

‘Tailoring the Plant to Fit the Soil’ in Stead of ‘Tailoring the Soil to Fit the Plant’ is an Alternate Environmental Sound Strategy to Acclimate Orthophosphate (Pi) Deprivation via Highly Coordinated Classical Pi-Starvation Induced Mechanisms

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Acclimation to orthophosphate (Pi) deprivation via highly coordinated Pi-starvation induced (PSI) classical mechanisms such as copious quantities of H⁺ and carboxylates (OAs) exudation, remodeling and modification of root architecture by increasing structural and functional plasticity, enhanced uptake rate and increased synthesis of Pi transporters would reduce or eliminate our current overreliance on expensive, polluting, and nonrenewable Pi-fertilizers. These complicated but elegant morphological, physiological, biochemical and molecular adjustments of Pi-starved plants provide an excellent example of how the unique flexibility of plant metabolism and energy transduction helps them to cope in a typically stressful environment. Pi-starved roots possess enhanced H⁺-ATPase and PEPCase which could result in increasing H⁺ efflux and OAs exudations in the root vicinity. This would lead to the rhizosphere acidification, which thereby contribute to the solubilization and assimilation of mineral Pi from environment. To visualize the dissolution of sparingly soluble Ca-phosphate and rhizospheric pH changes (*in situ*), genetically diverse *Brassica* cultivars were grown on agar media. Newly formed Ca-phosphate was suspended in agar containing other essential nutrients. With NH₄⁺ applied as the N source, the precipitate dissolved in the root vicinity and this was ascribed to acidification. No dissolution was occurred with NO₃⁻-nutrition. In order to observe the pH changes at the media-root interface (rhizosphere), an image analysis was carried out after embedding the roots in agar containing bromocresol purple as pH indicator. Efficient cultivar ‘Brown Raya’ showed greater decrease in pH than P-inefficient ‘B.S.A’ in the culture media. Hydroponically grown cultivars were compared with respect to P-utilization efficiency (PUE), P-stress factor (PSF), and Ca- and P-uptake at P-starvation. PUE, and Ca- and P-uptake correlated significantly ($P < 0.01$) with biomass accumulation, indicating that higher P-uptake of efficient cultivars was because of their higher Ca-uptake, which in turn was related to their better P-acquisition and PUE. Remodelling of root architecture of efficient cultivars helped the cultivars to establish a better rooting system, which provided basis for tolerance under P-starvation.

Key words: *Brassica*, *Bromocresol purple*, *H⁺-efflux*, *Rhizospheric pH changes and acidification*, *PUE*

1 INTRODUCTION

Plants are frequently phosphorus (P) starved due to extremely low soil bioavailability of this macronutrient (< 1 μM). A pH optimum for Pi uptake of 4.5–5.0 indicates preferential plant uptake of H₂PO₄⁻ over HPO₄²⁻ (Raghothama and Karthikeyan, 2005; Vance et al., 2003). Although total soil P content typically varies from 500 to 2000 ppm, total bioavailable P, as measured by soil extractants may be only a few ppm. Phosphorous is one of the least available, least mobile, mineral nutrients to the plants in many cropping environments, based on its contribution to the biomass as a macronutrient (Goldstein et

al., 1988; Raghothama and Karthikeyan, 2005). Many soils have large reserves of total P, often hundred-time more than the P available to the crops (Shenoy and Kalagudi, 2005). P is needed in metabolic processes such as energy transfer, signal transduction, macro-molecular biosynthesis, photosynthesis, respiration, etc. Therefore, direct availability of P determines the plant growth. Sub-optimal levels of P can lead to yield losses to the tune of 5% to 15% of the maximal yields. P deficiency is more critical in highly withered soils of tropics and subtropics (2 billion ha), as well as calcareous/alkaline soils of Mediterranean basin (Hinsinger, 2001) and limit plant growth affecting more than 5.7 billion ha worldwide (Vance et al., 2003) due to absolute P-deficiency in soils or fixation of P-fertilizer in form of hardly-soluble Fe- or Al- or Ca-phosphate complexes. Attempts at amelioration of this situation by additional P fertilizing is becoming an increasingly uneconomical and ecologically unsound

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practice, as the efficiency of the added P fertilizer is as low as about 10% (Werft and Dekkers, 1996). Moreover, water run-off, soil erosion and leakage in highly fertilized soils of industrialized countries may cause environmental problems such as eutrophication and hypoxia of lakes, rivers and marine estuaries. This has led to a search for more environment-friendly and economically feasible strategies for improving crop production in low P soils. In ideal situations, such strategies should enable the efficient use of P already present in the soil, by the cultivars.

Attempts to improve mineral phosphate solubilization (MPS) by enhancing capabilities of MPS rhizospheric bacteria have not been particularly successful because of limitations such as poor ecological fitness, low metabolite production, variability in inoculant-delivery systems, and inconsistent performance in field applications (Mark et al., 2003). An alternate strategy is to enhance the P use efficiency (PUE) of the plants. The latter strategy necessitates identification and deployment of plant traits that limit or enhance the uptake and utilization of P (Narang et al., 2000). Plants that are efficient in absorption and utilization of the absorbed nutrients greatly enhance the efficiency of applied fertilizers. A more comprehensive understanding of the morphological, physiological and molecular basis of mineral nutrient uptake and utilization in plants is leading to strategies for development of better nutrient-efficient cultivars suited for optimal production with less fertilizer inputs. Adaptation of such cultivars with higher nutrient use efficiency is relatively easy, since no additional costs are involved, and no major changes in cropping systems are necessary. Also, nutrient efficient varieties contribute to sustainability in many other ways. They have a greater degree of disease resistance (thereby reducing the pesticide usage) due to enhanced membrane function and cell integrity, a greater ability to develop deep roots to penetrate sub-soil in infertile soils and greater seedling vigor which in turn gives higher seed yields (Graham and Welch, 1996). According to Gerloff (1977) cultivars can be classified as P efficient (higher yielding than other cultivars under low P supply) and/or responsive (higher yielding than other cultivars under high P supply). Current breeding strategies allow selecting for P responsiveness but not for P use efficiency (PUE), therefore, when selection for PUE is an important breeding objective, breeders will have to look at alternative breeding strategies to meet this objective (Ortiz-Monasterio et al., 2002). PUE is the ability of crop cultivars to function well under low available-P concentrations. According to an estimate, the PUE can be enhanced by 25%, employing the current knowledge about the PUE of crop species (Werft and Dekkers, 1996). To increase PUE, its component traits such as P-acquisition (absorption/uptake), translocation (transport/partitioning/remobilization), and internal utilization must be improved. Inter- and intra-specific variations for these traits are known to be under genetic and physiological control, but modified by the plant-environment interactions. In order to improve PUE, it is

important to explore genetic variation for all the associated traits. Plants growing in apparently infertile environments are often classified as 'efficient' rather than being viewed in the ecological context as the plant best capable of utilizing the available resources.

