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Differential responses of Duo grass (*Lolium* × *Festuca*), a phosphorus hyperaccumulator to high phosphorus and poultry manure treatments

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Use of suitable plants to extract and concentrate excess phosphorus (P) from contaminated soil serves as an attractive method of phyto-remediation. Plant species vary considerably in their potential to assimilate different organic and inorganic P substrates. Duo grass (a hybrid of *Lolium* × *Festuca)* seedlings were grown in liquid nutrient media supplemented with various concentrations of potassium dihydrogen phosphate (KH_2PO_4) and phytate to study their P-accumulation potential. Plants that received extra P showed significantly greater plant biomass and accumulated more shoot P compared to the plants that were supplied with normal P. Duo was also grown in poultry manure amended soil and liquid media to evaluate their ability to grow and accumulate biomass in poultry manure impacted soils. Thus this grass species may be utilized as a P hyper-accumulator for phyto-extraction of excess P into their biomass from soils. Duo grass can utilize both organic (phytate), as well as, inorganic P from the growth medium as evidenced in the results.

Key words: Duo grass, phosphorus, phosphorus substrates, phytate, phosphorus accumulation, phytoremediation.

INTRODUCTION

Phosphorus, a major plant mineral nutrient, plays a pivotal role in many cellular processes including energy transfer, protein activation, and metabolic regulation. Plant absorbs P from the soil in the form of inorganic phosphate. In natural ecosystems, phosphorus (P) availability often limits plant growth because of its relatively low concentration in soil (Bieleski, 1973). This is because a considerable portion of soil P (20 to 80%) exists as organic forms and is unavailable for plant uptake (Holford, 1997). Inositol penta- and hexa-phosphates (phytates) constitutes the major component of the organic P in most soils.

To meet plant P requirement and to enhance the fertility of soils, enormous quantities of inorganic P fertilizers and animal manures are applied to soil often resulting in soil P-buildup. This excess soil P results in P-runoffs to water sources; leading to water pollution through eutrophication and algal blooms (Sharpley et al., 2000). There are several measures being considered to minimize P transport from agricultural soils to water bodies. An environmentally friendly method is the vegetative management of excess P using suitable plants that can extract P from contaminated soil and concentrate it in their foliar parts (Starnes et al., 2008).

These plant varieties termed as hyperaccumulators can hyperaccumulate P contents between 0.8 and 1.45 (%) in dry matter are useful for management of excess soil P (Novak and Chan, 2002). In this way, harvesting of the P-

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Abbreviations: KH_2PO_4, Potassium dihydrogen phosphate; **K_2SO_4,** potassium sulphate; **P,** phosphorus.

enriched biomass and removal from the system will result in a decrease in soil P concentrations. Extensive variation in the uptake and removal of P exists among the various crops species. Sharma et al. (2007) screened several crop species belonging to vegetables and herbs for their P accumulation potentials. According to their report (Sharma et al., 2007), cucumber (*Cucumis sativus* L.), squash (*Cucurbita pepo* var. *melopepo*), and sunflower (*Helianthus annuus* L.) demonstrated high shoot P accumulation (>1.0 % in shoot biomass).

Utilization of grasses for phytoremediation of P from animal manure impacted soils (Novak and Chan, 2002; Delorme et al., 2000; Frossard et al., 2000) and also from wastewater and animal lagoons (Abe and Ozaki, 1998) was already reported. Belanger et al. (2002) reported that variations exist among Timothy (*Phleum pratense* L.) genotypes on tissue P concentrations. Two cultivars of annual ryegrass, Marshall and Gulf also showed exceptional P-accumulation potential (1% dry wt) from soil as well as from hydroponic media (Sharma et al., 2004; Sharma and Sahi, 2005). These two genotypes also showed the capability of P accumulation from poultry manure containing media (Starnes et al., 2008). In a recent research, Huang et al. (2012) studied the effects of excess supply of P on the growth, P accumulation, and physiological characteristics of 2 ecotypes of *Polygonum hydropiper* L.

Duo (*Lolium* × *Festuca*) is a Festulolium grass increasingly used for forage and as turf grass. This grass is a hybrid derived from the cross between Meadow Fescue (*Festuca pratensis* Huds.) and a ryegrass (perennial or annual - diploid or tetraploid). Even though ryegrasses (*Lolium perenne* L. and *Lolium multiflorum* L.), are characterized by high yield, good palatability and digestibility, they are sensitive to various stresses. Duo grass is more superior to other existing cultivars of fescue and ryegrass due to the successful combinations of desirable traits from both parents. Duo grass shows enhanced winter hardiness and excellent tolerance against drought, which are inherited from meadow fescue. We are interested to study the suitability of this hybrid grass for P-removal from high P contaminated sites.

