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# *In-Vitro* Plantlet Regeneration from Shoot Tip of Field-grown Hermaphrodite Papaya (*Carica papaya* L. cv. Eksotika)

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## ABSTRACT

An experiment was carried out on plantlet regeneration from shoot tip of hermaphrodite field-grown papaya (*Carica papaya* L. cv. Eksotika). BAP concentrations in combination with NAA were assessed for shoot induction and proliferation. For rooting, pretreatment in different indol-3-butyric acid (IBA) concentrations, followed by culture on medium with and without vermiculite supplement were evaluated. MS medium containing 1.0 mg $L^{-1}$ 6-benzylaminopurine (BAP) and 0.05 mg $L^{-1}$ α-naphthaleneacetic acid (NAA) produced the highest mean number of shoots (73.3) per explant. The number of emerging shoots per explant differed significantly from all other treatments including the control. A highest rooting percentage (95%) was obtained on shoots pretreated in 1.0 mg $L^{-1}$ IBA. Culture on medium supplemented with vermiculite, exhibited a mean number of 1.4 roots per shoot and mean root length of 2 cm. A protocol for *in-vitro* plantlet regeneration from shoot tip of hermaphrodite field-grown papaya (*Carica Papaya* L. cv. Eksotika) is developed.

**Key Words:** Eksotika papaya; 6-benzylaminopurine; α-naphthaleneacetic acid; Indole-3-butyric acid

## INTRODUCTION

*Carica papaya* L. belongs to the family *Caricaceae.* It is an important fruit species in the world. In Malaysia, the most popularly grown cultivar is the Eksotika. Eksotika papaya, which resulted from cross between Subang 6 and the Hawaiian Sunrise Solo was released by MARDI in 1987 (Chan, 1987). The cultivar is better than its counterpart the Hawaiian Sunrise Solo. Both cultivars produce fruits that contain total soluble solid (TSS) in the range of 13 - 15%, but the Eksotika is bigger in size. It has a firm textured orange red flesh and the fruit weighs on average about 600 - 800 grams (Chan, 1989; Tokimin, 1989). Its sweet and delicious taste makes it especially good when taken fresh as dessert. Other common forms of consumption of Eksotika fruits are as jams, juice, soft drinks and as fillers for sauces. The Eksotika has also a high content of papain, an important product of papaya used as a meat tenderiser, which is in great demand in the international market (Rohani, 1994).

Botanically, papaya plant has three different sex types: male plant producing staminate flower, female plant producing pistillate flower and hermaphrodite plant producing bisexual flower. Papaya plants can be propagated sexually by using seeds. However, the setback of propagating by seeds is the production of non-true-to-type planting materials due to the segregation of off springs at the second filial generation. Therefore vegetative propagation via micro-propagation is an alternative to obtain true-to-type plants on a large scale.

Micro-propagation of other cultivars of papaya has been achieved using shoot tips and axillary buds (Rajeevan & Pandey, 1986; Reuveni *et al.,* 1990). Although reports are available on the whole plant multiplication of Eksotika papaya, its *in vitro* propagation of Eksotika papaya from field-grown materials has not been reported. The present paper describes plantlet regeneration from shoot tips of hermaphrodite field-grown papaya cv. Eksotika.

## MATERIALS AND METHODS

**Plant material and sterilization procedure.** Plant materials for this research were shoot tips obtained from hermaphrodite field-grown plants of *Carica papaya* L. cv. Eksotika. They were obtained with the courtesy of the Department of Agriculture, Serdang, Selangor, Malaysia. The shoot tips were sterilized using 0.2% Benlate solution for 15 min followed by 70% ethanol for 10 minutes and 0.1% $HgCl_2$ for five minutes. They were rinsed with sterile distilled water for five times. The shoot tips were next immersed in 30% clorox solution for 20 min and finally rinsed with sterile distilled water for five times.

