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Sterilization and Germination of Papaya (*Carica papaya* L.) Seed and Response to LEDs

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ABSTRACT

Seeds obtained from two commercially available hybrid papaya (*Carica papaya* L. cv. 'Rainbow' and 'Sunrise Solo') were removed from the fruit at the ripe stage of development. Seeds were left to soak overnight then washed in running tap water to remove as much of the fruit as possible and the gelatinous aril attached to seeds. Seeds were physically rubbed against a conventional kitchen sieve to remove all the arils. The naked seed were surface sterilized in a solution of 0.1% mercuric chloride + 2 drops of Tween-20 for 5 min, rinsed 3 times in sterilized distilled water (SDW) then sprayed with 80% ethanol – sufficient to cover the seeds but not soak them – for 1 min. Using this protocol 100% sterilization and 100% germination were possible. Sterilized seeds were rinsed three times in SDW under clean-bench conditions then plated, in Petri dishes, on simple Murashige and Skoog medium with 3% sucrose and placed under different light conditions: positive control (100% white), negative control (darkness) and two combinations of light-emitting diodes (LEDs) with different red (R) and blue (B) ratios (30% R + 70% B; 70% R + 30% B). Although seed germination was extremely high (97%-100%) independent of the treatment, 70% R + 30% B resulted in extremely long hypocotyls while and 30% R + 70% B showed highly stunted seedlings; profuse somatic embryogenic callus formed under 70% R + 30% B. Using our protocol, 100% papaya seed germination can be achieved on Murashige and Skoog rather than on Vacin and Went medium.

Keywords: LED, light-emitting diode; **MS**, Murashige and Skoog; **VW**, Vacin and Went

INTRODUCTION

Papayas (*Carica papaya* L.) are most conventionally propagated by seed (reviewed by Teixeira da Silva *et al*. 2007). Seed germination is affected by many factors, including the type of substrate, environmental factors such as oxygen, water, temperature and for some plant species, light (Hartmann *et al*. 2002). *C. papaya* seed germination is slow, erratic and incomplete (Chacko and Singh 1972). Papaya seeds, like seeds of many other tropical and sub-tropical species, are considered to recalcitrant, i.e. they retain high moisture content during maturation, they do not withstand desiccation, and they require high moisture content for germination (Chin and Roberts 1980; Hofman and Steiner 1989).

C. papaya seeds are enclosed within a gelatinous sarcotesta (aril, or outer seed coat which is formed from the outer integument) which can prevent germination, although dormancy is also observed in seeds from which the sarcotesta has been removed (Lange 1961; Yahiro 1979). Removal of the sarcotesta improves germination (Perez *et al.* 1980; Sangakkara 1995). The sarcotesta contains several phenoic compounds which inhibit papaya seed germination (Tokuhisa *et al*. 2007). The flesh of papaya fruit contains inhibitors that can prevent germination but drying freshly extracted seeds increases germination (Yahiro 1979). Pre-soaking the seeds in water for 24 h promotes germination (Riley 1981). Washing seeds could improve germination but this depended on the soil type used (Okeyo and Ouma 2008). Shade drying resulted in higher seed germination than oven drying while seeds from ripe fruits germinated more than those from over-ripe fruits (Sangakkara 1995).

Light-emitting diodes (LEDs) are new artificial light sources which have considerable biological actives (Nhut and Nam 2010). Plant growth responses under different blue (B) to red (R) LED ratios were different. Huan (2004) indicated that callus proliferation of *Cymbidium* Twilight Moon 'Day Light' was best under 75% R + 25% B compared to 100% R, 100% B, 50% R + 50% B, and 75% B + 25 % R treatments. At various R:B ratios, Nhut (2002) proved that strawberry plantlets grew better under the ratio of 70% R + 30% B. This is the first study on the effect of LEDs on seed germination.

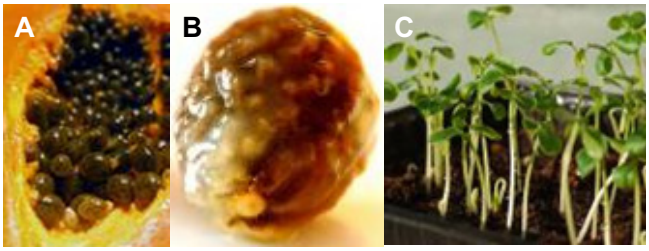

Fig. 1 'Sunrise Solo' papaya sliced to reveal seeds (**A**). One seed with sarcotesta still attached (**B**). Seedlings after 2 weeks germination *in vitro* acclimatized to soil conditions (**C**).

This experiment was established to find a simple sterilization procedure for papaya seeds of two popular export cultivars. Moreover, ideal germination conditions were also tested.

MATERIALS AND METHODS

Chemicals and reagents

All plant growth regulators (PGRs) were purchased from Sigma-Aldrich (St. Louis, USA) and were of tissue culture grade. All other chemicals and reagents were of the highest analytical grade available and were purchased from Wako (Japan), unless specified otherwise.

