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Microbial Phytases and Phytate: Exploring Opportunities for Sustainable Phosphorus Management in Agriculture

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Abstract

Myo-inositol phosphates (phytates) are important biological molecules produced largely by plants to store phosphorus. Phytate is very abundant in many different soils making up a large portion of all soil phosphorus. This review assesses current phytase science from the perspective of its substrate, phytate, by examining the intricate relationship between the phytate-hydrolyzing enzymes and phytate as their substrate. Specifically, we examine available data on phytate's structural features, distribution in nature and functional roles. The role of phytases and their localization in soil and plant tissues are evaluated. We provide a summary of the current biotechnological advances in using industrial or recombinant phytases to improve plant growth and animal nutrition. The prospects of future discovery of novel phytases with improved biochemical properties and bioengineering of existing enzymes are also discussed. Two alternative but complementary directions to increase phosphorus bioavailability through the more efficient utilization of soil phytate are currently being developed. These approaches take advantage of microbial phytases secreted into rhizosphere either by phytase-producing microbes (biofertilizers) or by genetically engineered plants. More research on phytate metabolism in soils and plants is needed to promote environmentally friendly, more productive and sustainable agriculture.

Keywords

Phytate, Phytase, Soil Bacteria, Biofertilizer

1. Introduction

Phosphorus is one of the key elements necessary for growth and development of all liv-

ing organisms. It is essential for biogenesis of phospholipids in cell membranes, nucleic acids, ATP and plays an important role in many regulatory and metabolic processes [1] [2]. Phosphorus is also an important component of many soils, where it is often found in both organic and inorganic forms. Plants utilize mostly inorganic soil phosphates for growth and development, but phosphate concentration in many agricultural soils rapidly declines as the demand for more agricultural products intensifies. Insufficient amounts of easily extractable inorganic phosphorus is one of the most critical factors limiting agricultural yields, a problem that is typically solved by widespread application of rock phosphate fertilizer. However, such approach is not sustainable long-term and will eventually lead to the depletion of world phosphorus reserves [3]. Indeed, some reports predict that rock phosphate deposits will be exhausted by the end of this century [4]. In addition, application of rock phosphate is not very efficient, as up to 80% of all fertilizer is quickly modified, immobilized or transformed into insoluble organic phosphorus derivatives and thus, becomes unavailable to plants. Furthermore, the massive use of phosphate fertilizers has a substantial negative impact on the environment, as runoff from the fields pollutes natural water reservoirs, where it can destabilize ecosystems through eutrophication and waterlogging.

In addition to inorganic phosphates, a substantial fraction of total soil phosphorus is present in organic form [5] [6]. While the exact numbers may vary from one soil type to another, many authors estimate that various organic forms of phosphorus may constitute 30% - 80% of the total soil P [7] [8]. In certain soil types, *myo*-inositol phosphate (phytate) is one of the major forms of organic soil phosphorus making up to 50% of all organic P in soil [8] [9] [10] [11]. For example, phytate concentration was shown to vary from 3.9% to 25.3% of total extractable P in carbonate-free Cambisol soils and calcareous czernosems, respectively [12]. Phytate is a relatively stable compound and is often found in precipitated forms and in immobilized aggregates. Such precipitates are difficult to solubilize due to phytate's chelating activity and the formation of inaccessible complexes with metal cations, amino acids, peptides and various mineral soil components [13]. The phytate-peptide complexes are at least partially resistant to proteolytic degradation in gastrointestinal tract of non-ruminant animals [14], which prevents extraction of these valuable nutritional factors from plant seeds. Hence, phytate is often considered an anti-nutritional factor for animals. Undigested by animals, insoluble complexes formed by *myo*-inositol phosphate and other compounds are excreted and accumulate in soil and water, shifting their ecological balance.