The aim of this study is to compare the growth attributes as well as the P accumulation of the Duo grass grown in the presence of varying concentration of two different P substrates supplied as phytate (IHP or organic P) and KH_2PO_4 (inorganic P) in the growth media. P accumulation of this grass in liquid media and soil amended with poultry manure was also determined.

MATERIALS AND METHODS

Germination of seeds

Seeds of Duo grass purchased locally were germinated on water-agar (0.8% w/v) medium in the dark at 20°C after surface sterilization with 0.1% (w/v) mercury chloride for 10 min. Germinated seedlings were grown for five days. After five days, seedlings were transplanted to filter sterilized half strength modified Hoagland's solution for an initial acclimation before subjecting to P treatments. Seedlings were acclimated by transplanting them to filter sterilized half strength modified Hoagland's solution and grown for a week before subjecting to P treatments.

Growth of grass seedlings in Hoagland's media with added P

To determine the effect of various P substrates on growth and P accumulation, Duo grass seedlings were grown aseptically in liquid Hoagland's media (Sharma and Sahi, 2005) supplemented with different P substrates. Two different P substrates inorganic or KH_2PO_4 and phytate as inositol hexaphosphate at three different concentrations, 2.5, 5 and 7.5 mM were used in the present study. All P substrates used in this study were purchased from Sigma Aldrich (99% purity, St. Louis MO, USA). P substrates were added individually to filter sterilized ½ strength liquid Hoagland's solution without P. Half strength Hoagland's solution contained 500 uM P was used as the control. Filter sterilized growth media (50 ml each) were dispensed into individual jars and acclimated Duo grass seedlings (five seedlings each) were aseptically transplanted to the media in jars and maintained in a growth chamber (Percival Scientific Inc. IA, USA) under 16 h light/8 h dark cycle (1800 to 2000 µmol m^{-2} s^{-1} of cool fluorescent light) at 20 ± 2°C with aeration. Growth media was changed every three days and seedlings were harvested after two, three, four and five weeks of treatment for analysis of biomass and shoot P content. Values represent means of three replicates ± standard error of the mean.

Growth of Duo grass in soil and liquid media amended with poultry manure

Poultry manure containing growth media was prepared by mixing of poultry manure (10, 25 and 50 g/L) in ¼ strength Hoagland's solution without P. Poultry manure collected from local farms (Bowling Green, KY, USA) was air dried, powdered in a mill and then sieved through #40 mesh screen. Powdered and sieved poultry manure was used for hydroponic and soil experiments. Manure used in the present experiment contained a P-concentration of 2 g/kg according to an earlier study (Data not shown). Nutrient solution was prepared by mixing sieved manure powder with Hoagland's solution (¼ strength) without P. Seedlings of Duo grass were transferred to (10 to 12 each) containers containing 20 ml of nutrient media and the seedlings were maintained in a Percival growth chamber at 25±2°C under 16 h photoperiod with aeration for five weeks. Seedlings were also grown in Hoagland's solution (1/4 strength) that contained 250 uM P as the control. Solution was replenished at 3 days interval and ten replicates were maintained for each treatment. Duo was also grown in soil mixed with various concentrations of poultry manure (0, 10, 25, and 50 g/kg of soil) in pots (1.5 kg/pot). Soil experiment was conducted in a greenhouse at 16 h photoperiod as described by Starnes et al. (2008). Soil used in this experiment (Western Kentucky farm, KY, USA) belonged to Pembroke series and had characteristics of Mollic epipedon was neutral to slightly alkaline. The water soluble P in soil is 4.9 mg/kg soil (Sharma and Sahi, 2005). Plants were watered three times a week and then harvested after 5 weeks of manure treatment and the total shoot P accumulation was determined as described in the next section. Values represent means of three replicates ± standard error of the mean.

