

Final Report of the work done on the Major Research Project entitled “In vitro conservation and reproductive biology of *Pittosporum tetraspermum* an endangered medicinal plant”.

(Period from February 1, 2010 to January 31, 2013)

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ANNEXURE – II

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Micropropagation

One of the major objectives of the project was to standardize various protocols for the micropropagation of *Pittosporum tetraspermum*. We have initiated tissue culture works in this direction. First experiment was to develop a micropropagation protocol from the nodal cuttings of this plant. Nodal cuttings were collected regularly from the field grown plants and culture works were initiated. The nodal explants did not show any bud break when cultured on a phytohormone free MS medium. However the the presence of a cytokinin in the medium was essential to obtain bud break as well as axillary shoot development and elongation from single node cuttings. The type and concentration of cytokinin significantly influenced the percentage of shoot regeneration and average shoot length. Of the three different cytokinins (viz. BA, Kn and TDZ, 1-8 mg/l each) tested, TDZ was more effective than Kn and BA in inducing shoot development for nodal explants. The percentage of shoot proliferation improved with increasing concentrations of TDZ up to the 6 mg/l (Table 1). This medium induced shoot proliferation in 65% of explants with an average number of 3.2 shoots per explant within 45 days. It took about 10–12 days for bud sprouting irrespective of the treatments. The responding cultures exhibited an early enlargement of the axillary bud followed by bud break. From each responding bud, a solitary shoot or two shoots elongated within 18–20 days. A TDZ concentration higher than 6 mg/l did not improve shoot proliferation, but reduced the percentage of bud break to a greater extent.

Callus induction

So far there is no previous report on tissue culture work in this plant. Our second aim was to develop callus regeneration protocol in this plant and there is no research in this direction has been carried out by any workers and this is probably the first attempt in this direction. Hence initiation, callus induction, and

regeneration are extremely difficult. More over this is a medium sized tree and we have faced several problems during experiments like exudation from cut ends, slow response, less response rate etc. as seen in most of the trees. When the internodal nodal cuttings, cotyledons and immature leaf pieces were inoculated on MS medium supplemented with various concentrations of 2, 4-D (0.5–4 mg/l) alone or in combination with BA (0.2-1.0 mg/l), callus growth was initiated in about two weeks and moderate growth followed for most of the cultures (Table 2). The immature leaf responded better than other two explant types and hence further studies were done with only immature leaves. The calli were ready for harvest after 6 weeks of incubation in the media. The calli were friable, organogenic in all concentrations of 2, 4-D. Of the various concentrations of 2, 4-D tried, optimum response was observed on MS medium supplemented with 3 mg/l 2, 4-D where callus production from immature leaf was 64% (Table. 2). To enhance the callus induction further, we have used low concentrations of BA (0.2-1.0 mg/l) alone with 3 mg/l 2, 4-D. The highest callus induction was observed on MS medium supplemented with 3.0 mg/l 2, 4-D and 0.8 mg/l BA. Here 81% cultures responded with high frequency of callus induction. For maintenance of viable calli it was necessary to decrease the 2,4-D concentration to 1mg/l. The leaf cultures responded better than internodal nodal cuttings culture in terms of percent cultures producing callus. The internodal calli produced calli only from the cut end whereas in leaf cultures the calli were produced from the brim of the cut area.

Shoot induction from callus

Callus from the multiplication medium was subcultured on MS medium supplemented with various concentrations of 6 benzyl amino purine (BA; 2–10 mM) or kinetin (Kn; 2– 10 mM) alone or supplemented with 1-naphthalene acetic acid (NAA; 0.5 or 1.0 mg/l) for shoot regeneration. Initial sign of callus regeneration was observed after 7 days by producing clusters of green meristemoids on the callus surface. These meristemoids develop in to shoot clusters within another two weeks. The callus derived from leaf explants showed significantly higher regeneration ability than that induced from other explants like cotyledon and internode (Table 3). Of the two cytokinins used for callus regeneration, BA was comparatively better. However, BA or Kn alone produced low frequency of shoot regeneration. BA alone at 6.0 mg/l produced the optimum 52%

