Phosphate (Pi) uptake and remobilisation within plant in *Brassica* spp. under different Pi availabilities input

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**Abstract.** Phosphorus (P) is one of six essential macronutrients in plants to ensure crop growth and productivity. Plants only acquire phosphorus in the form of inorganic phosphate (Pi) known as orthophosphate, mainly in the form of $\text{H}_2\text{PO}_4^-$. The high demand of Pi in fertilisers for agriculture use contributed to environmental pollution. Therefore, it is important to understand the Pi uptake from the soil and remobilisation within the plant to increase P use efficiency (PUE). In this study, the experiments were undertaken to look at the variation of Pi uptake by *Brassica* spp. under different soil Pi availability. Two soil Pi concentrations, P1 (0 g L$^{-1}$) and P4 (0.225 g L$^{-1}$) were selected to represent the low and high Pi treatment, respectively. *B. napus* plants grown in compost with both high and low Pi treatments were harvested at four different parts of the plant (leaf, stem, flower, and pod silique and seed) at six different growth stages, namely two leaf stage, four leaf stage, flowering stage, first flower opens stage, seed filling stage and maturity stage. The results of free Pi concentration in P4 (0.225 g L$^{-1}$) were significantly higher than P1 (0 g L$^{-1}$) (P $\leq$ 0.01). Remobilisation of Pi determined by total P contents of plants treated with P1 and P4 both showed similar trends, increased P contents up to 50% of plant P at harvest 5 (seed stage), and loss of P at leaf, stem and flower tissues during senescence at maturity stage. Under deficiency, leaf Pi concentration which determined the internal Pi redistribution showed Pi was redistributed from source (mature organs) to sink (developing tissues) Pi tissues. These results provide insights on plant nutrient status and can be used for further investigation for crop optimal production under low Pi availability.

**Keywords.** Phosphate, phosphorus, *Brassica* spp., Pi remobilisation, phosphorus use efficiency (PUE).

1. Introduction

Phosphorus (P) is a major component of all living cells. It is involved in several key cellular functions, including energy transfer by adenosine triphosphate (ATP). It is also a vital component in plant cell membranes, as the part of phospholipids making up the phospholipid bilayer. Another important role of P in cells is the sugar phosphate backbone of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is an essential part of the process of carrying the genetic code from one generation to the next, providing the blueprint of all aspects of plant growth and development [1,2]. Plant roots only acquire P from the rhizosphere solution as inorganic phosphate (Pi) [3]. Phosphorus in the form of Pi has a low mobility in the soil. Therefore, phosphate (Pi) fertilisers are used in agricultural production systems to
maintain crop yield and quality. The main source of Pi fertiliser is from mine rock phosphate which the high demand and use of the resources for current usage makes the existing rock phosphate reserves could be exhausted in the next 20-80 years, where the supply could no longer to keep up with demand [4,5,6].

The highly use of P by human activities that result in excessive P concentrations in the environment from the sewage and industrial wastes can eventually cause pollution and eutrophication in freshwater aquatic systems [7]. Eutrophication in these P limited environments causes oxygen concentrations to be depleted in the water and reduces the ability of animals to live in these habitats [8]. Therefore, the proper P management is important to mitigate the depletion of P reserves, increasing P recovery and recycle a larger proportion of Pi from the society and environment [9,10].

Higher plant species differ in their efficiency of Pi uptake from the soil solution, especially during low Pi availability [11]. Therefore, plants have developed phenotypic adaptation strategies to adapt to low Pi environments, including maximizing the soil exploration through root proliferation and elongation, root exudation of organic acids to increase Pi availability, and mutual association with micorrhizal fungi [12, 13, 14, 15, 16].

Low phosphate (Pi) availability in soil is a limiting factor to a growing plant. Investigation of the nutrient status and how plant remobilise Pi during deficiency is important for future studies to develop crops which could grow optimally and provide a high yield with minimal use of fertiliser. In this study, experiments were undertaken to look at the variation in Pi uptake by Brassica spp. under different soil Pi availabilities. First, B. rapa plants were grown in compost with a range of Pi fertiliser treatments to investigate the response to Pi availability. Second, two soil Pi concentrations were selected to represent the low Pi and high Pi treatment. B. napus plants were grown in compost with these two treatments and harvested at different parts of the plant and at six different growth stages. Free Pi concentration and total P content were determined to assess the nutrient status of the plant.

