Research Article

Acclimatization of *in vitro* clonal propagated pineapple plantlets (*Ananas comosus*) utilizing different potting media and fertilizers in ex-vitro environmental interaction

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Abstract | Pineapple is originally indigenous to local Paraguayans in South America. In Pakistan, natural environmental conditions are not suitable for growth of pineapple plant. A quick protocol for development and acclimatization was focused on through microshoot tip tissue culture therapy. The explants were propagated using MS medium fortified with different concentrations of KIN and BAP alone or in combination with NAA or IBA. The best frequency for shoot induction was observed by MS+Kin1.0mg/l while MS+BAP 1.0mg/l +NAA 0.1mg/l and MS+Kin 1.5mg/l +NAA 0.5mg/l showed best plant proliferation with 100% shoot formation. Afterwards, the well grown plants were treated with soil and distinctive blends of potting mixtures i.e. leaf manure, compost, coir, sand and red wood bark in differentiating proportions. Potting mixture with composition of red wood bark coir, sand and compost, soil, sand showed best results for the parameters of plant height and number of leaves. Different fertilizers including Hoagland's solution, DAP, NPK and urea were applied. Well adapted plants were then shifted into open fields for multiplication purpose. In Pakistan, soon there can be virus free production of fruitful pineapple plants by microshoot tip tissue culture therapy.

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Introduction

Pineapple (*Ananas comosus*) is listed in the category of the most economical and tropically important fruits (Duval *et al.*, 2001). At present pineapple is the third most significant fruit after mangoes and bananas in the picture of worldwide tropical fruit production. The average production of conventional material

is 4-5 propagules per year, which takes considerable time to produce adequate planting material (Amin *et al.*, 2005). The traditional (vegetative) method of propagation is not meeting the international market demand. Moreover, the importation of the usual matter for direct planting by farmers may not fulfill the quarantine necessity and is also costly (Danso *et al.*, 2008).

Pineapple has got vitamins (A, B and C) and minerals like calcium, phosphorus, iron (Singh and Yadav, 1980). The leaves of pineapple are used to produce the textile fiber in the Philippines, commonly used as the material for women's formal wear in the country. The fiber is also used as a constituent for wallpaper and other furnishings. Pineapple fruit has many advantages including skin, hair and health benefits. For pineapple, the technique of micropropagation can be thoughtout to be trouble free, but the rate of multiplication is so little that it would take 96 months to produce sufficient propagules from a single parent plant (Almeida *et al.*, 2002). In traditional breeding methods, the selection by clones was tiresome and so as to grow pineapple plant diversity with required qualities several generations of back crossing are required. Primitive techniques of hybridization for good pineapple diversity are clumsy and time taking for a vegetatively propagated plant (Mhatre, 2007).

In Pakistan, the need to increase and bring in this worthy crop is great. Pineapples can be grown and the production can be made highly practical by intercropping with bananas or coconuts. Using planting pieces taken from the parent plant, pineapple is propagated by asexual methods. Though, pineapple‘s micropropagation has more benefits than old methods of propagation (Yanes *et al.*, 2000). For virus free planting material production, this method is operated (Jain and Häggman, 2007). Tissue culture of crown or shoot tips of pineapple was effectively done (Hamad and Taha, 2008). Asexually pineapple is propagated via various parts of the plant such as suckers, crowns or slips. Plant proliferation by tissue culture is carried out by 4 steps, which include culture introduction, multiplication, rooting and acclimatization. One of the significant steps of micropropagation is the last step. The major complicated aspect of *in vitro* propagation is *ex vitro* plant formation (Adjei and Alderson, 1998). After the shifting process from the *in-vitro* state to greenhouse or open fields, a significant number of micro-propagated plants do not continue to exist. An adaptation process is needed by nearly all species grown in *in-vitro* conditions to make certain that adequate plants sustain and proliferate dynamically when shifted to soil (Hazarika, 2003). If the fertilizer and water are applied open handedly and root system remains as integral as possible after transplanting, plants grow at a faster rate (Brainerd and Fuchigami, 1982).

To build up micropropagation methods, a great research has been done, including mass production of pineapple plant by multiplication, culture media and use of growth enhancers for *in-vitro* rooting and propagation of pineapple plants. Zepeda and Sagawa (1981) had grown *in-vitro* pineapple plant. Thus, for rapid mass scale plant development, the method of micropropagation has become a trustworthy and customized technique (De Wald *et al.*, 1988; Jain and Häggman, 2007). Under the environment of compromised gas exchange, low light intensity, high air humidity, plants were grown during the phases of *in-vitro* culture and sucrose was added as the energy source to the medium. The above mentioned factors makes the process of acclimatization complicated for the micropropagated plants by contributing to the blockage of photosynthetic effectiveness and this situation may increase high losses for the duration of

shifting to the *ex-vitro* conditions (Pospóšilová *et al*., 1999).