cultures with 10.5 shoots per one gram callus. Among the various concentrations of BA tried with 0.2 and 0.5 mg/l NAA for callus regeneration, 6.0 mg/l BA in conjunction with 0.5 mg/l NAA produced optimum response (Table 3). Here, 77% cultures responded with an average number of 18.2 shoots per one gram callus derived from leaf explants. The shoot buds originated either singly or in clusters. Hence it is confirmed that synergistic effect of BA or Kn together with NAA produced highest shoot regeneration in *P. tetraspermum*.

Rooting and field transfer

Well developed shoots measuring a size of about 1.5-2.0 cm in length were used for root induction. The shoots were cultured on half strength MS medium fortified with either IBA (1.0-4.0 mg/l) or NAA (1.0-4.0 mg/l). There was no root induction on MS basal medium. On all the concentrations of IBA and NAA root initiation was observed. However, IBA was comparatively better in terms of percent rooting and mean number of roots. Among all treatments, 3.0 mg l⁻¹ IBA produced highest response. Here 86% shoots produced roots with an average number of 2.2 roots per shoot. White, healthy roots were emerged from the basal region of the shoots. In the present study half strength MS medium was more suitable for inducing roots. Plantlets with roots were transplanted to the plastic cups and eventually to the clay pots. Of the 50 plants transferred to soil 45 (90%) survived.

Table 1. The effect of various cytokinins (viz. BA, Kn and TDZ) on shoot proliferation from single node cuttings culture of *Pittosporum tetraspermum* on MS medium. The observations were taken 45 days after culture.

Plant growth regulators (mg l ⁻¹)			Percent response	Average no. of shoots	Average length of shoots (cm)
BA	KN	TDZ			
0.0	0.0	0.0	0.0	0.0±0.0	0.0±0.0
1.0	-	-	26	1.4±0.3	1.6±0.3
2.0	-	-	31	1.3±0.2	1.5±0.3
3.0	-	-	38	1.1±0.3	1.4±0.4
4.0	-	-	48	1.8±0.4	1.6±0.5
5.0	-	-	41	1.6±0.1	1.8±0.3
6.0	-	-	37	1.7±0.2	1.6±0.2
7.0	-	-	32	1.9±0.3	1.5±0.2
8.0	-	-	29	1.7±0.2	1.6±0.5
	1.0	-	19	1.3±0.3	1.7±0.4
	2.0	-	24	1.1±0.3	1.6±0.4
	3.0	-	28	1.0±0.1	1.9±0.3
	4.0	-	33	1.5±0.4	2.0±0.2
	5.0	-	38	1.5±0.3	2.1±0.2
	6.0	-	36	1.8±0.4	2.3±0.1
	7.0	-	34	1.9±0.2	1.7±0.1
	8.0	-	25	1.7±0.2	1.9±0.5
-		1.0	31	1.8±0.1	1.6±0.1
-		2.0	37	1.9±0.5	1.8±0.2
-		3.0	45	2.0±0.3	2.2±0.2
-		4.0	51	2.2±0.4	2.4±0.4
-		5.0	58	1.5±0.4	2.3±0.3
-		6.0	65	3.2±0.3	2.1±0.2
-		7.0	55	1.9±0.1	2.4±0.3
-		8.0	43	1.8±0.3	2.2±0.5

Table 2. Effect of various concentrations of 2, 4-D on callus induction from immature leaf explants of *P. tetraspermum*. Medium: MS; Culture period: 45d