**Explant preparation and treatments.** After sterilization, the outer leaves were removed and 5 mm long shoot tips were excised. The shoot tip explants were cultured on MS (Murashige & Skoog, 1962) medium supplemented with 500 mg $L^{-1}$ casein hydrolysate, 30 g $L^{-1}$ sucrose, 0.2% of phytagel (modified from Chan & Teo, 1994) and different concentrations of BAP (0, 0.1, 1.0 & 2.0 mg $L^{-1}$) in combination with NAA (0, 0.05, 0.1 & 0.2 mg $L^{-1}$) for shoot induction. Proliferated shoots were separated into single shoots and cultured on MS medium without growth regulators (MSO) for one week, prior to rooting, to remove

any residual effects of BAP and NAA. The shoots were then pretreated on MS medium containing different concentrations of IBA (0.0, 0.1, 1.0 & 2.0 mg $L^{-1}$) for one week to stimulate root initiation. Finally the shoots were cultured on MS medium with or without vermiculite for further root development. The pH of the media was adjusted to 5.7 and the cultures were kept at 25 ± 2°C in the growth room with a daily fluorescence lighting of 16 h providing an intensity of 15.8 μmol$m^{-2}$ $s^{-1}$.

**Parameters observed.** Parameters observed in the shoot induction stage were mean number of shoots produced per explant, mean shoot height (cm) and percentage of explants that responded to form callus (%). Data were collected every two weeks until the twelfth week of culture, while growth characteristics were observed every week. Parameters on rooting that were recorded were percentage of explants producing root (%), mean number of roots per explant and mean root length (cm). The data on rooting were collected until the eighth week of culture.

**Experimental design and statistical analysis.** The experiment was a single factor experiment arranged in a Completely Randomized Design (CRD) with four replications and each replication per treatment contained ten explants. Data were analyzed using the analysis of variance (ANOVA) and Duncan New Multiple Range Test were employed for comparison of treatment means.

## RESULTS

**Induction of multiple shoots from shoot tips.** Within two to three weeks of culture on the induction medium, the outer leaves covering the shoot tip swelled and opened up. After eight weeks of culture, minute shoots proliferated from the shoot tip (Plate 1). The multiple shoots developed further after 12 weeks of culture (Plate 2). Combinations of BAP and NAA tested affected the mean number of shoots produced per explant, shoot height and the percentage of explants that responded to form callus after 12 weeks of culture.

Significant differences were noted among the treatment combinations on mean number of shoots produced per explant (Fig. 1), mean shoot height (Fig. 2) and the percentage of explants formed callus (Fig. 3). The combination of 1 mg $L^{-1}$ BAP with 0.05 mg $L^{-1}$ NAA (B2N1) produced the highest mean number of shoots per explant (73.3) and resulted in a mean shoot height of 0.9 cm (Fig. 1 & 2). On medium containing 0.1 mg $L^{-1}$ BAP and 0.05 mg $L^{-1}$ NAA (B1N1), the mean shoot height reached 1.2 cm but the number of shoots produced was low (24.3). Treatments containing 0 to 0.2 mg $L^{-1}$ NAA but without BAP produced less than two shoots per explant (Fig. 1). Increasing the BAP concentration up to 1.0 mg $L^{-1}$ resulted in increased number of shoots produced per explant. However, the number of shoots produced per explant decreased when the BAP concentration was increased to 2.0 mg $L^{-1}$.

The presence of NAA at higher concentrations (0.1 & 0.2 mg $L^{-1}$) either alone or in combination with BAP, caused significant callus formation at the base of the explants (Fig. 3). At 0.05 mg $L^{-1}$ NAA and less, either alone or in combination with BAP there were less or no callus was formed. The highest amount of callus (90%) was obtained in treatment containing 0.20 mg $L^{-1}$ NAA without BAP. This was followed by treatment containing 0.20 mg $L^{-1}$ NAA with 0.1 mg $L^{-1}$ BAP at 60%.

**Rooting of shoots.** After a week of pretreatment on medium containing different concentrations of IBA, the shoots were transferred on hormone-free MS medium with and without vermiculite supplement. The cut end or basal portion of the shoot became swollen and whitish followed by the emergence of roots by the second week of culture. Within three to four weeks, the roots elongated and developed further. Root formation was most established by the eighth week of culture (Plate 3A & B).

