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Research Article

EFFICIENT PROCEDURE FOR CLONAL PROPAGATION OF *CARICA PAPAYA* L. VIA SHOOT TIP EXPLANT

***Sanjeev Kumar Ambasta and Seema Kumari**
Rajendra Agricultural University, Extension department, Pusa, Samastipur, Bihar
**Author for Correspondence*

ABSTRACT

An efficient protocol for *in vitro* propagation of *Carica Papaya* L. developed in the project on experimentation. Callus Induction optimized by manipulation of growth regulators during organogenesis. Various kinds of plant growth hormones such as BAP concentrations in combination with NAA, Kinetin, IBA were preferred for use to initiate cultures. Cultural buds were preferred to shoot tips and in vitro propagation of *carica papaya* L. because of its unbranched nature. Maximum no. of surviving buds and growing cultures were recorded at M.S. medium supplemented with Kinetin, 50µm and NAA, 10µm.The best multiplication rate of shooting was observed within a month of transfer of shoots in MS medium supplemented with BAP 2.5µm concentrations .The basic proliferation medium. Strong stout rooting system was observed in ½ MS medium with IBA 10 µm and 3% sucrose. Rooted plants were usually planted to poly bags containing soil rite, vermiculite and garden soil in (1:1:1) combination .The plantlets showed 80 % survival under field condition. The objective and aim of this work is to minimize conventional techniques of asexual propagation of *carica papaya L* in grafting and root cuttings existed in the past are often tedious and impractical when carried and in large scale including producing quality plant material which is healthy at commercial level as well as Standardize a suitable micro propagation techniques using shoot tip explants.

Key Words: *Agar, Auxin, Micropropagation, Mother Plant, Papaya*

INTRODUCTION

The chymopapain contained is papaya softens tight muscles and is the reason it is the main ingredient in meat tenderizers. The fruit contains vitamin A, B and C. It is a tonic, laxative, digestive, and rejuvenative. Ripe fruit is very useful in digestive disorders and it cures all sorts of stomach trouble and liver disorder. It is thermogenic, aphrodisiac, stomachic, digestive carminative, diuretic, anti-haemorrhoidal and cardio tonic. Papaya plant has three difference sex types: male plant producing staminate flowers, female plant producing pistillate flower and hermaphrodite plant producing bisexual flower. The papaya is short-lived, fast-growing, woody large herbs to 10 or 12 feet in height. It generally branches only when injured. All parts contain latex. The yellow green or deep purple trunk is straight and cylindrical with prominent leaf scars. Its diameter may be from 2 or 3 inches to over a foot at the base. Foliage leaves emerge directly from the upper part of the stem in a spiral on nearly horizontal petioles 1 to 3-1/2 feet long. The blade deeply divided into 5 to 9 main segments. The life of a leaf is 4 to 6 months. Flowers are fleshy, waxy and slightly fragrant. Some plants bear only short-stalked female flowers or bisexual flowers on short –stalks while others may bear male flowers. Some plant may have both male and female flowers. Fruit are two types of papayas, Hawaiian and Mexican. The Hawaiian varieties are papayas commonly found in super markets. These pear shaped fruit and have yellow skin when ripe Mexican papayas are much larger than the Hawaiian types and weight upto10 pounds and more than 15 inches long. A ripened papaya is juicy, sweetish and somewhat like a cantaloupe in flavor. The fruit (and leaves) contain papain whip helps in digestion and used to tenderize the meat. The seeds are round –shaped seed have a spicy flavor somewhat reminiscent. The set back of propagating by seed is the production of non – true – to –type planting materials due to 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.

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Classification

Kingdom	-	Plantae
Subkingdom	-	Tracheobionta
Super division	-	Spermato phyta
Division	-	Magonliophyta
Class	-	Mangnolispsida
Subclasses	-	Delleniidae
Order	-	Violales
Family	-	*Caricaceae*
Genus	-	*Carica* L
Species	-	*Carica papaya* L

Though the exact area of origin is unknown, the papaya is believed native to tropical America and cultivation spread throughout south and central America, Southern Mexico up to about 1959.Successful commercial production today is primary in Hawaii, tropical Africa, the Philippines, India, Malaya, Australia, apart from the widespread the papaya is one of the leading fruits of Southern Mexico and 40% of that country's crop is produced in the state of Veracruz on 14,800 corers (6,000ha) yielding 120,000 tons annually.