Although inositol phosphate can be viewed as a rich source of phosphorus in soil, plants are largely unable to utilize it from the rhizosphere due to the low phytate-hydrolyzing (phytase) activity in plant roots [15]. Phytases release inorganic phosphate from phytate to generate low-phosphorylated *myo*-inositols. Phytases are synthesized by many microorganisms, including various bacteria, fungi, micromycetes and other microbes often collectively called biofertilizers due to their ability to promote plant growth. Secreted microbial phytases hold a high potential for biotechnology due to their often higher specific activity towards phytate [16] [17] and can potentially be used to increase soil phosphorus availability for plant nutrition. In light of this, a promising approach in plant biotechnology is to generate transgenic plants engineered to secrete

microbial phytases into rhizosphere. In theory, this approach can provide substantial amounts of phosphorus for plant nutrition, which would in turn increase plant productivity and their nutritional value for animal consumption [18]. In addition, such genetically modified plants could potentially help solve ecological problems by reducing phytate accumulation in soil and water [19] [20].

Several excellent reviews on microbial phytases have recently been published and are highly recommended [5] [6] [19]-[25]. This review focuses on phytase science from the perspective of its substrate, phytate. Specifically, we evaluate the most recent data on phytate's structure, distribution in nature and function, its role in plant nutrition. We further discuss promising environmentally friendly and cost effective strategies to increase soil phosphorus bioavailability through the use of biofertilizers or generation of transgenic plants capable of secreting microbial phytases into rhizosphere. The advantages and drawbacks of each approach, as well as the likely direction of future research, are also discussed.

2. Structural Features of Inositol Phosphates

Inositol phosphates were first identified in biological systems over 100 years ago [26] [27]. For a long time they remained the subject of intense scientific debates largely because of lack of clear understanding of their dimensional structures. Specifically, the exact three-dimensional model of inositol was unclear until studies using nuclear magnetic resonance and X-ray diffraction analysis demonstrated the vast structural diversity of different isoforms [28]. We now know that the exact conformation of inositol (cyclohexane-1,2,3,4,5,6-hexol) varies depending on bond location, leading to the formation of multiple stereoisomers [1] [29]. Hydroxyl groups of stereoisomers are oriented either axially or equatorially, thus resulting in nine possible inositol conformations [29]. The names of all nine inositol stereoisomers are typically highlighted in italics. *Myo*-inositol with one axial and five equatorial hydroxyl groups has the most stable conformation (the "chair") (**Figure 1**) [1] [30] [31] [32].

When hydroxyl groups in inositol ring are replaced by phosphate residues the molecule becomes a phosphorylated alcohol-inositol phosphate. Depending on the number of phosphates, several different compounds can be formed-from inositol-monophosphate to inositol-hexakisphosphates. For example, *myo*-inositol hexakisphosphate is the phosphate salt of *myo*-inositol, in which all six hydroxyl groups are substituted by phosphate residues (**Figure 2**). According to the official nomenclature, this compound is *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen) phosphate, but often it is also called *myo*-inositol hexakisphosphate or simply phytate. The official abbreviation for this

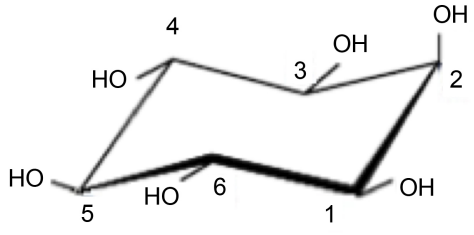

Figure 1. *Myo*-inositol "chair" conformation.

compound is $InsP_6$ or IP_6. While sometimes phytate is also called phytic acid, this terminology is not generally applicable to other stereoisomers of phosphorylated inositol.