The adaptation of micropropagated plants to the new ecological surroundings, primarily to the high level light intensity and low comparative moisture is allowed by the structural and physiological modifications during acclimatization. Consequently, the plants show autotrophic behavior and grow as other normal plants (Drew *et al*., 1992). In comparison to traditional propagation, *in-vitro* culture practices comprise of a vital constituent of biotechnology and possess the ability not just the production of innovative plants in relatively small period but also to develop the existing cultured varieties (Akbar *et al*., 2003). The protocol has been standardized for the establishment of culture multiplication, rooting and hardening of the plants.

Acclimatization is a key step to successful *in-vitro* production. On the other hand, the advantage of any micropropagation method can only be entirely apprehended by triumphant shifting of plants from tissue culture containers to surrounding circumstances in *ex-vitro* conditions (Hazarika *et al*., 1996). Due to the dissimilarities between the *in-vitro* and *ex-vitro* environments acclimatization is referred as a crucial phase of micropropagation (Read and Fellman, 1984). The process of acclimatization was effectively attained by the selection of suitable substrate (Normah *et al*., 1995) and through fertilizer's utilization (Tavares *et al*., 2008). During the acclimatization, the selection of appropriate substrate decreases the plant mortality rate (Almeida *et al*., 2002; Moreira *et al*., 2006). Acclimatization is not always an easy step though, since in many species, low rates of survival were obtained. In large quantity, fine quality planting material is needed for marketable farming. Many pineapple diseases show the main trouble of the pineapple production in most profitably growing areas. Consequently, for the mass production of plant, multiplication at *in-vitro* level would be a useful substitute technique. As it leads to the production of large number of disease free consistent planting materials independent of the season in a relatively shorter period, it has relative benefits over the conventional processes. Therefore, the advancement of tissue culture techniques was led by the necessity to get better production of chosen privileged genotypes of pineapple.

2. Materials and Methods

2.1. Origin of explant

Actively growing shoot tips approximately 1 to 2 cm were harvested from the vineyard of Bagh-e-Jinnah, PHA, Lahore, Pakistan and brought to the laboratory for meristem excision.

2.2. Microshoot tip tissue cultures of pineapple

One piece of sterilized explant was taken. Meristem tips were excised aseptically under stereomicroscope with 50-100X magnification. With the help of sharp and fine forceps, meristem with one or two leaf primordial (upto 0.5 mm) was excised. These meristems were inoculated in MS medium fortified with BAP and KIN under aseptic condition of laminar air flow cabinet and culture room and labeled the inoculated test tubes with date, name of explant and medium. Later these tubes were incubated at suitable conditions. For this process, the temperature in the culture room was maintained at 20 ± 2ºC. The culture was incubated for about 16 hours with light intensity of 2000-3000 lux. Plants also received dark period of 8 hours.

2.3. Acclimatization in green house

The *in vitro* developed plants were initially dipped in any anti-fungal solution for 5-10 minutes to avoid fungal attack. Then different treatments were given to micro-plants.

2.4. Treatment with sand

The plantlets were treated with different types of sand that including sand treated with dry oven, boiled sand, autoclaved sand and normal or un-autoclaved sand and were placed under this condition in pots for a time period of at least 30 days with regular watering and their survival rate was observed. For this purpose, sand was heated in dry oven in laboratory at 180ºC for 3 hours, cooled, contained and then used for the plant treatment. The other type of sand used was taken and heated in autoclave at 121ºC, 15 psi for 15-20 minutes. Similarly another type of sand used for the experiment was the boiled sand for which the water was boiled at 100ºC and then sand was added into it, boiled, strained and then allowed to cool for further use. All these types of sand were compared with the normal sand.

2.5. Treatment with potting mixtures

Later on, the impact of multiple potting mixtures on their survival, plant proliferation including plant height and number of leaves was observed under optimum state of light, temperature and dampness for a period of 2 months. Following are the 8 different potting mixtures in different ratios given to the plants which involved: Treatment 1 contained soil: leaf manure (70:30), treatment 2 had compost: leaf manure (70:30), treatment 3 contained soil: leaf manure: compost (33:33:33), treatment 4 had soil: leaf manure, coco peat (33:33:33), treatment 5 consisted of sand: leaf manure: coco peat (10:45:45), treatment 6 had sand: compost: coco peat (10:45:45), Treatment 7 contained sand: coco peat: red wood bark (10:45:45) and treatment 8 had sand: compost: soil (33:33:33).