Plant biomass and tissue P analysis

Following the P treatment, grass seedlings were harvested and

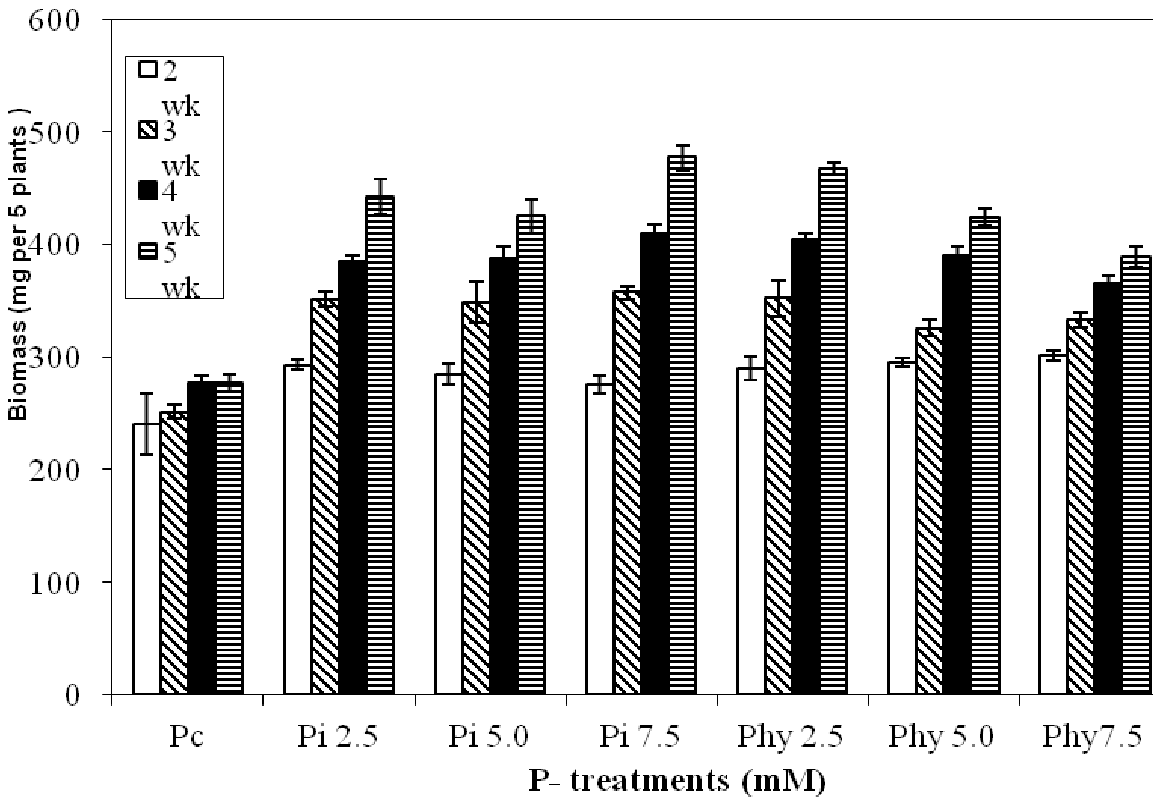

Figure 1. Effect of different P substrates on biomass of Duo grass. Duo grass seedlings were grown for 5 weeks in 1/2 strength Hoagland's solution containing 500 μM P (Pc) or P supplied either as KH_2PO_4 (Pi) or phytate (Phy) in varying concentrations (2.5 to 7.5 mM). Values represent mean of three replicates ± standard error of the mean.

rinsed with deionized water to remove traces of growth media and blotted dry. Plants were also divided into root and shoot material and dried in a hot-air oven at 70°C for three days for estimation of P concentration. Total P concentration of oven-dried tissues was measured by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) following digestion with concentrated nitric acid (Starnes et al., 2008).

RESULTS AND DISCUSSION

Biomass of grass seedlings grown in various concentrations of KH_2PO_4 and phytate over a period of five weeks was shown in Figure 1. Biomass of grass seedlings subjected to P-treatments at all concentrations showed an increase compared to the control. This effect was more pronounced in the IHP treatments. Among the various Pi treatments, increase in fresh weight of Duo grass seedlings was recorded only with an increase in the concentration of KH_2PO_4 from 5.0 to 7.5 after 5 weeks. In other treatments, significant difference was not noticed. In phytate treatments, 2.5 mM phytate resulted in more biomass than the higher concentrations of 5.0 and 7.5 mM (Figure 1).

