Availability of the P_i to Banana (*Musa acuminate* var. Dwarf Cavendish) Explant *in vitro*

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Abstract: Phosphorous deficiency, due to precipitation and fixation in the MS (Murashige and Skoog, 1962) medium can be a limiting factor for growth and development of banana (*Musa acuminate* cv. Dwarf Cavendish) explants. It was found that after mineral precipitation, the standard plant tissue culture MS medium had lost about 20 % of original P_i during the first week of culture. Although, an appropriate concentration of the all other elements (except concentration of Na_2HPO_4 0 to 4 *mM*) were supplied the same concentrations as MS medium, the decrease in P_i availability observed in the depressed rate of growth, especially in the 0, 1 *mM* Na_2HPO_4 treatments (the limitation of P_i availability was the main cause of the inhibition of the growth). Better response was found with 2 *mM* Na_2HPO_4 in respect of growth rate, total uptake of mineral and concentration of mineral in the leaf. The PO_4^{2-} uptake is proportional to the P_i availability. P_i availability is influenced by P_i diffusion and diffusion is the dominant process in mineral availability *in vitro*. Low rate diffusion of P_i through the gelled medium is main reason for their low uptake and their deficiency *in vitro*. The rate of P_i diffusion increased as a result of increasing its concentration. But in contrary to the Fick's law, the rate of mineral diffusion in the gelled tissue-culture media is not directly proportional to the initial concentration. The rates of mineral diffusion and mineral availability were not similar. PO_4^{2-} and Ca^{++} diffused slowly, whereas, K^+ and Mg^{++} diffused rapidly. PO_4^{2-} movement through the gelled medium by diffusion and its availability and its uptake by explants are discussed in terms of growth rate *in vitro*.

Key words: Banana · Diffusion coefficient · *In vitro* culture · P_i availability · P_i precipitation · Uptake

INTRODUCTION

Banana (*Musa spp*.) is the most important tropical fruit, which is from 1972 on, the "shoot tip culture" techniques have been used for the accelerated multiplication [1, 2, 3, 4, 5, 6, 7, 8, 9]. While micropropagation of *Musa* spp. is well understood [6, 10, 11] and even relatively commercialized, there are still some certain problems, which limit its widespread use [12]. One of the major shortcomings of tissue culture raised plantlets is decline the rate of explant growth after a short time (two or three weeks after culture), necessity of frequently transfer of explant to a fresh culture medium, consequently high expenses and contamination [5, 10, 13]. One of the standard media used in banana tissue culture is MS revised medium, however, the problem is that MS medium precipitates on keeping, which may not be noticed when agar is presented [14]. The limiting factor for maximum mineral uptake and optimum growth is that minerals are not enough available in the explants. Availability and uptake of minerals by explant, especially P_i, is recognized as being important in plant tissue culture

[1, 15]. The basic assumption is that diffusion is the main mechanism of mineral uptake [16, 17, 18]. If a small portion of mineral diffuse only around the explant, mineral depletion rapidly occurs and growth stops. This is prevalent for P_i deficiency for many plants *in vitro* [19, 20, 21, 22, 23]. The P_i uptake by the *Ptilotus exaltatus in vitro* was corresponded with P_i depletion in the medium [18]. With a low P_i supply, P_i depletion occurs soon and uptake and growth decrease or cease.

Phosphorous has been reported to cause increased nutrient uptake [24], increase leaf photosynthesis [17], reduced transplant mortality [21], improved water relations and chlorophyll content [18, 19], increased contents of DNA and RNA [24], overall growth [22, 23] and finally bud production in banana cultures [25]. If P_i diffusion through the gel is limiting P_i availability (and hence explant growth), increase in the mineral concentration in the medium will result in a proportionate increase in total mineral available to the explant. Furthermore, P_i availability to the explants is related to the P_i movement in the medium. Therefore, it was hypothesized that P_i uptake and hence growth, are proportional to the initial

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concentration of P_i in the medium. In this work the ratio of initially P_i supply and P_i diffusion: its solubility (available) and its uptake are widely discussed in terms of mineral movement by diffusion and explants growth.

