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SHORT COMMUNICATION

IN VITRO SELECTION OF HILL MAIZE (*ZEA MAYS* L.) HYBRIDS FOR LOW PHOSPHATE TOLERANCE

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Genotypic response of hill maize hybrids *viz.* Vivek-4, Vivek-5, VL-42 and Him-129 to low concentrations of phosphate (low-P) was studied *in vitro*. An induction in anthocyanin accumulation and increase in total protein content were observed with decreasing concentration of P in the medium. SDS-polyacrylamide gel electrophoresis of *in vitro* cultured plants showed the over expression of 4-6 polypeptides under P deficient levels than to sufficient. Thus, results thereby indicated that many proteins to be specifically synthesized *de novo* under Pi deficiency as part of the adaptive mechanism under low P condition. Amongst the four hybrids of maize Him-129 and Vivek-5 showed relatively good response in terms of anthocyanin accumulation in stem and higher total protein content along with activation of few extra new bands of polypeptides under P deficiency and so, proved their tolerance against low P stress.

***Key words*: *In vitro*, maize, phosphorus deficiency, SDS-electrophoresis, shoot tip culture**

Maize (*Zea mays* L.) is an important cereal crop which provides food for human beings, feed for animals and raw material for several agro-based industries. Due to low phosphorus (P) efficiency, high growth rate and large biomass production, it requires more P (Mujeeb *et al.* 2008). Heavy fertilization of P is a traditional approach to increase the crop production (Kleinman *et al.* 2002). In gross fertilizer consumption, India ranks fourth in the world, after the U.S., U.S.S.R. and China. Furthermore due to heavy fertilization, good quality phosphate ore are rapidly depleting and would last by 2060 (Oelkers and Valsami 2008). Further, erosion of Pi rich soil particles by surface water may reach into aquatic body. Increased Pi concentration in aquatic systems resulted in eutrophication and degradation of the environment. To anticipate phosphate crisis and inability of farmers to purchase expensive P fertilizers has threatened agricultural productivity and demands effective strategy to deal with this problem (Abelson 1999).

Wide variations were reported in different crops in their ability to uptake and utilize P referred to as ''genetic shortage'' (Ming *et al.* 2002). Generally, the Pi concentration in the soil, 10 µM or less, more so in hills, results in Pi starvation for plant growth and survival (Chen *et al.* 2009). Many morphological, biochemical and molecular changes in plants occur during P deficiency (Raghothama and Karthikeyan 2005). The enzyme of "bypass reactions" that circumvent Pi and adenylate requiring steps in glycolysis are also activated under Pi deficiency (Xu *et al.* 2004). Thus, a better understanding of limited factors of plants P nutrition and how plants sense and respond to phosphate deficiency

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may provide the knowledge and biotechnological tools to develop strategies for more efficient utilization of P by plants (Abel *et al.* 2002). Another strategy is selection and breeding of crops for P use efficiency that can reduce production costs, minimize environmental pollution and contribute to maintenance of world P resources globally (Zhang *et al.* 2009). Keeping the above facts in view, present study was mainly aimed to select low phosphate tolerant hill maize hybrid(s) *in vitro*.

In vitro shoot tip culture was carried out as described earlier (Joshi *et al.* 2009). Seeds of four hill hybrids namely Vivek-4, Vivek-5, VL-42 and Him-129 were obtained from V.P.K.A.S. (ICAR), Almora, and were surface sterilized in 0.1% $HgCl_2$ solution supplemented with 2-3 drops of detergent for 2 minutes and then washed 5 times with sterilized distilled water. To facilitate germination, seeds were placed in the MS medium in 450 ml glass bottles (Jam bottles) and were maintained at 28±2°C in B.O.D incubator.