Significant differences were observed on the percentage of root formation (Fig. 4), mean number of roots produced per shoot (Fig. 5) and mean root length (Fig. 6) obtained on shoots pretreated in different concentrations of IBA followed by culture on medium with and without vermiculite compared to shoots without IBA pretreatment and placed on medium without vermiculite. About 90% Ninety percent rooting was observed on shoots pretreated in 1.0 mg $L^{-1}$ IBA and then cultured on medium with vermiculite and it differed significantly from all the other treatment combinations. Pretreating shoots in IBA, followed by culture on medium with or without vermiculite resulted in root formation. Meanwhile, shoots without IBA pretreatment but placed on medium containing vermiculite still produced root, but the frequency was low (4.5%). Shoots without IBA pretreatment placed on medium without vermiculite supplement did not produce root at all. A highest mean number of roots produced per explant (1.7) was produced on shoots pretreated in 1.0 mg $L^{-1}$ IBA and then cultured on medium without vermiculite, which differed significantly from the other treatment combinations.

Shoots pretreated with 1.0 mg $L^{-1}$ IBA followed by culture on medium with vermiculite and on medium without vermiculite produced roots reaching up to 2 cm in length. In both treatments, the mean root lengths did not differ significantly from each other but were significantly different from the rest of the treatment combinations.

## DISCUSSION

The production of shoots from axillary buds and shoot tip explants is the most applicable and reliable method of *in vitro* propagation. In this study, different concentrations and combinations of BAP and NAA tested affected shoot induction and proliferation. The presence of BAP and NAA in the medium promoted and enhanced the number of shoots produced from the field-grown shoot tip explants. BAP breaks the apical dominance and caused shoot

**Plate 1. Proliferation of minute shoots from field-grown shoot tip of Eksotika papaya after eight weeks of culture**

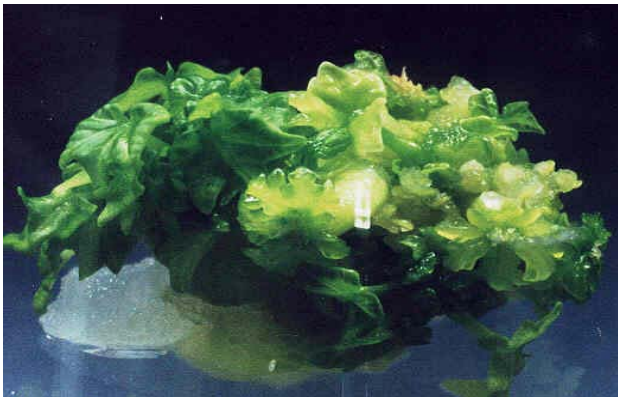
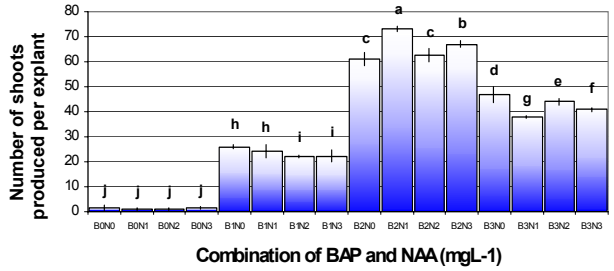


**Plate 2. Further development of the proliferated shoots after twelve weeks of culture**

**Fig. 1. Effect of BAP in combination with NAA on mean number of shoots produced per explant after twelve weeks of culture**




initiation, while the presence of NAA enhanced meristem cells to elongate (George, 1993; Anonymous, 2001). The combination of 1 mg $L^{-1}$ BAP with 0.05 mg $L^{-1}$ NAA produced the highest mean number of shoots (73.3) per explant (Fig. 1), that reached a mean height of 0.9 cm after twelve weeks of culture (Fig. 2). According to Drew (1988) a six fold increase in shoot number was obtained after 15 weeks culture of axillary buds of papaya on medium with 2