In a study conducted in ryegrasses, Sharma and Sahi (2005) noticed that dry weights of Gulf and Marshall grasses increased with respect to increasing concentrations of soil P (0 to 10 g KH_2PO_4 /kg of soil) and later a decline in biomass was noticed beyond a concentration of 10 g P/kg soil. In this study, phytate at a concentration of 2.5 mM resulted in greatest biomass in grass seedlings that was comparable to the biomass accumulated at 7.5 mM of KH_2PO_4 (Figure 1). According to Richardson et al. (2000), the shoot dry weight of wheat plants grown with phytate was the same as the plants grown without P in the growth media.

All the KH_2PO_4 and phytate treatments caused an increase of P content in shoots with respect to controls (Table 1). The maximum increases were noticed after five weeks at 7.5 mM KH_2PO_4 and 2.5 mM phytate (17.2 and 17.4 g Kg^{-1} dry weight respectively). In the case of KH_2PO_4 treatments, a significant increment in shoot P uptake was noticed in grass seedlings corresponding to the duration of treatment. P accumulation also showed a corresponding increase with an increase in the concentration of KH_2PO_4. Shoot P concentration of 12.2 g/kg tissue dry weight was recorded in Duo after two week treatment with 2.5 mM KH_2PO_4, which increased to 13.8 g after five weeks (Table 1). In agreement with the present data, Sharma and Sahi (2005) reported that in annual ryegrasses shoot P accumulation (7.8 to 11 g/kg dry weight) was proportional to increasing concentration of

Table 1. Phosphorus accumulation in the shoots of Duo grass. Duo grass seedlings were grown for 5 weeks in half strength liquid Hoagland's solution containing 500 µM P (Pc) or P supplied either as KH_2PO_4 (Pi) or phytate (inositol hexaphosphate, IHP) in varying concentrations (2.5 to 7.5 mM). Values represent mean of three replicates ± standard error of the mean.

Treatment (mM)	Shoot P content (g kg^{-1} DW)			
	2 weeks	3 weeks	4 weeks	5 weeks
Pc	7.3 ± 0.2^d	8.6 ± 0.12^d	9.1 ± 0.41^d	9. 5± 0.19e*
Pi (2.5)	12.2 ± 0.20^b	11.8 ± 0.15^b	12.6 ± 0.51^b	13.8 ± 0.30^c
Pi (5.0)	13.3 ± 0.55^a	14.2 ± 0.20^a	11.9 ± 0.15^b	15.1 ± 0.11^b
Pi (7.5)	13.9 ± 0.08^a	12.1 ± 0.71^b	13.8 ± 0.17^a	17.2 ± 0.24^a
Phytate (2.5	9.8 ± 0.45^c	10.1 ± 1.73^c	13.4 ± 0.20^a	17.4 ± 0.23^a
Phytate (5.0)	10.6 ± 0.23^c	11.2 ± 0.20^b	9.9 ± 0.23^d	10.7 ± 0.28^d
Phytate (7.5)	9.3 ± 0.35^c	9.6 ± 0.25^c	10.6 ± 0.25^c	10.6 ± 0.28^d

*Mean followed by same letters are not statistically significant at p<0.05% level

of KH_2PO_4 in soil and in solution culture wherein the grass seedlings accumulated P in excess of 2 % of dry weight in their shoot in the presence of 5 g/L KH_2PO_4 in the growth medium. Since KH_2PO_4 is easily soluble, grass seedlings showed enhanced capability to assimilate and absorb P from this P source.

For phytate-supplied seedlings, highest shoot P uptake was noticed at the lowest concentration of phytate (2.5 mM) after five weeks of treatment, which was 17.4 g/kg shoot dry weight. According to the results, concentration of phytate greater than 2.5 mM did not result in a significant increment in shoot P uptake in the Duo grass seedlings (Table 1). Seedlings that grew in the presence of 5.0 and 7.5 mM phytate for five weeks showed P concentration of 10.7 and 10.6 g P/kg dry weight, respectively, in their shoots. Enhanced phytate concentration of 5.0 and 7.5 mM in the growth medium did not result in an appreciable increase in shoot P concentration. This may be due to the insoluble nature of phytate and grass seedlings could not absorb excess P from the growth medium as easily as evidenced in the case of KH_2PO_4. Richardson et al. (2000) reported that wheat seedlings had only limited ability to obtain P from phytate as the growth and P uptake by plants supplied with phytate was significantly reduced and was comparable to plants grown in the absence of P. Tarafdar and Claassen (1988) showed that Egyptian clover (*Trifolium alexandrinum* L.) could use phytate as a source of P in solution culture. It has also been reported that white lupin (*Lupinus albus* L.) can acquire sufficient P for growth and development from organic P sources (Adams and Pate, 1992). Wheat plants supplied with phytate as P-source also showed lesser shoot P content than the other treatments (Richardson et al., 2000). It is evident from the study that grass seedlings were not able to fully utilize the phytate or organic P and convert it into aboveground biomass at greater concentration as evidenced in our data (Table 1); but, this plant could very well utilize phytate concentrations less than 5 mM from the liquid media without much toxic effect.