For culture initiation, MS medium (Murashige and Skoog 1962) supplemented with 2 mg l^{-1} BAP and 500 mg l^{-1} casein hydrolysate along with 3% sucrose, 0.6% agar and pH adjusted to 5.8 (autoclaved at 15 psi for 20 minutes) was used. Five days old 3-5 cm long sections of shoot tips were excised from the 1st nodal region and placed horizontally in the medium (Fig.1a). Cultures were maintained in tissue culture chamber at 16 hr photoperiod, 150 µmol $m^{-2}s^{-1}$ light intensity and 25±2°C temperature.

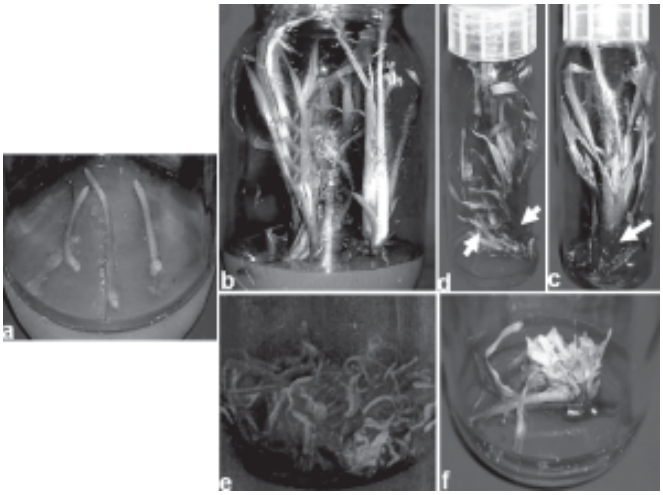

Fig. 1. *In vitro* shoot tip culture in maize. (a) Inoculation of 5 days old shoot tips induced on MS with 2 mg l^{-1} BAP and 500 mg l^{-1} casein hydrolysate (b) Shoot induction on MS with 2 mg l^{-1} BAP and 500 mg l^{-1} casein hydrolysate (c) Subculturing under MS with 35 mg l^{-1} phosphorus (d) Subculturing under MS with 30 mg l^{-1} phosphorus (e) Subculturing under MS with 20 mg l^{-1} phosphorus (f) Subculturing under MS with 10 mg l^{-1} phosphorus.

About 15-30 days old multiple shoots were taken and transferred to MS medium supplemented with different concentration of P (i.e. 35 mg l^{-1}, 30 mg l^{-1}, 20 mg l^{-1} and 10 mg l^{-1}) in the form of KH_2PO_4 (i.e. 153 mg l^{-1}, 131.8 mg l^{-1}, 87.9 mg l^{-1} and 43.95 mg l^{-1} respectively). The control was taken as 170.0 mg l^{-1} KH_2PO_4 (i.e. 38.68 mg l^{-1} P). They were subcultured biweekly and genotypic response was observed in terms of anthocyanin pigmentation and browning in leaves. The purple color of anthocyanin was quantified using following equation as described elsewhere (Yang *et al.* 2008).

$$\text{Total Anthocyanins} = A_{530} \text{ X dilution factor/ } 98.2$$

where, A_{530} was the absorbance in the diluted sample and dilution factor was 10. The factor 98.2 was the molar absorptive value for the acid-ethanol solvent.

To check the protein expression under different concentration of P, soluble proteins were extracted from 1 gm leaves following the method of Yun and Kaeppler (2001). The amount of protein was estimated by the Bradford method (Bradford 1976). Equal amount (25 µg) of soluble proteins was resolved on one-dimensional 10 % SDS-PAGE using a vertical slab gel system and the discontinuous system as described previously (Laemmli 1970).