Background Art of the Work/Prior History

Micro – propagation of papaya has been achieved using shoot tips and axillary buds (Rajeevan and Pandey, 1986 and Reuveni *et al.,* 1996).

Litz and conover (1978) were the first to report to procedure based on shoot tip culture for the propagation of adult papaya. Litz RE, Conover RA (1981) also reported failure to re –establish apical dominace in shoots after 12-13 subcultures.

Litz and Conover (1980), used 2micromolar BAP and 1 micromolar NAA to enchance cell division on peduncles of papaya before transferring to another medium for callus induction.

Bhojwani and Razdan (1983) stated that in some species of papaya that 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 of papaya culture that required auxin and cytokinin, the auxin should be used sparingly to avoid callus growth.

According to Epstein and Ludwig muller (1993); De klerk *et al.,* (1997) IBA is a synthetic auxin that has relatively higher stability than IAA (endogenous auxin) on adult papaya.

De klerk *et al.,* (1997) reported that NAA is also a stable auxin, which stimulates callus formation of papaya out induces fewer root.

Hartmen *et al.,* (1997) reported on *Carica Papaya* that IBA has greater ability to promote rooting and induces less callus formation (De klerk *et al.,* 1997).

According to Teo and chan (1994) were reported that micro – cutting of papaya dipped in 12.3 mm IBA followed by culture on medium consisting of distilled water plus 10g/l agar and achieved 59.5% rooting.

Drew (1988) and Reuveni *et al.,* (1990) used 0.2 –2mg/l. IBA in their rooting media for papaya but could not produce good quality roots.

Kataoka and Inoue (1992) rooted micro cutting of papaya by dipping in 2500 mg/l IBA and then planted them on vermiculite medium under exvitro condition.

Medhi, Ali Al and Lemoyne Hogan (1976) obtained proliferating plants from tissues of papaya isolated from 5-6 cm seedling on a simple medium with kinetin.

De Bruijne, Delanghe and Van Rijck (1974) were formulated for use with explants from mature papaya tissue and causes rapid callus induction from petiole segments.

The basal salts and organic mixture of Murashige and Skoog (1962) was used together with different concentration of plant growth substances in latin square design.

Papaya is normally propagated by seed. To start a plant, extract the seeds from ripe papaya and wash them to remove the gelatinous covering. They are then dried dusted with a fungicide and planted as soon

as possible. Plants the seed in warm (80^0), sterile potting mix. Seeds should be planted in sterile soil as young papaya seedlings have a high mortality rate from damping off. Gibberellic acid can be used to speed up germination in some seasons. Seedling being flowering 9-12 months after they germinate. Papaya plants can also be grown from cuttings, which should be hardened off for a few days and then propped up with the tip touching moist, fertile soil unit roots form. Vegetation propagation do not exist for large scale production, thus clonal propagation by tissue culture is much required.

MATERIALS AND METHODS

Tissue culture methods allow many desirable features to be retained. A superior parent plant can be selected as a source material without destroying it, identical progeny can be produced at high rates throughout the year.

Selecting Healthy Mother Plant

To carry out this experiment, we visited the different field of papaya of different institute like Orissa University of Agriculture and Technology (OUAT), Bhubaneswar and Directorate of Horticulture, Bhubaneswar as part of training and collected different kinds of mother plants for papaya (*carica papaya* L.) in addition to Rajendra Agricultural University, Pusa, Samastipur, Bihar.

