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MICROPROPAGATION OF MEDICINALLY IMPORTANT WEED, *Acorus calamus*, RHIZOME EXPLANTS WITH INDIGENOUS NATURAL GROWTH REGULATORS

Bilal Muhammad Khan[1*], Jehan Bakht[2], Safdar Hussain Shah[2] and Mohammad Shafi[3]

ABSTRACT

A novel, cost-effective and indigenous micro-propagation protocol for the endangered A. calamus was successfully developed. Tang II (Natural Growth Substances) in a concentration of 20 ml L^{-1} was found as the best growth regulator for both shooting and rooting of the explants. It required 3.38 days to bud-break, showed 86.66% bud-break, 1.26 shoots per explant and produced the lengthiest shoots (2.15 cm) in rhizome explants at the end of first week after inoculation. Furthermore, Tang II (H) which required 3.92 days to bud-break stood out as the best growth regulator even at the end of fourth week after inoculation. It also revealed the highest bud-break of 93.33%, 1.25 mean number of shoots per explant and the lengthiest shoots (11.33 cm on the average). Similarly, it required 10.94 mean number of days for root initiation, initiated roots in 66.66% of explants, recorded 3.05 average number of roots per explant and 3.75 cm average root length. Moreover, the survival rate of micro-propagated plantlets, which were transferred to soil to be grown under natural conditions was 83.00%.

Key words: *Acorus calamus,* rhizome explant, micro-propagation, tissue culture.

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INTRODUCTION

Acorus calamus, generally known as Sweet Flag, is a member of the family Araceae (Adoraceae). It is also known as *Acorus odoratus* and *Calamus aromaticus.* The plants included in this family are rhizomatous or tuberous herbs. In Pakistan, it is almost confined to

[1]Dept. of Biotechnology, Abdul Wali Khan University, Mardan Pakistan
[2]Institute of Biotechnology and Genetic Engineering, [3]Department of Agronomy, The University of Agriculture, Peshawar, Pakistan
*Corresponding author's email: ranezai@yahoo.com

District Swat in Khyber Pakhtunkhwa Province where it is found as a weed on the banks of streams and in marshy places. The local people identify the plant by the name of "Skha Waja". Several important biological activities such as antifungal (Lee *et al*., 2004; Lee, 2007), antibacterial (McGraw *et al*., 2002; Phongpaichit *et al*., 2005), allopathic (Nawamaki and Kuroyanagi; 1996), anticellular and immunosuppressive (Mehrotra *et al*., 2003) have been attributed to the rhizomes, roots and essential oil extracted from these plant parts. It can also be used as an insecticide as its essential oil has been reported to possess antigonadal activity in insects (Koul *et al*., 1977 a, b; Saxena *et al*., 1977; Schmidt and Brochers, 1981; Mathur and Saxena, 1995).

Overwhelming increase in human population, growing trend of urbanization and overexploitation of natural resources has resulted in rapid disappearance of natural flora in certain areas (Alam and Ali, 2009). According to a report, over 580 flowering plants in Pakistan carry a 'threatened' or endangered status (Nasir, 1991); the number climbs up to 709 plants according to another study (Chaudhri and Qureshi, 1991). A number of species have gone extinct in the Hindukush-Himalayan regions because of over-collection (Shinwari, 2010). Hence, the major contribution towards making extinct some of the flora of Pakistan and bringing others to the brink of extinction comes from the practices of local collectors, vendors, herbal drug dealers and others. Resultantly, there is a need to devise ways for sustainable harvesting of medicinal plants from the wild, train local collectors in suitable collection techniques, train the people in growing medicinal plants, do away with some of the middlemen in the trading chain (Shinwari, 2010), and to encourage research activities in the development of *in vitro* propagation techniques for the conservation of endangered species.

Micro-propagation or clonal propagation is a technique which can be employed to deal with the situation of rapid disappearance of many important plant species. The traditional methods of asexual propagation lost ground to this technique due to the following reasons: (a) millions of genetically alike plants can be produced per year from a very small portion of plant tissue, (b) desired resistance can be produced in micro-propagated plants, (c) rapid proliferation can be achieved as the technique is season independent, and (d) valuable germplasm can be stored over long periods of time by employing this technique.

A. calamus is a reported endangered species in the flora of Pakistan (Hamayun *et al*., 2006) and it carries the same 'endangered' status in other regions of the world too (FRLHT, 2000'; Dušek *et al*., 2007; Rajasekharan *et al*., 2010; Bhagat, 2011; Verma and Singh,

2012; Mazher and Sharma, 2012; Kareem *et al*., 2012). Micropropagation through tissue culture is arguably most suited in such instance to produce disease-free plants on a large scale to overcome the demands of pharmaceutical industry and as a tool for the conservation of the plant itself. This technique, in fact, has been employed using different parts of *A. calamus* as explant (Verma and Singh, 2012; Ahmed *et al*., 2010; Bhagat, 2011; Sandhyarani *et al*., 2011; Devi *et al*., 2012; Anu *et al*., 2001; Lee and Han, 2011; Ahmed *et al*., 2007). The above mentioned studies have reported positive response of *A. calamus* explants to micropropagation.

All the previous studies, however, were conducted using commercially available synthetic growth regulators. These often pose the problems of unavailability and prove uneconomical, particularly in developing countries like Pakistan which completely rely on the import of these products as these are not manufactured domestically. Keeping this in mind, we used indigenous natural growth regulators as substitute for their commercially available counterparts in our experiment. These growth regulators were extracted from local plants by Prof. Dr. Safdar Hussain Shah (Director, Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar-Pakistan) and were generously provided to us for conducting our experiment. A striking feature of these growth regulators is their ability to initiate both shoot formation and root formation. This makes them even more cost-effective in comparison with the commercially available growth regulators. Additionally, this helps minimize the problem of contamination associated with the transfer of shoots to rooting medium.

MATERIALS AND METHODS

Plant material

The present study was conducted at the 'Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan'. Fresh plant material was collected from different localities of District Swat, Khyber Pakhtunkhwa and were grown in green house at the same institute. Disease and pest free healthy plant material which showed better biomass yield was used in this part of our research work.

Rhizome Explants

Fresh rhizome from healthy and disease free plants were selected as explant. The scales were removed and the rhizome was cut into small portions ranging in size from 1 – 1.5 cm through sterilized surgical blade and was then carefully transferred to the culture flask.