**Fig. 2. Effect of BAP in combination with NAA on mean shoot height (cm) attained after twelve weeks of culture**

B0N0=MSO, B0N1=0.05 mg$L^{-1}$ NAA, B0N2= 0.10 mg$L^{-1}$ NAA B0N3=0.20 mg$L^{-1}$ NAA, B1N0=0.10 mg$L^{-1}$ BAP, B1N1=0.10 mg$L^{-1}$ BAP + 0.05 mg$L^{-1}$ NAA, B1N2=0.10 mg$L^{-1}$ BAP + 0.10 mg$L^{-1}$ NAA, B1N3=0.10 mg$L^{-1}$ BAP + 0.20 mg$L^{-1}$ NAA, B2N0=1.0 mg$L^{-1}$ BAP, B2N1=1.0 mg$L^{-1}$ BAP + 0.05 mg$L^{-1}$ NAA, B2N2=1.0 mg$L^{-1}$ BAP + 0.10 mg$L^{-1}$ NAA, B2N3=1.0 mg$L^{-1}$ BAP + 0.20 mg$L^{-1}$ NAA, B3N0=2.0 mg$L^{-1}$ BAP B3N1=2.0 mg$L^{-1}$ BAP + 0.05 mg$L^{-1}$ NAA, B3N2=2.0 mg$L^{-1}$ BAP + 0.10 mg$L^{-1}$ NAA, B3N3=2.0 mg$L^{-1}$ BAP + 0.20 mg$L^{-1}$ NAA

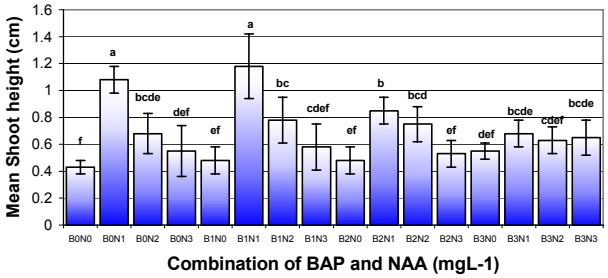
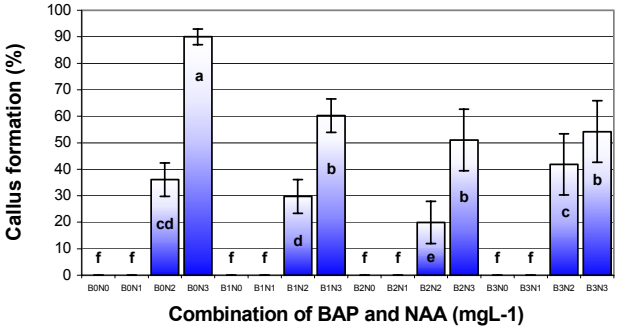



**Fig. 3. Effect of BAP in combination with NAA on percentage of explant that responded to form callus**

B0N0=MSO, B0N1=0.05 mg$L^{-1}$ NAA, B0N2= 0.10 mg$L^{-1}$ NAA B0N3=0.20 mg$L^{-1}$ NAA, B1N0=0.10 mg$L^{-1}$ BAP, B1N1=0.10 mg$L^{-1}$ BAP + 0.05 mg$L^{-1}$ NAA, B1N2=0.10 mg$L^{-1}$ BAP + 0.10 mg$L^{-1}$ NAA, B1N3=0.10 mg$L^{-1}$ BAP + 0.20 mg$L^{-1}$ NAA, B2N0=1.0 mg$L^{-1}$ BAP, B2N1=1.0 mg$L^{-1}$ BAP + 0.05 mg$L^{-1}$ NAA, B2N2=1.0 mg$L^{-1}$ BAP + 0.10 mg$L^{-1}$ NAA, B2N3=1.0 mg$L^{-1}$ BAP + 0.20 mg$L^{-1}$ NAA, B3N0=2.0 mg$L^{-1}$ BAP B3N1=2.0 mg$L^{-1}$ BAP + 0.05 mg$L^{-1}$ NAA, B3N2=2.0 mg$L^{-1}$ BAP + 0.10 mg$L^{-1}$ NAA, B3N3=2.0 mg$L^{-1}$ BAP + 0.20 mg$L^{-1}$ NAA




µm BAP and 0.5 µm NAA. Chan and Teo (1994) obtained 2 shoots per explant from axillary buds of papaya cultured on M.S medium containing 0.1 mg $L^{-1}$ BAP for eighteen weeks. However, these shoots when transferred into liquid medium produced 82 times more shoots ten weeks later.