MATERIALS AND METHODS

Preparation of Plant and Medium: Young suckers selected from healthy banana (*Musa acuminate* cv. Dwarf Cavendish) and outer layers of leaves and the corm tissue are removed. What remains is a piece 10 cm long and 6 cm in diameter. The piece is wiped clean with 75% alcohol. Under aseptic conditions, the sheaths and bases of the leaves are trimmed to expose the meristematic region. For initiating *in vitro* cultures, shoot spices (5×20 mm) were excised from sample. Four uniform shoot tips (3-5 mm) was subculture aseptically in each container. To measure the effect of phosphorous on growth and multiplication rate, an appropriate concentrations of all macro and micro elements (except phosphorous) were supplied the same concentrations as MS (Murashige and Skooge, 1962) medium [26]. Phosphorous was supplied at seven levels 0, 1, 1.5, 2, 2.5, 3 and 4 *mM* Na_2HPO_4. The medium was supplemented with thiamine 0.4 mg l^{-1}, myo-inositol and L-tyrosine both at 100 mg l^{-1}, sucrose 30 g l^{-1} and agar (Difco BiTek™ agar) 8 g l^{-1}. The medium pH was adjusted to 5.6 by HCl 0.5 N and NaOH 0.5 N before autoclaving. Growth regulators including BA (benzyladenine) 4 mg l^{-1} and adenine sulfate 80 mg l^+ were added. Then medium was autoclaved by 101 KPs, 120° C for 15 min. Thirty ml of solution in each 250 ml polycarbonate container was dispensed (with 5 replicates of each treatment and the control). All explants were kept in a growth room at a temperature of 25 ± 2°C, with 55% relative humidity and 16 h 50 ìm^{-1}S^{-1} cool white fluorescent light. The fresh weight and dry weight (as growth rate), multiplication rate, root formation, shoot elongation and also plant appearance were recorded every two weeks. Furthermore, total uptake of minerals, mineral concentrations in the leaf was measured at weeks 6 and 8. Containers did not occupy fixed positions on culture shelves but were moved around randomly during visual examination every week.

Measuring Mineral Diffusion: A double layer gel system was developed to study the effects of initial concentrations of minerals on the mineral diffusion within the culture vessel. The media used were based on de Fossard [27] preparation four relative concentrations (0, 0.2, 1 and 2 as zero, low, medium and high) of minerals

were used. To each medium 30 g l^{-1} sucrose and 8 g l^{-1} Bacto Bitek ™ agar, were added. Medium pH was adjusted to 5.6 by HCl 0.5 N and NaOH 0.5 N before autoclaving. Thirty ml aliquot was disposed into each 250 ml autoclavable polycarbonote screw capped containers and were autoclaved at 103 KP$_a$ and 121°C for 15 minutes. Again, 30 ml of autocalved medium containing no mineral was dispersed upon the firstly set bottom layer. To control the effect of mass flow, containers were sealed. All containers were incubated at constant room temperature (25±°C) during experiment. Sample collection was done by the Cork bore (No. 3 dim.1 cm) randomly from the surface of the top layer at two times (day one and day five). The volume of provided samples was equal to 10 ml that is exactly 1/3 of total medium volume and 5 uniform pieces of cuttings. To survey the mineral diffusion through different distances (6 to 30 mm) at two different times (day one and day five), instead of 30 ml, 150 ml of gel was poured out for making an approximate 3-cm thickness top layer. The cutting of top layer was performed from distances 6, 12, 18, 24, 30 mm from top-non-mineral layer after one day and five days. Gel sample collection was done by the Cork bore (No. 3 dim.1 cm) randomly from the surface of the top layer at day five. All cuttings were uniform and the same size and 10 ml volume.

Measuring Soluble (Available) P_i: To determine the proportion of phosphorous available (at all seven treatments: 0, 1, 1.5, 2, 2.5, 3 and 4 *mM* Na_2HPO_4), to the explants the relative amount of water soluble phosphorous in the medium was measured. The most appropriate method of estimation of available phosphorous in the gel medium was recognized as water extraction (per. com Williams, 2006). This method could distinguish between the insoluble (unavailable) or (total precipitation, fixation and unexchangable P_i) and the soluble proportion, which was considered available to the explants.

Measuring Total Uptake: Subtracting the residual proportion from total initially supplied (non-planted medium) gave a value for the amount of total mineral taken up by the four explants per container. Total uptake of individual minerals was measured as mg/30 mg ml at week 8. In each treatment 3 replicates were used and treatment means were plotted.

Mineral Analysis: Analysis of most macro and microelements (P, S, K, Mg, Ca and Fe) of all samples (plant and gel materials) was carried out by the Inductivity

Coupled Plasma Emission Spectrometry (ICP-ES). All samples digested according to technique used by (SCD) Sealed Chamber Digest [28].

Data Analysis: Four shoot tips were inoculated per container and four containers were maintained for each treatment. Each unit in the container was considered a replicate and data were analyzed for a factorial experiment involving five P_i levels and four mineral concentration treatments. All calculations were determined by using the computer program Excel Microsoft and data was analyzed by ANOVA and means subjected to LSD test at 5% level using NEVA [29]. In each treatment 3 replicates were used and treatment means were plotted by using the computer program Excel Microsoft.