2. Materials and Method

2.1. B. rapa physiological responses to Pi availability (six treatments) in compost mixture.

Seeds of B. rapa (R-o-18) were sown in 1 L pots containing a peat (Attgrow) and sand mix (75:25). The following nutrients were added to the mix: 0.4 g L⁻¹ ammonium nitrate (Yara UK Limited, Grimsby, UK), 0.75 g L⁻¹ potassium nitrate (Yara), 2.25 g L⁻¹ ground limestone (Attgrow), 2.25 g L⁻¹ ground magnesian limestone (Attgrow), and 0.4 g L⁻¹ fritted trace elements (wm255; Librel ® BMX, Bradford, UK). To alter the Pi concentration in the compost mix, single superphosphate (J. Arthur Bowers, Lincoln, UK) was added to aliquots of the above mix at 0.0, 0.075, 0.15, 0.225, 0.45 and 1.35 g L⁻¹ of compost mix (Fig. 1A). Based on preliminary experiments, the plants were watered with deionised water three times a week to ensure no other nutrients could affect the growth of the plants. After the seeds germinated, they were thinned to reduce them to one per pot.

The experiment was conducted in a glasshouse at the University of Reading, UK and the glasshouse temperatures were maintained between 20-25 °C. Each of the six treatments, P1 (0 g L⁻¹), P2 (0.075 g L⁻¹), P3 (0.15 g L⁻¹), P4 (0.225 g L⁻¹), P5 (0.45 g L⁻¹), and P6 (1.35 g L⁻¹) had five replicates. A split plot design was used as the experimental design and the plants were harvested at three different time points (14, 28, and 42 days after sowing). The total number of pots was 90. Plants were harvested by cutting just above the level of the compost (shoot).

2.2. B. napus physiological responses to Pi availability (two treatments) in different growth stages in compost mixture.

Seeds of B. napus (Canard) were sown in 1 L pots containing peat and sand mix (75:25). The nutrients were added according to section 2.1. Initially several seeds were sown per pot to ensure enough material for harvesting at the early growth stages; harvest 1 (four seedlings) and harvest 2 (two seedlings). For all other harvests, only one seed was allowed to grow per pot (harvest 3-6). The experiment was conducted in a controlled environment growth room at the University of Reading, UK and the growth
conditions were set as per section 2.1 (Fig. 1B). Each of the two treatments, which represent the low and high external Pi concentrations (P1= 0 g L\(^{-1}\) and P4= 0.225 g L\(^{-1}\)) had five replicates. A split plot design was used as the experimental design and the plants were harvested at six different growth stages; i) two-leaves, ii) four-leaves, iii) flowering, iv) first flower opens, v) seed, and vi) maturity (Fig. 2). Samples were freeze dried as described in section 2.3. Samples were analysed for Pi and total P concentrations.

2.3. Plant Pi analyses

Two drying methods were used for analysing free Pi concentration, oven-dried and freeze-dried. In the oven-dried method, plants were cut just above the compost to obtain whole shoots. Shoots of the plant were collected and weighed to obtain fresh weight before being dried at 60 °C and reweighed for the dry weight. In the freeze-dried method, shoots or specific plant parts were cut and directly flash frozen in liquid nitrogen. The samples were kept in a -80 °C freezer before being dried in a freeze dryer (Applied Vacuum Engineers, Bristol, UK). The samples were ground using laboratory mill (Perten Instruments Warwick, UK) to ensure representative plant samples were used for Pi analysis. The Pi was extracted using 1% acetic acid and homogenised 3 times with a TissueLyser (Qiagen, Manchester, UK) at a frequency of 30 Hz for 60 secs. The quantification of Pi concentration in tissues was measured by releasing the cellular contents into water by repeated freeze-thaw cycles followed by quantifying Pi by the molybdate-blue assay using Thermo Scientific Multiscan (Thermo Scientific, UK) according to the procedure by Ames (1966) \[17\] with some modifications. A total of 300 µL of reagent was added; 10 µL of sample, 80 µL of deionised water and 210 µL of colour substrate, incubated at 37 ºC for 30 min. The Pi concentration was measured at 620 nm using Ascent Software Version 2.6.

2.4. Total P analysis

For total P analysis, 0.1 g of ground dry sample was pre-digested with 2 mL of ultra-pure water and 8 mL of trace element grade concentrated nitric acid (HNO\(_3\)). Then the samples were digested in MARS XPRESS digestion tubes (CEM Corporation, Buckingham, UK) using trace element grade concentrated nitric acid (Fisher Scientific) and ultra-pure water (HNO\(_3\)-H\(_2\)O). Digestions were accomplished by MARS6 microwave digestion system (CEM Corporation) at 140 °C for 45 min. Total P concentration was quantified using a molybdate-blue assay according to the procedure by Ames (1966) after the samples were filtered through filter paper into a 50 mL centrifuge tube.

