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Effect of Nutrient Medium pH on *in vitro* Shoot Multiplication of Banana

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ABSTRACT

Nutrient medium provides an ideal condition during *in vitro* development of plant tissue. The pH of medium is important factor of successful micro propagation besides different medium constituents and other growth components. The experiments were conducted to monitor the changes over time, decline of medium pH and to determine the effect of medium pH on shoot multiplication rate of banana var. Grand Naine. The medium pH was adjusted from pH 5.00 to 6.00 before addition of agar. Results of the experiments concluded that, change of medium pH significantly affects shoot multiplication rate, average weight of shoot clumps and number of roots per shoot. The gelling strength of medium is also affected by changes in pH. The decline in medium pH also observed during storage, but the medium stored in dark condition do not showed change of medium pH. However, prolonged incubation of explants deflects medium pH changes to acidic.

Keywords: Medium pH, medium autoclaving, agar, multiplication rate.

INTRODUCTION

The plant nutrient medium pH has been shown to be very important to many aspects of shoot development and growth. Tissue culture media provide ideal conditions not only for growth of plant cells, but also for bacteria and fungi. It is therefore necessary to sterilize media to kill these microbes prior to inoculation of explants. The plant nutrient media are commonly sterilized by autoclaving at 121°C and pressure of 105 kPa for 15 to 20 minutes. Similar to soil pH, nutrient medium pH may also influence nutrient uptake by plant tissue (Ramage & Williams, 2002), such as ammonium uptake being facilitated with a stable pH of 5.5 (Thorpe *et al.*, 2008). The decline and fluctuations in medium pH may be due to medium constituents, heat sterilization, ion exchange and environmental conditions (Owen *et al.,* 1991; Skirvin *et al.,* 1986). Additions of synthetic or natural organic acids generally increase medium buffering ability (Thorpe *et al.,* 2008).

The medium pH fluctuations have been reported for crabapple and pear on MS medium (Singha *et al.,* 1987). According to Skirvin *et al.* (1986) and Thorpe *et al.* (2008), *in vitro* nutrient absorption by explant is a function of ion exchange, where deposition of free hydrogen ions (H^+) and hydroxyl ion (OH^-) in the medium may contribute to acidic or alkaline medium pH. In contrast, photo-oxidation induced chelating events that bind free iron, reducing iron availability, may also influence medium pH (Hangarter & Stasinopoulos, 1991).

Medium pH fluctuations can involve many factors, and can eventually become problematic for tissue culture. However, the interactions between explant tissue and its growing medium are not well studied. To ensure an optimal shoot culture development and provide high quality shoots for later development, medium pH can be a key indicator in determining optimal subculture time. Medium pH may be further utilized as a diagnostic tool for some abnormal growth symptoms, such as necrosis, caused by low pH induced nutrient deficiency.

The medium pH affects the uptake and utilization of medium components such as macro elements and microelements and growth regulators. Generally, 5.6–5.9 pH is adjusted before autoclaving of medium are recommended for tissue culture of the majority of plant species (George, 1993).

The low pH affects the gelling strength of the agar; banana shoots inoculated on this nutrient media failed to propagate properly. High acidic medium inhibits shoot growth through destabilization of growth hormones and precipitation of phosphate and ion salts (Dodds & Roberts, 1990). The main objective of this research work is to study the effect of change in medium pH on *in vitro* propagation of banana.

MATERIAL AND METHODS

Plant material:

The experiments were carried out at Genuine Biosciences, tissue culture research laboratory, Osmanabad, Maharashtra -413501. In this study, cultures of banana var. Grand Naine were used.