An effective genotypic variation in anthocyanin concentration was observed under low P stress (Fig. 2). In P sufficient condition *viz.*, 38.68 mg l^{-1} P (170 mg l^{-1} KH_2PO_4) cultured explants of all hybrids showed good multiple shoot proliferation (Fig. 1b). In the MS medium containing 35 mg l^{-1} P (153 mg l^{-1} KH_2PO_4) multiple shoots showed good growth at par with control as in Him-129 and Vivek-5. However, slight reduction in growth was observed in VL-42 and Vivek-4. No browning or anthocyanin pigmentation was observed in any variety (Fig. 1c). In the MS medium containing 30 mg l^{-1} P (131.8 mg l^{-1} KH_2PO_4), poor multiple shoot proliferation and anthocyanin accumulation was observed in Him-129

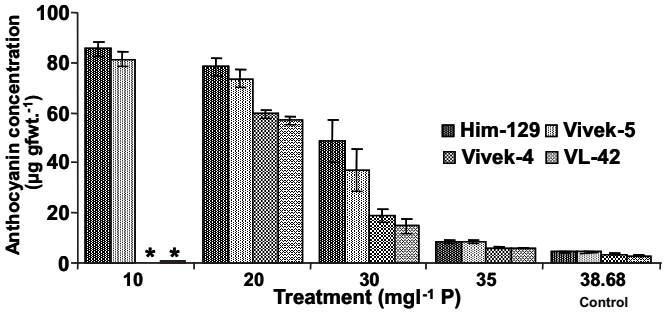

Fig. 2. Anthocyanin concentration in µg/ g frwt. of the multiple shoots of *Zea mays* cv. Him-129, VL-42, Vivek-4, Vivek-5. '*' indicates negligible values. Data are mean values of n=3 ±SD.

and Vivek-5. Poor growth and low anthocyanin accumulation was observed in VL-42 and Vivek-4 (Fig. 1d). In the MS medium containing 20 mg l^{-1} P (87.9 mg l^{-1} KH_2PO_4) poor growth and high anthocyanin accumulation was observed in Him-129 and Vivek-5. However, very poor growth, low anthocyanin pigmentation and browning were observed in VL-42 and Vivek-4 (Fig. 1e). In the MS medium containing 10 mg l^{-1} P (43.95 mg l^{-1} of KH_2PO_4) very poor shoot proliferation, high anthocyanin accumulation and browning of leaves were observed in Him-129 and Vivek-5. In contrast, severe browning was observed in VL-42 and Vivek-4 and the plants ultimately died (Fig. 1f).

P nutrition is one of the most significant factors for healthy growth of plants. Reports have confirmed that phosphate deprivation results in poor proliferation of aerial parts which becomes progressively greater with time and finally lead to accelerate the process of senescence (Usuda and Shimogawara 1992). Anthocyanins are generally glycosylated and therefore extremely soluble compounds usually found in vacuoles (Robinson 1991). This capability allows anthocyanins to bind and transport reactive monosaccharides produced during P starvation (Hammond and White 2008). Anthocyanins protect leaves from the stress of photoinhibitory light fluxes by absorbing the excess photons (Gould 2004). Anthocyanins are, in addition, excellent scavengers of free radicals, greater than those of ascorbate and á-tocopherol (Wang *et al.* 1997). Recently, it was reported that nuclear localized WRKY transcription factor resulted in impaired plant responses to P starvation and accumulation of anthocyanin (Chen *et al.* 2009).

Spectrophotometric analysis showed that total protein content was increased with the decrease in the amount of P in culture medium (Fig. 3). Highest amount of protein was observed in Him-129 followed by Vivek-5 and lowest amount of protein was observed in Vivek-4 followed by VL-42 under P deficiency. It was showed earlier that protein expressed under phosphate starved conditions indicates the extensive changes in gene expression and /or protein (Yan *et al.* 2001). Several proteins were found to be increased under phosphate starvation because of *de novo* synthesis of proteins (Raghothama 1999).