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Original Research Paper

Plant material and seed reparation

Fruits of two commercially available hybrid papaya (*Carica papaya* L. cv. 'Rainbow' and 'Sunrise Solo') cultivars were purchased from a local supermarket with guaranteed import quality and with no apparent surface infection or markings. Seeds were removed from the fruit at the ripe stage of development and were left to soak for 24-48 h (Bhattacharya and Khuspe 2001) then washed in running tap water to remove as much of the fruit as possible and the gelatinous aril attached to seeds. Chemical scarification (0.1 N HCl, 0.1 N HNO_3 and concentrated H_2SO_4), as recommended by Bhattacharya and Khuspe (2001), was not performed. A simple floatation test (Okeyo and Ouma 2008) was done to determine seed viability; seeds that floated as well as any seed with split seed coats were discarded as these were not considered to be viable or were considered to be damaged. Seeds were physically rubbed against the inside of a conventional kitchen sieve to remove all the remaining arils. After dabbing between commercially-available kitchen paper towels, naked seeds were surface sterilized according to Experiment 1 and then placed on different media in Experiment 2 or under different light sources in Experiment 3. To avoid possible loss in seed germination as a result of contamination by microorganisms in the greenhouse, experiments were conducted *in vitro*. *In vitro* seed germination is much faster than *ex vitro* germination (Bhattacharya and Khuspe 2001).

Experiment 1

Seeds from each cultivar (50 seeds for each cultivar and for each treatment, arranged as 5 batches of 10 seeds per Petri dish), after preparation as described above, were sterilized in one of three protocols: P1: seeds were placed in a solution of 0.1% mercuric chloride ($HgCl_2$; following advice for sterilization of papaya immature ovules; Kavitha and Chezhiyan 2005) + 2-3 drops of Tween-20 for 5 min, rinsed 3 times in sterilized distilled water (SDW) then sprayed with 80% ethanol – sufficient to cover the seeds but not soaked – for 1 min. Seeds were once again rinsed 3 times in SDW. P2: As for P1 except that all sterilization steps were conducted with double the amount of time. P3: As for P1, except that 4.5% sodium hypochloride (NaOCl; recommended by Bhattacharya and Khuspe 2001; Farzana *et al*. 2008) was used to substitute $HgCl_2$. Seeds of both cultivars from P1, P2 and P3 were placed on M1 medium (previously optimized in Experiment 2).

Experiment 2

Seeds from each cultivar (50 seeds for each cultivar and for each treatment, arranged as 5 batches of 10 seed per Petri dish), after prepared as described above, were sterilized by P1 following optimization of the sterilization procedure, then placed on one of three media: M1: Murashige and Skoog (MS) (1962) medium containing 3% sucrose and 8 g/l Bacto agar (Difco Labs., USA). M2: As for M1 but half-strength MS micro- and macronutrients were used. M3: As for M1, except that Vacin and Went (VW, 1949) medium was used instead. No media contained any PGRs.

Experiment 3

Seeds from each cultivar (50 seeds for each cultivar and for each treatment, arranged as 5 batches of 10 seed per Petri dish), after prepared as described above, were sterilized by P1 following optimization of the sterilization procedure, then placed on M1 following optimization of the medium. Seeds were then cultured under one of three light sources: L1: heat fluorescent lamps (HFL; positive control). L2: complete darkness (negative control); L3: LEDs at 70% red (R) and 30% blue (B). L4: As for L2, except that the R: B LED ratio was 3: 7. After 2 weeks, seedling length was measured when germinated seeds were left on the same medium.

Culture conditions

All media in Experiments 1-3 were adjusted to pH 5.8 (recommended by Bhattacharya and Khuspe 2001; Farzana *et al*. 2008) with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 21 min. Seeds were half embedded into the medium, 5 per Petri dish, which were sealed with Parafilm® and incubated at 25°C under a 16-h photoperiod with a light intensity of 45 μmol/m^2/s provided by plant growth fluorescent lamps (Plant Lux, Toshiba Co., Japan). For Experiment 3, the LEDs were as described in Nhut (2002).

Statistical analyses

In Experiments 1-3, seeds were considered to have germinated when the radicle protruded from the seed coat. Seeds were observed daily and seed germination was quantified as number of days from plating. Experiments were organized according to a randomized complete block design (RCBD). Data was subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's multiple range test (DMRT) using IRRISTAT version 3.0.

RESULTS AND DISCUSSION

Sterilization procedure 1 resulted in the highest level of germination (100%) and the lowest level of contamination for both cultivars while sterilization procedures 2 and 3 were relatively ineffective resulting in less than optimal germination and high levels of contamination (bacterial and fungal) (**Table 1**). Full-strength MS medium was superior to half-strength MS medium and VW medium for both cultivars (**Table 2**). Using optimized sterilization procedure P1 and medium M1, seeds were germinated under different light conditions, with some interesting results: although germination percentage was close to 100% in all light conditions and for both cultivars, shoot length was drastically stunted under blue LEDs and highly elongated under red LEDs (**Table 3**).

