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Remobilization of seed phosphorus reserves and their role in attaining phosphorus autotrophy in maize (*Zea mays* L.) seedlings

Muhammad Nadeem[1,2,3]*, Alain Mollier[1,2], Christian Morel[1,2], Loïc Prud'homme[1,2], Alain Vives[1,2] and Sylvain Pellerin[1,2]

[1]INRA, UMR 1391 ISPA, F-33140 Villenave d'Ornon Cedex, France; [2]Sciences Agro, UMR 1391 ISPA, F-33170 Gradignan, France; [3]Department of Environmental Sciences, COMSATS Institute of Information Technology, Vehari, Pakistan

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Abstract

Successful remobilization of seed reserves is the driving force behind seedling establishment for maximum final crop outcomes. The remobilization of stored maize seed phosphorus (P), and its allocation towards growing seedlings is critical for P-autotrophy during early ontogeny. We aimed to (1) evaluate the time frame of the origin of P utilized by maize seedlings, including the heterotrophic, transitional and autotrophic phases; and (2) compare P and carbon (C) dynamics in both seed and seedling compartments during the same phases. Using isotopic signatures (^{32}P), we identified different P fluxes (P-heterotrophy, P-transition and P-autotrophy) and determined the proportion of P fluxes from heterotrophic seed P and external P uptake during 23 d of early ontogeny. The P-heterotrophic growth phase lasted from the first to the fourth day after sowing, when seedlings were entirely made up of heterotrophic P originating from remobilized seed P pool. In our experimental conditions, the P-transitional phase, when growing seedlings were supported by both heterotrophic and autotrophic P, lasted from the fifth to the fifteenth day after sowing. Thereafter, seed P reserves were exhausted and seedlings depended entirely on external P uptake, indicating the P-autotrophic stage. Although seed P reserves were remobilized earlier than C reserves, the length of the three growth phases for P and C was similar in the maize seedlings.

Keywords: autotrophy, carbon, heterotrophy, maize, mineral nutrition, phosphorus

*Correspondence
Email: muha.nadeem@gmail.com

Introduction

Seedlings go through different growth phases based on stored seed and external nutrient supplies (Scaife and Smith, 1973). Seedlings are completely dependent on stored endosperm and scutellum nutrients during very early growth. Success or failure of the developing seedlings during early ontogeny is directly proportional to the restoration of transcriptional and translational machineries (Loreto *et al.*, 2001; Mei and Song, 2008; Finkelstein, 2010) and to many other factors, including successful remobilization of stored seed reserves that are accumulated throughout the ripening period (Le Deunff, 1975; Lawrence *et al.*, 1990), seedling root growth (Fan *et al.*, 2003; Zhu and Lynch, 2004; Enns *et al.*, 2006), soil conditions, water and temperature (Miller, 2001; Manz *et al.*, 2005; Louarn *et al.*, 2008).

At the beginning, the seedling is dependent on heterotrophic nutrition that comes from remobilized seed reserves. This is followed by a transitional stage when seedling nutrient requirements are fulfilled by seed reserves and external supplies from the soil. Seedling growth is then supported by external nutrient supply by root uptake and photosynthesis, indicating the autotrophic growth period. The seed reserves are exhausted and no longer play a role in nutrition, indicating autotrophic growth (Whalley *et al.*, 1966; Bourdu and Gregory, 1983; Deleens *et al.*, 1984; Bathellier *et al.*, 2007).

Earlier studies on maize showed that seed C reserves are largely concentrated in the endosperm rather than in the scutellum (Deleens *et al.*, 1984; Bathellier *et al.*, 2007; Nadeem *et al.*, 2011, 2012a). The role of seed C remobilization and the redistribution of metabolites have been thoroughly studied by many researchers (Bedi *et al.*, 2009; Nadeem *et al.*, 2011, 2012a, b). Evidence of the succession of heterotrophic to autotrophic carbon metabolism in newly growing maize seedlings has been

reported in earlier studies (Cooper and MacDonald, 1970; Deleens *et al.*, 1984).