Changes of pH in the rhizosphere (the volume of soil around the roots that is influenced by the activity of plant roots), are by far the best documented chemical interaction occurring at the soil-root surface. Plant species and cultivars can substantially change their rhizospheric pH by releasing H^+ or OH^- to compensate for an unbalanced cation-anion uptake at the soil root interface. Various origins of pH changes are (i) cation-anion balance, (ii) carboxylates/organic acids (OAs) release, (iii) root exudation and respiration, and (iv) redox-coupled processes. Enhanced H^+ and OAs exudations are crucial biochemical adjustments as PSI response to enhance the solubilization and acquisition of sparingly soluble P-sources [rock-P or $Ca_3(PO_4)_2$] or insoluble inorganic P-complexes.

The dissection of the mechanisms underlying the nutrient acquisition, transportation, assimilation, utilization and remobilization under the shady umbrella of membrane biophysics, molecular biology and plant physiology will help to understand the scavenging of Pi from unavailable bound P reserves. With similar objectives, genetically diverse *Brassica* cultivars were compared to elucidate classical plant traits contributing to P-acquisition and PUE as PSI response.

2 MATERIALS AND METHODS

2.1 *In situ* P-starvation induced (PSI) dissolution of Ca-phosphate precipitate in root vicinity and pH stat experiments

2.1.1 Plant material, preparation of the agar medium and culture conditions

The tested cultivars were genetically diverse with respect to P-acquisition and P-use efficiency (PUE) for biomass synthesis and grain yield and categorized as P-efficient ['Brown Raya' and 'Con-1'] and P-inefficient ['B.S.A'] cultivars on the basis of their P-acquisition and PUE from sparingly soluble P-sources [rock-P and $Ca_3(PO_4)_2$] under P-stress environment in an earlier study (Akhtar et al., 2006ab). Ten grams of agar was dissolved in 900 ml demineralized water by heating. After cooling the solution to no lower than 50 °C nutrient media containing either NH_4^+ -N or NO_3^- -N were prepared. The NH_4^+ -medium was supplemented with the following nutrient salts (mM): $CaCl_2$ [8], K_2HPO_4 [2], $NH_4H_2PO_4$ [4], $MgSO_4 \cdot H_2O$ [0.5], and [in μM]: Fe(III)-EDTA (Ferric Dihydrogen Ethylene Diamine Tetra-acetic Acid) [50], H_3BO_3 [25], $MnSO_4 \cdot H_2O$ [2], $ZnSO_4 \cdot 7H_2O$ [2], $CuSO_4 \cdot 5H_2O$ [0.5], KCl [50], H_2MoO_4 [0.5]. After the addition of K_2HPO_4 to the $CaCl_2$ solution a white precipitate of Ca-phosphate was formed. Then the pH was adjusted with 1 N NaOH to 6.1. The medium was made up

to 1 litre, and one part of media was modified containing adequate/sufficient level of P (200 μM P) and supplemented with 60 ppm bromocresol purple indicator. Bromocresol purple is purple at pH 6.8 and above and turns yellow at pH 5.2. The NO_3^- -medium contained 2 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, but only 6 mM CaCl_2 and 4 mM NaH_2PO_4 instead of $\text{NH}_4\text{H}_2\text{PO}_4$. The medium also contained a white precipitate of calcium phosphate and was adjusted to pH 6.1. For the observation of pH changes, apart from the before mentioned media, an additional set of media containing deficient/stress level of P (20 μM P) was also prepared. All sets of media were kept liquid at 50 $^\circ\text{C}$ in a water bath. 75 ml of the medium, which was stirred to prevent the precipitate from settling, were poured into plastic Petri dishes (10 cm diameter). Seeds of *Brassica* cultivars were soaked for half a day in water and then placed in the Petri dishes near the edge, where a slot allowed the shoot to grow out of the dish. The covers of the dishes, also supplied with a slot at the edge, were held on with tape. The dishes were set on a slope, so that the roots grew downwards, mainly in the agar medium and less on its surface. The plantlets were kept under controlled conditions. The culture conditions were as follows: temperature 25 $^\circ\text{C}$; light intensity 40 $\mu\text{mol m}^{-2} \text{S}^{-1}$; relative humidity 50%; light/dark 14/10 hr. Petri dishes were wrapped with non-transparent foil to prevent the growth of algae.

2.1.2 Observation of P-deprivation induced pH changes and mobilization of calcium phosphate in root vicinity and root image analysis

P-stress induced pH changes were compared in hydroponically grown cultivars with differing ability to mobilize sparingly soluble P sources [rock-P and $\text{Ca}_3(\text{PO}_4)_2$] in an earlier study (Akhtar et al., 2006a). However, *in vivo* P-starvation induced root mediated pH changes (appearance of typical patterns along the roots of the various colors of the pH indicator) and dissolution of newly formed calcium phosphate was visualized and their images were recorded in the present study (Fig. 1). Biochemical adaptations occurring in root involve extrusion of H^+ and carboxylates (OAs) into rhizosphere via plasmalemma H^+ ATPase and anion channels [chloride channels (CLC), and nutridrug and toxin extrusion proteins (MATE proteins)], respectively. Release of H^+ and carboxylates decrease rhizospheric pH and can mediate the solubilization and acquisition of unavailable bound P forms especially under alkaline calcareous environments.

2.2 P-acquisition and internal P-utilization efficiency of genetically diverse *Brassica* cultivars

2.2.1 Plant material, growth conditions and biomass assay

Different cultivars tested were; 'Brown Raya', 'Con-1', 'Toria' and 'B.S.A'. Seeds were germinated in polyethylene lined iron trays containing pre-washed riverbed sand and irrigated with distilled water for seed germination and seedling establishment. Seven day old uniform sized seedlings were transplanted in foam plugged holes in thermopal sheets floating on continuously aerated 200-L

half strength modified Johnson's solution (Johnson et al., 1957) in 2 polyethylene lined tubs (1 x 1 x 0.3 m) on day zero after transplanting (DAT=0). Plants were maintained in each tub by randomly repeating ten plants of each cultivar in equally spaced numbered holes. The solutions were modified to maintain deficient (20 μM) and adequate (200 μM) P levels using $\text{NH}_4\text{H}_2\text{PO}_4$. The pH of the solution was monitored daily and maintained at 5.5 ± 0.5 . The solution was renewed every 5 days to maintain concentrations being exhausted because of plant uptake.

