their combinations. Levels of media pH were adjusted to 5.8 before autoclaving. All treatments were cultivated in Binder climatic chamber under the conditions indicated above. The percentage of rooted shoots and the number of roots/plantlet were recorded after 3 weeks of cultivation.

Rooted plants were transferred to pots containing a mixture of peat and vermiculite (1 : 1 v/v) and grown at high humidity (achieved by covering the plants with polyethylene bags), under the same conditions of light and temperature as those used for the *in vitro* culture. After development of about 10-15 leaves, the plants were moved to the greenhouse.

All experiments had a completely randomized design and were repeated at least twice. Each treatment consisted of 4 replicates and each replicate consisted

of 4 - 8 explants. Data are mean values ± SE. The results were compared through ANOVA and DMRT.

Results and Discussion

The composition of the base salts is of great importance for regeneration of adventitious shoots and *in vitro* micropropagation of plants. Currently, MS medium is most often used for micropropagation, but sometimes it can be suboptimal. We have used three basic media MS, WPM and B5 for callus induction on leaf explants in this work. It was shown that the use of BA as a cytokinin was ineffective and did not lead to the callus initiation on leaf explants, regardless of the basal medium. It was, therefore necessary to use a stronger cytokinin like TDZ, to induce callus formation. TDZ had a critical role in the induction of callus, since calluses were not forming when leaf explants were placed in media without TDZ. However, high concentrations of TDZ in the medium (1 - 2 mg/l) also did not lead to callus initiation (Table 1).

The formation of calluses was observed on 100% explants after 6 weeks when MS and WPM media were supplemented with 0.1 - 0.2 mg/l TDZ and 0.05 - 0.1 mg/l NAA. However, these calluses differed in color and consistency, depending on the salt composition of the medium (Table 1). On MS, loose yellow calluses predominantly appeared on the surfaces of the leaf cuts (Fig. 1A). On WPM medium, calluses were compact, white and mainly appeared in the leaf veins (Fig. 1B). The effective formation of callus was observed on leaf explants in all variants of the experiments only when the concentrations of TDZ did not exceed 0.2 mg/l.

B5 medium was not favorable for callus formation on leaf explants of *P. andersonii*. A slight callus formation was observed on some explants only in response to treatments of 0.1 - 0.2 mg/l TDZ. But these calluses quickly became necrotic and died in the following passages.

For induction of shoot regeneration, explants with calli, generated on MS and WPM media were cut (about 5 × 5 mm) and cultivated on MS or WPM media with 0.1 or 0.2 mg/l TDZ and 0.1 or 0.05 mg/l IBA, respectively (Table 2). All treatments were transferred to light. The cultivation of calli on shoot regeneration media resulted in the appearance of green spots on their surface in some treatments. These green spots subsequently were converted to shoot primordia (Fig. 2A). The formation of shoots with several leaves was observed in 4 weeks (Fig. 2B). The first shoot appeared after 14 days of cultivation on MS containing 0.2 mg/l TDZ and 0.05 mg/l IBA.

Table 1. Effects of medium composition, various concentrations of TDZ and NAA on callus induction percentages of *P. andersonii* after 40 days.

Basal medium	Plant growth regulators conc. (mg/l)		Callus induction (%)*	Callus characteristics
	TDZ	NAA		
MS	2.0	0.05	0 ± 0	Light yellow, loose
	1.0	0.05	0 ± 0	
	0.5	0.05	12.6 ± 0.9	
	0.2	0.05	89.7 ± 5.9	
	0.2	0.1	100 ± 0.0	
	0.1	0.05	100 ± 0.0	
B5	2.0	0.05	0 ± 0	Loose, rapidly browning
	1.0	0.05	0 ± 0	
	0.5	0.05	0 ± 0	
	0.2	0.05	0 ± 0	
	0.2	0.1	17.0 ± 1.7	
	0.1	0.05	9.5 ± 4.8	
WPM	2.0	0.05	0 ± 0	White, tight
	1.0	0.05	0 ± 0	
	0.5	0.05	0 ± 0	
	0.2	0.05	87.1 ± 3.5	
	0.2	0.1	100 ± 0.0	
	0.1	0.05	100 ± 0.0	

*Two independent experiments with 4 replicates per treatment. Each replicate contains 5 explants (total 40 explants); data (means ± SE).

When the yellow calli generated on MS were transferred to MS or WPM regeneration media, the calluses became green and shoot-primordia appeared. But these shoots were hyperhydrated and did not develop further. Hyperhydration in these treatments did not decrease even when the amount of sucrose in the medium was decreased to 1% and the agar concentration increased to 1%.