Duo grass was also grown in soil and aqueous media mixed with various concentrations of poultry manure to study their efficiency to uptake P (Figure 2). According to the results of this study, not much significant difference in P-content was noticed at various levels of soil manure additions than the control. This might be due to the effect of different interacting factors in soil that will affect the availability of P to plants. In hydroponic manure treatment, the greatest shoot P-content of 11 g/kg dry weight was noticed in plants subjected to 10 g/L treatment with respect to the control. Beyond this level, plant P-accumulation showed a decrease. Starnes et al. (2008) reported that ryegrass cultivars accumulated more P at the highest poultry manure concentration (50 g/L) in soil. In hydroponic system, manure is readily available as it is in a liquid form and plant roots are exposed to the nutrients than they are in soil. This might one of the reasons that resulted in more tissue P accumulation in grass seedlings subjected to hydroponic treatment.

In conclusion**,** as part of our quest for the identification of potential P hyperaccumulator plants to use for phytoremediation of high-P soil, Duo grass was used in the present study to assess its P extracting capabilities. Taken together these results, Duo grass can be considered as a good candidate for P phytoremediation because of its combinations of favorable attributes like high productivity, winter hardiness, and less susceptibility to many biotic and abiotic stresses than the ryegrasses and also due to its ability to tolerate and assimilate poultry manure which could be utilized for remediation of P contaminated sites.

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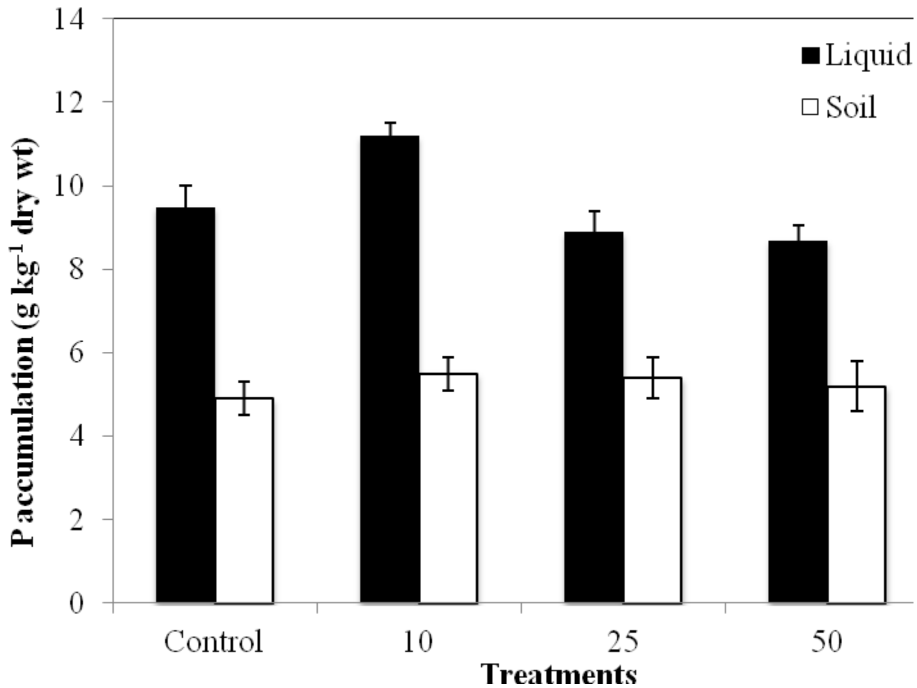

Figure 2. Phosphorus accumulation in the shoots of Duo grass seedlings grown for five weeks in Hoagland's solution (liquid) and in soil amended with various concentrations of poultry manure. Poultry manure (10, 25 and 50 g/L) was added to ¼ strength Hoagland's solution and control received no manure (contained 250 µM P). Duo grass seedlings were also grown in soil amended with poultry manure (0, 10, 25 and 50 g/kg soil). Values represent mean of three replicates ± standard error of the mean.

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