In papaya, pre-sowing treatment influences seed germination ability. Nagao and Furutani (1986) and Furutani and Nagao (1989) preconditioned papaya seed at 24°C before transfer to 32°C, increasing seed germination. Papaya seed germination is enhanced when soaked in KNO_3 for 30 min (Furutani *et al*. 1993; Nishina *et al*. 2004). Bhattacharya and Khuspe (2001) concluded that pre-sowing treatment of soaking papaya seeds in gibberellic acid (GA_3; 200 ppm) for 24 h resulted in highest germination percentage in soil compared to α-naphthalene acetic acid (NAA) and KNO_3 treatment. However, they also proved that *in vitro* conditions were better for seed germination of papaya than an *in vivo* environment, shown by more aseptic seedlings and shorter germination period. *In vitro* experiment, sterilization

Table 2 Experiment 2, in which *in vitro* seed germination medium was optimized (see materials and methods for details).

Medium	Rainbow	Sunrise Solo
	Germination %	
M1	100 a	100 a
M2	96 a	94 a
M3	68 b	72 b

n = 5 × 10 (total = 50) for each treatment. Different letters within a column and for each cultivar indicate significant differences ($P < 0.05$) using DMRT.

Table 1 Experiment 1, in which sterilization procedure was optimized (see materials and methods for details).

Sterilization treatment	Rainbow		Sunrise Solo	
	Germination %	Contamination %	Germination %	Contamination %
P1	100 a	0 c	100 a	0 b
P2	92 a	12 b	68 b	20 a
P3	68 b	26 a	76 b	24 a

n = 5 × 10 (total = 50) for each treatment. Different letters within a column and for each cultivar indicate significant differences ($P < 0.05$) using DMRT.

Table 3 Experiment 3, in light conditions were tested after sterilization and medium were optimized (see materials and methods for details).

Light treatment	Rainbow		Sunrise Solo	
	Germination %	Shoot length (mm)	Germination %	Shoot length (mm)
L1	100 a	23 c	100 a	38 b
L2	98 a	65 b	100 a	52 b
L3	100 a	110 a	96 a	88 a
L4	100 a	4 d	100 a	8 c

n = 5 × 10 (total = 50) for each treatment. Different letters within a column and for each cultivar indicate significant differences ($P < 0.05$) using DMRT.

is a strict requirement. The first step in all sterilization methods used in this study was washing the seed under running tap water. This is an important state, proved by several previous studies. Suksa-Ard and Kataoka (1996) indicated that germination of dried seed was remarkably enhanced by immersing under running tap water compared with soaking in standing water. Bhattacharya and Khuspe (2001) highly recommended that freshly extracted seeds should be washed before sowing in order to improve seed germination. According to Kavitha and Chezhiyan (2005), sterilants and the duration of exposure affected the survival rate of treated embryos: surface sterilization with 0.1% $HgCl_2$ for 5 min was best for 'Sunrise Solo' and 'CO. 7' papayas. NaOCl effectively kills bacteria, and is one of the most popular chemicals used for surface sterilization (Abdul-Baki 1974). Vejsadova (2006) indicated that the germination of orchid (*D. incarnata* subsp. s*erotina*) seeds sterilized by 0.5% NaOCl was >50% inhibited compared with $Ca(OCl)_2$. NaOCl also inhibited seed germination in papaya and also did not prevent total contamination from occurring (P3 in **Table 1**).

No studies indicate the importance of media choice on the germination success of papaya seeds.

LEDs are considerable as an interesting alternative light source for plant growth, especially for *in vitro* propagation of cotton (Li *et al*. 2010). This lighting system has valuable properties: small size, specific narrow-bandwidth wave length emissions, cool emitting surfaces and longevity and with positive effects on plant growth of several species. For example, Huan (2004) indicated that 100% red LEDs was the most effective for callus induction from protocorm-like body segments of *Cymbidium* Twilight Moon 'Day Light' while Nhut (2002) showed that the best R: B LEDS ratios for plantlet growth *in vitro* of strawberry cv. 'Akihime' was 70% R + 30% B, for *Eucalyptus citriodora*, *Cymbidium* Great Katy 'Love Me', *Phalaenopsis* Gallant Beau 'George Vazquez', banana 'Nam Dinh', and *Spathiphyllum* 'Merry' were 80% R + 20% B. 50% R +50% B LEDs was the best ratio for the growth of upland cotton (*Gossypium hirsutum* L.) plantlets *in vitro* (Li *et al*. 2010). LEDs in this study were as effective as control light, although they led to elongation of shoots (**Table 3**).

The papaya data set was previously presented as a poster in Norway (Teixeira da Silva 2005).

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