2.2.2 P-utilization efficiency (PUE), P-stress factor (PSF), and Ca-and P-uptake

Plants were cultured to classify cultivars for PUE and biomass accumulation. On 25 DAT, four plants of each cultivar were harvested while remaining six plants of each cultivar were harvested on 31 DAT. Plants of each harvest were immediately washed in distilled water and blotted dry using filter paper sheets. After separating the plants into roots, shoots and stems, samples were immediately put in craft paper bags and air dried in the laboratory for twelve hours before oven drying. Samples were dried at 70 $^\circ\text{C}$ for 48 hours in a forced air oven and then weighed. Dried samples were cut into pieces and finely ground in a Wiley Mill to pass through a 1mm screen (20 mesh). Uniform samples were then digested in HCl acid after dry ashing. P-concentrations (mg P g^{-1} dry matter) were measured by the vanadate-molybdate yellow color method (Chapman and Pratt, 1961) using a UV-visible spectrophotometer. Data presented here is of plants harvested at 31 DAT. P-uptake (mg plant^{-1}) was calculated on root and shoot bases by multiplying P-concentration in the respective tissue with its dry matter, and on a whole plant basis by adding the two. Relative reduction in shoot dry matter (SDM) due to P-stress (PSF) was determined on percent basis for each cultivar at each harvest using the following expression.

$$\text{PSF (\%)} = \frac{\text{SDM}_{\text{adequate P}} - \text{SDM}_{\text{deficient P}}}{\text{SDM}_{\text{adequate P}}} \times 100$$

PUE of the cultivars was determined at each harvest using the following formula.

$$\text{PUE (g}^2 \text{SDM mg}^{-1} \text{shoot-P)} = \frac{\text{SDM (g plant}^{-1})}{\text{Shoot P concentration (mg g}^{-1})}$$

Ca-concentration (mg g^{-1}) is determined by atomic absorption spectrophotometry.

2.3 Experimental set up, carboxylates exudation sampling technique and observation of PSI modification in root architecture

Full experimental details are described in an earlier study (Akhtar et al., 2006a). A brief summary is provided here. As mentioned earlier, the cultivars used in this study were genetically diverse with respect to PUE for biomass synthesis and categorized into 2 groups (Akhtar et al., 2006ab) on the basis of their PUE under P-stress environment. Group II cultivars (Non-efficient): 'B.S.A' and 'Toria', and group I cultivars (Efficient): 'Brown

Raya' and 'Con-1'. The seeds were sown on moist river sand and germinated in a dark chamber at 25°C. six-day-old, uniformly sized seedlings were transferred to a complete nutrient solution for 7 days using KH_2PO_4 as a P-source. The pH of the solution was daily monitored and maintained at 5.5 ± 0.5 . The solution was renewed every 3 days in order to maintain the nutrients exhausted by plant uptake. The seedlings were grown in a cultivation chamber (CFH-405; Tomy Seiko Co., Tokyo, Japan; Fig. 4A,C) at a cycle of 14 h/20°C night and 10 h/25°C day and a light intensity of $40 \mu\text{mol m}^{-2} \text{S}^{-1}$ (approximately 3800 lx). The relative humidity of the chamber was adjusted to 60 %. After 7 days in a complete solution, the seedlings were transferred to an aerated solution with (+P) or without (-P) 0.20 mM P in 3.5-L pots. Each cultivar was transplanted in three pots maintaining three plants per pot using factorial completely randomized design (CRD) and each set of experiments was repeated at least twice. The plants were grown for an additional 12 days in cultivation chamber. Plant roots were washed with deionized water and then submerged in 300 ml of aerated solution with 0.5 mM CaCl_2 (pH = 5.5) for 4 h and 8 h, respectively and root exudates were collected for organic anions (OAs).

The solution was evaporated to about 5 ml under reduced pressure at 45°C on a rotary evaporator (Vacuum controller NVC-1100, Eyela, Tokyo Rikakikai Co., Ltd., Japan). Solution containing root exudates was allowed to pass first through a cation exchange column (16 mm x 14 cm) filled with 5 g Dowex 50 W x 8 (100-200, H^+ form) resin (Muromachi Kagaku Kogyo Co., Ltd, Tokyo, Japan) and then through an anion exchange column filled with 2 g Dowex 1 x 8 resin (0.15-0.06 mm, Cl^- form) without adjusting the pH as shown in Fig. 4B. This procedure was carried out under non-sterile conditions and the OAs retained in the anion exchange resin were eluted with 8N formic acid. The eluent was concentrated to dryness under reduced pressure using a rotary evaporator. The residue was re-dissolved in 1 ml ultra pure water adjusted to pH 2.1 with HClO_4 and filtered with 0.45 μm filter. The OAs were detected by HPLC (LC-6A, Shimadzu, Kyoto, Japan) equipped with the ion-exclusion column Chemoo-pack Nucleosil 5C18, 4.6x250 (6 A), Japan). Results of carboxylates are presented earlier (Akhtar et al., 2006a). Images were recorded to compare PSI modification in root architecture as presented in Fig. 4D. To confirm the PSI remodeling in root architecture, another experiment was carried out under controlled conditions (Fig. 4E) and root images were recorded (Fig. 4F).

2.4 Statistical Analysis

Data were subjected to statistical analyses according to standard procedures (Steel & Torrie, 1980) using 'MSTAT-C' computer program and the methods described by Gomez and Gomez (1984). Treatment means were separated using Duncan's Multiple Range Test (DMRT). Correlation coefficient (r) values were determined among various parameters using treatment means. $P < 0.01$ was considered statistically significant.