Phosphorus is the main mineral involved in photosynthesis and a major part of seed P reserves (80%) is stored in maize scutellum tissues (Nadeem *et al.*, 2011, 2012a). The P reserves are primarily stored in the form of phytic acid, which refers to any salt of *myo*-inositol hexakisphosphate (Shears and Turner, 2007), recommended abbreviation insP_6 according to the International Union of Pure Applied Chemistry. Soon after imbibition, the activity of phytase (*myo*-inositol hexakisphosphate phosphohydrolase) starts to hydrolyse insP_6 (Laboure *et al.*, 1993; Steiner *et al.*, 2007), which, in turn, triggers the cleavage of one or more phosphate groups. InsP_6 is converted into non-phytate P and remobilized to the growing seedling (Nadeem *et al.*, 2012b). The transition from the P-heterotrophic phase to self-nutrition by external P uptake during early growth stages is important as it has long-term effects on final crop yield. The stored seed P reserves are used during the P-heterotrophic phase and to achieve P self-nutrition. A further responsibility of seedling roots is to support the transition from P-heterotrophic to P-autotrophic nutrition by external P uptake. As soon as the stored seed reserves are exhausted, these reserves start to accumulate in seedling leaves and roots. The flow of phloem in seedling roots is therefore important to supply nutrients to establish autotrophic growth (Enns *et al.*, 2006). Developing seedling roots are therefore very important in fulfilling seedling P demand by exogenous P uptake from the root zone. The leaves are the main centres of energy production through photosynthesis, for which P is one of the main nutrients required (Usuda and Shimogawara, 1991; Xu *et al.*, 2007).

Although our knowledge of the supply of nutrients from the maize seed reserve to the developing seedlings is substantial (Bourdu and Gregory, 1983; Deleens *et al.*, 1984; Bityutskii *et al.*, 2002; Wang *et al.*, 2005; Mei and Song, 2008; Nadeem *et al.*, 2011, 2012a, b; White and Veneklaas, 2012), not much quantitative knowledge is available about the seed reserves and assimilation-derived P for root and shoot development during heterotrophic and autotrophic phases of maize seedlings. The objective of the current research was to: (1) evaluate the time frame of the origin of P utilized in maize seedlings, including heterotrophic, transitional and autotrophic phases; and (2) compare P and C dynamics in both seed and seedling compartments during the same phases.

Materials and methods

Experimental layout and seedling growth conditions

The research was carried out in controlled environmental conditions during 2010 (March–April), at INRA Bordeaux, France. Remobilization of C and P stored in maize (*Zea mays* L.) seeds and external P uptake dynamics were monitored for a period of 23 d after sowing. Nine imbibed homogeneous seeds were placed at 2 cm sowing depth in small plastic pots containing perlite as a growth media. A total of 45 pots were used for 14 seedling harvests during the 23-d early growth period. A complete nutrient solution (Bhadoria *et al.*, 2004), containing all micro- and macronutrients, was added to each pot (290 ml pot^{-1}) to reach the optimum saturation capacity of the perlite. The nutrient solution consisted of 500 μM P as $NaH_2PO_4.2H_2O$, 1 mM NO_3 as $Ca(NO_3)_2.4H_2O$, 0.2 mM K as KCl, 0.1 mM Mg as $MgSO_4.7H_2O$, 46 μM B as H_3BO_3, 9.1 μM Mn as $MnCl_2.4H_2O$, 0.8 μM Zn as $ZnSO_4.7H_2O$, 0.3 μM Cu as $CuSO_4.5H_2O$, 0.5 μM Mo as $(NH_4)_6Mo_7O_{24}.4H_2O$; 2 mg l^{-1} Fe was added as Sequestrene-138 Fe (water-soluble granules with 6% Fe in chelated form). The pH of the nutrient solution was adjusted to 6.4. The nutrient solution had a high P concentration (500 μM) to enable us to determine external P uptake by developing seedling roots. The nutrient solution was labelled with radioactive [^{32}P]orthophosphate ions to quantify the absolute and relative proportion of external P uptake by seedling roots and remobilized seed P reserves in reaching P-autotrophy. Determination of radioactivity background noise was carried out from an additional three pots which were irrigated with unlabelled nutrient solution. All the pots were placed under light with an 18:6 h day and night photoperiod. Irrigation was carried out on the basis of water loss by evapotranspiration from pots during each day throughout the growth period. Temperature (air and inside the pots) was measured with copper constantan thermocouples (0.2 mm diameter). Relative humidity was recorded by a relative humidity probe and a photosynthetically active radiation (PAR) sensor was used to record the PAR. The average air temperature, relative humidity and PAR were 25°C, 43% and 246 μmol m^{-2} s^{-1}, respectively.

