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Effect of presoaking treatments on *in vitro* seed germination of papaya to facilitate axenic explant production

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Abstract
Studies were taken up to standardize *in vitro* seed germination of papaya cultivars CO 2 and TNAU papaya CO 8. As compared to other surface sterilisation treatments, lowest contamination was recorded in seeds disinfected with 5% sodium hypochlorite for one minute and sown under *in vitro* supplemented with Murashige and Skoog culture medium. Sowing the seeds under *in vitro* with seed coat removal was found to be better for enhancing *in vitro* germination. The influence of pre soaking of seeds was also studied by subjecting the seeds to varied chemical treatments involving GA_3, KNO_3, thiourea and boric acid. Presoaking of seeds in 500 ppm GA_3 for 12 hours had resulted in higher germination percentage, seedling height and seedling vigour index whereas early emergence of seedlings was influenced by 10% KNO_3. The *in vitro* generated seedlings can serve as the source of axenic explants for plant regeneration and further biotechnological studies.

Keywords: Papaya, *in vitro* seed germination, presoaking, seed coat removal

Introduction
Papaya botanically known as *Carica papaya* is a member of family Caricaceae and is an important tropical fruit crop treasured for its high nutritive value. Papaya fruits possess many pharmacological properties. It acts as digestive, antioxidant, antimicrobial, anticarminative, anticancerous and hepatoprotective. The immunological attributes of papaya leaves helps to improve the platelet count in dengue fever patients (Subenthiran *et al*, 2013) [17]. The proteolytic enzymes from papaya namely papain and chymopapain are widely used in brewing, meat and textile industries (Luis Madrigal *et al.*, 1980) [13].

The cultivated papaya varieties can be grouped into two major sex forms namely dioecious and gynodioecious. The dioecious varieties segregate into male and female forms and gynodioecious varieties segregates as female and bisexual (hermaphrodite) forms. In popular dioecious varieties namely CO 2 and TNAU papaya CO 8 released from Tamil Nadu Agricultural University, Coimbatore, the farmer has to necessarily plant four to six seedlings per hill and later resort to thinning of excess male seedlings and retain about 80 – 85% of female plant population in the field to get good economic returns.

Papaya cultivar CO 2 papaya is yellow pulped while the TNAU Papaya CO 8 is red pulped and both the varieties are suitable for fresh fruits, papain extraction or fruit processing to develop value added products like jam, ready to serve beverages and tutti frutti preparations. Since papayas are generally propagated through seeds, maintaining a huge seedling population increases the cost of cultivation. Therefore, shoot tip cultures of papaya through *in vitro* clonal multiplication of desired sex forms could be helpful to avoid such enhanced costs in early field maintenance of excess male plants. It is therefore necessary to develop tissue culture protocols. It is possible to detect sex form of the seedlings using SCAR markers (Urasaki *et al.*, 2002; Liao *et al.*, 2017) [19, 12] to avoid male plants but high cost of testing still makes this technique not so viable so far.

If the seedlings are first grown under *in vitro* and multiplied *in vitro*, it may be possible to clonally develop huge female population or bisexual population. Early screening with markers at a limited level could help to reduce the cost as once the seedlings are identified then it is possible to clonally multiply them. The advantage of young seedlings over the mature shoot tips of papaya is that tissue culture becomes difficult in the latex laden mature tissues. Further, in field grown seedlings the chances of fungal or viral infections (like that of Papaya Ring

Spot Virus) are possible. Some reports state that *in vitro* grown seedlings are known to possess high vigour for clonal multiplication in certain varieties of papaya (Efendi, 2017 [7] and da Silva, 2014) [7, 6] and in other crops which includes date palm (Mondal *et al*., 2017) [14] and morinda (Shekhawat *et al*., 2015) [16]. Such *in vitro* grown seedlings can be pathogen free and can serve as a source of explants for rapid propagation and callus induction for further *in vitro* manipulations.