3 RESULTS AND DISCUSSION

3.1 Demonstration of dissolution of calcium phosphate in the root vicinity

Soon after the roots started to grow, the root vicinity became translucent in the NH_4^+ -containing agar substrate, at first in the basal parts. Evidence for the dissolution of the Ca-phosphate was obtained after only 3 days, and the transparent zone extended downwards as plant growth continued. In Fig. 1A, dissolution of the precipitated Ca-phosphate is clearly visible around roots of *Brassica* cultivar 'Con-1' after 8 days of growth. This visible evidence for the dissolution of sparingly soluble Ca-phosphate around plant roots is due to the acidification of rhizosphere by root exuded protons. Dissolution of Ca-phosphate was observed when plants were fed with NH_4^+ as an N source, but not with NO_3^- . In the NO_3^- medium, root growth was faster than in the NH_4^+ -medium but no dissolution of calcium phosphate was observed. It is well established that forms of N has the most important influence on rhizosphere pH. Quantitatively, net ion fluxes such as uptake of inorganic cations and anions, extrusion or (sometimes) uptake of H^+ and carboxylates exudation are involved in plant growth. Extra cellular pH changes and intracellular pH stabilization is affected by cation/anion uptake and N assimilation, and internal charge balance involves the accumulation of inorganic and organic (carboxylates) anions. The driving force for nutrient uptake by root cells is H^+ extrusion, mediated by the activity of a plasma membrane bound H^+ pumping ATPase, which creates an outward positive gradient in electro potential and pH between the cytosol (pH 7-7.5) and the apoplast (pH 5-6). This electrochemical potential gradient provides the energization for anion uptake by proton-anion cotransport and for cation uptake via uniport or proton-cation countertransport. Excess uptake of anions over cations therefore leads to net removal of $\text{OH}^-/\text{HCO}_3^-$ in the rhizosphere and to an increase in rhizosphere pH. NO_3^- -nutrition (excess uptake of anions over cations) plus NO_3^- reduction and assimilation ($3\text{NO}_3^- \longrightarrow 2\text{OH}^-$) promoting OH^- release and an increase in rhizospheric pH. An increase of the rhizosphere pH mediated by NO_3^- -fertilization may enhance P availability either by lignad exchange of HCO_3^- with P absorbed to Fe and Al oxides and of practical significance under acidic conditions. In contrast, excessive uptake of cations is charge balanced by a net release of H^+ and consequently leads to rhizosphere acidification as outlined in Fig. 2. NH_4^+ -nutrition (excess uptake of cations over anions) plus NH_4^+ assimilation by root tissues ($3\text{NH}_4^+ \longrightarrow 4\text{H}^+$) leads to enhanced net H^+ extrusion and decrease in rhizospheric pH. In neutral to alkaline soils, NH_4^+ induced rhizosphere acidification can increase the availability of P out of acid soluble Ca-phosphate. Uptake of inorganic cations, especially K^+ , can be high in NO_3^- -nutrition where charge balance during N assimilation results in accumulation of carboxylate anions, such as malate or citrate. The alternative strategy for NO_3^-

uptake by plants involves net influx of H^+ , equivalent to OH^-/HCO_3^- release, and relatively low concentrations of carboxylate anions and inorganic cations such as K^+ . During NH_4^+ -nutrition, N assimilation results in large scale production and H^+ extrusion and in biosynthesis of relatively lower concentrations of carboxylates and inorganic anions as outlined in Fig. 2. Apart from these processes that are directly associated with N assimilation, carboxylates accumulation can take place with 'excess cation influx', i.e. exchange of cations such as K^+ for H^+ . All these processes collectively form the basis of internal

electric charge balance and biophysical (membrane transport) and biochemical pH stat. Superimposed on them, there can also be extrusion of OAs, i.e. carboxylates plus H^+ extrusion, as reported in an earlier study (Akhtar et al., 2006a). Because most P_i is transported as $H_2PO_4^-$, cotransport involves a cation. Acidification of cytoplasm which occurs upon P_i -addition to P-deficient cells suggests H^+ is the cotransport product for the vast majority of plants. Ample quantities of OAs and H^+ are exuded to solubilize bound P from inorganic and organic complexes.