The developing seedlings were sampled on 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23 d after sowing. At each sampling date, three pots were selected randomly and six seedlings were selected from each treatment. After each sampling, the seedlings were placed at 4°C for 1 min in 0.5 mM $CaSO_4$ solution to remove perlite from the roots. After 1 min, the seedlings were dipped in high P solution (500 μM) containing 0.5 mM $CaSO_4$ at 4°C for 4 min to desorb all ^{32}P from roots (Rubio *et al.*, 2004). Thereafter, the seedlings were placed on a glass plate and separated with a scalpel into different parts, namely seed, seedling (coleoptile+mesocotyl, roots and leaves) and total seedling (seed+seedling). After recording the fresh biomass of each seedling part, the samples were placed in a lyophilizer for 24 h to measure their lyophilized biomass.

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Chemical analysis

Phosphorus was determined in seed endosperm, scutellum and seedling parts (leaves, roots and coleoptile+mesocotyl) by the malachite green colorimetric technique (Van Veldhoven and Mannaerts, 1987). Each sample was ground separately in a mixer mill (Retsch MM400 mixer mill, Resch GmbH, Han, Germany). One part of the ground sample was weighed and then P content was measured colorimetrically after P mineralization with HNO_3. Total seedling P content is the sum of the P in seedling leaves, roots, seed, coleoptile and mesocotyl.

The two P fluxes in growing seedlings (one from the seed P reserves and the other external P uptake) were identified by labelling the P in the external nutrient solution with ^{32}P isotope. The radioactivity was measured by liquid scintillation cocktail (Insta-gel Plus Packard, PerkinElmer, Boston, Massachusetts, USA) using a Packard Rd 2100 scintillation counter (Canberra Industries, Meriden, Connecticut, USA) and the standard time was 20 min. The radioactivity-induced background noise was zero.

Carbon contents in seedling roots and leaves were analysed with a C analyser using the dynamic flash combustion technique (Flash EA 1112 series, Thermo Electron S.A., Courtaboeuf, France). The second part of the ground sample (0.03 g) of the roots, leaves, coleoptiles + mesocotyl was taken in small tin capsules and combusted by an autosampler at 900°C to release C in gaseous form. The concentrations of C of roots, leaves and coleoptile + mesocotyl were then measured using a separation column.

Statistical analysis

Each mean value is the result of measurements of six seedlings with three replicates. Statistical analyses were done with R version 2.9.1 (R Development Core Team, 2009). Mean values were compared using Student's *t*-test at the 95% probability level.

Results

Kinetics of germinating seed C and developing seedling C reserves

Seed C content and total C accumulated in the seedling were similar for the first 2 d after sowing, showing that no C was lost from the germinating seed (Fig. 1a). Seed C contents started to be remobilized from the second day after sowing and thereafter a regular decrease in seed C contents was observed (Fig. 1a). Maximum seed C reserves (76% of initial C stock) were remobilized up to the thirteenth day after sowing. Only a small change (4% of initial C stock) in seed C contents was observed after the thirteenth day and from then on, seed C contents remained significantly unchanged until the final seedling harvest and represent about 20% of the initial C content (Fig. 1a).

Total seedling C accumulation remained significantly ($P<0.05$) static during 2 d of early ontogeny (Fig. 1a). Thereafter, a decrease was observed in total seedling C accumulation during the first week (from day 2 to day 7). This seedling C accumulation decrease represented 11% of the total initial seedling C stock at time zero. On the seventh day after sowing, the seedlings had two visible leaves (total leaf length= 19 ± 3 cm) and roots were 133 ± 2 cm long. Starting from the following harvest (i.e. from the ninth day after sowing), the total C contents of the seedling started to increase slowly. At the final seedling harvest, the total seedling C content was 125 (± 0.3) mg, i.e. a net accumulation of 14 (± 1.7) mg of C since sowing.