2.6. Treatment with fertilizers

After this treatment, the survived plants were further exposed to the treatment with fertilizers including Hoagland's solution, DAP (Di-ammonium phosphate), Urea and NPK (nitrogen, phosphorous, potassium).The fertilizers were given to the plants in such a way that a specific number of plants e.g. five plants surviving after treatment with potting mixtures were treated with fertilizers and relative increase in plant height and leaf number were observed. The plants treated with fertilizers were supplied with dosage of fertilizers at specific intervals and watered. The plants giving maximum growth and positive response toward previously mentioned treatments and fertilizers were taken under consideration for further analysis, field trials and multiplication purpose.

2.7. Statistical analysis

For this experiment, Completely Randomized Design with 5 replicates was utilized. The data for each and every degree of freedom was subjected to analysis of variance (ANOVA) using the COSTAT V.63: statistical software (Cohort software, Berkely, California). The mean values were compared with the least significant difference test following Duncan Range Test at 5% level.

3. Results

The present study deals with the factors that the effect the acclimatization of *in-vitro* propagated pineapple plant which included the influence on survival, plant height and number of leaves of the *in-vitro* propagated pineapple plant by treating with sand, potting mixtures and fertilizers.

3.1. Microshoot tip tissue cultures

The technique of plant tissue culture presents a well-organized way for pineapple's swift *in-vitro* clonal propagation (Murashige, 1974). It also makes it promising to produce disease free and consistent propagules (Mathews and Rao, 1980). In present study, for pineapple production through microshoot tip culture therapy, different concentrations of cytokinin alone and in combination with

auxin were used to inoculate the meristems in MS media and better results were calculated. On initial steps, two plant hormones at different concentrations BAP (0.5, 1, 1.5, 2) and KIN (0.5, 1, 1.5, 2) were used in coordination with MS media and number of shoots per plantlet was observed as depicted in Figure 1. MS medium plus KIN 1.0mg/L showed best shoot formation within minimum number of days (Figure 8a) as also reported in *Ananas comosus'* Queen' by Ibrahim *et al.* (2013). Later on, the plantlets were subjected to the treatment of different combinations of MS media supplemented with both cytokinin and auxin like BAP+NAA, KIN+NAA and BAP+IBA at different concentrations. The best shoot length was observed in combination of MS media supplemented with BAP+NAA at concentration of 1.0 mg/L and 0.1 mg/L (Figure 2) and the hormonal combination that showed the best number of shoots was in MS+KIN 1.5 mg/L + NAA 0.5 mg/L as shown in Figure 8b.

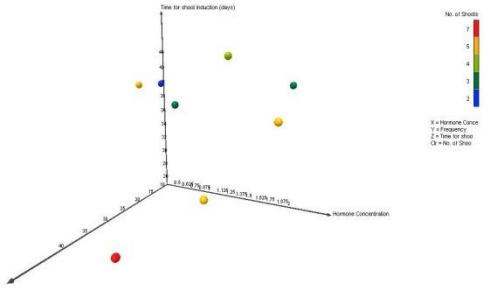

Figure 1: Effect of different concentrations of cytokinins (BAP and KIN) in mg/L on shoot formation of micropropagated *A. comosus*

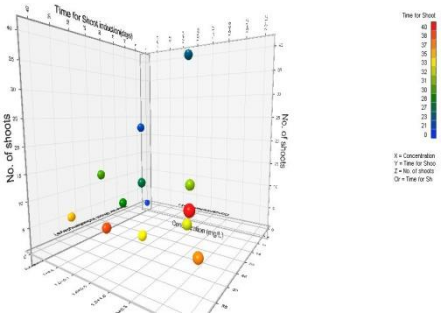

Figure 2: Effect of different combinations of cytokinin and auxin on multiplication of shoots of *A. comosus*

3.2. Acclimatization

Acclimatization is a critical phase before the transplantation of mericlones for field trials. Pineapples require an extensive hardening phase until it attains an appropriate size (Teixeira *et al*., 2001) due to slow growth nature. In present study, the mericlones (*in vitro* grown plantlets) were shifted to green house for hardening. In greenhouse, plantlets were treated with different types of sand including sand sterilized dry oven, boiled sand, autoclaved sand and un-autoclaved sand in pots for a time period of at least 30 days with regular watering and their survival rate was observed (Figure 8c-f). From every type of sand utilized in the process, 100% survival rates were observed. Usman *et al*., (2013) hardened the pineapple plantlets by using riverside sand as substrate for acclimatization by substituting perlite.