Media preparation:

Full strength Murashige and Skoog medium (Murashige and Skoog, 1962) was used throughout experiments. All stock solutions of Murashige and Skoog's (1962) medium were prepared in advance, each stock bringing it to room temperature and required quantity of each stock is added one by one in a beaker and final volume make up by adding distilled water. In all MS media components supplemented with 3% sugar and 0.6% agar as gelling agent. BAP 3 mgs/l and $AdSO_4$ 10 mgs/l for shoot multiplication and IBA 2 mgs/l and 0.2% charcoal for *in vitro* root induction were incorporated throughout experiments. To study the effect of change in medium pH, which alters the shoot morphogenesis, experiments were design in such way that, pH of media adjusted to 5.00, 5.20, 5.40, 5.60, 5.80 and 6.00 before autoclaving. All the media were boiled to dissolve agar and 30 ml media was dispensed in 300 ml capacity jar bottles. The media were then sterilized at 121°C for 20 minutes. The prepared media were stored in dark as well as in light store room and used for conducting experiments.

Establishment of Explants:

The sucker explants of banana var. Grand Naine were collected and carried to laboratory. These suckers were trimmed to size 60mm length x 30mm wide. The explants was washed several times using raw water to remove all dirt and were treated with 0.2 % bavistin along with a drop of tween-20 for half an hour. All the required initiation media, solutions such as 70% ethyl alcohol, 0.1% $HgCl_2$, 10% sodium hypochlorite and sterilized distilled water were taken to laminar air flow chamber for further surface sterilization procedure. The explants were surface sterilized with 70% ethyl alcohol for 45 seconds, 10% sodium hypochlorite for 10 minutes and followed by $HgCl_2$ for 5 minutes. The explants were rinsed with sterilized water for 4 to 5 times to remove excess of sterilents. The explants were then inoculated on initiation medium after removing outer leaf layer.

Cultural conditions:

The fresh cultures were incubated in dark for a week in growth room at 28±2°C. After a week all the cultures were kept in a temperature controlled incubation room and intensity of light (1200 lux) was provided by using white florescent tubes. All the cultures were assessed daily for bacterial/ fungal contamination. Data was recorded at 4

weeks of incubation. The contamination free explants obtained from initiation stage was further sub cultured on shoot multiplication medium containing benzyl amino purine (BAP) 3.0 mgs/l, sucrose 30.0 gms/l and agar 6 gms/l. The *in vitro* developed explant material was used for conducting all experiments.

***In vitro* shoot multiplication and rooting:**

Shoots were multiplied by the method of enhanced release of axillary buds (Murashige, 1974). The *in vitro* multiplied shoot cultures maintained by routine subculturing on already standardized multiplication medium comprising of MS-basal salts, 3 mg/l BAP, for all the experiments. The serial subculturing was performed after every 4-5 weeks by separating and transferring the shoots onto fresh nutrient medium. Rooting was carried out on rooting medium comprising of MS basal salts and supplemented with IBA (2 mg/l). Micro shoots from 4 weeks old cultures were used to make cuttings of approximately 3-4 cm long for root induction. The lower leaves were removed from micro shoots and inoculated for rooting. Root induction and growth was recorded after 4 weeks of incubation period.

Checking of medium pH:

The media pH Measurements were carried out by immersing of the electrode before addition of solidifying agent (agar). The pH meter was calibrated using buffer pH- 4.0, and pH- 7.0. The media pH was adjusted to 5.00, 5.20, 5.40, 5.60, 5.80, and 6.00 before addition of agar and sterilized in an autoclave for 20 min at 121ºC. The pH of media was also checked after autoclaving of different media. The pH of used agar medium after incubation of culture also checked and the data was recorded. The pH of shoot multiplication medium was checked to see the effect of change of pH on shoot multiplication rate and weight of shoot clumps. The pH of root induction medium was checked to observe the influence of pH change on average weight of plantlet, length of root and number of roots. To find out the effect of pH on gelling strength and clarity of agar was also studied.

Statistical analysis:

All experiments were repeated three times. Data collected in the experiments were subjected to analysis of variance (ANOVA) using a factorial design and evaluated using the statistical program OPSTAT-Statistical Software. The statistical analysis based on mean values per treatment was made using ANOVA technique for completely randomized design and random block design (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Change in medium pH after autoclaving and storing:

Steam sterilization in autoclaving influences change in medium pH. Media pH found to be declined according to each final adjusting pH before addition of agar. Medium of pH 5.20 showed a greater extent of pH fluctuations, decline in pH by unit 0.81 (Table-1 & Figure-1).