2.5. Data analysis

Statistical data analyses were performed using Genstat (17th Edition, VSN International, Oxford, UK). First, the distribution and variance of the data were plotted to determine whether the data were distributed normally, and variance was evenly distributed across the data set. Data that had even variances and normal distribution were subjected to further analysis. Data that were not normally distributed and/or had unevenly distributed variance were subjected to log10 transformation. The data were analysed using a Residual Maximum Likelihood (REML) procedure \[18,19\]. Further least significant difference (LSD) analysis was performed and the data were plotted using Microsoft Excel 2010.
Figure 1. A. *Brassica rapa* plants grown in 1 L plastic pots containing compost and sand media (75%/25%) in the glasshouse. B. *Brassica napus* plants grown in 1 L plastic pots containing compost and sand media (75%/25%) in the controlled environment room.

Figure 2. *Brassica napus* plants at different stages of growth. A. Two-leaf stage B. Four-leaf stage C. and D. First-flower opens stage E. and F. Middle-flowering stage G. Seed stage H. Mature plants.
3. Results

3.1. *B. rapa* plant growth responses to six different compost P concentrations

The shoot biomass of plants significantly increased as the amount of added Pi increased from 0 to 0.225 g L\(^{-1}\) of compost, after which there was no significant change in shoot biomass (Fig. 3). For all three harvests, the effect of Pi treatment was significant (Table 1). In the first harvest (day-14), the overall difference in shoot biomass was not as great as with later harvests (Fig. 3A). This might be due to plants still using their internal resources to support their early stage of growth. On the second harvest (day-28), the shoot biomass of plants significantly increased from P1 to P2 (P<0.05). Treatments P2, P3, P4 and P6 were not significantly different from each other, but P5 gave the highest shoot biomass (Fig. 3B). The plants showed different growth response phenotypically, where the P1 plant was the smallest and P6 plant was the biggest compared to plants from other treatments (Fig. 4). For the third harvest (day-42), the shoot biomass increased significantly from P1 to P2 (Fig. 3C). There was no significant difference between the remaining treatments in the third harvest. Overall, the relationship between yield (shoot biomass) and added Pi showed a quadratic curve divided into three segments, ascent, peak and descent representing three zones of deficiency, sufficiency and toxicity, respectively.

Table 1. Restricted Maximum Likelihood analysis (REML analysis) of *Brassica rapa* growth at six different Pi treatments.

| Harvesting period | Fixed term | Wald statistic | n.d.f | F statistic | d.d.f | Prob. > F |
|-------------------|------------|----------------|-------|-------------|-------|-----------|
| 1\(^{st}\) Harvest | Pi treatment | 27.45          | 5     | 5.49        | 18.9  | 0.003     |
| 2\(^{nd}\) Harvest | Pi treatment | 56.11          | 5     | 11.22       | 19.1  | <0.001    |
| 3\(^{rd}\) Harvest | Pi treatment | 27.99          | 5     | 5.6         | 20    | 0.002     |

n.d.f= numerator degrees of freedom
d.d.f= denominator degrees of freedom
Figure 3. Shoot dry weights of Brassica rapa plants growing in compost with increasing additions of phosphate harvested after 14 days (A), 28 days (B), and 42 days (C). Plants were grown under glasshouse conditions. Each data point represents mean ± SEM (n=5). Data were analysed using a REML procedure, followed by Fisher’s unprotected least significant difference (LSD) test, so that data points with different letters are significantly different (P<0.05). Equations for fitted lines are (A) $y = -0.0086x^2 + 0.0215x + 0.0127$, $R^2 = 0.7748$ (B), $y = -0.3704x^2 + 0.5771x + 0.0786$, $R^2 = 0.8677$ (C) $y = -1.8931x^2 + 3.0857x + 0.7368$, $R^2 = 0.482$. 
Figure 4. Growth response of *Brassica rapa* plants to different additions of phosphate (Pi) to the compost (P1= 0.0, P2= 0.075, P3= 0.15, P4= 0.225, P5=0.45 and P6= 1.35 g L$^{-1}$ of compost mix) at 28 days after sowing. Scale bar: 10 cm.