Furthermore, the survived plantlets were treated with eight potting mixtures having sand, soil, compost, red wood bark, and leaf manure and coco peat in various ratios for about 60 days (Figure 3). After this, the plantlets were analyzed for their survival rate with potting mixtures in green house conditions. The observed survival rate ranged from 60 to 100% during the time period of 60 days. As evident by Figure 8g, treatment 5 (sand 10: leaf manure 45: coco peat 45), treatment 7 (sand 10: coco peat 45: red wood bark 45) and treatment 8 (sand 33: compost 33: soil 33) showed maximum survival rates of the plantlets.

After observing the survival rates of plantlets, the parameter of plant height was taken under consideration. After 2 months, the maximum increase in plant height was observed in plants with treatment 8 as shown in Figure 4. Later on, in addition to the plant height, number of leaves per plantlet was also analyzed. After 2 weeks of plantlet shifting, treatment 5 (sand 10: leaf manure 45: coco peat 45) showed a good increase in number of leaves per

plantlet. Similarly, after 4 weeks, treatment 7 and after 6 weeks, treatment 8 (Figure 8h-i) showed best results for increase in number of leaves respectively as shown in Figure 5.

Different fertilizers including Hoagland's solution DAP (di-ammonium phosphate), NPK and urea were applied and the survival rates for the plantlets were observed (Figure 8J and 8I). The survival rate ranged from 50 to 100%. Later on the plantlets were analyzed for the parameters of plant height and number of leaves per plantlet against all these fertilizers as depicted in Figure 6 and Figure 7 respectively. In case of plant height, treatment 7 and 8 grew well in Hoagland's solution and NPK (Figure 8k). In case of multiple leaves, treatment 7 gave highest number of leaves in Hoagland's solution. Similarly, Farahani, (2013) studied the effect of fertilizers on development of pineapple's micropropagated plantlets.

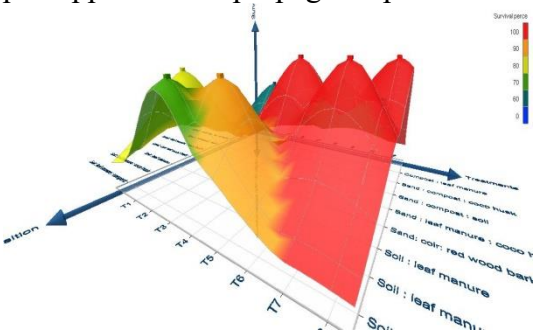

Figure 3: Effect of different potting mixtures (treatments) on survival percentage of *in-vitro* propagated plantlets of *A. comosus* in greenhouse

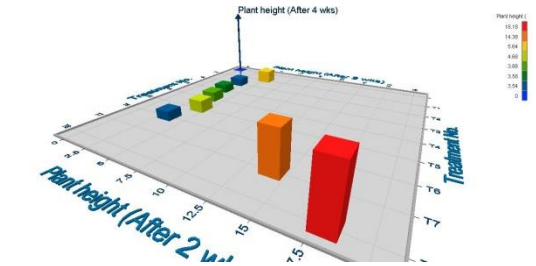

Figure 4: Effect of different potting mixtures on average increase in plant height of *in-vitro* propagated *A. comosus*

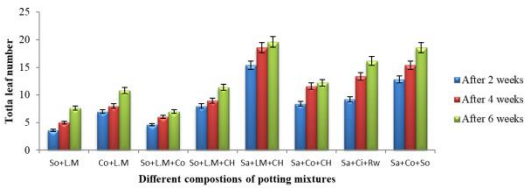

Figure 5: Effect of different potting mixtures (treatments) on number of leaves of *A. comosus*

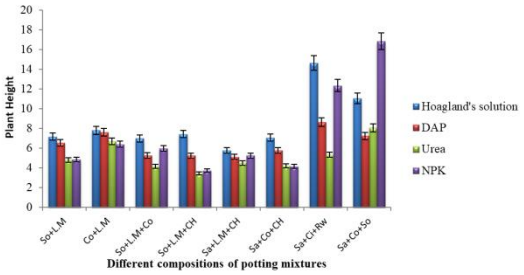

Figure 6: Effect of Hoagland's solution, DAP, Urea and NPK on plant height of plantlets of *A. comosus* survived from different potting mixtures