Table 1: Effect of sterilization on change in Ph

Final pH adjusted	pH after autoclaving	pH decline
5.00	4.32	0.68
5.20	4.39	0.81
5.40	4.77	0.63
5.60	4.83	0.77
5.80	5.16	0.64
6.00	5.31	0.69

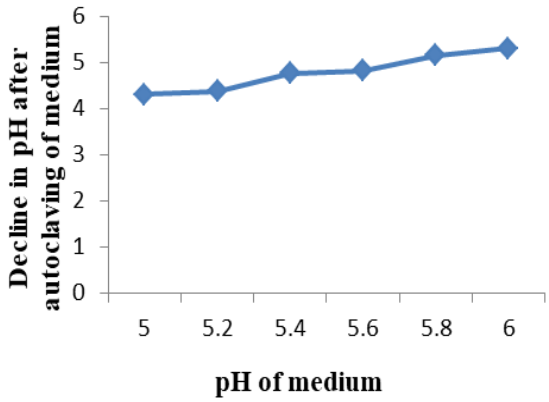

Figure 1: Effect of sterilization on change in pH

Furthermore, no alteration of pH change during storage of medium in dark which is good sign for maintain stable media pH than storage in light. Medium of pH 5.8, when kept in the dark condition showed no change in pH upto 30 days of incubation. In contrast to this, when medium of same pH stored in light condition showed change in pH of medium. It shows that during culture storage, light can slightly decrease culture medium pH over the incubation

times (Table 2). Similar results were observed by Chien *et al* (2015) in *Pseudotsuga menziesii* shoot culture.

Table 2: Effect of storage conditions on pH of medium. (Medium pH 5.8)

Days of storage	pH of medium dark storage	pH of medium light storage
5	5.17	5.17
10	5.17	5.17
15	5.17	5.16
20	5.17	5.15
25	5.17	5.13
30	5.17	5.12

Change in gelling strength of medium:

The pH determines many important aspects of structure and activity of biological macromolecules. To investigate the changes of medium pH on gelling strength and clarity, the entire media fortified with 6 gm/l agar. Observation were recorded after 1 week of freshly prepared sterilized medium. The gelling strength of medium is dependent on pH value. It was observed that the medium started to solidify when medium pH reaches 5.4, although it was soft. The results of our experiments showed that the nutrient medium of pH 5.8 and 6.0 was ideal for gelling with clarity (Table-3). The pH value less than 5.8 shows no satisfactory gelling. The medium became too hard when pH adjusted to 7 or higher, which probably affected the nutrition uptake and also showed a tendency of cracking itself. Our results are supported by investigations of Shi *et al.* (2017) on tissue culture of apple.

Table 3: Effect of medium pH on gelling strength and clarity of medium.

pH of medium	Gelling strength	Clarity
5.0	_	+
5.2	_	+
5.4	+	++
5.6	++	++
5.8	+++	++
6.0	+++	++
7.0	++++	+++

Where, + not satisfactory, ++ satisfactory, +++ good ++++ hard and - no gelling.

Influence of pH on shoot multiplication:

The pH of the culture medium is an important factor for growth and development of shoots *in vitro*. (Mimura *et al,* 2000). The proliferation efficiency of banana was affected by the medium pH within the range from 5.0 to 6.0. The multiplication rate and average weight of shoot clump of effective shoots were influenced by change in medium pH (Table-4). It was observed that pH from 5.6 to 5.0 significantly lowered the multiplication rate with average weight of shoot clump. It was observed that multiplication medium when adjusting pH to 5.8 (before autoclaving) served as best for multiplication rate of 1:2.36 with average weight of shoot (4.81 mg) (Table-4 & Figure-2).