With this background, experiments were carried out to standardize *in vitro* culture of papaya seedlings of CO 2 and TNAU Papaya CO 8 varieties so as to serve as source of explants for axenic culture. Seed germination in papaya is slow and erratic and exhibits dormancy on storage. Hard seed coat imposes dormancy in papaya seeds. Freshly extracted seeds have higher germination but desiccation induces dormancy in seeds (Lange, 1961) [10]. Papaya seeds are intermediate between orthodox and recalcitrant in seed storage as it germinates immediately after extraction but withstands partial drying without loss of viability (Vozzo, 2002) [22].

Materials and Methods

The seeds of cultivars CO 2 and TNAU papaya CO 8 were collected from the sibmated female trees maintained at University orchard, Horticultural College and Research Institute, Tamilnadu Agricultural University, Coimbatore. All the chemicals used in this study were of analytical grade and the growth regulator was of tissue culture grade. Healthy and mature seeds were selected by discarding floats and the seeds were subjected to the following experiments to determine the effective treatment for *in vitro* seed germination of papaya.

Experiment I: Disinfection of explants

Attempts were made to disinfect the papaya seeds by treating with 0.5% carbendazim for 15 minutes and 0.1% streptomycin sulphate for 15 minutes. Further seeds were surface sterilized with 70% ethanol for 30 seconds under laminar hood. The seeds were subjected to following secondary sterilization treatments.

D_1 : Sterile distilled water (control)
D_2 : 0.1 % Mercuric chloride for 1 minute
D_3 : 0.1 % Mercuric chloride for 2 minutes
D_4 : 0.1 % Mercuric chloride for 3 minutes
D_5 : 1% Sodium hypochlorite for 1 minute
D_6 : 1% Sodium hypochlorite for 2 minutes
D_7 : 1% Sodium hypochlorite for 3 minutes
D_8 : 5% Sodium hypochlorite for 1 minute
D_9 : 5% Sodium hypochlorite for 2 minutes
D_{10} : 5% Sodium hypochlorite for 3 minutes

In order to remove the traces of sterilizants, the seeds were washed in sterile distilled water thrice and inoculated in full strength Murashige and Skoog medium (1962) with 3% sucrose and 2.8g/L phytagel. The experiment was replicated four times with twenty five explants each. Contamination percentage and germination percentage were recorded.

Experiment II: Effect of seed coat on *in vitro* seed germination of papaya

The seeds were sterilized according to the results of Experiment I and were subjected to the following treatments.

S_1: With seed coat (control)
S_2: Seed coat removed

Observations recorded on germination percentage, days taken for first germination and days taken for fifty per cent germination. The experiment was replicated four times with twenty five explants each.

Experiment III: Effect of presoaking chemicals on *in vitro* seed germination

In both cultivars, the seeds were subjected to the following presoaking chemical treatments for 12 hours. The treatments were as follows.

G_1 : Water (control)
G_2 : 250 ppm GA_3
G_3 : 500 ppm GA_3
G_4 : 5 % KNO_3
G_5 : 10 % KNO_3
G_6 : 1000 ppm Thiourea
G_7 : 2000 ppm Thiourea
G_8 : 0.5 % Boric acid
G_9 : 1 % Boric acid

Each treatment was replicated thrice with thirty experimental units per replication. After pre-soaking the seeds were subjected to the best treatments recorded in the experiment 1 and experiment 2 to standardize the *in vitro* germination protocol. The parameters *viz.*, germination percentage, days taken for first germination, days taken for 50% germination, seedling height and seedling vigour index were recorded. The seedling height and germination percentage were measured on 30^{th} day.

Observations recorded

Contamination percentage: The ratio of number of seeds contaminated to the total number of seeds inoculated and expressed in percentage.

Germination percentage: The ratio of number of seeds germinated to the total number of seeds inoculated/sown and expressed in percentage.