RESULTS

Phosphorous Diffusion in the Gel: The relative diffusion of individual elements (e.g. PO_4^-) through gel increased as a result of increasing the levels of mineral supply, but the rate of diffusion was not proportional to the initial concentration. The pattern of diffusion of minerals varied with each other. The highest one, as fast elements, was recognized for K^+, Mg^{++}, the lowest one, as slow elements, was known for PO_4^- and Ca^{++} the other elements (e.g. Fe^{++}) was realized as intermediate. In contrary to the Fick's law, the rate of diffusion is not directly proportional (Fig. 1).

Phosphorous Available (Soluble) *in vitro*: Increasing Na_2HPO_4 concentration significantly (P=0.05) increased the amount of soluble P_i. But increasing is not proportional to the initial P_i supply in the medium. It was found that more than 15% of original P_i was precipitated almost in the all treatments. As the amount of P_i supply increased the amount of precipitation significantly (P=0.05) increased (Table 1).

Plant Growth *in vitro*: Banana growth (FW and DW) significantly (P=0.05) increased with increasing concentration of Na_2HPO_4 from 0 to 2 *mM* then decreased up to 4 *mM* (Fig. 2). This showed that banana cultures required phosphate up to a certain level. If the content was low, growth decreased and if it was high, growth decreased. There was little growth over the first two weeks then the growth rate increased rapidly up to week 6 (Fig. 3). The FW growth rate response to the level of P_i paralleled the final weight but the rate of DW growth during the last two weeks was similar for all levels of P_i (except the 0 *mM*). The best shoot growth (as fresh

Table 1: Effect of Na_2HPO_4 concentration in the medium (mM) on Pi residual, net P_i, soluble, diffusion, uptake and multiplication rate (No. month^{-1}) of banana (*Musa acuminate* cv. Dwarf Cavendish) at week 8 growth

P_i supplied (*m*M)	P_i residual (based mg)	Net P_i (based mg)	P_i soluble (mg)	P_i diffusion (mg/day/30cm)	Total uptake (mg)
0.0	0.52	0.21	0.10	0.1	0.00
1.0	1.11	0.98	0.50	0.5	0.30
1.5	1.27	1.08	0.59	0.8	0.45
2.0	1.43	1.27	0.63	0.9	0.59
2.5	1.59	1.35	0.75	1.0	0.65
3.0	1.67	1.03	0.84	1.1	0.70
4.0	1.69	1.03	0.92	1.2	0.75
LSD= 0.05	0.48	0.65	0.28	0.23	0.42

weight and dry weight) was observed in the 2 *mM* Na_2HPO_4 treatment (Fig. 2). Whereas the most rooted explants were obtained in the low 1 *mM* Na_2HPO_4 treatment (data no shown). In other words, banana cultures preferred a low Na_2HPO_4 concentration for the formation and growth of roots. Root development was poor in 2 *mM* Na_2HPO_4 treatment. When concentration was lower (1mM Na_2HPO_4) the shoots rooted well but growth was inhibited and phosphorous deficiency was observed.

Uptake of Individual Minerals: Total uptake of minerals significantly (P=0.05) increased with Na_2HPO_4 up to 2mM, (except iron). Beyond 2mM, uptake decreased for all minerals except phosphorus which continued to increase up to 4mM Na_2HPO_4 at week 8 (Fig. 4). High (more than 2mM) Na_2HPO_4 applications increased significant (P=0.05) the concentration of leaf sodium, decreased the concentration of leaf potassium, but had no significant (P=0.05) effect on magnesium and sulfur. With the exception of phosphorus and sodium, the pattern of uptake of individual minerals in response to Na_2HPO_4 level parallels the growth response.

Plant Mineral Concentrations: The final concentration (% dry weight) of macro and micro minerals in the plant paralleled the uptake pattern increasing up to 2 *mM* Na_2HPO_4 then decreasing, except for phosphorus and sodium which continue to rise. Of the microminerals only iron showed a marked decline in concentration beyond 1 *mM* Na_2HPO_4 (Fig. 5). No relationship was found among leaf nutrient levels and growth quality.