Table 4: Effect of medium pH on shoot multiplication of banana after 4 weeks of culture

pH of medium	Shoot multiplication rate	Average weight of shoot clumps (mgs)
5.0	1: 1.24	3.92
5.2	1: 1.26	3.79
5.4	1: 1.57	3.97
5.6	1: 1.71	4.21
5.8	**1: 2.36**	**4.81**
6.0	1: 2.26	4.76
$CD_{0.05}$	1: **0.34**	**0.26**
SE	1: **0.11**	**0.08**

In vitro root induction is an important stage of micropropagation. In our study, the effect of pH on root induction was also significantly influenced, different medium with different pH were analyzed for rooting (Table-5). Longer roots were observed at the pH 5.8 and 6.0 while shorter roots were formed on media having pH ranges from 5.0 to 5.6. Medium pH 5.8, showed more number of roots (3.68) as compare to others with average root length of 8.26 cm. (Table-5 & Figure-3).

Medium pH was changing due to excretion of some compounds from the explant. Typically, *in vitro* explant incubation exhibited a curvilinear relationship with pH over time. In this study we observed that, as subculturing duration increases the pH of medium declines (Table-6). Overall, it was observed that a 21-days subculture may be most suitable for maintenance medium pH level, medium freshness and desirable growth of banana explants. Therefore, we would suggest sub culturing at 20-25 days for banana as a best subculture period.