On medium containing 0.1 mg $L^{-1}$ BAP and 0.05 mg $L^{-1}$ NAA, the mean shoot height reached 1.2 cm (Fig. 2) but the number of shoots produced was 24.3 (Fig. 1), which was lower than that produced in treatment containing 1 mg $L^{-1}$ BAP and 0.05 mg $L^{-1}$ NAA. Meanwhile, treatments containing 0 to 0.2 mg $L^{-1}$ NAA but without BAP produced less than two shoots per explant (Fig. 1). Increase in BAP up to 1.0 mg $L^{-1}$ increased the number of shoots produced per explant. The results show that 1 mg $L^{-1}$ BAP was the optimum level for shoot multiplication from field-grown

**Fig. 4. Effect of IBA pretreatment followed by culture on medium with and without vermiculite supplement on percentage of root formation after eight weeks of culture**

I0S0=Without IBA and without vermiculite, I0S1=Without IBA and with vermiculite I1S0=0.1 mgL$^{-1}$ IBA and without vermiculite, I1S1=0.1 mgL$^{-1}$ IBA and with vermiculite, I2S0=1.0 mgL$^{-1}$ IBA and without vermiculite, I2S1=1.0 mgL$^{-1}$ IBA and with vermiculite, I3S0=2.0 mgL$^{-1}$ IBA and without vermiculite, I3S1=2.0 mgL$^{-1}$ IBA and with vermiculite

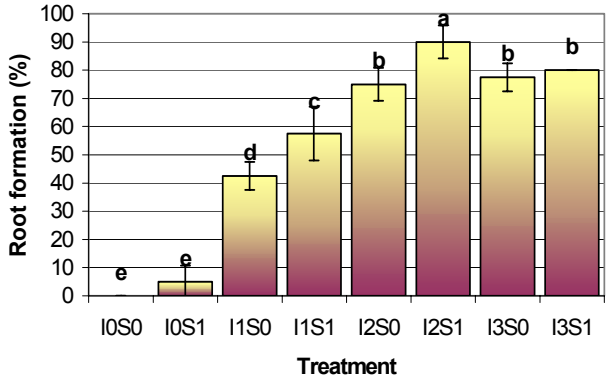
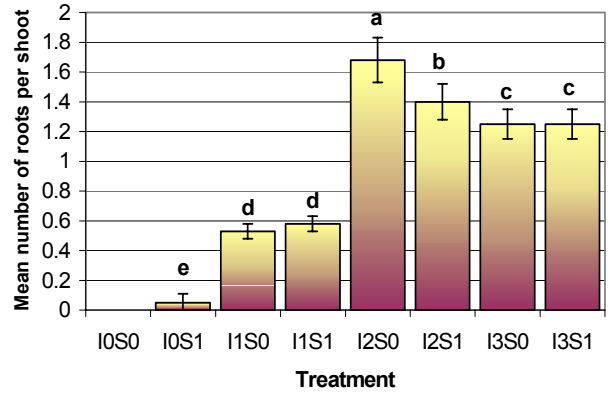



**Fig. 5. Effect of IBA pretreatment followed by culture on medium with and without vermiculite supplement on mean number of roots produced per shoot after eight weeks of culture**

I0S0=Without IBA and without vermiculite, I0S1=Without IBA and with vermiculite I1S0=0.1 mgL$^{-1}$ IBA and without vermiculite, I1S1=0.1 mgL$^{-1}$ IBA and with vermiculite, I2S0=1.0 mgL$^{-1}$ IBA and without vermiculite, I2S1=1.0 mgL$^{-1}$ IBA and with vermiculite, I3S0=2.0 mgL$^{-1}$ IBA and without vermiculite, I3S1=2.0 mgL$^{-1}$ IBA and with vermiculite




shoot tips of papaya cv. Eksotika (Fig. 1). The number of shoots produced per explant decreased on increasing the BAP concentration up to 2.0 mg L$^{-1}$. Anderson (1975) observed that higher BAP concentration (between 2.5 - 20 mg L$^{-1}$) supported a poor rate of shoot multiplication in Rhododendrons.