Explants Sterilization

In order to surface sterilize the explants, these were washed thoroughly in running tap water to remove traces of dirt and then kept in liquid detergent (0.2% Tween 20) for 5 minutes. The explants were then again washed in running tap water to remove any traces of the detergent and were subsequently rinsed with distilled water (3 to 5 times). The explants were then dipped in 0.3% Fluconazole solution (fungicide) for 5 minutes and were then rinsed 3 to 5 times with distilled water followed by treatment with 0.1% mercuric chloride solution for 6 minutes in a Laminar Flow Unit (LFU). Finally, the explants were rinsed 5 to 8 times with autoclaved distilled water.

Culture Media

MS medium reported by Murashige and Skoog (1962) with 3% sucrose, 0.8% agar, 100 mgL^{-1} inositol and growth hormones was used as the basal medium for the inoculation of explants.

Stock Solutions

Macronutrients, micronutrients, iron source and vitamins were prepared in stock solutions of one liter each.

Growth Hormones

We used indigenous natural growth regulators as substitute for their commercially available counterparts in our experiment. These growth regulators were extracted from local plants by Prof. Dr. Safdar Hussain Shah (Director, Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar-Pakistan) and were generously provided to us for conducting our experiment. These growth regulators included Tang, Tang II and Islam, each of which was used in two concentrations (10 $ml.L^{-1}$ and 20 $ml.L^{-1}$). Additionally, MS medium devoid of any growth regulator was used as negative control and BAP (2 $mg.L^{-1}$) as reference growth regulator in our experiment.

Explant Inoculation

The surface used was sterilized by switching on the UV lamp of LFU at least 6 hours before explant inoculation. It was subsequently cleaned with 70% ethanol. The explants of suitable size were inoculated, under aseptic conditions, in test tubes containing MS medium augmented with different concentrations of growth hormones used in the study. The mouth of test tubes was then flamed, covered with aluminum foil and tightly sealed with Para-film to exclude entry of external air. The test tubes were then properly labeled before transferring to growth room.

Shoot Proliferation and Rooting of Micro-shoots

There was no distinction of separate shooting and rooting media in our experiment. This was due to the coupled shooting and rooting ability of growth hormones used in the study.

Culture conditions

The incubation temperature of the growth room was 25±3°C having a photoperiod of 12 hours and 2000-2500 lux light intensity.

Acclimatization of Micro-Propagated Plants

Well rooted plants were carefully taken out from the test tubes and rinsed with warm water to remove traces of agar. These were subsequently planted in pots kept in growth room having a mixture of sterilized soil and sand in a ratio of 3:1. Polythene bags with small holes were used to cover the pots for maintaining highly humid conditions. On alternate days the plantlets were irrigated with MS half strength salt solution. After two weeks the pots were taken out of the growth room and the plantlets were exposed to natural conditions for 3 to 4 hours daily by removing the polythene bags. After 20 days, these were transferred to a glass house where the pots were kept for another 20 days. The plants were finally transferred to soil to be grown under natural conditions.

Data Collection

The data were taken weekly for the following parameters for four complete weeks:

a) Number of days to bud-break
b) Percent bud-break
c) Number of shoots per explant
d) Shoot length (cm)
e) Number of days to root initiation
f) Percent response
g) Number of roots per explants
h) Root length (cm)

RESULTS AND DISCUSSION

The indigenous growth regulators used in the present study were Tang, Tang II and Islam which were used in concentrations of 10 $ml.L^{-1}$ (L) and 20 $ml.L^{-1}$ (H). Four parameters (days to bud-break, % bud-break, number of shoots per explant, and shoot length) were considered for shooting data and another four (days to root initiation, % response, number of roots per explant, and root length) for rooting data in our experiment. Data was taken at the end of each week for complete four weeks after inoculation. The results here are reported week-wise for the above mentioned parameters. Complete data for shoot induction in rhizome explants is given in Table-1 and that for root initiation is shown in Table-2.

One Week after Inoculation

The explants were observed for shoot induction and root initiation, and the data and pictures were taken at the end of first week after inoculation.

i) Shooting

The least days were required by explants on MS media devoid of any growth regulator (Negative Control) with mean requirement of 3.00 days to bud-break. It was followed by Tang (L), Tang II (H), Islam (H), Tang (H), Tang II (L) and Islam (L) with mean requirement of 3.17, 3.38, 3.68, 3.70, 3.81 and 4.41 days, respectively, to bud-break. BAP (2 $mg.L^{-1}$), taken as reference control in the study, showed the maximum mean requirement of 4.75 days to bud-break.

The best result at the end of first week after inoculation was recorded for Tang II (H) with 86.66% bud-break, which was closely followed by Islam (H) which measured 83.33% bud-break. These were followed by Islam (L), Tang II (L), Tang (H), Tang (L) and BAP (2 $mg.L^{-1}$), which measured 80.00%, 73.33%, 66.66%, 56.66% and 53.33% bud-break. MS media without any growth regulator with 13.33% bud-break represented the least % bud-break at the end of first week after inoculation.

Tang II (L) gave the most number of shoots among the tested growth regulators with 1.27 mean number of shoots per explant germinated. It was followed by Tang II (H), Islam (H) and Tang (H) with 1.26, 1.24 and 1.10 mean number of shoots per explant at the end of first week after inoculation. Negative control, BAP (2 $mg.L^{-1}$) and Tang (L) showed the least number of shoots with each having only 1.00 shoot per explant.

As expected, MS media devoid of any growth regulators recorded the lowest reading of 0.95 cm mean shoot length. Tang II (H), on the other hand produced the lengthiest shoots with mean shoot length of 2.15 cm. It was followed by Islam (L), Islam (H), Tang (H), Tang II (L), Tang (L) and BAP (2 $mg.L^{-1}$) which recorded 1.92 cm, 1.83 cm, 1.70 cm, 1.45 cm, 1.10 cm and 1.09 cm mean shoot length respectively.

ii) Rooting

Islam (L) was the only growth regulator that initiated root formation in the first week of inoculation requiring 5.00 days for this purpose. The percent response of this growth regulator was calculated to be 3.33% at the end of first week after inoculation. Furthermore, this growth regulator produced 2.00 roots per explant responded and the average root length was recorded to be 0.1 cm.