Figure 2a shows the transition from a heterotrophic source of C (seed C reserves) to an autotrophic source

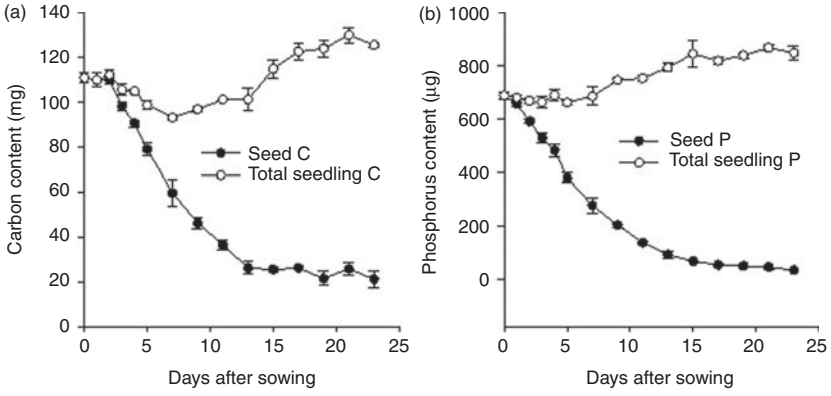

Figure 1. (a) Dynamics of seed C (●) and accumulation of total seedling C (○); (b) dynamics of seed P (●) and total seedling P accumulation (○) in germinating maize seeds and developing seedlings during early ontogeny. Data points indicate means of three replicates and bars represent the standard error of the mean.

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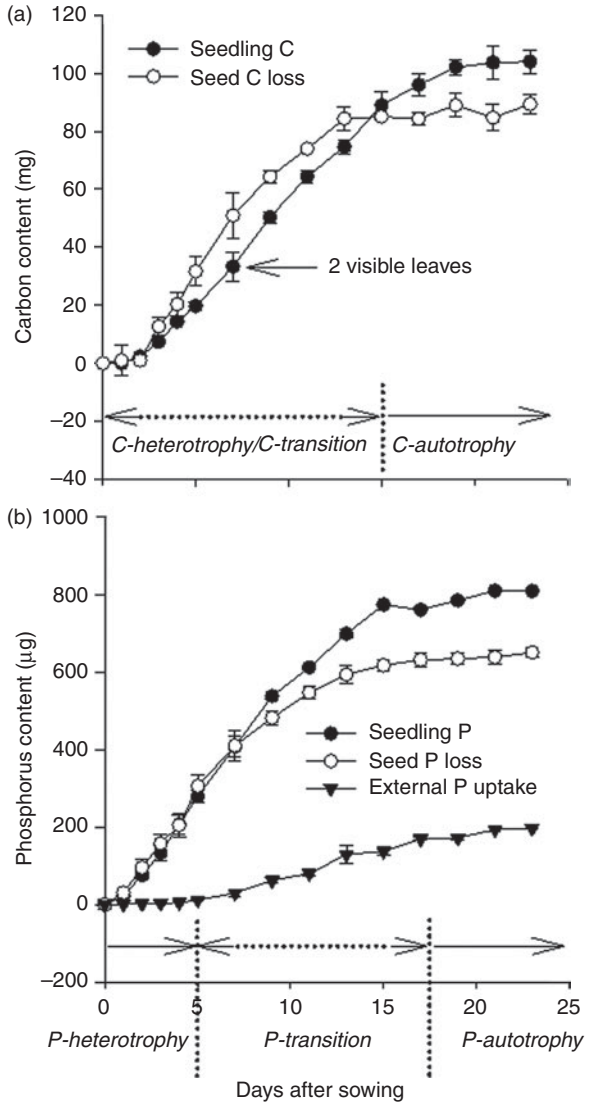

Figure 2. (a) Dynamics of C in seedling (●) and seed C loss (○); (b) seedling P accumulation (●), dynamics of P in seed (○) and external P uptake (▼) in germinating maize seeds and developing seedlings during early ontogeny. Data points indicate means of three replicates and bars represent the standard error of the mean.