3.2. Changes in shoot Pi concentration with increasing compost Pi concentration in *B. rapa*

Shoot Pi concentration was significantly affected by the application of increasing amounts of Pi in *B. rapa* (Fig.5). For first harvest 14 days after sowing, data were recorded as a single value because of insufficient amount of sample biomass for measuring Pi concentration. Shoot Pi concentrations were at the highest value on the third harvest for every Pi treatment. The application of Pi treatments generally increased the shoot Pi concentration, but the highest value on the third harvest for every Pi treatment. The application of Pi treatments generally increased the shoot Pi concentration, but the highest value was observed under treatment P5 (Fig. 5). The lowest shoot Pi concentration was observed at P1 for all harvested periods, as expected due to no added Pi (0 g L$^{-1}$) in the compost mixture.
Figure 5. Shoot phosphate (Pi) concentration of Brassica rapa plants growing in compost with increasing additions of Pi harvested after 14 days (A), 28 days (B), and 42 days (C). Plants were grown under glasshouse conditions. For harvest after 14 days, data are a single value due to low biomass for measuring shoot Pi. Data points for all other data represent mean ± SEM (n=5). Data were analysed using a REML procedure, followed by Fisher’s unprotected least significant difference (LSD) test, so that data points with different letters are significantly different (P<0.05).

3.3. Changes in biomass and total P content of B. napus tissues grown with two different rates of P during development

The effects of the additional Pi on B. napus growth and Pi related traits during development were evaluated using the line Canard grown in compost with no added Pi (P1) and with 0.225 g L⁻¹ added P (P4). Samples were harvested at six different growth stages; i) two-leaves (harvest 1), ii) four-leaves (harvest 2), iii) flowering (harvest 3), iv) first flower opens (harvest 4), v) seed filling stage (harvest 5), and vi) maturity (harvest 6), with the plants broken down into four tissues; i) leaf, ii) stem, iii) flower and iv) pod silique and seed (Fig. 6 and Fig. 7). No plants were harvested at day 7 (harvest 1) and day 9 (harvest 2) due to insufficient amount of sample for measuring Pi concentration.
The yield of filled pod, silique and seed of *B. napus* in the pot study was approximately 38.1 (±0.5)% of the total plant biomass at maturity while the combination of leaf and stem tissue constituted roughly 60% of plant biomass at maturity, where leaf and stem recorded about 16% and 42% of plant biomass, respectively (Fig. 6). The maximum individual tissue biomass accumulation in *B. napus* occurred at maturity stage in both treatments P1 and P4 with 4.561 and 4.952 g, respectively in pod silique and seed tissues of the plant (Fig. 6).

Total P contents of plants treated with P1 and P4 both showed similar trends, where pod silique and seed of *B. napus* contained over 50% of plant P at harvest 5 (seed stage). Leaf, stem and flower showed a loss of P during senescence at maturity stage due to redistribution of P to the seed (Fig. 7).

![Figure 6](image-url)

**Figure 6.** Total biomass per plant of *Brassica napus* at six growth stages; two-leaf stage (1), four-leaf stage (2), flowering stage (3), first flower opens stage (4), seed filling stage (5) and maturity stage (6) at four different tissues; leaf (red), stem (orange), flower (blue) and pod silique and seed (green) grown in compost with no added phosphate (P1) and 0.225 g L⁻¹ (P4). Plants were grown under control environment laboratory conditions. At the early stage of harvest (harvest 1 and 2), whole plant was used due to low biomass. Data points for all other data represent mean ± SEM (n=5).
Figure 7. Total P content and distribution between tissues throughout the growth cycle of *Brassica napus* plants; two-leaf stage (1), four-leaf stage (2), flowering stage (3), first flower opens stage (4), seed filling stage (5) and maturity stage (6) at four different tissues; leaf (red), stem (orange), flower (blue) and pod silique and seed (green) grown in compost with no added phosphate (P1) and 0.225 g L\(^{-1}\) (P4). Plants were grown under control environment laboratory conditions. At the early stage of harvest (harvest 1 and 2), whole plant was used due to low biomass. Data points for all other data represent mean ± SEM (n=5).

3.4. Changes in Pi concentration of B. napus tissues grown with two different rates of P availability during development

Overall, Pi concentration of plants growing in P4 were significantly higher than P1 (P ≤ 0.01). Leaf and stem Pi concentration increased at early stages of growth until harvest 4 for both treatments (P1 and P4). The concentration of Pi decreased in all plant parts until maturity stage (harvest 6). The highest Pi concentration was recorded in P4 stem with 6.085 µmol g\(^{-1}\) DW at flowering stage (harvest 3). At first flower open stage (harvest 4), tissue Pi concentration declined in the leaf and stem tissues by about 30-40% as the plant remobilised P from leaf, stem and flower to the developing seed, moving from source to sink tissues (Fig. 8).