Two Weeks after Inoculation

Shoot induction and root initiation was observed in rhizome explants and the data and pictures were taken at the end of second week after inoculation.

i) Shooting

MS media augmented with Tang II (H) showed mean requirement of 3.92 days which turned out to be the least number of

days required for bud-break. BAP (2 mg.L^{-1}), on the other hand, with a mean requirement of 5.75 days was the growth regulator with maximum number of days required for bud-break. It was followed by Tang (L), Islam (L), Tang II (L), Tang (H), Islam (H) and Negative control with 5.40, 5.21, 4.65, 4.22, 4.14 and 4.00 days required for bud-break respectively.

MS medium without any growth hormone showed the least percent bud-break of 16.66%. Islam (L) and Tang II (H), on the other hand, gave the highest bud-break of 93.33% each. These were followed by Islam (H), Tang II (L), Tang (L), Tang (H) and BAP (2 mg.L^{-1}) with 90.00%, 86.66%, 80.00%, 73.33% and 66.66% bud-break respectively.

Tang II (L) gave the best result with a mean of 1.53 shoots per explant. It was followed by Tang II (H) and Islam (H) which recorded 1.25 mean number of shoots per explant each, two weeks after inoculation. These in turn were followed by BAP (2 mg.L^{-1}), Tang (H), Tang (L) and Islam (L) with 1.15, 1.13, 1.08 and 1.07 mean number of shoots per explant respectively. Negative control with a single shoot per explant revealed the least number of shoots per explant.

Tang (H) which measured an average shoot length of 5.79 cm stood out as the hormone producing the lengthiest shoots. It was followed by Islam (H), Islam (L), Tang II (H), Tang II (L), BAP (2 mg.L^{-1}) and Tang (L) which recorded average shoot length of 5.47 cm, 5.31 cm, 4.90 cm, 4.04 cm, 2.68 cm and 2.15 cm respectively. The least lengthy shoots were observed in negative control with mean shoot length of 1.65 cm.

ii) Rooting

The data recorded at the end of second week after inoculation suggested that Islam (H) was quicker in initiating root formation requiring 9.66 days. It was followed by Islam (L), Tang II (L), Tang II (H) and BAP (2 mg.L^{-1}) with mean requirement of 9.75, 10.00, 10.00 and 10.66 days respectively for root initiation. With a mean requirement of 11.87 days Tang (H) turned out to be the slowest in initiating root formation.

Negative Control and Tang (L) failed to initiate rooting according to the results noted at the end of second week after inoculation. Tang II (H) produced the best result with 52.50% response while BAP (2 mg.L^{-1}) revealed the least response of 10.00%. Other growth regulators in descending order on the scale of percent response were Islam (L), Islam (H), Tang (H) and Tang II (L) which recorded 42.50%, 42.50%, 29.17% and 25.83% response respectively.

BAP (2 mg.L^{-1}) with a single root per explant responded was the growth regulator which produced the minimum number of roots

per explant. MS media augmented with Tang II (H), on the other hand, revealed the maximum number of roots (2.80) per explant responded. It was followed by Islam (H), Islam (L), Tang II (L) and Tang (H) which showed 2.66, 2.61, 2.00 and 1.62 mean number of roots per explant.

The lengthiest roots (1.21 cm on the average) were produced by explants inoculated on media supplemented with Tang II (H). It was followed by Islam (L), Islam (H), Tang (H) and Tang II (L) with 1.19 cm, 1.10 cm, 1.01 cm and 0.80 cm average root length respectively two weeks after inoculation. On the contrary, the least lengthy roots (0.26 cm) were observed in explants grown on media with BAP (2 $mg.L^{-1}$).

Three Weeks after Inoculation

The induction and initiation of shoots and roots was noted in rhizome explants and the data and pictures were taken at the end of third week after inoculation.

i) Shooting

Media with Tang II (H) required the least number of days for bud-break (3.92 days). It was followed by Negative Control, Tang (H), Islam (H), Tang II (L), Islam (L) and BAP (2 $mg.L^{-1}$) with an average requirement of 4.00, 4.22, 4.60, 4.65, 5.21 and 5.75 days respectively for bud-break. Media supplemented with Tang (L) required 5.84 days which was the most shown by any MS media for bud-break.

MS media devoid of any growth regulator showed the least percent bud-break of 16.66%, three weeks after inoculation. Islam (L), Tang II (H) and Islam (H), on the other hand, provided the highest percent bud-break of 93.33% each. These were followed by Tang II (L), Tang (L), Tang (H) and BAP (2 $mg.L^{-1}$) which recorded 86.66%, 83.33%, 73.33% and 66.66% bud-break respectively.

Tang II (L) revealed the most number of shoots (1.61) per explant germinated. It was followed by Islam (H), Tang II (H), Islam (L), BAP (2 $mg.L^{-1}$), Tang (H) and Tang (L) with an average of 1.35, 1.25, 1.17, 1.15, 1.13 and 1.08 shoots per explant respectively. Negative Control fell behind having a single shoot per explant.

Tang (H) with an average shoot length of 10.01 cm recorded the lengthiest shoots. It was followed by Tang II (H), Islam (H), Islam (L), Tang II (L), Tang (L) and BAP (2 $mg.L^{-1}$) which recorded 8.55 cm, 8.11 cm, 7.76 cm, 7.67 cm, 6.02 cm and 5.91 cm mean shoot length respectively. MS media without any growth hormone with an average shoot length of 3.12 cm revealed the shortest shoots in length.

ii) Rooting

The most days (13.36 days on the average) were taken by Tang (H) for initiating root formation in rhizome explants. The least requirement of days for this purpose (10.57 days on the average),

however, was demonstrated by explants inoculated on media with Islam (H). It was followed by Tang II (H), Islam (L), BAP (2 mgL^{-1}) and Tang II (L) with mean requirement of 10.94, 11.43, 12.50 and 13.33 days respectively for root initiation in rhizome explants of *A. calamus*.

Negative Control and Tang (L) once again did not produce any roots in the rhizome explants. MS media containing Tang II (H), on the other hand, initiated roots in 66.66% explants at the end of third week after inoculation which was the best result in regard to this parameter. It was followed by Islam (L), Islam (H), Tang (H) and Tang II (L) which recorded 58.33%, 56.66%, 46.66% and 45.00% response respectively at the end of third week after inoculation. With a percent response of 13.33%, BAP (2 $mg.L^{-1}$) was the growth regulator which initiated root formation in the least number of explants inoculated.