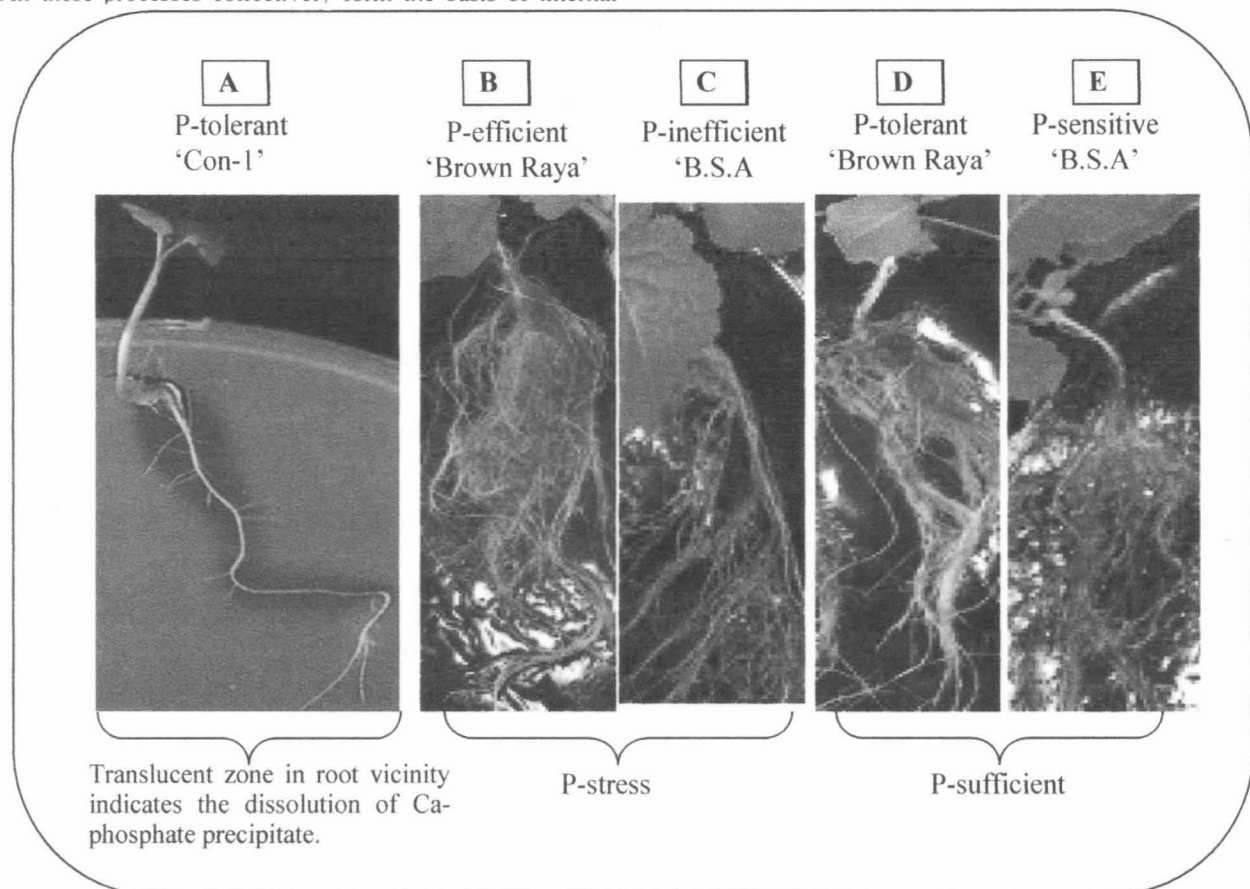


Fig. 1 (A) P-tolerant *Brassica* cultivar 'Con-1', 8-day-old, growing in agar media containing calcium phosphate precipitate and nutrients including NH_4^+ as an N source. Translucent zone in root vicinity indicates the dissolution of Ca-phosphate precipitate. (B, C, D, E) comparison of the rhizospheric pH of two genetically diverse *Brassica* cultivars, growing in agar media containing calcium phosphate precipitate plus nutrients and additionally supplemented with 60 ppm bromocresol purple (pH indicator). The original purple colour of the medium turned yellow in translucent areas, indicating a decrease in pH due to H^+ efflux in the root vicinity.

3.2 Observation of P-starvation induced pH changes in the root vicinity

When the agar medium contained bromocresol purple in the vicinity of the roots turned yellow, indicating acidification of the rhizosphere in the NH_4^+ -medium (Fig. 1B,C,D,E). The rhizospheric pH change can be directly demonstrated by embedding roots in agar with an indicator such as bromocresol purple. The indicator had no harmful effects on the growing roots at the concentration used. Cultivars and P-levels showed significant differences in rhizospheric acidification and at stress P-level this

acidification was more prominent. The efficient cultivar 'Brown Raya' showed a greater decrease in the rhizospheric pH than P-inefficient 'B.S.A' cultivar (Fig. 1B,C,D,E). It can be assumed on the basis of this observation that H^+ -efflux is one of the strategies adopted by *Brassica* cultivars to increase P-availability in the rooting media.

H^+ release into the rhizosphere is a common response of plant cells to counter intracellular acidity. Generally, H^+ release results from the activity of a plasma membrane H^+ -ATPase. This enzyme uses ATP to pump H^+ out of

***P*-deprivation induced root mediated changes in rhizosphere chemistry**

Cation uptake > anion uptake

(NH_4^+ nutrition; N_2 fixation, nutrient deficiency e.g. P)

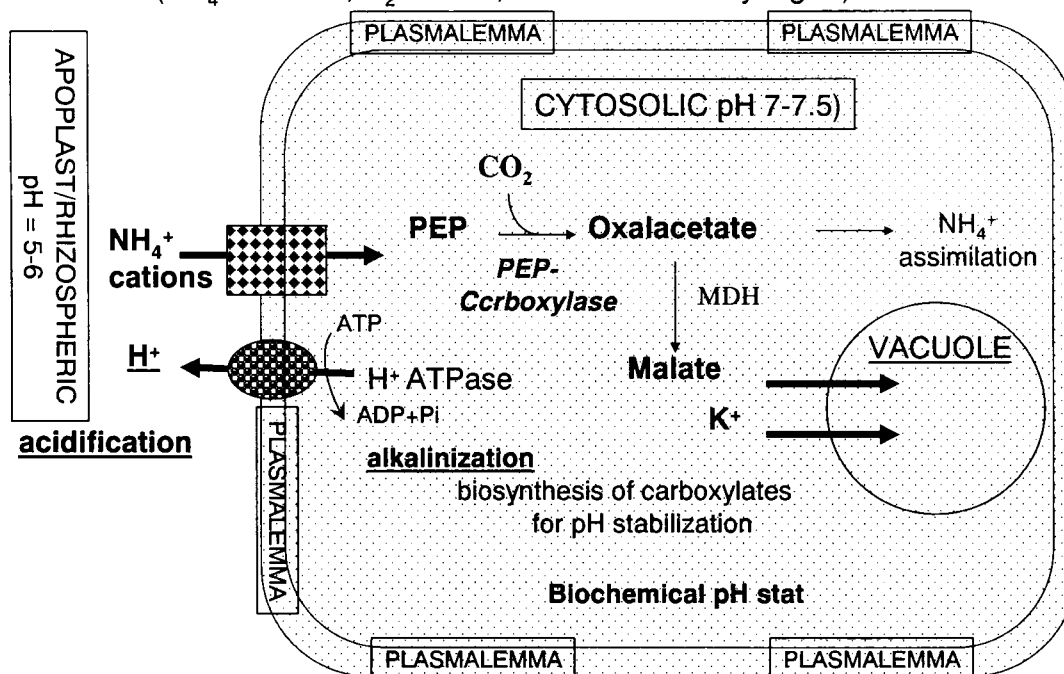


Fig. 2 Schematic representation for extracellular pH changes and intracellular pH stabilization as affected by cation uptake > anion uptake, N assimilation and P-starvation induced inhibition of nitrate uptake associated with high inherent uptake rates for cations.

cell, thereby creating pH and electrical potential differences across the plasmalemma. Yan et al. (2002) reported that enhanced catalytic activity of a plasma membrane H^+ -ATPase of P-stressed cluster roots in white lupin that might be responsible for the increase of H^+ -extrusion. Only the youngest P-deficient cluster roots had significant H^+ extrusion and H^+ -ATPase activity. It is noteworthy that Sakano (2001) addresses the potentially problematic generation of H^+ during OA-synthesis via glycolysis in his revised pH-stat hypothesis of the plant cell. By contrast to the 'feed-forward' fashion of the nonplant system, plant glycolysis is regulated by a feedback process. This feedback regulation only permits glycolytic activity when the cytoplasm is alkaline enough to stimulate PEPC, an enzyme with an alkaline pH optimum (Sakano, 2001). As Sakano pointed out that active H^+ extrusion is a possible response of the plant cell to prevent the inhibiting effect of cytosolic acidification.