The normal development of shoots and their elongation in the absence of hyperhydration were observed only in the case when white, tight calli generated on WPM medium were transferred to MS. After transferring these calli became green, and shoots primordia, developing normally, appeared. The maximum number of shoots regenerated on the media containing 0.2 mg/l TDZ and 0.05 mg/l IBA (Table 2).

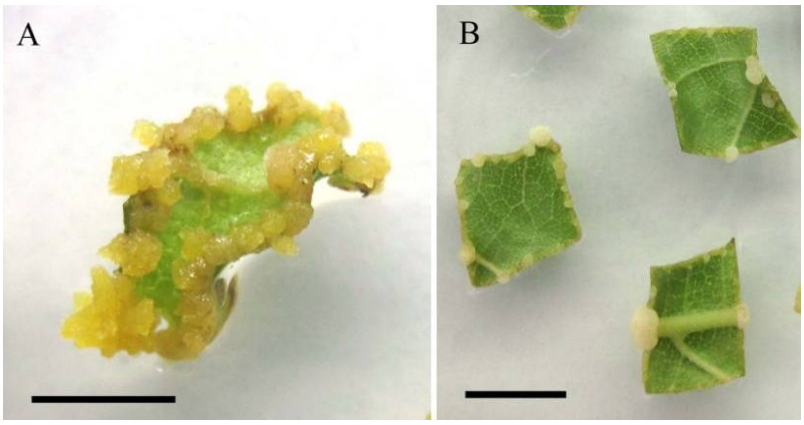

Fig. 1. Callus induction on leaf explants of *P. andersonii*. (A) Yellow calli initiated from leaf explants of *Parasponia* culture on MS containing 0.2 mg/l TDZ and 0.05 mg/l NAA. (B) White calli initiated from leaf explants of *Parasponia* culture on WPM medium. Scale bars are 1 cm.

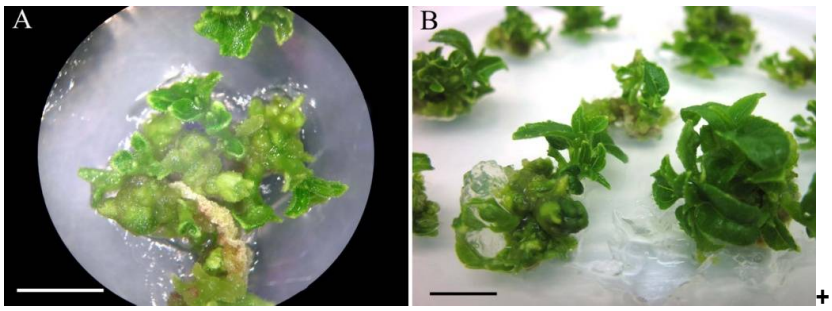

Fig. 2. Shoot regeneration of *P. andersonii*. (A) Shoots regenerated on MS containing 0.2 mg/l TDZ and 0.05 mg/l IBA. (B) Well developed multiple shoots (4 weeks after calli transfer). Scale bar 1 cm.

Adventive organogenesis was also observed when white, compact calli were transferred from WPM medium to a regeneration medium based on WPM salts. However, in this case the generated primordia and shoots were not viable, they turned yellow and died.

Hyperhydration of shoots was probably associated with a high concentration of TDZ in the medium. Therefore, BA (0.2 mg/l) was added instead of TDZ to the medium for further cultivation and elongation of shoots.

In rooting experiments, we used the normally developed, non-hydrated shoots. Two basic media, MS and WPM containing auxins NAA or IBA (or their combination) were used for rooting of *Parasponia* shoots. Rooting experiments showed that root growth and development *in vitro* were accelerated with

decreasing salt concentrations in the media. Significantly better results were obtained on WPM medium than on MS (Table 3).

Table 2. Effect of basal medium and growth regulators on multiple-shoot induction from white calluses, generated on WPM medium (4 weeks after callus inoculation).