Plant growth regulators (mg l ⁻¹)		Rate of callus induction	Percent callusing	Fresh weight (mg)	Dry weight (mg)
2, 4-D	BA				
0.0	0.0	-	0.0	0.0	0.0
0.5	0.0	++	35	211.3±3.4	24.4±2.8
1.0	0.0	++	47	228.4±4.7	27.8±3.1
2.0	0.0	++	56	232.6±5.6	29.5±2.2
3.0	0.0	++	64	241.8±4.3	23.8±1.8
4.0	0.0	++	58	224.2±3.2	21.6±1.9
3.0	0.2	++	68	276.4±6.4	25.6±1.6
3.0	0.4	++	76	291.6±5.3	27.7±1.8
3.0	0.6	+++	78	322.7±7.1	38.4±2.1
3.0	0.8	+++	81	342.4±3.2	41.3±2.3
3.0	1.0	++	76	308.5±4.5	32.9±2.2

Table 3. Effect of various concentrations of BA alone or in combination with IAA on in vitro shoot regeneration from leaf derived calli of *P. tetraspermum*. Medium: MS; Culture period: 45 d

Plant growth regulators (mg l ⁻¹)			Percent callus forming shoots	Mean number of shoots per gm callus	Mean shoot length (cm)
BA	Kn	NAA			
0.0			0.0	0.0	0.0
2.0			38	6.2±1.3	0.9±0.2
4.0			44	8.4±2.4	0.8±0.3
6.0			52	10.5±2.8	0.7±0.2
8.0			42	10.8±2.2	0.9±0.1
10.0			38	9.3±1.8	1.1±0.3
6.0		0.5	77	18.2±2.1	1.3±0.2
6.0		1.0	63	16.5±2.6	1.2±0.1
	2.0		23	8.2±1.3	0.6±0.2
	4.0		32	9.4±2.4	0.7±0.3
	6.0		37	9.5±2.8	0.6±0.2
	8.0		39	9.8±2.2	0.8±0.1
	10.0		26	9.3±1.8	0.8±0.3
	8.0	0.5	44	11.2±2.1	0.9±0.2
	8.0	1.0	49	14.5±2.6	1.0±0.1

Table 3. Influence of IBA and NAA on rooting of in vitro formed shoots on half strength MS medium. Observations were taken 45 d after culture.

Auxins (mg/l)		Percent rooting	Mean root number	Mean root length (cm)
IBA	NAA			
0.0	0.0	0.0	0.0	0.0
1.0		66	1.3±0.3	1.3±0.6
2.0		77	1.8±0.2	1.1±0.5
3.0		86	2.2±0.4	1.6±0.7
4.0		73	1.2±0.5	1.1±0.9
	1.0	54	1.5±0.4	1.2±0.3
	2.0	63	1.7±0.4	1.0±0.5
	3.0	69	1.8±0.5	1.1±0.4
	4.0	43	1.4±0.3	1.2±0.6

Reproductive Biology

Study site

The work was carried out for over three years on a natural population growing in the grass land and shola forests of Vaghamon hills, Kottayam district, Kerala, India. The approximate age of the marked trees varied from 10-26 years. The approximate height of the trees ranged from 5-12 meters.

Phenology

Phenological observations were recorded monthly with respect to leaf fall if any, leaf flush, flowering (Including floral bud formation), and fruiting (Period between fruit formation until seed dispersal) on the selected plant in the studying area. The intensities of these phenological events was estimated using the semi-quantitative scale of Fournier (Fournier 1974) and identification of the morphological patterns was made according to the classification proposed by Newstrom (Newstrom et al. 1994).

Floral biology

Fresh flowers of *P. tetraspermum* were collected for floral biological studies. Type of inflorescence and the average number of flowers/inflorescence were checked. Length of sepals, petals, stamens and gynoecium were estimated. Number of stamens, their arrangement, and nature of stamens were also observed. The nature of stigma, style and the position of ovaries were identified by using a dissection microscope. Number of locules and the number of ovules per locules were identified by taking the free hand sections of ovary. Number of pollen grains per flower and number of ovules per ovary were studied by various methods given by Kearns and Inouye (Kearns and Inouye 1993). Pollen size was measured with an ocular micrometer under light microscope following the procedure of McKone and Webb (McKone and Webb 1988). Pollen nature was determined by acetolysis (Shivanna and Rangaswamy 1993). Photographs were taken by using a photomicroscope (Labomed, India) attached to a camera (Nikon D70).