H^+ extrusion and carboxylates exudation are highly dependent on P supply. Sas et al. (2001) reported that on an equimolar basis, H^+ in P-deficient plants was 2-3 folds greater than carboxylate exudation and different mechanisms are involved in H^+ release and OAs exudation. Neumann et al. (1999) showed that exposure of P-stress induced cluster roots to the anion channel-blockers ethacrynic- and anthracene-9-carboxylic acids inhibited citrate exudation by 40–60%. Vance et al. (2003) reported

that through *in situ* hybridization, PEPC and MDH transcripts are localized to cluster root apices and elongation zones, indicating the enzymes necessary for malate and perhaps citrate synthesis occur in the root tips. This data coupled with that of Neumann et al. (1999) suggest that P-starvation induced rapid efflux of OAs, similar to Al_3^+ -tolerance, may involve selective anion channel proteins at sites of exudation. Since malate and citrate are fully dissociated in the cytosol and because cell membranes are in essence impermeable to ions, it is not surprising that the release of OAs from root tips in either P-stress or Al toxicity might involve some type of channel proteins.

Irrespective of N source, P-deprivation induced rhizospheric acidification can increase the P availability in neutral and alkaline calcareous soils. Inhibition of NO_3^- uptake as a response of P-stress associated with high inherent uptake rates for Ca^{2+} and Mg^{2+} may finally lead to excess uptake of cations, compensated by enhanced net extrusion of H^+ . Protons are provided by increased biosynthesis of OAs via nonphotosynthetic CO_2 fixation by PEP carboxylase in the root tissue. The remaining OAs may be stored in the vacuoles of the root tissue, translocated to shoot, or released into the rhizosphere with H^+ or K^+ as counterions. Carboxylate exudation charge balanced by H^+ extrusion may additionally contribute to rhizosphere acidification.

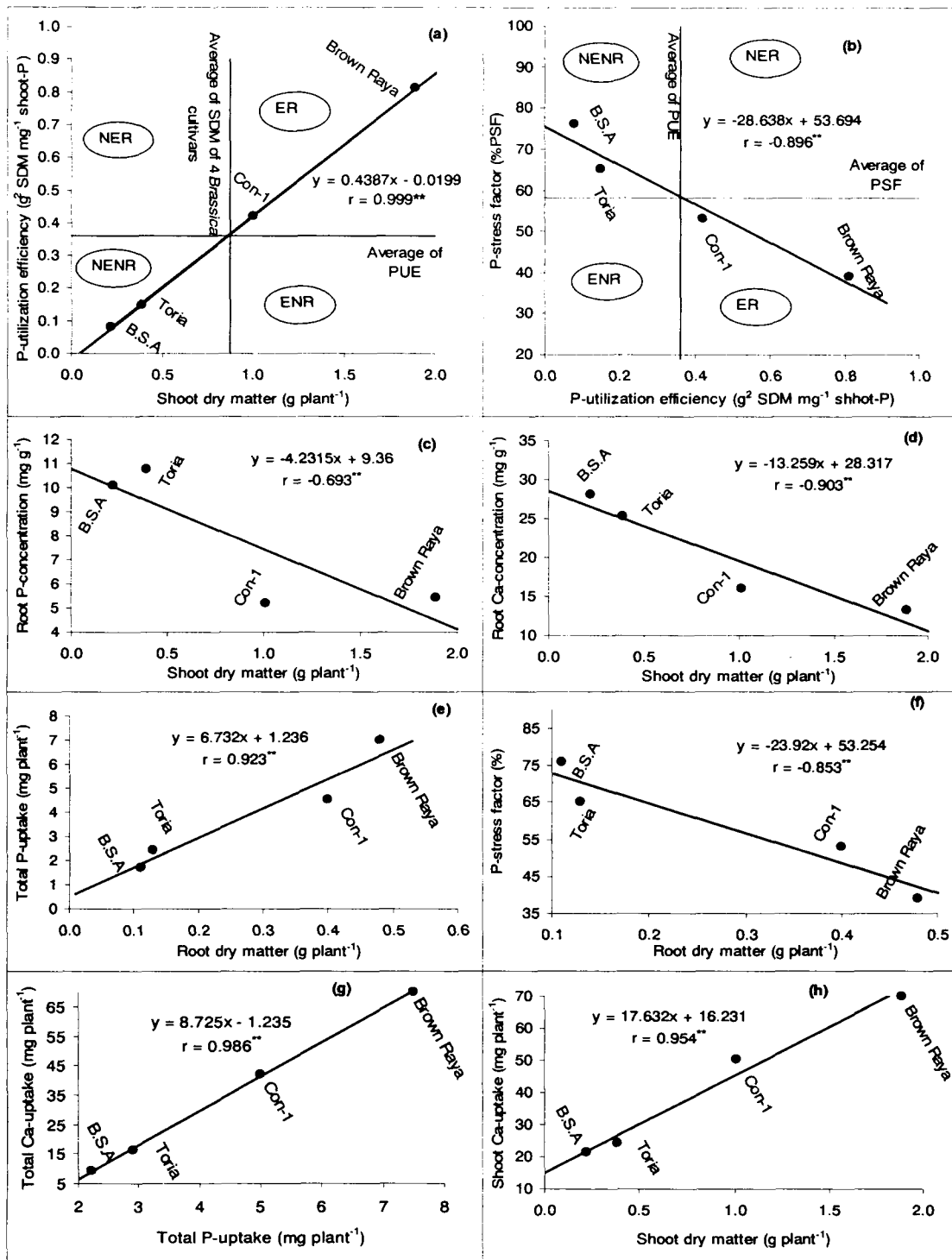


Fig. 3 (a) Ordination plot to classify cultivars for P-utilization efficiency (PUE) as a function of shoot dry matter (b) P-stress factor and (c, d, e, f, g & h) relationship between various growth parameters and biomass accumulation of four *Brassica* cultivars at stress P. ER: Efficient and Responsive; NER: Non-efficient but Responsive; ENR: Efficient but non-responsive; NENR: Non-efficient and non-responsive. ** = Significant at P = 0.01.

3.3 P-utilization efficiency (PUE), P-stress factor (% PSF), and P-and Ca-uptake

The cultivars can be categorized as efficient-responsive 'ER' ('Brown Raya' & 'Con-1') to non-efficient-non-

responsive 'NENR' ('Torja' & 'B.S.A'), while there was no cultivar that could be characterized as efficient-non-responsive 'ENR' and non-efficient-responsive 'NER' in this study (Fig. 3a). The synthesis of biomass per unit of

absorbed P (PUE) under P-stress conditions is a useful criterion for assessing tolerance of cultivars to P-deficiency stress (Shenoy and Kalagudi, 2005; Egle et al., 1999). Statistically significant differences ($P < 0.01$) of PUE under both the conditions of P supply indicated variability among tested cultivars to utilize absorbed P for biomass synthesis. A significant negative correlation existed between P-stress factor (%PSF) and PUE of the cultivars ($r = -0.896^{**}$; Fig. 3b) observed under low P supply implying that the cultivars which were inefficient utilizers of the absorbed P under P-stress were more sensitive to such conditions. Thus the parameter seems to be a useful index of P-stress tolerance of *Brassica* cultivars. The cultivars such as 'Brown Raya', and 'Con-1', which produced higher biomass under P-stress due to their higher PUE may prove useful where economic constraints restrict fertilizer application.