With 2.88 mean number of roots per explant responded, Tang II (H) turned out to be the growth regulator which produced the most number of roots per explant. It was followed by Islam (L), Islam (H), Tang II (L) and Tang (H) with 2.82, 2.78, 2.50 and 2.18 mean number of roots respectively per explant responded. BAP (2 $mg.L^{-1}$), on the other hand, produced only a single root per explant.

BAP (2 $mg.L^{-1}$), with an average root length of 0.32 cm, turned out to be the growth hormone which produced the shortest roots. On the contrary, Tang II (H) produced the lengthiest roots with an average root length of 2.30 cm. It was followed by Islam (H), Islam (L), Tang (H) and Tang II (L) which revealed an average root length of 2.11 cm, 1.83 cm, 1.83cm and 1.41 cm respectively.

Four Weeks after Inoculation

At the end of fourth week after inoculation, the induction and initiation of shoots and roots was measured in rhizome explants and the data and pictures were taken (Fig. 1).

i) Shooting

Tang II (H) which required 3.92 days to bud-break stood out as the best growth regulator in regard to this parameter. It was closely followed by Negative Control with a mean requirement of 4.00 days to bud-break. This in turn was followed by Islam (H), Tang II (L), Tang (H), Islam (L) and BAP (2 $mg.L^{-1}$) which required 4.60, 4.65, 5.08, 5.21 and 5.75 days respectively for bud-break. With a requirement of 5.84 days, however, Tang (L) turned out to be the slowest among the tested growth regulators in initiating bud-break.

MS media devoid of any growth regulator, with a percent bud-break of 16.66%, was the media which initiated bud-break in the least number of explants inoculated. Islam (L), Tang II (H) and Islam (H), on the contrary, revealed the highest bud-break of 93.33% each. These were followed by Tang II (L), Tang (L), Tang (H) and BAP (2

mg.L^{-1}) with percent bud-break of 86.66%, 83.33%, 76.66% and 66.66% respectively.

The most number of shoots per explant (1.65) was recorded for MS media with Tang II (L). It was followed by Islam (H), Tang II (H), Islam (L), Tang (H), BAP (2 mg.L^{-1}) and Tang (L) which revealed 1.39, 1.25, 1.17, 1.17, 1.15 and 1.12 mean number of shoots respectively per explant germinated. Negative Control, on the other hand, produced the least number of shoots per explant (a single shoot).

MS media without any growth regulator produced the shortest shoots measuring 4.10 cm on the average. The lengthiest shoots, on the other hand, were recorded for Tang II (H) with an average shoot length of 11.33 cm. It was followed by Tang (H), Tang II (L), Islam (H), Islam (L), Tang (L) and BAP (2 mg.L^{-1}) which measured an average shoot length of 10.50 cm, 9.52 cm, 8.63 cm, 8.59 cm, 7.21 cm and 5.93 cm respectively.

ii) Rootinga

MS media devoid of any growth regulator failed to initiate root formation even at the end of fourth week after inoculation. Islam (H), with a mean requirement of 10.57 days, was the quickest among the tested growth regulators in initiating root formation. It was followed by Tang II (H), Islam (L), BAP (2 mg.L^{-1}), Tang (H) and Tang II (L) with mean requirement of 10.94, 12.11, 13.33, 13.36 and 14.92 days respectively for root initiation. Tang (L), on the other hand, was the slowest in root initiation with an average requirement of 21.00 days for this purpose.

BAP (2 mg.L^{-1}) initiated root formation in only 13.33% explants and hence was the growth regulator which produced roots in the least number of explants inoculated. Islam (L) and Tang II (H), on the contrary, initiated root formation in the majority of explants inoculated where each of these showed a percent response of 66.66%. These were followed by Tang II (L) and Islam (H) with a percent response of 56.66% each. These in turn were followed by Tang (H) which revealed a percent response of 46.66%, and Tang (L) with a percent response of 16.66%, four weeks after inoculation.

BAP (2 mg.L^{-1}) and Tang (L) produced the lowest number of roots per explant responded, i.e., a single root each. Islam (L), on the other hand, recorded the highest number of roots (3.11) per explant responded. It was followed by Tang II (H), Islam (H), Tang II (L) and Tang (H) with an average of 3.05, 2.78, 2.50 and 2.36 roots respectively per explant responded.

The data recorded at the end of fourth week after inoculation revealed that Islam (H) produced the lengthiest roots with an average root length of 3.80 cm (Fig. 32). It was followed by Tang II (H), Tang (H), Islam (L), Tang II (L) and Tang (L) which recorded an average root

length of 3.75 cm, 3.23 cm, 3.11 cm, 2.55 cm and 1.40 cm respectively. The shortest roots (0.41 cm on the average) were produced by BAP (2 mg L^{-1}).

Acclimatization of Micro-propagated Plants

The micro-propagated plantlets which were transferred to soil to be grown under natural conditions (Fig. 2) showed a survival rate of 83.00%.

Commercially available synthetic growth regulators, though, were used in all the previous studies which frequently pose the glitches of inaccessibility and prove uneconomical. These problems associated with commercial growth hormones are more evident in developing countries like Pakistan which entirely rely on the import of these products as these are not manufactured locally. It is for this reason that we used indigenous natural growth regulators in place of synthetic hormones in our study. These growth regulators were extracted from indigenous plants by Prof. Dr. Safdar Hussain Shah (Director, Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar-Pakistan) who munificently provided these to us for use in our experiment. Their ability to initiate both shoot and root formation makes these distinct from their commercially available counterparts, and in turn makes these even more cost-effective. Furthermore, this aids in minimizing the problem of contamination linked with the transfer of shoots to rooting medium.

Three different growth regulators (Tang, Tang II, and Islam) were used in our experiment, each at two concentrations, i.e. 10 ml L^{-1} (L) and 20 ml L^{-1} (H). Moreover, MS media devoid of any growth regulator was used as negative control and media augmented with BAP (2 mg.L^{-1}) as reference media for determining the potential of these growth hormones against the synthetic ones. Four parameters (days to bud-break, % bud-break, number of shoots per explant, and shoot length) were considered for shooting data and another four (days to root initiation, % response, number of roots per explant, and root length) for rooting data in our experiment. Data was taken at the end of each week for complete four weeks after inoculation.