Preparation of Explants

The explants were collected from field grown female plants of *carica papaya*. Small size *Shoot (*2-3cm in length) consisting of the top young unexpanded leaves developing on the main stem were collected. The larger leaves were trimmed off and remaining apical tissue was agitated in distilled water. During the collection explants, a special care was taken to avoid damage to the plant. The explants were directly placed in beaker containing distilled water upon brought to the laboratory. Then treated with 5% teepol (a disinfectant detergent) for about 10 to 15 minutes to minimize the flow of latex. This tissue was then rinsed in 70% ethanol for one minute and was surface sterilized in 0.1 % $Hgcl_2$ for 15 to 20 minutes followed by three rinses in sterile distilled water. The exposed surface of the tissue were then trimmed leaving only the apex (2x2 mm) which was placed into nutrients media.

Preparation of culture media

For papaya micro propagation, MS (Murasige and Skoog's) based media supplemented with various concentration of growth regulators were used. Two types of growth regulators, an auxin and a cytokinin were added to the papaya growth media. They were supplemented with sucrose as carbon source at a concentration of 2%. The pH of the media was adjusted to 5.78-5.8 with either 1 N NaOH or 1 N HCl prior to autoclaving for 20 min at 120^0c and 15psi. Agar was added to the medium after measuring the pH. The stock solution of MS media was placed in table 1.

Plant -Growth Regulators used

Plant growth regulators used for micro propagation of papaya are BAP, IAA mainly used along with MS basal salt for initiation and formation of callus. The NAA and IAA with half strength MS used for rooting of the papaya plantlets. IAA (Indole –3-acetic acid) and IBA (indole – 3 –butryic acid) NAA (1-naphthalene acetic acid) was prepared by dissolving 20 mg of their regulators at 100% alcohol first then added double distilled water and volume make to 80 ml.BAP (6-benzyl amino purine) / (kinetin) was prepared by 20 mg dissolved in 0.5 ml of 0.1 N Hcl first then added double distilled water and volume make to 80ml.

Sterilization and Preparation of the Culture Media

A sterile and clean container of was taken and was rinsed with distilled water.All the stocks is stock A,B,C.D,E, F,G,H solution and myoinositol, sucrose added to the container at a desired concentration. Make up the volume to 1 liter by adding sterile distilled water. pH of the medium adjusted to 5.8, by adding 0.1% NaOH or 0.1%HCl. After checking pH Agar-Agar (6-8 gm/l) was added to the medium and boiled. Then the media were poured into the test tubes or bottles at appropriate volume. That were tubes are rapped with non-absorbent cotton plug and packaged with polythene bags and autoclaved was made in a high speed sterilizer with 1.06 kg/cm^2 for 15 min.

Table 1: Composition of Murashige and Skoog is Medium

Stock solution and Constituents	Concentration in the stock solution	Volume to be taken/litre of medium (ml)
Stock A		10
$Cacl_2 – 2H_2O$	44gm/l	
Stock B		freshly prepared
NH_4NO_3	1.65gm/l	
KNO3	1.9 gm/l	
Stock C		
KI	8.3 mg/e	10
$Cocl_2$	2.5 mg/l	
Stock D		10
KH_2PO_4	17 gm/l	
H_3BO_3	620 mg/l	
$NA_2M_oO_4$	25 mg/l	
Stock E		10
$MgSO_4 7H_2O$	37 gm/l	
$MnSO_4. H_2O$	1.69 gm/l	
$CuSO_4. 5H_2O$	2.5 mg/l	
$ZnSO_4. 7H_2O$	860 mg/l	
Stock F		05
FeSO4. $7H_2O$	5.57 gm/l	
Na_2EDTA	7.45 gm/l	
Stock G		05
Glycine	400mg/l	
Stock H		
Nicotinic Acid	64 mg/l	
Thiamine Hcl	32 mg/l	05
Pyrridoxine Hcl	64 mg/l	
Others		freshly added
Myoinositol	100mg/l	
Sucrose	30 gm/l	
Agar	6-8 gm/l	
PH 5.8		

Establishment of Shoot Apices in Culture

After sterilization the outer leaves were removed and 2cm long shoot tips were excised. The shoot tips explants were cultured on MS (Murashige & Skoog, 1962) medium supplemented with 30 g/l sucrose, and different concentration of kinetin (0,0.1,0.02,0.05,0.08 µm) in combination with NAA (0, 0.01,0.05, 0.1 and 1.5 µm). The culture are maintained at $25^0C \pm 2^0C$ with 16h light (3500 Lux)/8h darkness and 60-70 % humidity. Callus formation was visible after 25-35 days of culture. Shoot tip which were growing very slowly during initial culture were transferred with a basal cut to fresh medium for accelerating growth.