The qualitative and quantitative requirements for cytokinin and auxin for maximum rate of shoot multiplication depends on the endogenous levels present in the plant system, which varies with the species, explant types and the phase of plant growth. Ajithkumar and Seeni (1998), studied clonal propagation on bael (*Aegle marmelos* L.) using cytokinin for induction of bud break. The concentration of 2.5 mg L$^{-1}$ BAP was essential to induce bud break and when the cultures were sub-cultured onto the same medium, three to eleven shoots were obtained. According to Bhojwani and Razdan (1983), BAP is the most useful, reliable and also the cheapest cytokinin. The higher levels of BAP produced more number of shoots whilst arresting the growth of individual shoot. For shoot elongation, an additional step is often required using a lower cytokinin level in the medium and combined with various auxins such as NAA, IAA or $GA_3$.

In the present study, more shoots were produced within a shorter time span compared to other results reported on multiple shoot formation from field-grown shoot tips of papaya. Davies (1987) stated that cytokinin and auxin are two major hormones involved in plant development. The presence of these both in the medium affects a wide range of growth and developmental process in plant tissues such as enhanced cell division, bud development, leaf enlargement and delay in leaf senescence. Litz and Conover (1980), used 2 µm BAP and 1 µm NAA to enhance cell division on peduncles of papaya before transferring to another medium for callus induction. Drew (1988) used 1 µm BAP and 0.25 µm NAA on axillary bud explants of papaya before transfer to a medium with 2 µm BAP and 0.5 µm NAA to enhance axillary shoot growth. In this study, higher NAA concentrations of 0.1 and 0.2 mg L$^{-1}$ resulted in significant callus formation at the base of the explants. Bhojwani and Razdan (1983) stated that in some species, synthetic auxins such as NAA and IBA have been preferred for shoot multiplication, as they show a strong tendency for callus formation. According to Bonga and Aderkas (1992), generally for shoot formation that requires auxin and cytokinin, the auxin should be used sparingly to avoid callus growth.

In this study, roots were initiated from the base of shoots and pretreatment in an auxin (IBA) was obviously necessary to stimulate root initiation. Exposure to 1.0 mg L$^{-1}$ IBA for one week followed by a transfer to a medium supplemented with vermiculite stimulated 90% of the shoots to produce roots, which was the highest compared to other treatment combinations (Fig. 4). Although pretreating with 1.0 mg L$^{-1}$ IBA followed by culture on medium containing vermiculite gave the highest percentage of rooting followed by culturing on medium without vermiculite (Fig. 5). However both treatments did not show any significant difference on the root length (Fig. 6).

Rooting is a crucial and difficult stage in micro-propagation of papaya, especially to get roots with good characteristics (Wilna, 1988). The quality of roots produced plays an important role for successful transfer of plantlet to the soil during acclimatization. In this study, abnormal root growth, whereby the roots grew upward, occasionally occurred on shoots pretreated with IBA followed by culture on medium with or without vermiculite (Plate 3C & D). Drew (1988) and Reuveni *et al.* (1990) used 0.2 - 2 mg L$^{-1}$

**Fig. 6. Effect of IBA pretreatment followed by culture on medium with and without vermiculite supplement on mean root length (cm) attained after eight weeks of culture**

I0S0=Without IBA and without vermiculite, I0S1=Without IBA and with vermiculite I1S0=0.1 mgL$^{-1}$ IBA and without vermiculite, I1S1=0.1 mgL$^{-1}$ IBA and with vermiculite, I2S0=1.0 mgL$^{-1}$ IBA and without vermiculite, I2S1=1.0 mgL$^{-1}$ IBA and with vermiculite, I3S0=2.0 mgL$^{-1}$ IBA and without vermiculite, I3S1=2.0 mgL$^{-1}$ IBA and with vermiculite