Pollen/ovule (P/O) ratio

Pollen ovule (P/O) ratio was calculated by dividing the number of pollen grains by the number of ovules/flower (Cruden 1977). The ovules present in the ovary were counted with the help of a dissecting microscope. Buds examined for

P/O ratio were near anthesis i. e. pollen was mature but the anther has not dehisced. The mean P/O ratio was generated from individual ratios of separate buds. A minimum of 12 buds from each specimen were collected.

Reproductive biology

For reproductive biological studies mainly isolated pollen grains from flower buds were used. Pollen viability test were conducted by using pollen grains from flower buds. *In vitro* hanging drop culture method (Brewbaker and Kwack 1963) was used to check the pollen viability and for this 25 flower buds (5 anthers/bud) were selected. Time taken for pollen tube germination, and the average length of pollen tubes were estimated. Pollen load on stigmatic surface were checked by treating the pistil in 8N NaOH. Presence or absence of self incompatibility was also checked by pollen load on stigmatic surface and pollen tube germination on stigmatic surface. Fruit set and seed set were observed regularly after pollination.

Breeding systems

The breeding system of *P. tetraspermum* was studied by conducting controlled pollination with flowers that had been previously enclosed as buds in paper bags. Various types of tests including self-pollination and cross pollinations were carried out (Radford et al. 1974). The bags were removed from the flowers only after the anthesis and the fruit development was monitored regularly. In addition to above treatments, the flowers were also marked to evaluate the percentage of fruit set under natural conditions. We also calculated the reproductive efficacy (the ratio between the percentage of fruits formed by natural pollination and the percentage of fruits formed by hand cross pollination; Bullock 1985). In order to observe pollen tube growth, flowers were hand cross pollinated and the pistils were fixed in 70% FAA at intervals of 8, 16, 24, 48, and 72h after pollinations, studied for pollen tube growth.

Floral visitors

During the flowering period detailed studies on floral visitors were done. All the plants were observed in the field for more than 20 hours each during the field trips. Floral visitors and their behaviour were recorded from 9.30 to 15.00 h. Type of floral visitors, nature of visitors, purpose of visiting, interaction with flowers were properly recorded. Some of the visitors were captured for identification.

Results

P. tetraspermum is a small evergreen tree found in evergreen forest and shola forests of Western Ghats especially in Vaghamon hills, one of the foot hills of Western Ghats. The plant grows up to 12 metre height. Bark is brown and the young branchlets are terete, pubescent and lenticellate. Leaves are simple, alternate, spiral and usually crowded at the apex. The populations of plant is very limited in each locality and is now in the threatened category.

Phenology

Figure 1 presents the details of phenological events. In *P. tetraspermum* total loss of foliage was never observed as the plant is an evergreen tree. The plant was covered fully with green leaf throughout the year (Fig. 2A). The flush was uniform throughout and there was no maximum or minimum leaf flush. About one month before flowering new leaves aroused from the tip of the buds. These new leaves appeared light green (Fig. 2B). The flower bud started appearing during the second week of January. Although the flowering started in February in most of the populations, some intermittent flowering occurred during January in some populations. But these flowers did not produce any fruits. Flowering started in February and continues till the third week of April (Fig. 2C). Flowering peak was observed from the first week of March to fourth week of March. Flowering showed a decline from the first week of April and complete disappearance of flowers were found in the third week of April. In some plants the flowering continued up to the month of July. However, these flowers produced very less fruits (less than 2%). Fruit initiation started early in the first formed flowers. Maximum flower to fruit ratio was observed during the peak flowering period (19%). Fruit development took more time and average time required for flowering to fruit dehiscence took about 3 months. Some fruits were observed on the plant even during the month of August. Mature fruits were dehisced and the seeds were dispersed automatically.