The maximum PUE was observed in 'Brown Raya' and minimum was in 'Sultan Raya' as former produced maximum SDM per unit P-uptake and later produced the lowest. PUE is the ability of the plant to grow and yield well at suboptimal P-availability situation. Cultivars grouped 'ER' ('Brown Raya' and 'Con-1') are the most desirable as per their performance at both P sources in the culture media (Fig. 3a). The most undesirable cultivars are the 'NENR' type ('Toria' & 'B.S.A') as per their poor performance even at adequate (+P) P supply. P-uptake and RDM of 'NENR' cultivars were inferior to 'ER' cultivars. P-concentration was almost similar or higher in 'NENR' cultivars than 'ER' cultivars. This means the greater P-efficiency was due to PUE rather than to differences in P-concentration. P-concentration and uptake in shoot had a highly significant and positive correlation with RDM and SDM suggesting that the cultivars with higher RDM accumulated higher amount of shoot P and produced higher SDM at stress P. Thus, under P-stress, better P-acquisition and PUE by the efficient cultivars for biomass synthesis collectively formed the basis of higher SDM production, evidencing that P-uptake and PUE are important plant traits for selecting low P tolerant cultivars. The cultivars having lower P-concentration in their roots (implying a smooth translocation of absorbed P to above ground parts) produced higher SDM ($r = -0.693^*$) at low P (Fig. 3c). Plants exposed to P-stress retained more P in their roots than shoots (Adu-Gyamfi et al., 1990) similarly to Gill and Ahmad (2003) who reported that efficient cultivars retained a relatively larger amount of stressed element in their roots, in a bid to develop a more efficient root system. Total P-uptake had a highly significant ($p < 0.01$) positive correlation with RDM (Fig. 3e) and SDM at stress P, suggesting that the cultivars with higher RDM accumulated higher amounts of P similar to Machado and Furlani (2004), who reported a positive correlation between SDM, RDM and P-uptake. A strong negative correlation (-0.853^{**}) existed between PSF and RDM of cultivars (Fig. 3f).

Negative correlation existed between root Ca-concentration and SDM of the cultivars ($r = -0.90^{**}$; Fig. 3d), whereas, the impact of root Ca-concentration was

positive on root P-concentration ($r = 0.85^{**}$). Cultivar differences were statistically significant for Ca-uptake on shoot, root as well as whole plant basis. The cultivars that had taken up higher Ca in their shoots also showed higher shoot P-uptake and were able to produce more SDM (Fig. 3g,h). Both Ca and P-concentrations of roots of cultivars had negative impact on SDM (Fig. 3c,d), implying that cultivars capable of smoothly translocating these ions to aboveground parts sustained higher SDM. The assumption could also be confirmed by the fact that cultivars, which depicted higher Ca and P-uptake in their shoots, produced higher SDM (Fig. 3g,h). Since a very strong positive correlation also existed between total Ca-uptake and total P-uptake of cultivars (Fig. 3g), it can be assumed that higher P-acquisition of efficient cultivars was because of their high Ca-uptake. By the principle of mass action, high Ca uptake by plants caused an increase in P-solubility from Ca-bound P sources—a phenomenon which can increase availability of indigenous P in calcareous soils. Cultivars that are efficient accumulators of Ca are desirable as they can acquire higher amounts of P from otherwise P-deficient soils. Significant cultivar differences of tissue Ca-concentrations and uptake at stress P, and their meaningful correlations with growth parameters also suggest their testing as criteria for P-efficiency of crops under P-stress.

3.4 PSI carboxylates exudation sampling protocol and remodeling and modification in root architecture to tolerate P-stress

Cultivars were grouped into two categories i.e., 'Brown Raya' and 'Con-1' (Efficient; group I) and 'Toria' and 'B.S.A' (Inefficient; group II) cultivars. Group I cultivars are relatively well adapted to P-limited conditions and accumulating biomass nearly twice as group II cultivars, when both were under severe P-conditions (Akhtar et al., 2006ab). The type and amount of exuded carboxylates (Citric, malic, and succinic acids) were reported earlier in detail (Akhtar et al., 2006a). Group I cultivars exuded 2-3 times more carboxylates than group II cultivars at P-starvation.

Adaptive changes in root growth and morphology increasing the surface area for nutrient absorption are most pronounced under conditions of P-starvation (Fig. 4D,F). Increases in root-shoot dry matter ratio, root branching, root elongation and fine root production have been related to modifications of sink strength associated with enhanced shoot-root partitioning of photosynthates and N compounds. A regulatory function of plant hormonal factors is suggested by changes in hormonal balances particularly of auxins, cytokinins, abscisic acid (ABA), and ethylene (Hare et al., 1997), and by altered responsiveness to hormonal signals (Borch et al., 1999).

P-starvation increases density and/or length of root hairs, but considerable differences exist between plant species and at cultivar level as visualized in Fig. 4D,E. Group I cultivars ('Brown Raya' and 'Con-1') showed more structural and functional plasticity and increased