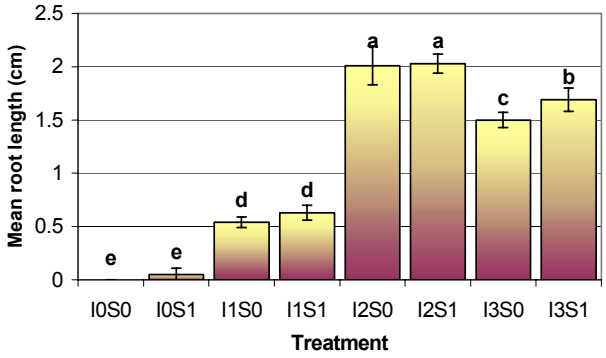
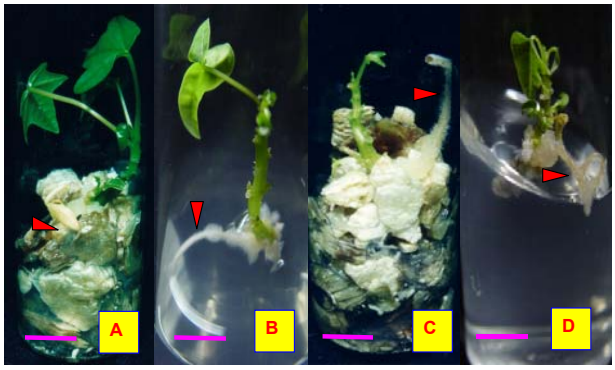



**Plate 3. Normal root growth on shoots pretreated in 1.0 mgL$^{-1}$ IBA followed by culture on medium with vermiculite (A); and without vermiculite (B); Abnormal root growth with root growing upwards on medium with vermiculite (C); and without vermiculite (D). Root formed (red arrow) Bar = 1 cm**




IBA in their rooting media for papaya but could not produce good quality roots. Plantlets with normal roots were obtained when shoots were dipped in 2.5 mg L$^{-1}$ IBA and then cultured on 0.1% agar medium without mineral nutrient. Whereas Kataoka and Inoue (1992) rooted micro-cuttings of papaya by dipping in 2500 mg L$^{-1}$ IBA and then planted them on vermiculite medium under *ex vitro* condition. Teo and Chan (1994) dipped micro-cuttings of papaya in 12.3 mm IBA followed by culture on medium consisting of distilled water plus 10 g L$^{-1}$ agar and achieved 59.5% rooting. However, some of the roots produced were thick and stumpy accompanied by callus formation at the basal end.

IBA is a synthetic auxin that has relatively higher stability than IAA (endogenous auxin) (Epstein & Ludwig-Muller, 1993; De Klerk *et al*., 1997). NAA is also a stable auxin, which stimulates callus formation, but induces fewer roots (De Klerk *et al*., 1997). IBA has greater ability to promote rooting (Hartman *et al*., 1997) and induces less callus formation (De Klerk *et al.,* 1997). In plant itself, endogenous auxin (IAA) is produced in the shoot tip and transported basipetally within the plant to stimulate root formation (Kotov, 1996). The IAA level increases considerably on first day and triggers the root formation. Nevertheless, the concentration lowers on the third day (Nordstrom *et al*., 1991) and sometimes the endogenous auxin is not enough to enhance root initiation. However, if exogenous IBA is applied it can be converted to IAA through an oxidation pathway (Epstein & Ludwig-Muller, 1993; De Klerk *et al*., 1999) to promote the rooting. In this study, the different concentrations of IBA as pretreatment acted likewise to enhance root formation on papaya shoots.

From the rooting experiment conducted in this study, pretreatment in 1.0 mg L$^{-1}$ IBA followed by culture on medium with or without vermiculite, were most suitable to trigger root initiation on shoots derived from shoot tip explants of papaya cultivar Eksotika.

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