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Multiplication of Shoots from Apices Establishment in Culture

Ms with different concentration of NAA (0.5, 5.0, 1.0 µm) and BAP (1, 2.5, 5, 10 µm.) were taken for multiple shoot production. After 40 days of culture in establishment medium, then transferred to above media combination. Maximum number of shoot within a month of transfer was obtained by BAP/NAA concentration 2.5/0.5 µm. Individual shoots were excised & planted fresh medium (NAA+BAP) at 20 days interval. The effects of various levels (1, 2, 5, 7, 8, 10 µm.) of IAA, IBA, NAA, and 2, 4-D were examined on rooting of shoots from difference subcultures.

Hardening

Hardened plantlets were carefully removed from the culture tubes and gently washed under running tap water. Before washing the plantlets were dipped in fungicide to remove any contaminants and adhering pieces of gelled medium.

Primary hardening

After dipping in fungicide, the plantlets transferred to net pot which contents mixture of garden soil, soil rite, vermiculite (1:1:1) .These net pot were transferred to poly house for 20 days.

Secondary hardening

After 20 days these were transferred to polythene bags filled with autoclave mixture of hardening mixture and were maintained in the Green house for 3-4 months under natural light with relative humidity of 90-10% and 25^{o}C temperature.

Prefield Hardening

The rooted plantlets after secondary hardening transferred to field condition.

Technical Aspect of In-Vitro Culture

Plant tissue culture is the technique which enables us to study the cells, tissue or organs by isolating them from the plant body and growing aseptically, in a suitable container, on an artificial nutrient medium, under controlled environmental condition.

Nutrient medium of carcia papaya L

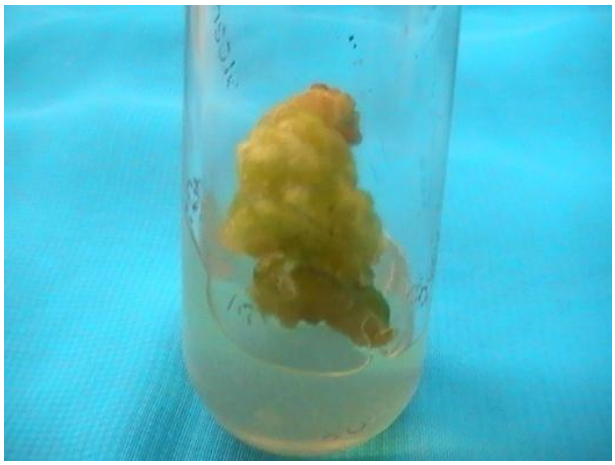

Figure 1: Vigorously growing callus in presence of BAP

Figure 2: Differentiation and shoot formation when high concentration of BAP and lower concentration of IBA

As experiment showed different types of effect of growth regulators (NAA,IBA,BAP) on growth and differences in plant tissue culture If the nutrient medium lacking a growth regulator the *carcia papaya* L pith tissue showed very poor growth. In the presence of a (BAP, Kn) a vigorously growing callus was formed (Figure 1). When transformed to in new medium containing NAA & IBA this callus differentiated and roots. If a portion of the same callus was planted on a medium with higher concentration of BAP and lower concentration (IBA) it differentiated and shoots (Figure 2) when starting work with a new system.

However most of the medium contain inorganic salts of major and minor elements, vitamins and sucrose. A medium with these integrants will be preserved to MS medium some times, growth regulates such as (Zeatin, IP) may also be added to the MS medium these all constituents are dissolved in distilled water, if necessary the medium is solidified with about 6-8 gm/l agars. The pH of the medium is adjusted around 5.8 slightly acidic. Now equal quantities of the medium are dispersed in cultured vials which are usually glass tubes or flasks the culture vials containing medium are plugged with non-absorbent cotton wrapped in cheese cloth such a closure allows the exchange of gases but does not permit the entry of micro-organisms in to the culture vials.