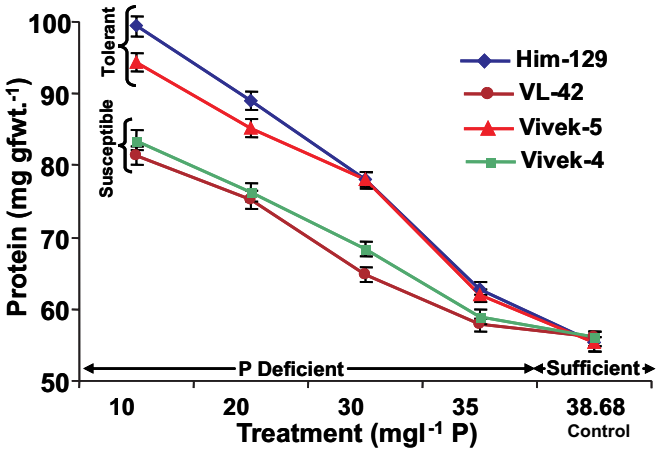

Fig. 3. Protein concentration in mg g^{-1} fwt. of the multiple shoots of *Zea mays* L. cv. Him-129, VL-42, Vivek-4 & Vivek-5. Data are mean values of n=3 ±SD.

As shown in Fig. 4, variations in electrophoretic protein banding patterns were analyzed under P sufficient (control) and deficient plants. Some specific bands with high relative intensity were detected in each pattern. At low concentration of P (i.e. 30 mg l^{-1}) four additional polypeptide bands between 10-15 kDa and one new band around 30 kDa were found over the control. Moreover, below 20 mg l^{-1} P additional four bands between 10-15 kDa, one band between 50-55 kDa and two bands between 80-85 kDa were found over the control. Similar banding pattern was observed at 10 mg l^{-1} P concentration. Some specific bands of high relative intensity were observed in each pattern suggesting that specific proteins were synthesized *de novo* in the P

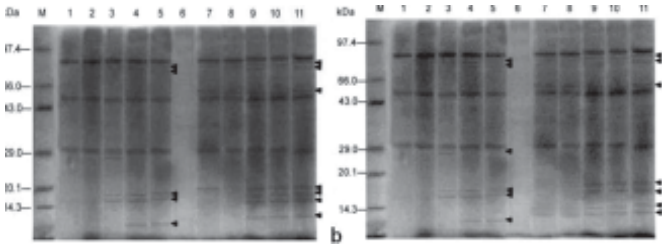

Fig. 4. SDS-PAGE of soluble protein in control and low phosphorus plants. (a) Lanes: 1, VL-42 Control; 2, 35 mg l^{-1} P; 3, 30 mg l^{-1} P; 4, 20 mg l^{-1} P; 5, 10 mg l^{-1} P; 6, Blank; 7 Him-129 Control; 8, 35 mg l^{-1} P; 9, 30 mg l^{-1} P; 10, 20 mg l^{-1} P; 11, 10 mg l^{-1} P (b) Lanes: 1, Vivek-4 Control; 2, 35 mg l^{-1} P; 3, 30 mg l^{-1} P; 4, 20 mg l^{-1} P; 5, 10 mg l^{-1} P; 6, Blank; 7 Vivek-5 Control; 8, 35 mg l^{-1} P; 9, 30 mg l^{-1} P; 10, 20 mg l^{-1} P; 11, 10 mg l^{-1} P.

deficient culture. Similarly, activated bands of polypeptides under P starvation were reported earlier in maize (Usuda and Shimogawara 1992) and *Brassica nigra* (Fife *et al.* 1990). Joh *et al.* (1998) reported that 47-52 kDa acid phosphatase subunits were expressed under P stress. Similarly 35-43 kDa intracellular and extracellular ATPase were activated under low P stress in maize (Yun and Kaeppler 2001). Intracellular ATPase accumulates in vacuoles and is thought to play a role in scavenging P for metabolic redistribution to improve the acquisition and redistribution of P, thus helping to promote growth under P deficient conditions. These reports showed that many genes specifically expressed under Pi-deficient conditions and their corresponding proteins may participate in the adaptive mechanism.

In conclusion, among four hill maize hybrids Him-129 and Vivek-5 showed relatively good response in terms of better shoot proliferation, purple coloration in stem, anthocyanin accumulation and higher total protein content along with appearance of a few extra new bands of polypeptides under P deficiency and so, proved their better performance under P limited conditions and were identified tolerant for low P stress.

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