The explants were observed for shoot induction at the end of first week after inoculation which revealed that the least days for bud-break were required by explants on MS media devoid of any growth regulator. It was followed by Tang (L), Tang II (H), Islam (H), Tang (H), Tang II (L) and Islam (L). BAP (2 mg.L^{-1}) showed the maximum mean requirement of days to bud-break. The data taken at the end of second week after inoculation revealed that MS media augmented with Tang II (H) showed mean requirement of the least number of days for bud-break. It was followed by Negative control, Islam (H), Tang (H), Tang II (L), Islam (L), and Tang (L). BAP (2 mg.L^{-1}), on the other

hand, was the growth regulator with maximum number of days required for bud-break. At the end of third week after inoculation, MS media with Tang II (H) required the least number of days for bud-break. It was followed by Negative Control, Tang (H), Islam (H), Tang II (L), Islam (L) and BAP (2 $mg.L^{-1}$). Media supplemented with Tang (L) required the most number of days for bud-break. Tang II (H) required the least number of days for bud-break according to the data taken at the end of fourth week after inoculation. It was closely followed by Negative Control which in turn was followed by Islam (H), Tang II (L), Tang (H), Islam (L) and BAP (2 $mg.L^{-1}$). Tang (L) turned out to be the slowest among the tested growth regulators in initiating bud-break.

The best result, at the end of first week after inoculation, in terms of percent bud-break was recorded for Tang II (H) which was closely followed by Islam (H). These were followed by Islam (L), Tang II (L), Tang (H), Tang (L) and BAP (2 $mg.L^{-1}$). MS media without any growth regulator represented the least percent bud-break at the end of first week after inoculation which justifies why this media required the least number of days for bud-break. After two weeks of inoculation, MS medium without any growth hormone showed the least percent bud-break. Islam (L) and Tang II (H), on the other hand, gave the highest bud-break. These were followed by Islam (H), Tang II (L), Tang (L), Tang (H) and BAP (2 $mg.L^{-1}$). MS media devoid of any growth regulator showed the least percent bud-break, three weeks after inoculation. Islam (L), Tang II (H) and Islam (H), on the other hand, provided the highest percent bud-break. These were followed by Tang II (L), Tang (L), Tang (H) and BAP (2 $mg.L^{-1}$). The percent bud-break in rhizome explants was also calculated at the end of fourth week after inoculation which suggested that MS media devoid of any growth regulator was the media which initiated bud-break in the least number of explants inoculated. Islam (L), Tang II (H) and Islam (H), on the contrary, revealed the highest bud-break. These were followed by Tang II (L), Tang (L), Tang (H) and BAP (2 $mg.L^{-1}$).

The data taken at the end of first week after inoculation indicated that Tang II (L) gave the most number of shoots per explant germinated among the tested growth regulators. It was followed by Tang II (H), Islam (H) and Tang (H). Negative control, BAP (2 $mg.L^{-1}$) and Tang (L) showed the least number of shoots per explant. Tang II (L) gave the best result in terms of shoots per explant in the second week of inoculation. It was followed by Tang II (H) and Islam (H) which in turn were followed by BAP (2 $mg.L^{-1}$), Tang (H), Tang (L) and Islam (L). Negative control with a single shoot per explant revealed the least number of shoots per explant. Tang II (L) revealed the most number of shoots per explant germinated in the third week of

inoculation. It was followed by Islam (H), Tang II (H), Islam (L), BAP (2 $mg.L^{-1}$), Tang (H) and Tang (L). Negative Control fell behind having a single shoot per explant. At the end of fourth week after inoculation, the most number of shoots per explant was recorded for MS media with Tang II (L). It was followed by Islam (H), Tang II (H), Islam (L), Tang (H), BAP (2 $mg.L^{-1}$) and Tang (L). Negative Control, on the other hand, produced the least number of shoots per explant.

As expected, MS media devoid of any growth regulator also recorded the lowest mean shoot length in the first week of inoculation. Tang II (H), on the other hand produced the lengthiest shoots, followed by Islam (L), Islam (H), Tang (H), Tang II (L), Tang (L) and BAP (2 $mg.L^{-1}$). After two weeks of inoculation, Tang (H) stood out as the hormone producing the lengthiest shoots. It was followed by Islam (H), Islam (L), Tang II (H), Tang II (L), BAP (2 $mg.L^{-1}$) and Tang (L). The shortest shoots were observed in negative control at the end of second week after inoculation. Tang (H) revealed the lengthiest shoots in the third week of inoculation. It was followed by Tang II (H), Islam (H), Islam (L), Tang II (L), Tang (L) and BAP (2 $mg.L^{-1}$). MS media without any growth hormone produced the shortest shoots in length. The average shoot length recorded at the end of fourth week after inoculation suggested that MS media without any growth regulator produced the shortest shoots. The lengthiest shoots, on the other hand, were recorded for Tang II (H) which was followed by Tang (H), Tang II (L), Islam (H), Islam (L), Tang (L) and BAP (2 $mg.L^{-1}$).

Islam (L) was the only growth regulator which initiated root formation in the first week of inoculation. The data recorded at the end of second week after inoculation suggested that Islam (H) was more quick in initiating root formation which was followed by Islam (L), Tang II (L), Tang II (H) and BAP (2 $mg.L^{-1}$). While Tang (H) turned out to be the slowest in initiating root formation. Negative Control and Tang (L) failed to initiate rooting according to the results noted at the end of second week after inoculation. The data taken at the end of third week after inoculation shows variation in the number of days required for root initiation among the different growth regulators used in the study. The most days were taken by Tang (H) for initiating root formation in rhizome explants. The least requirement of days for this purpose, however, was demonstrated by explants inoculated on media with Islam (H). It was followed by Tang II (H), Islam (L), BAP (2 $mg.L^{-1}$) and Tang II (L). MS media devoid of any growth regulator failed to initiate root formation even at the end of fourth week after inoculation. Islam (H) was the quickest among the tested growth regulators in initiating root formation. It was followed by Tang II (H), Islam (L), BAP (2 $mg.L^{-1}$), Tang (H) and Tang II (L). Tang (L), on the other hand, was the slowest in root initiation.