(photosynthesis) in a growing maize seedling. Up to the second day after sowing, C loss from the seed and C accumulation in the seedling were the same, i.e. no C accumulated in the seedling. From the second day after sowing, C contents in the seedling increased exponentially even though the leaves had not yet emerged, indicating that remobilized C stored in the seed fulfilled all seedling C requirements. Loss of C from the seed was due to both respiration and export to newly growing seedling tissues. The 3-day-old seedling had 11.2 (±1.5) cm long roots and a 1.4 (±0.1) cm long coleoptile plus the mesocotyl with enclosed leaves. The seedling had a root length of 133 (±22) cm and two visible 19 (±3) cm long leaves on the seventh day after sowing. The C accumulation rate in the seedling was almost constant, whereas seed C loss decreased from day 7 to day 15 after sowing (Fig. 2a). Photosynthetic activity started to contribute significantly to seedling C accumulation from day 7 after sowing. This corresponds to the transitional phase from C-heterotrophy to C-autotrophy. The difference between C loss from the seed and C accumulation in the seedling decreased from day 7 after sowing (Fig. 2a). The difference became almost zero on the fifteenth day after sowing when seedling accumulation and loss of C from the seed were quantitatively the same. From the fifteenth day after sowing, C loss from the seed stopped and accumulation in the seedling was due to photosynthesis only. This corresponds to the autotrophic C stage.

Germinating seed P contents and seedling P accumulation

Total seedling P contents remained significantly unchanged until the seventh day and started to increase thereafter (Fig. 1b). A regular increase in total seedling P accumulation continued until final seedling harvest. Seed P content started to be remobilized from the first day after sowing. Thereafter, a regular decrease in seed P content was observed up to the fifteenth day after sowing, when almost 92% of the original P contents stored in the seed were remobilized. Thereafter, no significant change in seed P reserves was observed (Fig. 1b).

Differences in initial and final seed P reserves at each sampling were calculated to determine the P loss. Seed P loss was assumed to be due to remobilized P exported to growing seedling compartments and possible P efflux from the seed. Phosphorus accumulation in the seedling was calculated as the sum of P in seedling leaves, roots and coleoptile plus mesocotyl at each seedling harvest date. Phosphorus loss from the seed and accumulation in the seedling overlapped until the seventh day after sowing (Fig. 2b). On the ninth day after sowing, accumulation of P in the seedling overtook P loss from the seed. The seedling had three visible leaves at this stage and thereafter P accumulation in the seedling was higher than P loss from the seed until the final seedling harvest (Fig. 2b). Seed P loss remained significantly unchanged from the fifteenth day after sowing onwards, as all seed P reserves were exhausted. The seedling roots started to uptake significant amounts of external P from the nutrient solution on the fifth day after sowing (Fig. 2b).

Seed heterotrophic P was the prime source for seedling leaf and root P nutrition during the first 10 days of early ontogeny (Fig. 3a, b). Although external P uptake was much lower than seedling P accumulation, a regular increase was observed in roots

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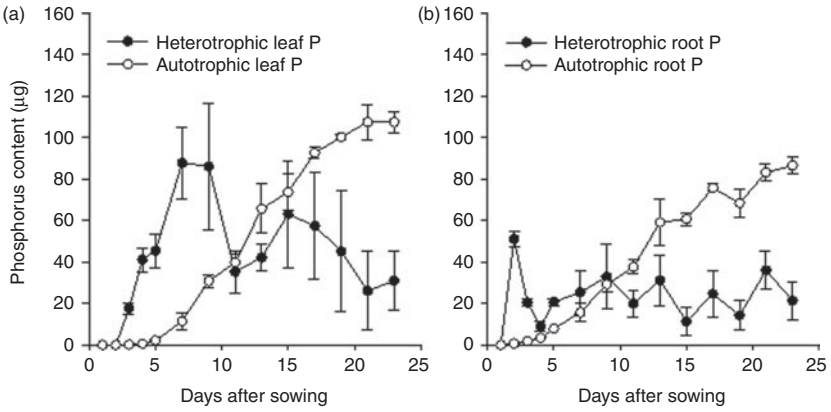

Figure 3. Seedling P reserves originating from heterotrophic (seed P, ●) and autotrophic (external P uptake, ○) P sources in (a) leaf and (b) root during early ontogeny. Data points indicate means of three replicates and bars represent the standard error of the mean.

and shoots until the last seedling harvest (Fig. 3a, b). At the beginning, leaf P originated mainly from heterotrophic P. The seedling root was the main sink for remobilized seed P reserves until the fourth day after sowing; thereafter external P uptake supported the seedling roots. Overall, heterotrophic P supply supported the seedling leaves and roots until the tenth day after sowing; from then on, external P was the main source of P used to fulfil seedling requirements.