Days taken for first germination: It is the number of days taken for the commencement of germination of seedlings and expressed in days.

Days taken for 50% germination: It is the number of days taken for 50% of seeds to germinate and expressed in days.

Seedling height: Seedling height was measured from the collar region of seedling to the highest tip of the plant and expressed in centimeters.

Seedling vigour index: The seedling vigour index was calculated according to Bewley and Black (1982). The formula for Seedling vigour index was Germination (%) x Seedling length (cm).

Statistical analysis

The experiment was carried out in completely randomized design (CRD) design. The data were analysed by estimating analysis of variance and working out the critical difference value. The percentage data was analysed using arcsine transformation and critical difference (CD) values were calculated for five percent probability (0.05) as per the statistical methodologies suggested by Panse and Sukhatme (1985).

Results

Experiment I: Disinfection treatments

Under *in vitro* conditions, among the two different seed disinfectants of different dose and duration, the seed disinfection treatment D_8 (5% sodium hypochlorite for 1 minute) had resulted in higher germination with lower contamination compared to other treatments (Fig. 1 and 2). Use of D_8 (5% sodium hypochlorite for 1 minute) resulted in 12.5% and 10.00% contamination in cultivars CO 2 and TNAU papaya CO 8. The germination percentage was 17.5% and 15.00% respectively. A sharp fall in contaminants is observed with higher concentrations of sodium hypochlorite compared to mercuric chloride in both cultivars CO 2 and TNAU papaya CO 8. Apart from disinfection, sodium hypochlorite has been found to break seed dormancy in certain agricultural crops (Chun *et al.*, 1997) [5]. Mercuric chloride is a strong anti microbial agent. It hindered cell division and often known to cause seed injury interfering with germination (Patra and Sharma, 2000) [15]. Higher dosage of fungicidal treatments resulted in hypertrophy (extensive thickening of primordia) of seeds.

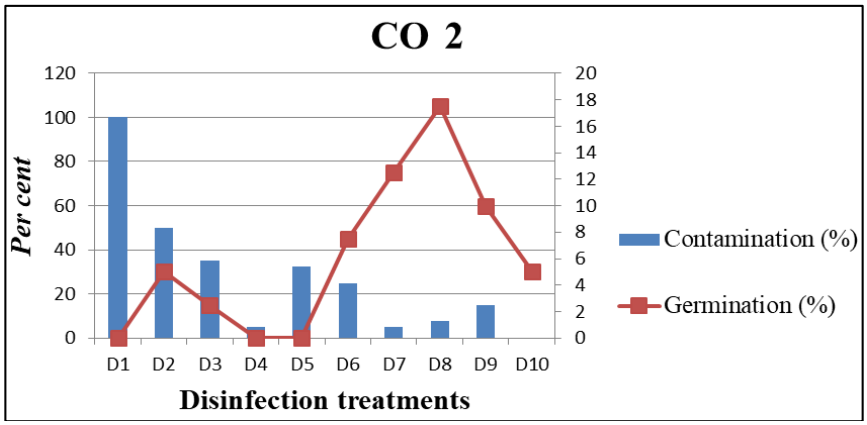

Fig 1: Effect of disinfectant treatments on contamination and germination percentage of papaya cultivar CO 2