DISCUSSION

This experiment examined some critical aspects of P_i availability and its uptake, which in turn affects banana growth *in vitro*. Diffusion coefficient in dilute solutions
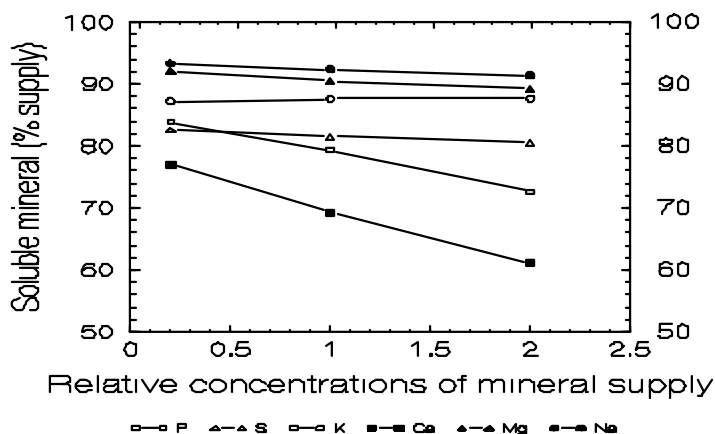
Fig. 1: Rate of availability of each macrominerals in the gelled medium as affected by four relative mineral concentrations (0X, 0.2X, 1X and 2X) after 1 day (30g/l/d). LSD (P=0.05)
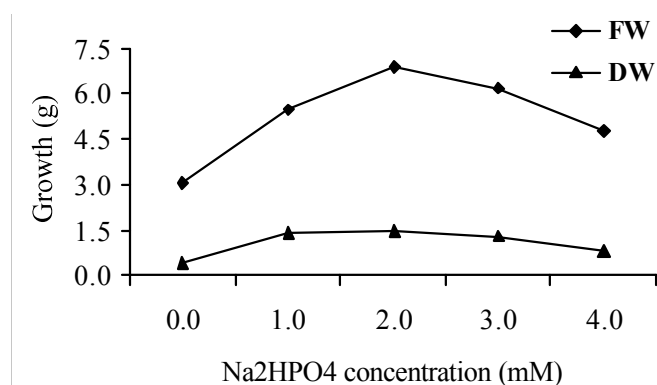

Fig. 2: Effect of Na_2HPO_4 concentration in the medium (mM) on FW (fresh weight) and DW (dry weight) as growth of four banana explant per vessel at week 8. LSD (P=0.05)
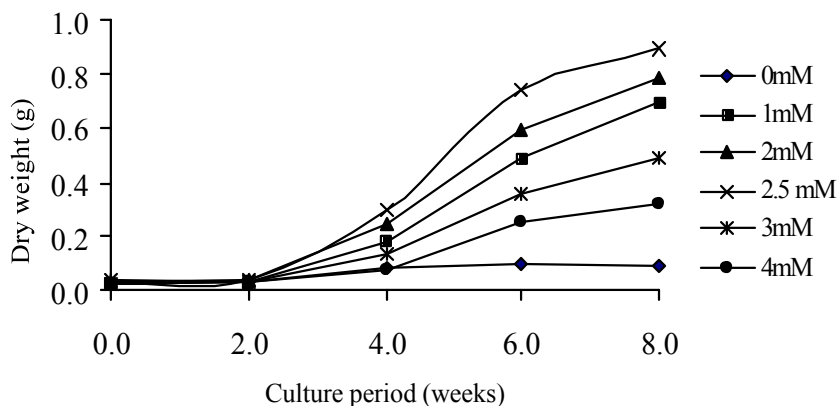