Discussion

Carbon heterotrophy to autotrophy

Maize seed C contents are mainly stored in the endosperm (Deleens and Brulfert, 1983; Deleens *et al.*, 1984) and are remobilized soon after imbibition during germination (Nadeem *et al.*, 2011, 2012a). The radicle and mesocotyl are the first seedling organs to develop from the germinating seeds and the remobilized C reserves are allocated to them (Nadeem *et al.*, 2011, 2012a). Loss of C from the seed starts to overlap C accumulation in the seedling from the third day after sowing, when the radicle starts to elongate. Many studies have shown that this difference is lost in seed respiration (Cooper and MacDonald, 1970; Deleens *et al.*, 1984; Bouaziz and Hicks, 1990; He and Burris, 1992). In our study, the growing seedling remobilized C from the seed for respiration plus for the formation of different seedling tissues (radicle, seminal roots, coleoptile and mesocotyl). The visual difference between seed C loss and seedling C accumulation increased until the seventh day after sowing (seed C loss = 33.3 mg; seedling C accumulation = 51 mg) and a decrease was observed thereafter. The first seedling leaf appeared on the third day after sowing. The 7-day-old seedling had two visible leaves (Fig. 2a). Deleens *et al.* (1984) reported that heterotrophic seed C is the prime source in the formation of leaves of 7-day-old maize seedlings. Thereafter, both sources of C (seed C reserves and photosynthesis) were used to fulfil the seedling C demand. Until the tenth day after sowing, both heterotrophic and autotrophic C supported the seedlings. Under our experimental conditions, the apparent reduction in seed C loss and carbon accumulation in the seedling refers to a shift from its dependence on a heterotrophic source to the transition period, as clearly identified by Deleens *et al.* (1984) using C isotopic techniques. The observed decrease in C loss from the seed and the accumulation of C in the seedling (Fig. 2a) indicated that the two visible leaves had started photosynthesis and that C had begun to accumulate in the seedling from an autotrophic C source.

Although the external C resource was not labelled with isotopic C to distinguish clearly the incorporation of C from photosynthesis, our results nevertheless suggest that the C-autotrophic stage was established when the seed C reserves reached a minimum 15 d after sowing (Deleens *et al.*, 1984). Autotrophic C export from cotyledons starts soon after the onset of photosynthetic activity in sunflower leaves (Lehmeier *et al.*, 2005). Loss of C from the seed was quantitatively zero until the second day after sowing. From the second day after sowing, the difference between seed C content and seedling C content increased, reflecting increasing demand by the seedling, and up until the seventh day, seed C loss was higher compared to C accumulation in seedling. This period is identified with a dotted arrow in Fig. 2a, showing a possible heterotrophic stage for C in growing maize seedlings. At the next seedling harvest (the ninth day after sowing), the seedlings had three visible leaves, i.e. an additional source for photosynthesis. The dotted arrow shows the possible limit between the transitional period and autotrophy for C. The 15-day-old seedlings were almost entirely dependent on autotrophic C resulting from photosynthesis, as the

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majority of seed C reserves (76%) were exhausted before this harvest date. Thereafter, the seedlings were entirely dependent on photosynthesis, as seed C reserves remained significantly unchanged (Fig. 2a) from day 15 until the last seedling harvest.

The study results strengthen the findings of Deleens *et al.* (1984) who reported that photosynthesis began just after leaf emergence. On the fifteenth day after sowing, seedlings had three visible leaves. Carbon accumulation in the seedling (89 mg) overlapped C loss from the seed C (85 mg) and seed C loss stopped. This stage indicates the complete dependence of the seedling on photosynthesis, and is evidence for autotrophic seedling growth. The estimated transition period for C could be between the fifth and the fifteenth day after sowing, as indicated by the dotted arrow in Fig. 2a.