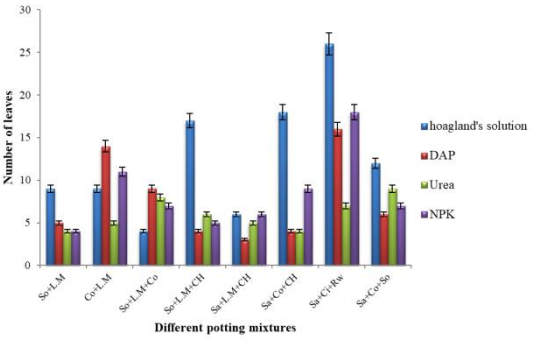

Figure 7: Effect of Hoagland's solution, DAP, Urea and NPK on number of leaves of plantlets of *A. comosus* survived from different potting mixtures

4. Discussion

Amin *et al.* (2005) reported that the hormonal combination gave best shooting results in pineapple. Moreover Firoozabady and Gutterson (2003) also stated that BAP and NAA combination in MS medium produced good shooting results but at a different concentration for pineapple production. Previously it was reported that a limited success can be obtained in case of plant *in vitro* grown

Fig. 8a-l: Effect of different hormones, potting mixtures and fertilizers on pineapple production. **a)** MS media fortified with KIN 1.0 mg/L with highest number of shoots. **b)** The best number of shoots in MS+KIN+NAA. **c)** Plants in normal sand. **d)** Plants in dry oven heated sand. **e)** Plants in autoclaved sand. **f)** Plants in boiled sand. **g)** Different potting mixtures used for acclimatization of plantlets in green house. **h)** Effect of different potting mixtures (treatments) on average increase in plant height of plantlets after 6 weeks. **i)** The highest number of leaves shown by treatment 8 (sand, compost and soil). **j)** Different fertilizers applied on plants survived by treatment with different potting mixtures in green house. **k)** Average increase in plant height and number of leaves after 4 weeks of application of Hoagland solution and DAP. **l)** Average increase in plant height and number of leaves after 4 weeks of application of urea and NPK.

plantlets (Mapes, 1973). According to the results of Lakshmi *et al*., (1974), pineapple plantlets developed through in vitro means were not able to go into multiplication of shoots phase.Cocopeat provides moisture availability and nutrition of the media and aeration is suplied through sand. The current results are in accordance with those described by Khayyat *et al.*, (2007) which indicates the superiority of cocopeat over other potting mixtures for growth of *Epipremnum aureum*. The greatest *Rosa indica* in media having cocopeat was also testified as well by Maloupa (2001). Similar observations were also stated by Sameei *et al*., (2004) who reported highest values of leaf number and area and shoot length in cocopeat included media for pineapple plantlets. Akbar *et al*., (2003) also hardened the plantlets by using garden soil, compost and sand (1: 1: 1 v/v) where 85% plants survived and later shifted to open fields.

The growing media are found to affect the plant survival rates and growth performance in the greenhouse (Vasane *et al*., 2006; Salifu *et al*., 2006).

5. Conclusion

It is concluded that micropropagated plantlets survived the green house conditions in every type of sand. Potting mixtures having sand, red wood bark, coir, leaf manure, coco peat and compost in experiment-based specified compositions showed distinguishingly better growth results. Hoagland's solution was concluded best for the plant height and number of leaves of plantlets exposed to application of potting mixtures. Well adapted plants were analyzed and then shifted into open fields for multiplication purpose. In Pakistan, natural environmental conditions are not suitable for pineapple production. Microshoot tip tissue culture therapy and selection of best potting mixture and fertilizers in *ex vitro* conditions can be very effective for virus free production of fruitful pineapple plants in future.

6. Acknowledgments

Authors would like to acknowledge Environmental Sciences Department for providing equipped lab and facilities in conducting present work.

7. Author's Contribution

All authors have made a considerable contribution to the concept, design, analysis and elucidation of data for the article.

8. Conflict of Interest

There was no conflict of interest among authors concerning the reporting of this article.

9. Novelty Statement

Several studies have reported the culture of pineapple in solid media. Solidifying agent is used on this media, which is an expensive chemical making the protocol costly. Present study was designed to shift the solid culture protocol to the liquid culture protocol, which is cost effective.

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