Fig. 3: Effect of Na_2HPO_4 concentration in the medium (mM) on dry weight of banana (Musa acuminate cv. Dwarf Cavendish) explant during 8 weeks of culture period. LSD (P=0.05)
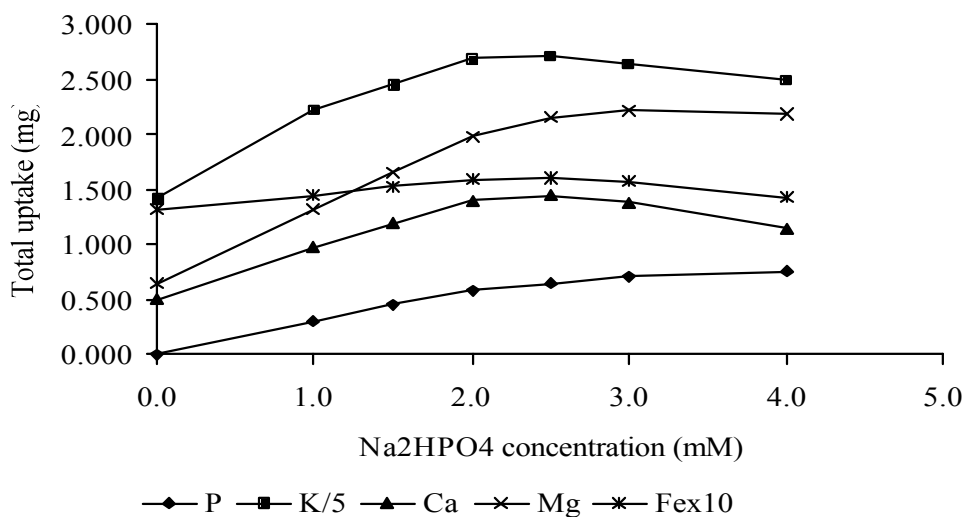
Fig. 4: Effect of Na_2HPO_4 concentration in the medium (mM) on uptake of each macro and micro minerals by four banana explant per vessel at week 8. LSD (P=0.05)
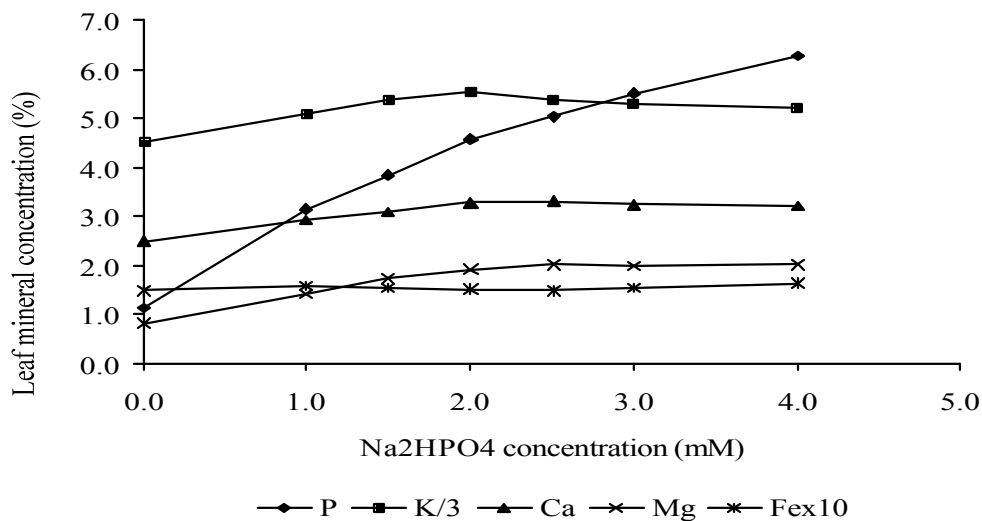

Fig. 5: Effect of Na_2HPO_4 concentration in the medium (mM) on concentration (%DW) of macro and micro minerals of banana explant at week 8. LSD (P=0.05)

can reasonably be taken as constant, whereas in gel it depends very markedly on concentration [30]. The diffusion coefficient which controls how fast P_i can diffuse, increases with increasing concentration and water content in the gel. The higher the water content, the greater the hydraulic conductivity, the greater the thermal conductivity and the more effective diffusion coefficients of solute in the gel. This is because $(HPO_4)^-$ as solute, heat and water itself all move by free water so diffusion becomes more free and direct as the water content increases. For example, Ackers and Steere [31] argued that the restricted diffusion coefficients in solution media are considerably lower than the free diffusion coefficients even in a 1% agar gel.

As the mineral concentrations increased, mineral diffusion increased, but the rate of diffusion of Ca^{++} and $(PO_4)^-$ was not directly proportional to their concentrations (Fig.1). This suggests that the rate of diffusion of $(PO_4)^-$ was less than other minerals. They could be fixed with the gel or precipitated with the other minerals, or low potential mobility of own ion. Fixation of $(PO_4)^-$ in gel can be regarded as a problem (become immobilized as diffusion proceeds, or as a problem in chemical kinetics in which the amount of $(PO_4)^-$ fixation depends on the amount of its supply) [32]. For example, Dalton et al, [14] found that 2 days after preparation of MS medium more than 13% of original P_i were precipitated and as the amount of P_i increased the amount of precipitation increased. It can be argued that chemical reactions of minerals within gel are often considerably dependent on the solubility and mobility of the reaction itself.

CONCLUSION

In vitro, if any ions transport by diffusion alone, it can be defined as available and no limitation. But P_i local depletion occurs, neither mass flow nor diffusion can supply the explant's P_i need. Thus, P defined as unavailable to explants. From this view point, P_i that adsorbed or precipitated becomes restricted and unavailable and other minerals are free (soluble) and they can transport become available.

ACKNOWLEDGMENTS

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