Transition from P-heterotrophy to P-autotrophy in maize seedlings

Phosphorus in seeds is primarily stored in the seed scutellum and the major form of this stored seed P is InsP_6 (Nadeem *et al.*, 2011, 2012a). The stored InsP_6 starts to be hydrolysed after imbibition and causes an increase in free orthophosphate in the seed (Nadeem *et al.*, 2011). This free orthophosphate, resulting from the hydrolysis of InsP_6 during seed imbibition, supplied P to developing seedlings during early ontogeny. The hydrolysis of InsP_6 is not an inhibiting factor for P supply to growing seedlings (Nadeem *et al.*, 2012b). Seed P contents are sufficient to sustain seedling P nutrition for several weeks after sowing (White and Veneklaas, 2012).

Different seed P status or exogenous nutrient P availability has no effect on seed P remobilization and external P uptake (Nadeem *et al.*, 2011, 2012a, b). On the fourth day, the calculated difference between loss of P from the seed and the accumulation of P in the seedling was quantitatively the same; thereafter the loss of P from the seed was less than the accumulation of P in the seedling (Fig. 2b). Thanks to ^{32}P labelling, it is very clear that the P heterotrophic stage lasted from sowing to the fourth day after sowing. The heterotrophic P supply was higher during the first 9 days after sowing. A significant external P uptake was observed in developing seedling roots from the fifth day after sowing. External P uptake increased quantitatively from the fifth day after sowing until the tenth seedling harvest (fifteenth day after sowing) when the seed P reserves were exhausted; this period thus indicates a transitional growth period with respect to phosphorus (Fig. 2b). The P-transition phase started in the seedling when only 44% of initial seed P reserves had been used by the seedling and 56% remained in the seed. The balance between the heterotrophic and the autotrophic P supply was quantitatively the same on the ninth day after sowing (Fig. 3) and the autotrophic P supply increased thereafter. From that point on, only external P supported the growing seedling, identifying it as an autotrophic stage with respect to P.

Interaction between remobilization of seed reserves of C and P

The growing maize seedlings develop their roots and leaves by consuming stored seed energy during early growth stages. Export of C towards seedling roots was twofold higher than to leaves during the first 5 d after sowing. Deleens *et al.* (1984) reported that a balance between heterotrophic and autotrophic C supply in maize seedling leaves was reached on the tenth day after sowing. Phosphorus transition from heterotrophy to autotrophy occurred simultaneously (at the fifth day after sowing) in seedling roots and leaves under our experimental conditions, whereas Deleens *et al.* (1984) reported that for C photosynthetic assimilates, the transition occurred several days later in roots than in leaves. A balance between heterotrophic and autotrophic P supply to the seedling was reached on the tenth day after sowing (Fig. 3). Leaves are the principal sites of photosynthesis, for which P is required. Our results demonstrated that both heterotrophic and autotrophic P are largely translocated towards leaves. The transitional phase is different for assimilation of newly photosynthetic C for roots and leaves in walnut (Maillard *et al.*, 1994) and sunflower (Lehmeier *et al.*, 2005) species. In our conditions, in developing maize seedlings, the P heterotrophic stage was shorter than the C heterotrophic stage (fifth day for P and seventh day for C), possibly due to differences in the quantity of C and P reserves in the seed. The transitional stage lasted for a similar time period for P and C (until the fifteenth day after germination for both). In general, the three C and P growth stages fall in almost the same time period. Research carried out on maize and walnut indicates that C accumulation and utilization starts several days later in roots than in leaves (Deleens *et al.*, 1984; Maillard *et al.*, 1994), whereas we observed no time delay; however, heterotrophic P was translocated to the leaves much more quickly than to the roots. Seed C, N and P follow similar remobilization kinetics, and P remobilization starts earlier than C in germinating maize seeds (Nadeem *et al.*, 2011). After the onset of P-autotrophy, the seedling leaves are the major sinks for seedling P reserves (Nadeem *et al.*, 2013).

Conclusion

In the above discussion, we clearly show that the three C and P growth phases occurred independently but in

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almost the same time period in maize seedlings. The heterotrophic stage was shorter for P than for C, while the transitional stage was longer for P than for C, due to the difference in seed C and P reserves. These results also strengthen the hypothesis that seed P reserves supply seedling leaves more than seed C reserves, so that the leaves can become photosynthetically active and acquire C from the surrounding environment. Further studies using both ^{14}C and ^{32}P labelling are now needed to confirm these findings.

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Conflicts of interest

None.

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