Tang II (H) produced the best result in terms of percent response while BAP (2 $mg.L^{-1}$) revealed the least response in the second week of inoculation. Other growth regulators in descending order on the scale of percent response were Islam (L), Islam (H), Tang (H) and Tang II (L). The data taken at the end of third week after inoculation indicates variation in the percent response of *A. calamus* rhizome explants inoculated on MS media augmented with different growth regulators. Negative Control and Tang (L) once again did not produce any roots in the rhizome explants. MS media containing Tang II (H), on the other hand, initiated roots in most number of explants at the end of third week after inoculation. It was followed by Islam (L), Islam (H), Tang (H) and Tang II (L). BAP (2 $mg.L^{-1}$) was the growth regulator which initiated root formation in the least number of explants inoculated. At the end of fourth week after inoculation, the growth regulator which produced roots in the least number of explants inoculated was again BAP (2 $mg.L^{-1}$). Islam (L) and Tang II (H), on the contrary, initiated root formation in the majority of explants inoculated. These were followed by Tang II (L) and Islam (H). These in turn were followed by Tang (H), and Tang (L).

BAP (2 $mg.L^{-1}$) with a single root per explant responded was the growth regulator which produced the minimum number of roots per explant in the second week of inoculation. MS media augmented with Tang II (H), on the other hand, revealed the maximum number of roots per explant responded. It was followed by Islam (H), Islam (L), Tang II (L) and Tang (H). At the end of third week after inoculation, Tang II (H) turned out to be the growth regulator which produced the most number of roots per explant. It was followed by Islam (L), Islam (H), Tang II (L) and Tang (H). BAP (2 $mg.L^{-1}$), on the other hand, produced only a single root per explant. BAP (2 $mg.L^{-1}$) and Tang (L) produced the lowest number of roots per explant responded in the fourth week of inoculation. Islam (L), on the other hand, recorded the highest number of roots per explant responded. It was followed by Tang II (H), Islam (H), Tang II (L) and Tang (H).

The data taken at the end of second week after inoculation specified that the lengthiest roots were produced by explants inoculated on media supplemented with Tang II (H). It was followed by Islam (L), Islam (H), Tang (H) and Tang II (L). On the contrary, the shortest roots were observed in explants on media with BAP (2 $mg.L^{-1}$). The average root length was also recorded at the end of third week after inoculation which showed that BAP (2 $mg.L^{-1}$) was the growth hormone which produced the shortest roots. On the contrary, Tang II (H) produced the lengthiest roots which was followed by Islam (H), Islam (L), Tang (H) and Tang II (L). The data recorded at the end of fourth week after inoculation revealed that Islam (H) produced the

lengthiest roots. It was followed by Tang II (H), Tang (H), Islam (L), Tang II (L) and Tang (L). The shortest roots were produced by BAP (2 mg L^{-1}).

Our results clearly indicated that Tang II in a concentration of 20 ml L^{-1} was the best among the tested growth regulators in terms of both shooting and rooting of *A. calamus* rhizome explants. This growth regulator required the least number of days for bud-break, initiated bud-break in majority of the explants, ranked third in terms of shoots produced per explant, and produced the lengthiest shoots of all. Moreover, it ranked second in the requirement of days for root initiation, initiated roots in the majority of explants germinated, ranked second in the number of roots per explant, and closely followed Islam (H) in producing the lengthiest roots. It is also clear from the results that when Tang II is used in a concentration of 10 ml.L^{-1}, it enhances the number of shoots produced per explant but significantly affects its ability in terms of all other parameters studied.

Well rooted plants were carefully taken out from the test tubes and rinsed with warm water to remove traces of agar. These were subsequently planted in pots kept in growth room having a mixture of sterilized soil and sand in a ratio of 3:1. Polythene bags with small holes were used to cover the pots for maintaining highly humid conditions. On alternate days the plantlets were irrigated with MS half strength salt solution. After two weeks, the pots were taken out of the growth room and the plantlets were exposed to natural conditions for 3 to 4 hours daily by removing the polythene bags. After 20 days these were transferred to a glass house where the pots were kept for another 20 days. The plants were finally transferred to soil to be grown under natural conditions where they showed a survival rate of 83.00%.

CONCLUSIONS

An innovative and cost-effective micro-propagation protocol was also developed for *A. calamus* and the preeminence of Tang II in a concentration of 20 ml L^{-1} for both shooting and rooting of the explants was established.

Table-1. Effect of Different Growth Regulators on Shooting in Rhizome Explants

Growth Regulator	Days to Bud-break (Mean ± S.D)	% Bud-break	No. of Shoots per Explant (Mean ± S.D)	Shoot length in cm (Mean ± S.D)
Week 01				
Negative Control	3.00 ± 0.95	13.33	1.00 ± 0.00	0.95 ± 0.31
BAP (2 mg L^{-1})	4.75 ± 0.92	53.33	1.00 ± 0.00	1.09 ± 0.46
Tang (10 ml L^{-1})	3.17 ± 0.60	56.66	1.00 ± 0.00	1.10 ± 0.26
Tang II (10 ml L^{-1})	3.81 ± 0.85	73.33	1.27 ± 0.25	1.45 ± 0.36
Islam (10 ml L^{-1})	4.41 ± 0.93	80.00	1.00 ± 0.00	1.92 ± 0.33
Tang (20 ml L^{-1})	3.70 ± 0.77	66.66	1.10 ± 0.10	1.70 ± 0.29
Tang II (20 ml L^{-1})	3.38 ± 0.82	86.66	1.26 ± 0.25	2.15 ± 0.44
Islam (20 ml L^{-1})	3.68 ± 1.14	83.33	1.24 ± 0.23	1.83 ± 0.43
Week 02				
Negative Control	4.00 ± 1.24	16.66	1.00 ± 0.00	1.65 ± 0.70
BAP (2 mg L^{-1})	5.75 ± 1.11	66.66	1.15 ± 0.28	2.68 ± 0.87
Tang (10 ml L^{-1})	5.40 ± 1.38	80.00	1.08 ± 0.08	2.15 ± 0.48
Tang II (10 ml L^{-1})	4.65 ± 1.14	86.66	1.53 ± 0.26	4.04 ± 0.47
Islam (10 ml L^{-1})	5.21 ± 1.19	93.33	1.07 ± 0.06	5.31 ± 0.77
Tang (20 ml L^{-1})	4.22 ± 0.89	73.33	1.13 ± 0.15	5.79 ± 1.23
Tang II (20 ml L^{-1})	3.92 ± 1.14	93.33	1.25 ± 0.24	4.90 ± 0.97
Islam (20 ml L^{-1})	4.14 ± 1.04	90.00	1.25 ± 0.24	5.47 ± 1.39
Week 03				
Negative Control	4.00 ± 1.44	16.66	1.00 ± 0.00	3.12 ± 1.10
BAP (2 mg L^{-1})	5.75 ± 1.11	66.66	1.15 ± 0.28	5.91 ± 1.21
Tang (10 ml L^{-1})	5.84 ± 1.10	83.33	1.08 ± 0.07	6.02 ± 0.95
Tang II (10 ml L^{-1})	4.65 ± 1.14	86.66	1.61 ± 0.30	7.67 ± 1.22
Islam (10 ml L^{-1})	5.21 ± 1.19	93.33	1.17 ± 0.09	7.76 ± 1.79
Tang (20 ml L^{-1})	4.22 ± 0.89	73.33	1.13 ± 0.05	10.01 ± 1.64
Tang II (20 ml L^{-1})	3.92 ± 1.14	93.33	1.25 ± 0.04	8.55 ± 1.37
Islam (20 ml L^{-1})	4.60 ± 1.21	93.33	1.35 ± 0.25	8.11 ± 1.45
Week 04				
Negative Control	4.00 ± 1.44	16.66	1.00 ± 0.00	4.10 ± 0.95
BAP (2 mg L^{-1})	5.75 ± 1.11	66.66	1.15 ± 0.28	5.93 ± 0.51
Tang (10 ml L^{-1})	5.84 ± 1.10	83.33	1.12 ± 0.11	7.21 ± 0.72
Tang II (10 ml L^{-1})	4.65 ± 1.14	86.66	1.65 ± 0.35	9.52 ± 1.50
Islam (10 ml L^{-1})	5.21 ± 1.19	93.33	1.17 ± 0.09	8.59 ± 1.91
Tang (20 ml L^{-1})	5.08 ± 1.37	76.66	1.17 ± 0.08	10.50 ± 1.65
Tang II (20 ml L^{-1})	3.92 ± 1.14	93.33	1.25 ± 0.04	11.33 ± 0.39
Islam (20 ml L^{-1})	4.60 ± 1.21	93.33	1.39 ± 0.29	8.63 ± 1.63