Tissue Pi concentration in P1 and P4 treatment showed a similar trend for leaf, stem, flower, and pod silique and seed tissues (Fig. 8). In flowering stage (harvest 3), stem Pi concentration was highest with 5.476 mg P g\(^{-1}\) DW in P1 treatment, the Pi concentration then began to decline at first flower opens stage
(harvest 4), seed filling stage (harvest 5) and maturity stage (harvest 6) with 1.841, 0.966, and 0.475 mg P g\(^{-1}\) DW, respectively. While in P4, stem Pi concentration was higher than P1 with 6.085, 3.429, 1.576, and 0.771 mg P g\(^{-1}\) DW in harvest 3, 4, 5 and 6, respectively. Tissues Pi concentrations declined as plants reach maturity (Fig. 8).

**Figure 8.** Tissue phosphate (Pi) concentration in *Brassica napus* plants; leaf (blue), stem (brown), flower (grey), and pod, silique and seed (orange) grown in compost with no added Pi (P1) and .225 g L\(^{-1}\) (P4). Plants were grown under controlled environment conditions and harvested at two-leaf stage (1), four-leaf stage (2), flowering stage (3), first flower opens stage (4), seed filling stage (5), and maturity stage (6). For harvest 1 and 2, data are not provided due to low biomass for measuring Pi. Data points for all other data represent mean ± SEM (n=5), except harvest 4 in treatment P4, (n=3).

4. Discussion

4.1. Growth response of *B. rapa* to increasing Pi availability

This study has shown that *B. rapa* grow differentially in response to external Pi availability (Fig 3.5). the effects elevated Pi availability on *B. rapa* growth were determined using the R-o-18 growth on compost mixture subjected to a range of increasing Pi availabilities. Plants were harvested after 14, 28 and 42 days. Shoot dry weights and Pi concentration in shoot biomass increased with time and Pi availability up to 0.45 g L\(^{-1}\) of added Pi (Fig. 3. and Fig. 5). From these results, significant differences in biomass and shoot Pi concentrations were observed between treatments P1 and P4, providing contrasting growth conditions with which to study responses. Measurements at these two soil Pi concentrations are suitable for assessing physiological measurements and modelling the response without the need to grow the plants at six different P concentrations [20]. This in line with previous studies in Brassicaceae where the P treatments can be determined based on growth response curves under certain conditions, providing a high P treatment represents the plant with a high and sufficient P, and low P treatment, where the P availability is not sufficient and low growth response is observed [20, 21, 22].
4.2. Evaluation of B. napus response and development under contrasting Pi availabilities

The growth and developmental responses of B. napus (Canard) to low and high Pi availability were conducted under controlled environmental conditions. The plants grown in pots were harvested at six different growth stages; i) two-leaves, ii) four-leaves, iii) flowering, iv) first flower opens, v) seed, and vi) maturity. Biomass, total P content and Pi concentration was recorded for individual plant tissues and the data was analysed. Under Pi deficiency (P1), leaf Pi concentration was reduced by 57% from harvest 3 to harvest 4 (Fig. 8). This showed that Pi was redistributed from older leaves to young organs. Internal Pi redistribution during Pi deficiency is consistent with previous work demonstrating that Pi is redistributed from source to sink tissues under these conditions [23]. In contrast, only a 26% reduction was observed in plants grown under the P4 treatment (Fig. 8). Pi remobilisation was determined by the amount of the total nutrient present in different organs of the plant at different growth development [24, 25]. In this study, B. napus total P content accounted over 50% in pod, silique and seed at maturity stage consistent with a study in wheat where remobilisation of P accounted for 56-63% of the grain P content [26].

In B. napus, total P accumulation continued until flowering stage but started to decline at maturity (Fig. 8). This is in contrast with previous work on B. napus, where P uptake continued until late maturity [27]. Total P content of B. napus peaked at harvest 5 (seed filling stage) (Fig. 8), in contrast with reports by Holmes (1980) and Barraclough (1989) who showed the maximum P accumulation occurred during late seed stage or at maturity [28, 29]. The differences results obtained might be due to natural variation in the species. Total P content was significantly lower in plants grown in this study compared to those grown by Rose et al. (2007), where total P contents were between 60 and 80 mg plant\(^{-1}\) compared to 25-30 mg plant\(^{-1}\) (Fig. 7). This might be associated with different soils (sandy loam v peat-based compost), pot size (2.5 L v 1 L) or fertiliser application rates used between the studies.

5. Conclusion

One of the strategies for plants in adaptation when facing with Pi deficiency is to optimise the acquisition and use of Pi in plant tissues. In Brassica napus, the redistribution and remobilisation of Pi from mature organs to the developing tissues occurred to make nutrient Pi available for growth showed how the plants cope with Pi deficiency as well as contributed to the phosphorus use efficiency (PUE).

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