Inositol phosphates are very strong natural poly-anion chelators of biologically important metal cations. Closely positioned phosphate groups in *myo*-inositol are able to form intramolecular links with metal cations Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+} and Mn^{2+}. A single phosphate group can also form more than one hydrogen bond with metal ions, creating stable chelating complexes (**Figure 3**) [23] [33] [34] [35]. The formation of phytate-metal complexes depends on the cation ionic radius. Bivalent cations with a long ionic radius, such as Ca^{2+} (0.99 Å) and Sr^{2+} (1.12 Å), are associated with two adjacent phosphate groups of phytate molecule. In contrast, cations with a short ionic radius, such as Mg^{2+} (0.65 Å), Fe^{2+} (0.74 Å) and Zn^{2+} (0.71 Å), can form hydrogen bonds with just one phosphate group of phytate [33]. In plant tissues and seeds phytate can form complexes with Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+} and Co^{2+} with relatively high affinity, with Ca^{2+}-phytate complex being the most common. Interestingly, phytate complexes with bivalent cations are formed in both acid and alkaline conditions and are found in either dissolved or precipitated states [23]. Phytate may also form complexes with trivalent iron and aluminum cations *in vivo* [36] [37].

Negatively charged phosphate residues in phytate can also bind positively charged

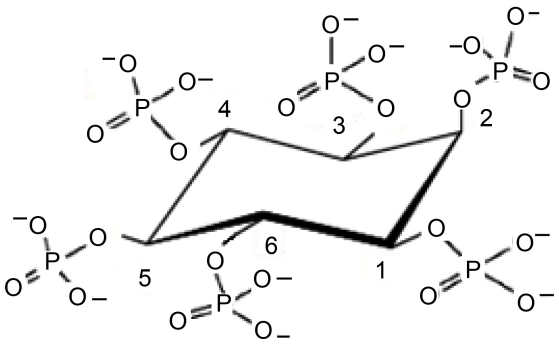

Figure 2. *Myo*-inositol 1,2,3,4,5,6-hexakisphosphate.

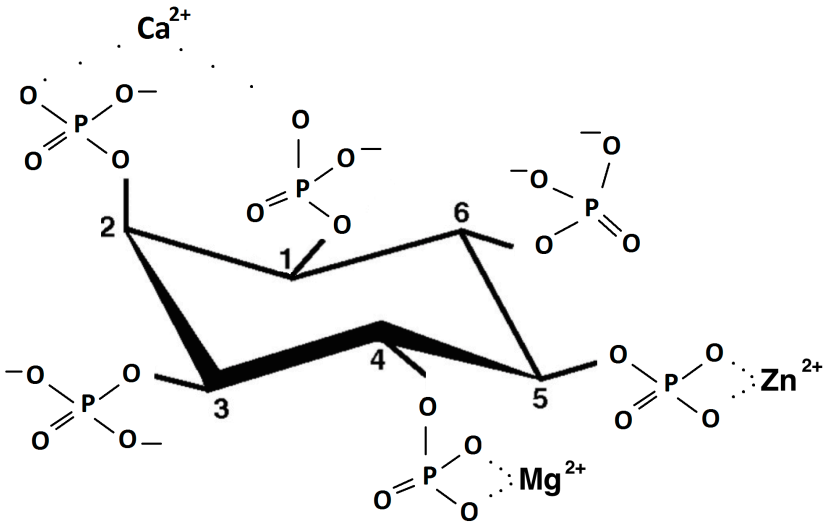

Figure 3. Phytate complex with bivalent metal cations.

amino acid residues in peptides and native proteins leading to the formation of stable protein-phytate complexes. A trimeric protein-metal cation-phytate complex can also be formed both in acid and alkaline pH conditions [23] [38]. Overall stability of such complexes largely depends on pH, concentration and cation type.

3. Phytate Distribution in Soil

The most wide-spread phosphorylated inositols in soil are *myo*-, *scyllo*-, *chiro*- and *neo*-inositol phosphates, of which *myo*-inositol hexakisphosphate is the most common. The other three phosphorylated stereoisomers can be ranked from more to less abundant in the order of *scyllo* > *chiro* > *neo*, but these are generally very rare in biological systems [39] [40]. Inositol phosphates with low phosphate group content (from monophosphate to tetrakisphosphate) are also uncommon in soil.