Culture media	Plant growth regulators	% explants responding	No. of shoots after 4 weeks*
MS	0.1 mg/l TDZ + 0.01 mg/l IBA	76	2.43 ± 0.20a
	0.1 mg/l TDZ + 0.05 mg/l IBA	69	3.43 ± 0.36bd
	0.2 mg/l TDZ + 0.01 mg/l IBA	75	2.14 ± 0.26a
	0.2 mg/l TDZ + 0.05 mg/l IBA	94	4.71 ± 0.29c
WPM	0.1 mg/l TDZ + 0.01 mg/l IBA	88	2.29 ± 0.18a
	0.1 mg/l TDZ + 0.05 mg/l IBA	77	2.57 ± 0.20a
	0.2 mg/l TDZ + 0.01 mg/l IBA	81	2.86 ± 0.26ab
	0.2 mg/l TDZ + 0.05 mg/l IBA	82	3.71 ± 0.29d

*Two independent experiments with 4 replicates per treatment, each containing 4 explants (total 32 explants); data (mean ± SE). Values followed by same letter are not significantly different at p = 0.05 (two-way ANOVA).

The rooting was not observed in the media containing IBA, whose concentration was more than 1 mg/l, regardless of salt composition in the medium. WPM medium containing 0.5 mg/l IBA without NAA was found as the best for rooting *Parasponia* shoots. There was 100% rooting after 3 weeks of cultivation, and the shoots had good root growth with a lot of roots on the plant (Fig. 3A, B; Table 3).

After this treatment, rooting of all the transplanted shoots was achieved within 2 weeks of cultivation. The addition of NAA to the rooting medium at any concentration did not lead to improvement of rooting, but often led to the formation of basal callus inhibiting the root induction.

For acclimatization, *in vitro* plants were transferred to plastic vessels containing a mixture of vermiculite/peat (1 : 1, v/v). These plants grew in a greenhouse, covered with a transparent plastic film to increase humidity. Humidity was reduced gradually during 2 months. However, despite the precautions taken, the survival rate of acclimatized plants was quite low. After 2 months of acclimatization, only 20% of the plants survived and were transferred to the greenhouse. Thus, the acclimatization was carried out and phenotypically normal, mature plants were obtained (Fig. 3C).

Table 3. Effect of basal medium, IBA, NAA concentrations and of these combinations on the rooting of *P. andersonii* shoots after 3 weeks.

Plant hormones used	MS		WPM	
	Response (%)*	No. of roots/shoot	Response (%)*	No. of roots/shoot
2 mg/l IBA + 0.2 mg/l NAA	0 ± 0a	0 ± 0	0 ± 0a	0 ± 0
1 mg/l IBA	73.3 ± 4.7b	4.4 ± 0.4	100 ± 0b	4.7 ± 0.2
1 mg/l IBA + 0.1 mg/l NAA	44.0 ± 6.7c	4.5 ± 0.2	86.8 ± 8.1bc	4.5 ± 0.2
0.5 mg/l IBA + 0.05 mg/l NAA	66.7 ± 5.9bd	4.3 ± 0.2	100 ± 0b	4.9 ± 0.3
0.5 mg/l IBA	79.3 ± 7.9b	4.8 ± 0.2	100 ± 0b	5.4 ± 0.2
0.2 mg/l IBA + 0.2 mg/l NAA	53.3 ± 4.7ced	4.0 ± 0.3	80.0 ± 8.2dc	4.0 ± 0.3
0.2 mg/l IBA	21.7 ± 1.2f	3.4 ± 0.2	53.3 ± 2.4e	3.6 ± 0.2

*For each medium, a single factor analysis of variance was performed between treatments. In columns "response" values followed by the same letter are not significantly different at $p \leq 0.05$ (one-way ANOVA).

Parasponia andersonii is a tropical woody plant with unique properties, which is an important species for studying of molecular mechanisms of a plant-rhizobium symbiosis. However, there are difficulties in the cultivation and propagation of this plant in the laboratory. Since it is a tropical plant, *P. andersonii* requires special conditions for its cultivation in greenhouses. The seeds of this plant germinate slowly with a high lethality of the seedlings. The development of effective methods of micropropagation and maintain *Parasponia in vitro* culture is an important step in overcoming these difficulties. There are reports about the using of stem segments as explants for the induction of *Parasponia* callus formation (Davey et al. 1993). However, leaves are the most favored explant source due to their abundance and ease of manipulation. The results of this investigation showed that leaf explants are a good source for multiplication of *P. andersonii*.

There is little information about the composition of basal salts used in the works dedicated to obtaining of *in vitro* cultures of *Parasponia.* Davey et al. (1993) had used MS salts to study the clonal micropropagation of *Parasponia*. Cao et al. (2012) had used salts of WPM medium. But in both works only the possibility of clonal micropropagation of *Parasponia* was demonstrated, but this issue has never been studied in detail. Present authors did not find any reports about callus formation and adventitious organogenesis from *Parasponia* leaves. Thus, the novelty of our work lies in the development of effective methods for micropropagation of *P. andersonii* including callus formation from leaf explants,

adventitious organogenesis, rooting of shoots and their acclimatization for field planting.