Floral biology

The floral traits of *P. tetraspermum* are presented in table 1. Inflorescence is a terminal raceme (Fig. 2C) and the total number of flowers per inflorescence varies from 96-217. Each day an average number of 10 ± 2 flowers per inflorescence open at the peak of the flowering period. Inflorescence has several

small branches and the flowers are arranged in an acropetal succession on it. Flowers are comparatively small, short pedicellate, complete, zygomorphic, bisexual, pentamerous, polypetalous, hypogynous and light cream in colour with an average length of 1.14 cm (Fig. 2D and E). The flower has a pungent odour. Calyx consists of 5 free sepals with an average length of 0.19 cm (Fig. 2F). Corolla consists of 5 whitish cream coloured petals with an average length of 0.78 cm (Fig. 3B). It took about three weeks from flower bud initiation to flower anthesis (Fig. 3A). Anthesis started at 08.30 h and the flowers were completely opened by approximately 09.30 h.

The androecium consisted of 5 stamens present in the form of a ring around the carpel and they are present at the same level of stigma (Fig. 3C). Average length of stamen is 0.72 cm (filament = 0.23 cm, and anther = 0.49 cm; Table 2). Anthers are basifixed and yellowish while the filament is short and creamy in colour (Fig. 3D). The yellowish anthers dehisced between 10.00-11.30 h. Anther dehiscence takes place through a longitudinal slit. Usually the anthers dehisce approximately 30 minutes after flower anthesis and shed 90% of the pollen within 2-3 hours. The flowers that do not set fruits abscise within 3-4 days. The petals wilted and fall down and the stamens dried from the basal part of the ovary. The ovary remains attached to the pedicel after pollination but the apical portion of the pistil i.e. stigmatic portion, dried up.

Acetolysis of the pollen revealed the pollen morphology. The pollen grains are triangular and trizonocolpate with $45 \pm 5.6 \mu\text{m}$ in size (Fig. 4B). Pistil is white cream in colour and differentiated into stigma, style and ovary, having an average length of 0.78 ± 0.4 cm at anthesis (stigma = 0.026 ± 0.003 cm, style = 0.35 ± 0.02 cm, ovary = 0.41 ± 0.04 cm; Fig. 3E). The stigma is wet, non-papillate and capitate. Stigma contains a thin film of exudates under the light microscope. Style is of medium size, having an average length of 0.35 ± 0.02 cm. Ovary superior, densely covered with papillate hairs and containing 3-8 ovules (Fig. 3E).

Reproductive biology

Acetocarmine staining showed $66 \pm 6\%$ fertile pollen at the time of anther dehiscence (Fig. 3F). *In vitro* pollen germination studies revealed that the pollen grains viability was $64 \pm 4\%$ at the time of anthesis (Table 1; Fig. 4A). Pollen viability showed a steady decrease 4 h after storage in the laboratory ($25 \pm 2^\circ\text{C}$)

conditions and viability reached less than $15\pm 2\%$ 12 h after storage in lab ($25\pm 2^\circ\text{C}$) conditions. However, $46\pm 3\%$ of the pollen grains stored at 4°C in a refrigerator remained viable after 24 h (Fig. 5). The number of pollen grains in an anther is 5246 ± 845 and per flower is 26230 ± 1021 .

Fruit set

Details of fruit set by various pollination experiments are presented in Table 3. Fruit set under open-pollination was poor and only 24% of the flowers set fruits with 58.3% fruits having seeds inside. None of the emasculated and bagged flowers as well as those bagged without emasculation sets fruits (Table 3). Therefore, it is confirmed that in this species there is no apomixes or autogamy. Additionally, none of the manually self pollinated flowers sets fruits, while over 57% of the cross pollinated flowers set fruits.