Aseptic conditions

The sugar content of the nutrient medium may support a luxuriant growth of many micro-organisms like bacteria and fungi, it is therefore extremes important to maintain a completely aseptic environment inside the culture vials, micro-organism can contaminate the medium is at least 3 ways. The micro-organisms present is the medium right from the beginning may be destroyed by sterilizing the properly plugged culture vials. It can be done by maintaining the temperature at 120ºC for about 15 minutes. The micro-organism may also be carried along with tissue that is being cultured, to prevent this, the plant material from which the tissue is to be excised is surface sterilized. The material may be surface sterilized with saturated chlorine water and then thoroughly washed with sterilized distilled water and be remove all traces of chlorine. Finally, precautions must also be taken the prevent the entry of micro-organism and while the plug of a culture vial is removed to transfer the tissue to the nutrient medium (inculcation). For this all operation from surface sterilization of the tissue up to inoculation are done in an aseptic environment. A culture made proper for the occasion (every tissue and organ has its special requirement for optimal growth) provides suitable medium for the organs with intact meristems to grow in organized fashion. A shoot segment with an intact apex may develop into a full plant with adventitious roots.

RESULTS

Tissue culture technique could offer valuable alternative and reliable procedure for mass propagation of homogenous and uniform plants for both commercial and research purposes.

Figure 3: Mother plant of *carica papaya* L.

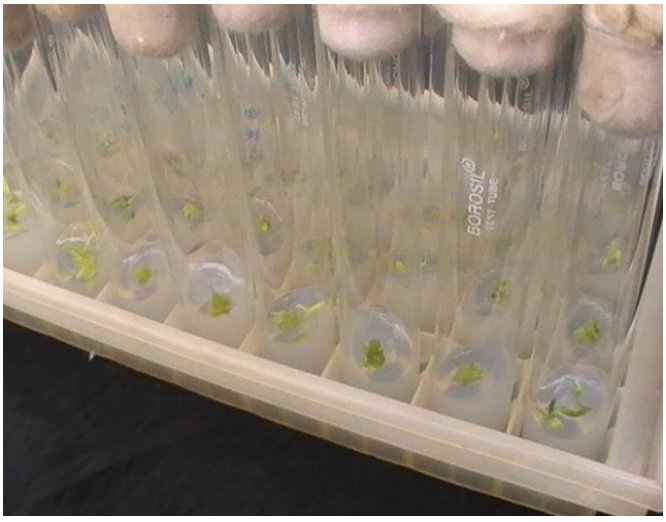

Figure 4: Initial culture after 07 days of initiation

Initiation of Shoot Tip Culture

The shoot tips are on initial Media which contain MS with NAA, Kinetin, BAP, About 20 explants were taken. After the incubation in the controlled Conditions of temperature and Humidity, it was observed that growth visible after 8 days of culture, but apical part were considered to be established only when the new

growth spread approximately to 2 cm diameter within 30-40 days of culture. Apical part which were growing very slowly during initial culture were transferred with a fresh medium (Kinetin/ Naphthalene acetic acid) of 50/10 µm for accelerating growth.

Table 2: Effect of NAA and kinetin on the establishment of shoot tip after 3 week of culture

Growth regulator (µm)		Shoot tips	
Naphthalene acetic acid	Kinetin	Survived (%)	Growing (%)
0.5	25	20	0
	50	30	20
	100	10	0
5.0	25	20	0
	50	40	20
	100	10	0
10	25	20	0
	50	60	40
	100	40	20

Shoot tip Survived grow better at kinetin 50 µm in combination with all levels of NAA. The survival rate of tip at kinetin 50 µm increased with increasing levels of NAA. Maximum number of surviving shoot tips (60%) and growing culture (40%) were recorded at Kin/NAA concentration of 50/10 µm. New growth was visible after 2 weeks of culture but the shoot tip were considered to be established only when the new growth to 2 cm diameter within 30-40 days of culture.