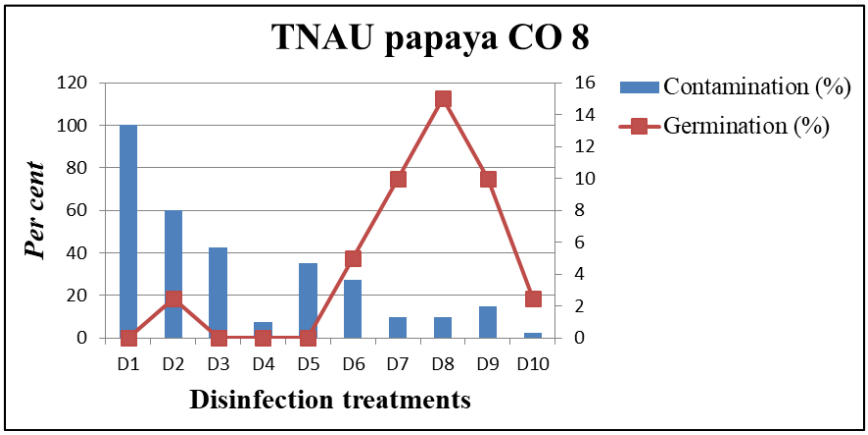

Fig 2: Effect of disinfectant treatments on contamination and germination percentage of papaya cultivar TNAU papaya CO 8

Experiment II: Effect of seed coat on *in vitro* seed germination of papaya

In the present study it was observed that in the both the varieties tested, seed coat removal enhanced the seed germination. The germination percentage of CO 2 without seed coat is 61.25% as compared 13.75% in control while in TNAU papaya CO 8, *in vitro* germination was 57.5% when seeds were without seed coat and 12.5% in control (Table 1).

Papaya seeds comprises a gelatinous sarcotesta arising from multi layered outer integument of ovule (Foster, 1943) [8], a compact hydroscopic mesotesta arising from sub epidermal layers of outer integument and a thin, tanniniferous inner epidermal layer which covers the two cotyledons and an embryo. Sarcotesta of papaya seed is known to acts as a protective layer to seed in natural conditions until it was decomposed by soil microbes thereby preventing the leaching of accumulated auxins and other inhibitors from seed which delays germination (Tseng, 1992) [18]. The reduced germination under *in vitro* condition especially when with intact seed coat could be because of failure to imbibe moisture and as well lack of microbial facilitation.

Under natural conditions, cracking of seed coat was the first step in the mechanics of papaya seed germination (Webster *et al.*, 2016) [21]. Seed coat cracking was facilitated by soil microbes. The days taken for first and as well 50 per cent germination is comparatively low in **S_2** (seed coat removed) than control.

In cultivar CO 2, the seeds devoid of seed coat germinated in 8.72 days compared to control which has taken 16.5 days for germination. Seeds implanted on the media without seed coat took 18.38 days for initiation of germination, whereas it was 25.13 days in control. Fifty *per cent* germination was attained in 18.38 and 25.12 days in treatments without seed coat and intact seed coat.

In TNAU papaya CO 8, seeds without seed coat germinated on 9.97 days and fifty per cent germination was reached in 20.63 days. Initial germination and fifty *per cent* germination was delayed to 18.42 days and 27.75 days in treatments with intact seed coat.

The seed coat impedes the embryo from germination unless it is longitudinally cracked over the micropyle. In some earlier studies also it was found that artificial rupturing of the seed

coat with pressure enhanced germination (Webster *et al.*, 2016) [21]. Removal of sarcotesta from seed facilitated germination regardless of the desiccation of seeds. Intact embryos of papaya devoid of seed coat resulted in higher germination upto 83% at 30 ºC compared to seeds with seed coat grown under *in vitro* conditions (Bhattacharya and Khuspe, 2001 and Chauhan *et al.*, 2014) [3, 4].

Table 1. Effect of seed coat removal on *in vitro* seed germination of papaya cultivars CO 2 and TNAU papaya CO 8.