The average flower and fruit production during the study period of 12 labelled individuals were presented in table 4. The average number of total flowers per plant varies in each year. Average number of total flowers per plant were 18102, 15438 and 23541 and ripening fruits per plant were 2302, 2234 and 2613 during the year 2009, 2010 and 2011 respectively (Table 4). However, the number of mature fruits per plant was 908, 838 and 1203 during the year 2009, 2010 and 2011 respectively. Fruit is a globose, glabrous capsule with 1.0 to 1.5 cm in diameter (Fig. 4D). Mature seeds are dark brown in colour and covered with a resinous viscous fluid (Fig. 4E). The ovoid capsule dehisced by 2 equal valves (Fig. 4F). Number of seeds varies from 3-8. However, most of the seeds contain 3-4 seeds only. Seeds measuring about 5.0 mm in diameter (Fig. 4F).

Floral visitors

The flowers are visited by honey bees, *Apis dorsata* and *Apis indica* and butter flies. Honey bees and butter flies visit the flower in the morning after anthesis. All these visitors play significant role in cross-pollination.

An average number of floral visitors in 12 different plants studied during the peak flowering period were presented in figure 6. The maximum floral visitors (12 honey bees and 2 butterflies) were recorded during 11:00-12:00 h. Based on our observations it can be realized that the effective pollinators are honey bees and butter flies. Cream colour and odour of flowers attract the pollinators towards the flower after anthesis. Occasionally it was noticed that

ants, house flies and spiders also visit the flowers during afternoon between 2pm-4pm. However, it is not clear whether these organisms help in pollination in any way.

So far we have not published some paper from the project. Similarly we have submitted some papers in some journals for the possible publication. In all the papers published/going to publish, the financial assistance from UGC will be acknowledged.

Table 1. Floral traits of *P. tetraspermum*.

Parameters	Observations
Inflorescence	Simple raceme
Flowering period	February-April
Flower	Hermaphrodite and zygomorphic
Flower colour	Cream
Odour	Mild fragrance
Nectar	Nil
Flower opening	8.30-9.30
Anther dehiscence	Bursting inwards by slits
Number of anthers/flower	5
Average number of pollen grains/flower	20800
Average number of ovules/ovary	3-8
Pollen-ovule ratio	1:2600
Pollen type	Trizonocolpate
Pollen shape	Triangular
Pollen size	45±5.6 µm
Pollen viability	64±4%
Stigma type	Capitate

Table 2. Flower characteristics in *P. tetraspermum*. Values indicate the average of 8 flowers from each of 12 plants studied.

Parameters studied	Measurement*
Flower length	1.14 ± 0.04cm
Length of calyx	0.19 ± 0.03cm
Length of corolla	0.78 ± 0.05 cm
Length of stamen	0.72 ± 0.04cm
Length of pistil	0.69 ± 0.05 cm

* Average of 8 flowers from each plant.

Table 3. Fruit and seed set in treated flowers. Values indicate the average of three independent experiments done in 12 plants.

Treatment	No of flowers pollinated	No. of fruits developed (%)	No of fruits having seeds (%)
Bagged without emasculatation	35	0.0	-
Bagged after emasculatation	35	0.0	-
Self-pollination	35	0.0	-
Cross pollination	35	20±2.3 (57)	16±2.1 (80)
Open pollination	50	6.0 ±1.8 (12)	4± 0.6 (66.6)

Table 4 Average flower and fruit production per plant during the study period of 12 labelled individuals.

Year	Average number of flowers/plant	Average number of ripening fruits/plant	Average number of mature fruits/plant
2009	18,102±1041	2302±123	908±32
2010	15, 438±1062	2234±76	838±32
2011	23,541±1232	2613±112	1203±21

Signature of the PI

Dr. Dennis Thomas T.