Table-2. Effect of Different Growth Regulators on Rooting in Rhizome Explants

Growth Regulator	Days to Root Initiation (Mean ± S.D)	% Response	No. of Roots per Explant (Mean ± S.D)	Root length in cm (Mean ± S.D)
Week 01				
Negative Control	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
BAP (2 mg L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Tang (10 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ±0.00
Tang II (10 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Islam (10 ml L^{-1})	5.00 ± 0.00	3.33	2.00 ± 0.00	0.10 ± 0.00
Tang (20 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Tang II (20 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Islam (20 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Week 02				
Negative Control	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
BAP (2 mg L^{-1})	10.66 ± 1.52	10.00	1.00 ± 0.00	0.26 ± 0.05
Tang (10 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Tang II (10 ml L^{-1})	10.00 ± 1.15	25.83	2.00 ± 0.52	0.80 ± 0.07
Islam (10 ml L^{-1})	9.75 ± 1.91	42.50	2.61 ± 0.19	1.19 ± 0.04
Tang (20 ml L^{-1})	11.87 ± 1.45	29.17	1.62 ± 0.11	1.01 ± 0.07
Tang II (20 ml L^{-1})	10.00 ± 1.55	52.50	2.80 ± 0.57	1.21 ± 0.04
Islam (20 ml L^{-1})	9.66 ± 1.96	42.50	2.66 ± 0.43	1.10 ± 0.02
Week 03				
Negative Control	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
BAP (2 mg L^{-1})	12.50 ± 1.87	13.33	1.00 ± 0.00	0.32 ± 0.05
Tang (10 ml L^{-1})	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
Tang II (10 ml L^{-1})	13.33 ± 2.43	45.00	2.50 ± 0.56	1.41 ± 0.21
Islam (10 ml L^{-1})	11.43 ± 2.46	58.33	2.82 ± 0.30	1.83 ± 0.35
Tang (20 ml L^{-1})	13.36 ± 2.20	46.66	2.18 ± 0.32	1.83 ± 0.30
Tang II (20 ml L^{-1})	10.94 ± 2.11	66.66	2.88 ± 0.60	2.30 ± 0.43
Islam (20 ml L^{-1})	10.57 ± 2.22	56.66	2.78 ± 0.57	2.11 ± 0.47
Week 04				
Negative Control	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00 ± 0.00
BAP (2 mg L^{-1})	12.50 ± 1.87	13.33	1.00 ± 0.00	0.41 ± 0.15
Tang (10 ml L^{-1})	21.00 ± 2.24	16.66	1.00 ± 0.00	1.40 ± 0.22
Tang II (10 ml L^{-1})	14.92 ± 3.75	56.66	2.50 ± 0.56	2.55 ± 0.25
Islam (10 ml L^{-1})	12.11 ± 2.77	66.66	3.11 ± 0.42	3.11 ± 0.47
Tang (20 ml L^{-1})	13.36 ± 2.20	46.66	2.36 ± 0.40	3.23 ± 0.48
Tang II (20 ml L^{-1})	10.94 ± 2.11	66.66	3.05 ± 0.71	3.75 ± 0.51
Islam (20 ml L^{-1})	10.57 ± 2.22	56.66	2.78 ± 0.57	3.80 ± 0.53