Multiplication of Shoots from Tips Established in Culture

Standardization of basis proliferation medium (BPM)

Naphthalene acetic (NAA) (0, 0.1 and 0.5 µm) and BAP (1, 2.5 and 5µm) were tried for multiple shoot production from shoots tips growing in establishment medium. After 40 days of culture in establishment medium, the shoots tips were transferred to above media combination.

Figure 5: Well developed multiple shoots (Maximum no. of shoots was observed in combination of BAP (2µM) and NAA (0.5 µM) along with MS media)

Table 3: Effect of cytokinin in the presence of 0.5 µm naphthalene acetic acid on the multiplication and growth of shoot of shoot tip origin. Measurements after 3 weeks of culture.

Cytokinin	Level(µm)	Character Number of shoot	Length of shoot(cm)	Length of the longest leaf(cm)
BAP	2	13.2	0.6	1.0
	4	12.2	0.5	0.7
	8	9.2	0.4	0.7
KIN	2	8.2	0.9	1.6
	4	5.6	3.8	1.3
	8	4.8	0.6	0.9
ZEA	2	4.4	0.9	1.5
	4	5.6	1.1	1.9
	8	4.6	0.8	1.3

Maximum number of shoot i.e. 13.2 was observed in BAP 2µm & NAA µm along with MS.

Maximum number of shoots (5 shoots/shoot tips) within a month of transfer was obtained by BAP/NAA concentration of 2.5/0.5 µm. Individual shoots were excised from the proliferating culture and planted in fresh basis proliferation medium at 20 interval. The shoot cultures have maintained by sub-culturing in BAP. However, little bacterial contamination was noticed, apparently with no detriment effect on growth with progressive subcultures

Regulation of shoot proliferation and growth

Difference levels of 6- Benzyl Amino purine (BAP), kinetin (kn) (2, 4 and 8µm) at NAA (0.5µm) were examined for their effect on the proliferation and growth of shoots. Shoots (5mm in length) used in this study were obtained from multiple culture in BPM at the end of first subculture.BAP at all levels induced higher proliferating rate other than cytokinines, Maximum number of shoots (13.2) was recorded at BAP (2µm) and NAA (0.5µm).Shoot became smaller and compact with increasing levels of BAP, produced higher proliferation rates whereas other cytokinines exerted favorable effects on shoot growth desirable for easy and rapid handling during sub- culturing and rooting.

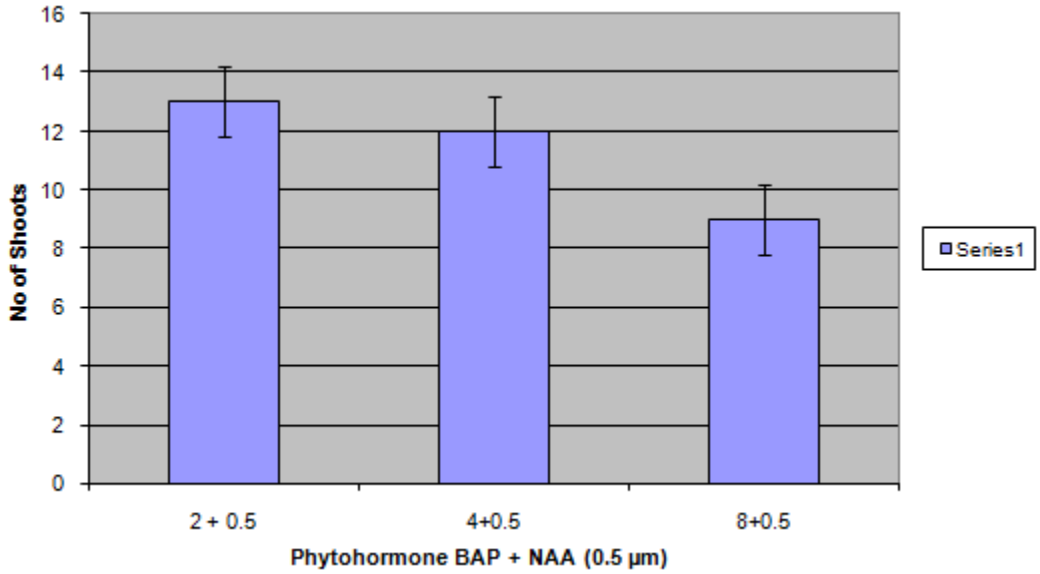

Figure 6: Effect of different concentration of BAP in the presence of 0.5µm NAA on the multiplication of shoot with reference table-3.