This work showed that the optimal combination of hormones for 100% induction of morphogenic callus was 0.1 - 0.2 mg/l TDZ together with 0.05 - 0.1 mg/l NAA. The use of BA in media did not lead to the initiation of callus formation. This result showed that *Parasponia* leaf tissue required moderate levels of a strong cytokinin to induce callus. TDZ is known as one of the most effective cytokinins for *in vitro* manipulation of many recalcitrant woody species (Huetteman and Preece 1993).

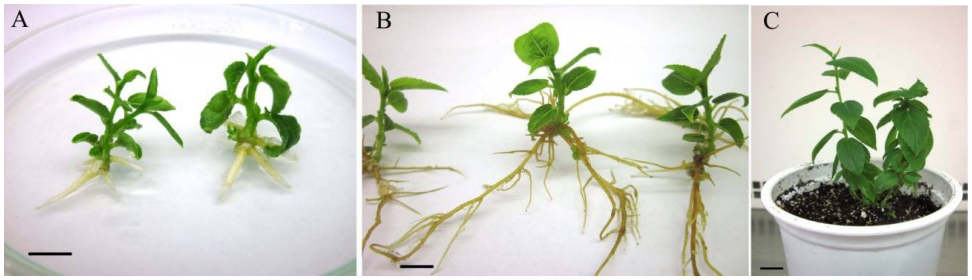

Fig. 3. Rooting and acclimatization of *P. andersonii* shoots. (A) Regenerated shoots, after 3 weeks of 0.5 mg/l IBA treatment. (B) Regenerated shoots, showing development of the root system (6 weeks after inoculation). (C) Hardened, three-month-old *P. andersonii* plant in greenhouse. Scale bars are 1 cm.

Davey et al. (1993) reported the induction of callus from explants of *Parasponia* nodes and internodes and the shoot regeneration from the resulting calli, when explants were cultured on MS supplemented with 0.1 - 2.0 mg/l BA, 0.05 - 2.0 mg/l IBA, and 0.01 - 1.0 mg/l GA_3. But present work demonstrated that *Parasponia* leaf explants required a stronger cytokinin than BA such as TDZ for the callus induction.

It was shown that TDZ increased the frequency of shoot regeneration from different types of tissue for various plant species, especially trees. The disadvantage of TDZ was that its excessive concentration in the medium and its gradual accumulation in the plant tissue may lead to hyperhydration (Huetteman and Preece 1993). Present work demonstrated that it was possible to overcome the problem of hyperhydration of *Parasponia* plants with the help of a two-step culture procedure. For this, callus was initially induced on a TDZ-containing primary WPM medium. Then the secondary medium MS containing BA was used to induce shoot elongation.

For root induction two methods are used: Inclusion of auxins in the rooting medium and dipping the base of shoots in a concentrated auxin solution. For

obtaining roots Davey et al. (1993) cultivated *Parasponia* shoots on a medium containing high auxin concentrations during one week and further cultivated them on a hormone-free medium. Cao et al. (2012) reported about the *Parasponia* shoots rooting on WPM medium, containing 1.0 mg/l IBA and 0.1 mg/l NAA. We demonstrated that the WPM medium containing 0.5 mg/l of IBA was the best medium for rooting of *Parasponia* shoots. An increase in the concentration of IBA in the medium of more than 1.0 mg/l, as well as the addition of NAA, were not very effective, since it delayed the formation of roots and led to the formation of basal callus. Browning and death of buds and leaves were reported when high auxin concentrations were being used in treatments for a long time (Mills et al. 1997).

Browning of the culture medium and death of explants due to phenolic compounds were a common problem for *in vitro* tissue culture of woody plants (Naik et al. 2000). This problem also occurred in the cultivation of *P. andersonii* leaf explants. In the present study, the browning problem was overcome by repeated transfer of the explants to a fresh medium.

The protocol for micropropagation of *P. andersonii* from leaf explants was developed. This method of generating multiple plants from leaf explants can be used as one of the methods of micropropagation of this unique, endemic tree. The new method of micropropagation, combined with *Agrobacterium*-mediated transformation, can be used to generate transgenic *Parasponia* plants.

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