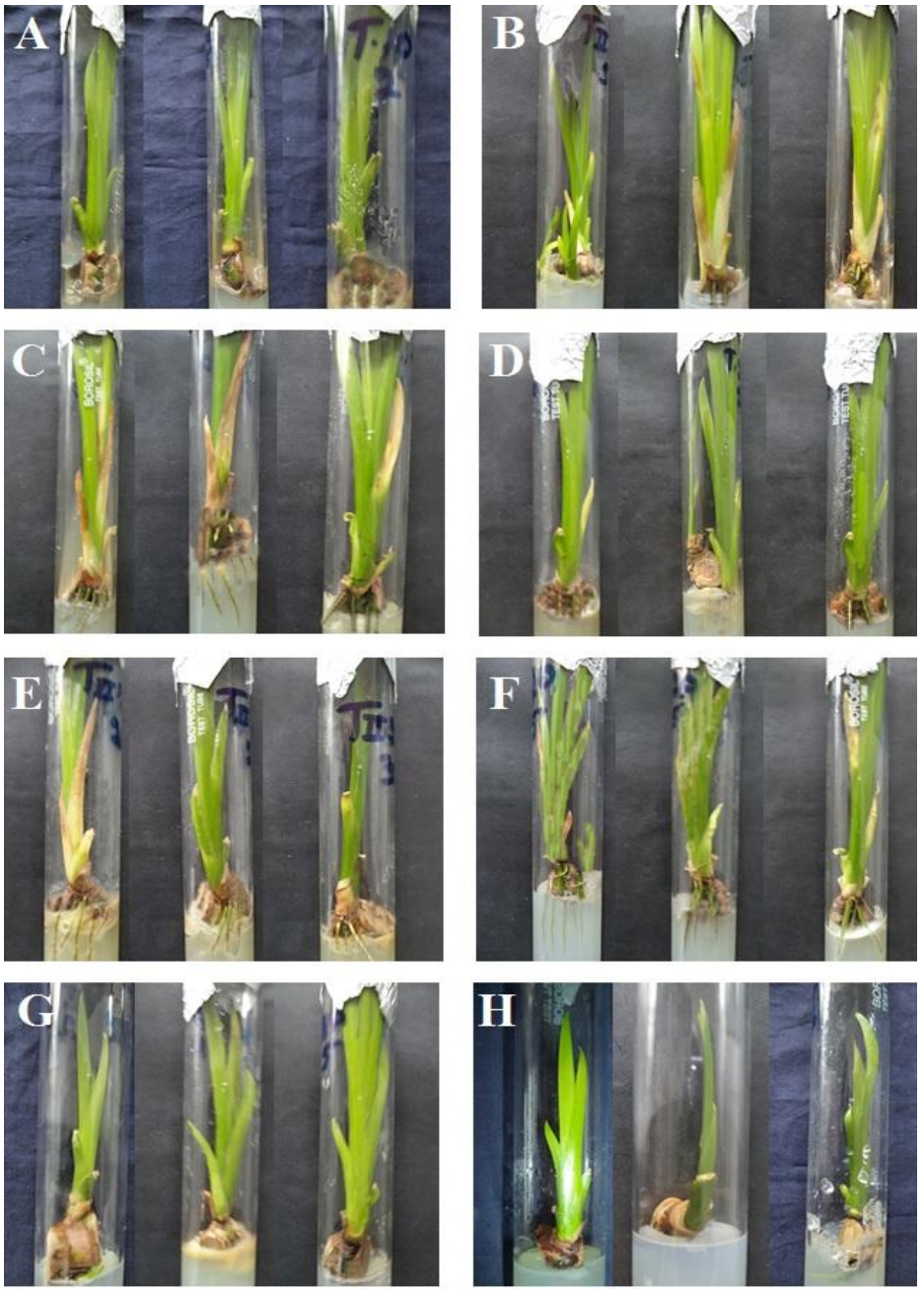

Figure 1. Rhizome explants four weeks after inoculation: A) Tang (L); B) Tang II (L); C) Islam (L); D) Tang (H); E) Tang II (H); F) Islam (H); G) BAP (2 $mg.L^{-1}$); and H) Negative Control.

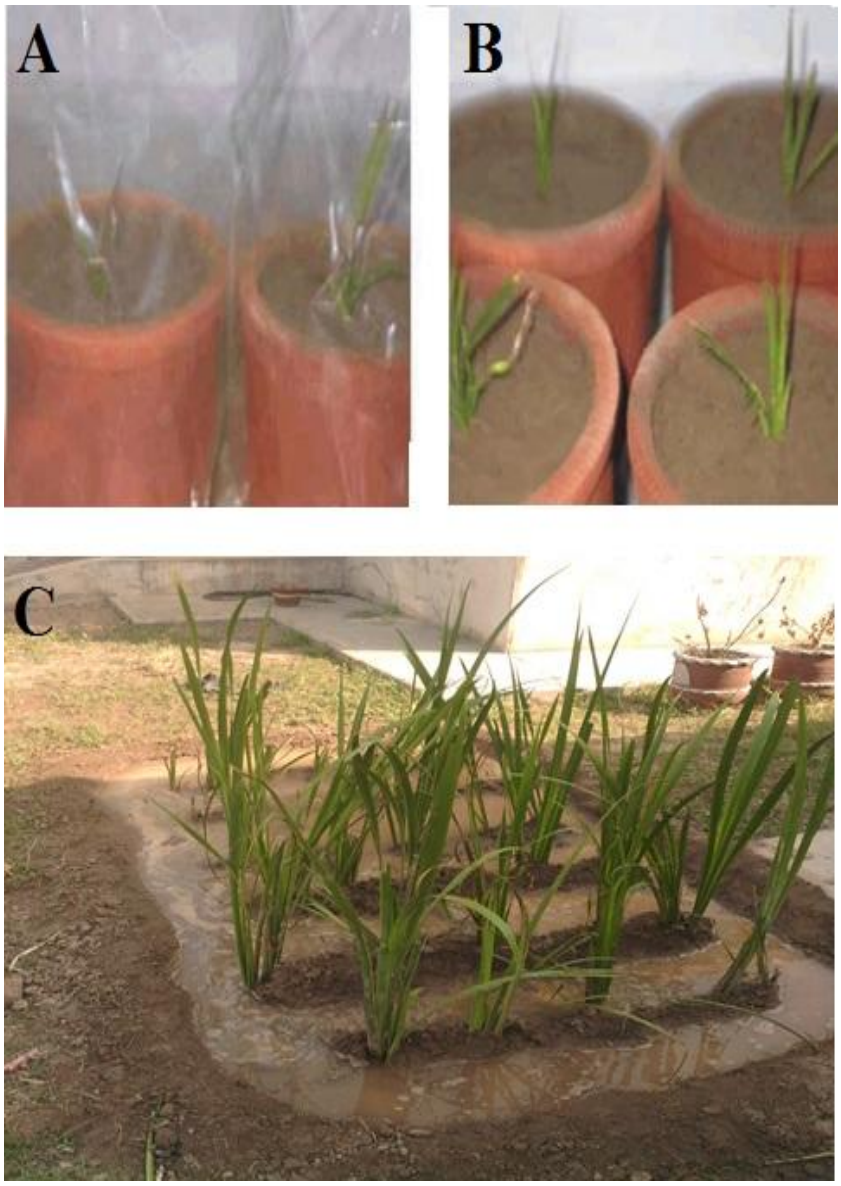

Figure 2. Acclimatization of the micro-propagated plants: A) Potted plants covered with polythene bags; B) Potted plants kept in the green house; and C) Acclimatized micro-propagated plants in field conditions.

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