Rooting

The effects of various levels of IAA, NAA, IBA were examined on rooting of shoots from different – Subcultures. IAA, NAA did not induce rooting and only IBA produced roots on shoots. Maximum number of shoots (90%) rooted with IBA (10µm) (Table4). Primary roots ranged from 9 to 12 in number and root system developed normally with secondary and tertiary branching.

Table 4: Effect of different concentration of Indole 3- butryic acid (IBA) along with half Ms medium on root induction

Basal media	Phytohormone IBA (µm)	Percentage of shoot producing root	Time taken in root induction (days)	Root length +SE(CM)
½ MS	1	47	22	2.11 ± 0.22
	2	56	20	2.32 ± 0.26
	5	65	19	2.5 ± 0.23
	7	73	18	2.63 ± 0.20
	8	80	16	2.78 ± 0.18
	10	90	14	3.52 ± 0.11

In IBA (10mg/l) along with half MS, 90% shoot produce root in less time i.e. days and highest root length 3.52. SE = Standard error of mean.

The rooted plant lets were taken from the rooting medium washed wells with distilled water then put into plastic pots with gardens soil, soil rite and vermiculite (1:1:1) on ratio for 3-4 weeks in green house. After acclimatization the plantlets were transferred to field.

DISCUSSION

The production of shoots from shoot tip explants is most applicable and reliable method of *in vitro* propagation of papaya combination of Naphthalene acetic acid, kinetic and 6-Benzyl amino purine was used which affect the callus formation and proliferation. Different concentration of NAA and kinetin tested affected the establishment of shoot tip after 3 week at culture. Maximum number of surviving shoot tips (60%) and growing culture (40%) were recorded at kn/NAA concentration of 50/10 µm (Table 2). Different concentration and combination of BAP, KIN, ZEA and NAA (0.5 µm) were tested effect shoot induction and proliferation shoots(5mm in length) were obtained from multiple culture in BPM at the end of firm culture (Figure 5). Maximum number of shoots (13.2) was recorded at BAP (2µm) & NAA 0.5µm) (Table 3 and Figure 6), shoots tip became smaller and compact with increasing levels of BAP, ZEA. The presence of BAP and NAA in the medium promoted and enhances the number of shoots produce from the field grown explants. BAP breaks the apical dominance and caused shoot induction. The qualitative and quantitative requirements for cytokinin and auxin for maximum rate of shoots multiplication depends on the endogenous levels present in plant system, which various with species to species explants types and phase of growth. The concentration of 2 µm BAP was essential to include bud break and when the cultures were sub- cultured on to the same medium, four to thirteen shoots were obtained (Table 3).Rooting from shoots was obtained by different concentration of IBA along with MS media. In IBA (10µm) along with half MS media,, 90%. Shoot producing roots in less time that is 14 days and highest root length (3.52) (table 4). Exposure to 10 µm IBA for one week followed by a transfer to medium supplemented with vermiculite stimulated 90% of the shoot to produce roots, which was the highest – compared to other treatment combination. Rooting is a crucial and difficult stage in micro propagation of papaya especially to gets with good characteristics. The quality of roots produced plays an important role for the successful transfer of plantlets to soil during acclimatization. In this study abnormal and normal root occur, where by the roots grew upward, occasionally occurred on shoots pretreated with IBA followed by culture on medium with or without vermiculture. The rooted plantlets ware taken from

the rooting medium washed with distilled water then potted into the plastic pots with combination of garden soil, soil rite, vermiculite for 3-4 weeks in green house. After acclimation the plantlets were transferred to field.

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