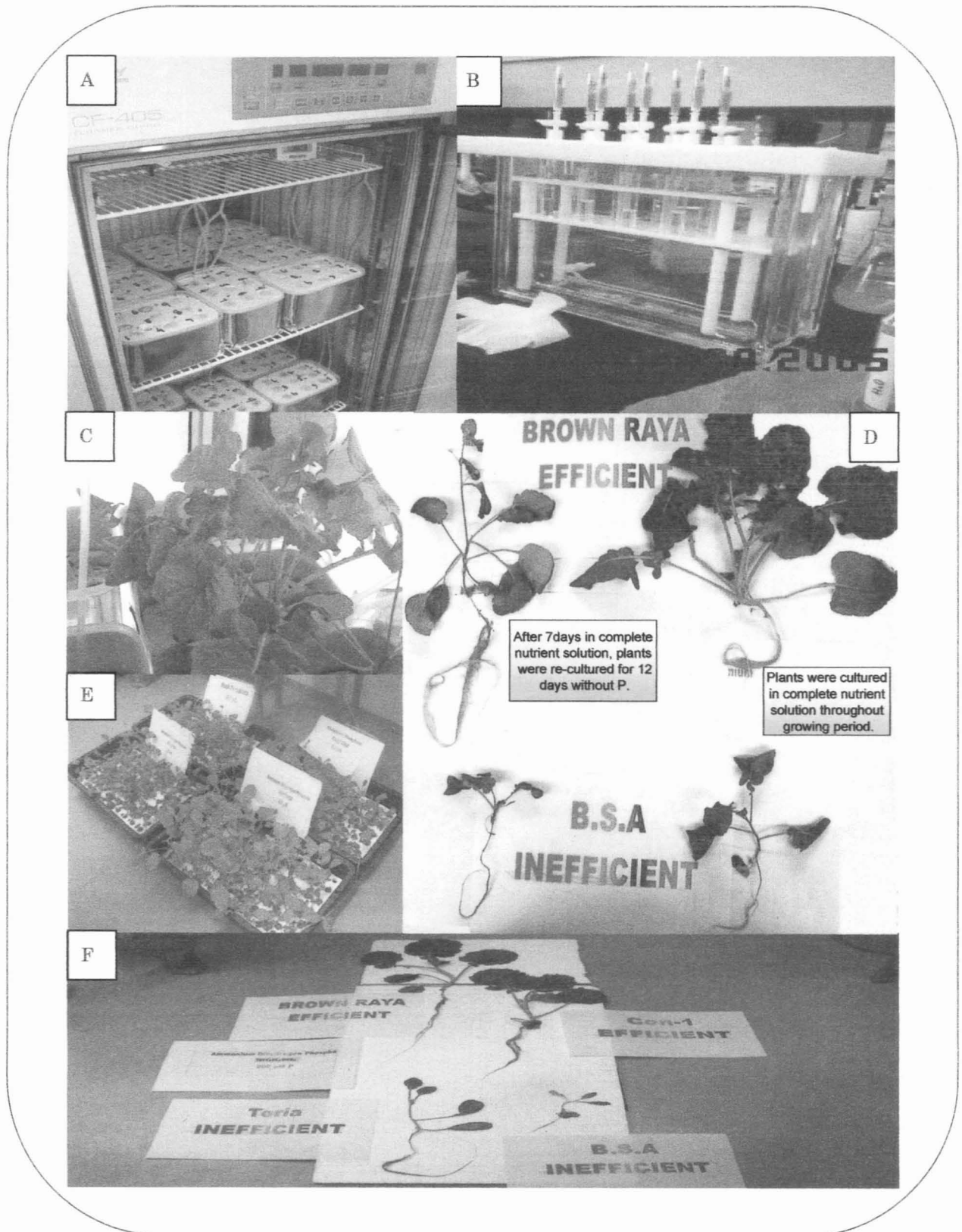


Fig. 4 (A, C, E) Plant cultivation techniques. (B) Extraction technique of exuded carboxylates using cation-anion exchange column filled with resins. (D, F) Remodeling and modification in root architecture of efficient cultivar 'Brown Raya' is an important genetic adaptation to explore P from P-deficiency stress environment.

surface area for P-absorption by growing long, thin roots with numerous and prolific development of long root hairs than group II ('Torla' and 'B.S.A') cultivars (Fig. 4D,F).

Root architecture denotes the spatial configuration of roots of different order and age, with the implication that the overall configuration has some functional significance

(Lynch, 1995). Efficient cultivars allocate more biomass to roots when Pi is limiting for their growth. Some of the observed difference between plants grown with high vs. a low of Pi may be ontogenetic, owing to comparisons of plants at different sizes, rather than a truly plastic response. However, there is also clear evidence that Pi supply has a direct effect on biomass partitioning, independent of ontogeny. Effects of Pi supply on biomass partitioning between roots and shoots are thought to involve a decreased production in and export of cytokinins from roots at a low Pi supply, possibly associated with a decreased rate of uptake and metabolism of nitrogen. Increased root production (root length), without a proportional increase in living-root biomass, i.e. enhanced root turnover, allows greater amounts of uptake of immobile soil resources, such as P. Fast root turnover is a very important trait of cluster-root-producing species, as discussed below (Shane and Lambers, 2005). Root hairs are a fairly common root structure, and increased root-hair length and numbers are considered to be an adaptation that enhances Pi acquisition and a plant's competitive advantage when soil Pi is limiting for growth (Bates and Lynch, 2001). Species and cultivars that develop more and/or longer root hairs are far more efficient at accessing Pi from soils, and thus show less of a growth response in P-fertilized soils than do species that lack these traits. This point was elegantly demonstrated in a comparison of cultivars of *Brassica*; cultivars with the capacity to form longer root hairs took up more P, and tended to yield better when Pi was limiting crop growth compared with cultivars having roots hairs half the length. Root-hair abundance and length is enhanced by P deficiency (Schmidt, 2001). The increased growth of root hairs observed for plants grown at low Pi availability can be mimicked in plants grown at high Pi supplies.

In response to P-starvation, plants have evolved various sophisticated biochemical, metabolic, morphological and transcriptional adaptations to enhance P acquisition and conserve Pi. These adjustments include bypass reactions of adenylate and Pi-dependent steps in respiratory pathways, changes of membrane lipid composition, rhizosphere acidification by H⁺-efflux, enhanced expression of high affinity Pi transporters as well as increased synthesis and secretion of carboxylates and acid phosphatases to increase Pi accessibility from insoluble complexes and organophosphates in recalcitrant soil matter; remodeling of root system architecture such as higher root/shoot ratio, lateral root growth, and increased root hair formation to accelerate soil P exploration. All these changes improve the capacity of the plant root system to better explore and mine the soil under P-deprivation.

4 CONCLUSIONS

Studies of plant responses to Pi-deprivation have revealed some remarkably adaptive mechanisms that contribute to the survival of Pi-stressed plants. Although these adaptations are not identical in all plants, certain

aspects are conserved in a wide variety of plants from very different environments. In the present study, analysis of P-starvation induced (PSI) morphological and physiological traits revealed that efficient cultivars such as 'Brown Raya' and 'Con-1' can scavenge mineral Pi better than P-inefficient 'B.S.A' and 'Toria' cultivars under P-deprivation via PSI elegant adaptations. To elucidate the underlying mystery of fascinating complex morphological, biochemical and molecular mechanisms whereby plants acclimate to Pi-stress are of crucial importance and an area of research priority. This could lead us to the development of rational strategies for engineering Pi-efficient transgenic crops that would require less exogenous Pi-fertilization and hence, paving way to economically and ecologically sustainable cropping systems.

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