S. No.	Parameters	Treatments	CO 2	TNAU papaya CO 8	MEAN
1.	Germination percentage (mean + SE in %)	S_1 – With Seed coat	13.75 ± 1.25	12.50 ± 1.44	13.13
		S_2 – Seed coat removed	61.25 ± 4.27	57.50 ± 6.61	59.38
2.	Days to first germination (mean + SE in %)	S_1 – With Seed coat	16.50 ± 0.32	18.42 ± 1.13	17.46
		S_2 – Seed coat removed	8.72 ± 0.94	9.97 ± 0.56	9.35
3.	Days to 50 % germination (mean + SE in %)	S_1 – With Seed coat	25.13 ± 0.38	27.75 ± 1.79	26.44
		S_2 – Seed coat removed	18.38 ± 0.13	20.63 ± 0.94	19.51

Experiment III: Effect of presoaking chemicals on *in vitro* seed germination

Presoaking of seeds was done with nine treatments and then the seeds were subjected to the best treatments recorded in the experiment 1 (5 % Sodium hypochlorite for 1 minute) and experiment 2 (seed coat removal) to standardize the *in vitro* germination protocol. The results of experiment III are as follows.

Germination percentage

It is evident from the data (Table 2) that, pretreatment with 500 ppm of GA_3 is efficient in improving the germination of papaya seeds compared to other treatments in both cultivars CO 2 and TNAU papaya CO 8. The *cv.* CO 2 had registered 91.67% seed germination under *in vitro*. Similarly, TNAU papaya CO 8 seeds recorded 88.33% seed germination under *in vitro*.

Days taken for first germination and 50% germination

In cultivar CO 2, the days taken for first germination are 9.79 days under *in vitro* while in TNAU papaya CO 8 a similar response was recorded (9.26 days). In both cultivars CO 2 and TNAU papaya CO 8 (from Table 2) pre-soaking in 10% KNO_3 resulted in early germination than other chemical treatments.

Among all presoaking treatments, 10% KNO_3 and 500ppm GA_3 was also found to reduce the days taken for 50% germination. The days taken for fifty per cent germination in *cv.* CO 2 was 14.47 days and in TNAU papaya CO 8 it was 14.59 days (Table 2).

Seedling height

The *in vitro* seedlings of *cv.* CO 2 attained 9.53 cm and TNAU papaya CO 8 reached 8.50 cm (Table 3) within 30 days. The seedling height is lowest in control treatment in both cultivars. It is demonstrated once again in this study, as use of 500 ppm GA3 for soaking seeds has remarked increase in shoot and root length of seedlings of both cultivars.

Seedling vigour index

It is clear from Table 3 that the presoaking treatments influence seedling vigour of papaya. Among the treatments, GA_3 at 500 ppm was found to improve seedling vigour of both cultivars CO 2 and TNAU papaya CO 8. The *cv.* CO 2 recorded the highest seedling vigour index of 855.56 while in the cultivar TNAU papaya CO 8 it was 750.83. The lowest seedling vigour index was registered in control treatment (water soaking).

Discussion

Higher seed germination can be attributed to the due to the involvement of GA_3 in the activation of cytological enzymes, increasing cell wall plasticity and better water absorption (Anburani and Shakila, 2008) [1]. The treatment results on early germination are in accordance with Bhattacharya and Khuspe (2002) [3].

GA_3 is also well known for promoting shoot elongation in seedlings by increasing nutrient mobilization and root activity. Potassium ion has been known to play a role in increased the water uptake (Barche *et al.*, 2010) [2] and nitrate ion could act as a source for amino acids, nucleic acid and chlorophyll. Further, KNO_3 has a role to play in breaking the seed dormancy, mobilation of sugar reserves and GA_3 in synthesis of hydrolysing enzymes that facilitates germination process (Lay *et al.*, 2015) [11]. Since the possibility for transmission of virus especially in seed coat removed seeds is highly unlikely, the *in vitro* seedling production and multiplication offers good scope for production of axenic plants or to serve as a source of axenic explants.