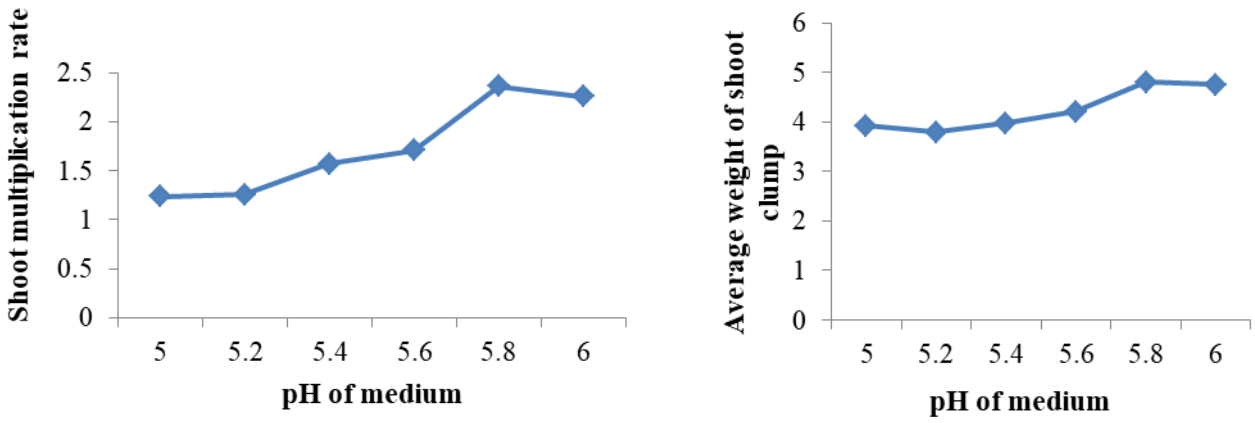

Figure 2: Effect of medium pH on *in vitro* shoot multiplication

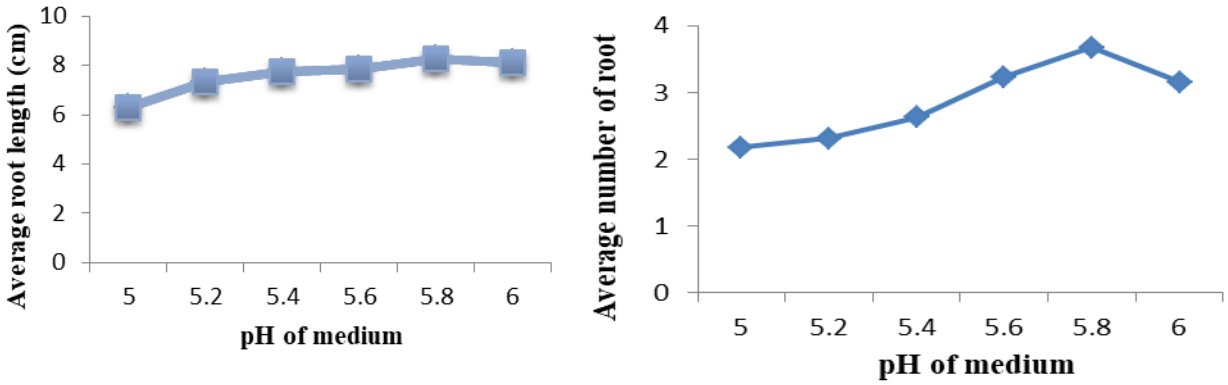

Figure 3: Effect of pH on root formation

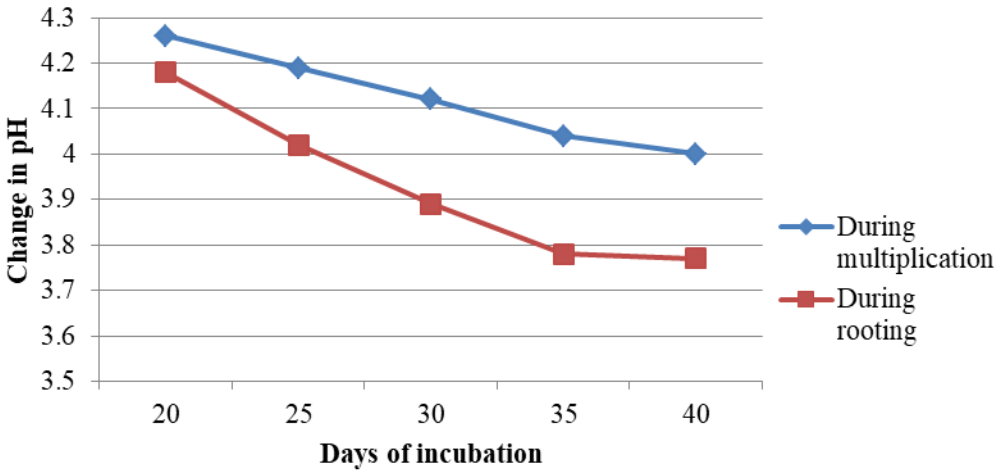

Figure 4: Effect of culture explants on medium pH

Table 5: Effect of medium pH on shoot multiplication of banana after 4 weeks of culture

Medium pH	Average root length (cm)	Average number of roots
5.0	6.30	2.18
5.2	7.33	2.32
5.4	7.76	2.63
5.6	7.83	3.24
5.8	**8.26**	**3.68**
6.0	8.10	3.16
$CD_{0.05}$	**0.76**	**0.46**
SE	0.24	0.14

Table 6: Effect of culture explants on medium pH (Adjusted medium pH 5.8)

Subculture Duration	pH of Used Medium (Multiplication Stage)	pH of Used Medium (Rooting Stage)
20 Days	4.26	4.18
25 Days	4.19	4.02
30 Days	4.12	3.89
35 Days	4.04	3.78
40 Days	4	3.77

CONCLUSION

In this research work, a wider pH range was studied to get a better understanding. Several factors could account for change in pH during media preparation. Fluctuation of medium pH can be influenced by enzymatic break down of sugars, release of phenolic compounds and photolysis on light-sensitive medium components. These factors effect on *in vitro* plant growth and developments. The initial pH of the medium could be varied due to medium components, such as plant growth regulator and the ways whether acid or alkaline solution was used to dissolve. The medium pH has considerable influence on uptake, utilization of nutrients, and physical strength of the medium.

Thus, the most important conclusion from this research is that to adjust medium pH 5.8 for *in vitro* propagation of banana var. Grand Naine. This ensures maximum explant growth performance in *in vitro* condition. Storage of medium in the dark resulted in less fluctuation of medium pH than storage under light.

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Conflicts of interest: The authors stated that no conflicts of interest.

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