Table 2: Effect of presoaking treatments on germination percentage and days taken for germination

	Germination (%)		Days taken for first germination		Days taken for 50% germination	
Treatments	CO 2	TNAU papaya CO 8	CO 2	TNAU papaya CO 8	CO 2	TNAU papaya CO 8
G_1 : Water (control)	58.33 (49.83) c	48.33 (44.04) c	16.52 c	16.93 cd	22.14 d	23.67 b
G_2 : 250 ppm GA_3	81.67 (65.01) b	78.33 (62.48) b	15.60 bc	17.24 d	21.08 cd	22.49 b
G_3 : 500 ppm GA_3	91.67 (76.13) a	88.33 (74.87) a	10.95 a	10.65 ab	15.76 ab	15.22 a
G_4 : 5 % KNO_3	76.67 (61.34) b	76.67 (61.33) b	15.29 bc	14.48 cd	20.86 cd	21.12 b
G_5 : 10 % KNO_3	78.33 (62.48) b	78.33 (62.91) ab	9.79 a	9.26 a	14.47 a	14.59 a
G_6 : 1000 ppm Thiourea	68.33 (55.85) bc	66.67 (54.88) bc	12.14 ab	17.84d	17.20 abc	23.71 b
G_7 : 2000 ppm Thiourea	75.00 (60.32) bc	73.33 (59.24) b	14.83 bc	13.45 bc	20.21 bcd	20.58 b
G_8 : 0.5 % Boric acid	66.67 (54.83) bc	53.33 (46.92) c	16.24 c	17.29 d	22.09 d	24.21 b
G_9 : 1 % Boric acid	71.67 (57.98) bc	63.33 (52.74) bc	15.97 c	16.63 cd	20.70 cd	22.94 b
Mean	60.41	57.71	14.14	14.86	19.39	20.95
CD	10.91 *	12.05 *	3.59 *	3.48 *	4.80 *	3.66 *
SEd	5.19	5.73	1.71	1.66	2.28	1.74

Note: Numbers in parentheses were arcsine transformed values
Treatment means followed with same letters were not significantly different
*Significant at 0.05 % level of significance

Conclusion

The study conducted to standardize *in vitro* seed germination of CO 2 and TNAU papaya CO 8 papaya revealed that that disinfecting the seeds with 5% sodium hypochlorite for 1 minute resulted in lowest contamination and higher germination levels. Removal of the hard seed coat of papaya favoured high and early germination under *in vitro* condition compared to seeds intact with seed coat. Presoaking of papaya seeds in 500 ppm GA_3 for 12 hours improves the germination percentage of papaya seeds. Although 10% KNO_3 was reported to increase the early germination, it has lower germination percentage compared to 500 ppm GA_3. Hence, presoaking of seeds with 500 ppm GA_3 is recommended for improving *in vitro* seed germination. Using this method, a large number of axenic plants can be produced in a short period of time economically. These *in vitro* grown plants could serve as source for axenic explants.

Table 3: Effect of presoaking treatments on seedling height and seedling vigour index

	Seedling height		Seedling vigour index	
Treatments	**CO 2**	**TNAU papaya CO 8**	**CO 2**	**TNAU papaya CO 8**
G_1 : Water (control)	3.37 g	4.70 f	196.39 e	227.17 e
G_2 : 250 ppm GA_3	8.38 b	7.72 b	684.64 b	604.47 b
G_3 : 500 ppm GA_3	9.33 a	8.50 a	855.56 a	750.83 a
G_4 : 5 % KNO_3	7.55 c	6.72 c	578.83 c	514.94 bc
G_5 : 10 % KNO_3	8.32 b	6.82 c	651.47 bc	533.97 b
G_6 : 1000 ppm Thiourea	6.28 e	5.53 e	429.36 d	368.89 d
G_7 : 2000 ppm Thiourea	7.72 c	5.80 d	578.75 c	425.33 cd
G_8 : 0.5 % Boric acid	5.53 f	4.53 g	368.89 d	241.78 e
G_9 : 1 % Boric acid	6.55 d	5.72 d	469.42 d	362.06 d
Mean	7.03	6.23	534.84	447.83
CD	0.23 *	0.16 *	100.64 *	104.54 *
SEd	0.11	0.08	47.90	49.51

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