Inositol phosphates can undergo epimerization, when two stereoisomers (for example, *myo*-inositol and *scyllo*-inositol) that differ by spatial location of only one chemical bond can turn into each other. Experiments with labeled carbon isotopes established that *myo*-inositol in certain soil conditions can be phosphorylated by soil microorganisms to yield *myo*-inositol hexakisphosphate [39]. However, natural chemical synthesis of phosphorylated inositol stereoisomers in soil is thought to be rare, mainly because such chemical reactions require prolonged heating in strongly acid or alkaline conditions. Instead, most soil inositol phosphates are assumed to be synthesized by living organisms, including plants and soil microbes.

Indeed, *myo*-inositol hexakisphosphates are widely distributed in various plant tissues (especially seeds), as well as in other eukaryotes. *Scyllo*-inositols mostly in monophosphate form are found in aleurone layer of barley seeds. D-*chiro*-inositol hexakisphosphates are present in small quantities in pine needles and in leaves of flowering trees [39]. Other inositol phosphate stereoisomers have not yet been found in plants. Phytate appears to be the main storage form of phosphorus and inositol in plants. Phytate makes up to 30% of all phosphorus fractions in roots, while its fraction increases up to 80% in seeds and cereal grains [21] [41] [42]. Phytate mostly accumulates at the last stages of plant life cycle and is returned to soil with seeds, where it is again made available to plants during germination via intrinsic phytases [43] [44]. Seed phytate can often be found in association with insoluble salts of potassium, magnesium and other metals and is stored in globular inclusions (globoids) of aleurone layer and in embryo vacuoles. For example, more than 80% of phytate in maize is present in embryo [45]. Typically, seed ripening and germination are accompanied by changes in pH, temperature, metal cation concentration that promote conversion of phytate complexes to a more soluble form [46].

4. Phytases as Biological Tools to Harvest Inorganic Phosphorus from Phytate

The enzymes phytases belong to the general class of phosphatases (EC 3.1.3) and hydrolyze phytate to release inorganic phosphorus [47]. Phytases are classified into several families with important differences in structure, substrate specificity, pH-optimum and mechanism of hydrolysis. Some of the histidine acid phosphatases (HAPs) have

low pH-optimum and a broad substrate specificity, while most known alkaline β-propeller phytases (BPPs, mostly of bacillar origin) are specific only to phytate molecule and its complexes. Phytate-degrading enzymes are commonly found in oilseeds and nuts, legumes and in cereal pollen grains. Phytase activity is abundant in cereal seeds (rye, triticale, barley, wheat) and in pseudo-cereal fruits (amaranth, buckwheat). Legumes and oilseeds have about 10 times lower phytase activity than grain seeds [48]. Phytases from plant seeds are often associated with membrane structures and are present in aleurone layer in cereals and in cotyledons in legumes, where large quantities of phytate are also found [48] [49]. Some stages of plant life cycle, such as seed germination, are characterized by increased level of phytase activity which is necessary to promote fast growth of seedlings [47]. Most purified and characterized plant phytases have acidic pH-optimum averaging around pH 5.0 and are stable up to 55˚C - 60˚C. For example, a maize phytase has maximum activity at 55˚C and pH 4.5, while a soybean phytase GmPhy has pH-optimum at pH 4.5 - 5.0 and is stable up to 60˚C [49].

The few plant phytases that can be found in roots are characterized by low hydrolytic activity and are not secreted into rhizosphere. Phytase activity of *Arabidopsis thaliana* roots represents less than 0.8% of total root acid phosphomonoesterase (phosphatase) activity [15]. Furthermore, this phytase does not appear to be an extracellular enzyme. Therefore, *A. thaliana* and other plants are mostly unable to grow on phytate as the only source of phosphorus in the agar medium [15]. Similarly, experiments with wheat in laboratory conditions established that low phytase activity in plant roots is the main factor limiting wheat ability to obtain phosphorus from phytate [50].

While many soil bacteria and fungi are known to produce extracellular phytate-hydrolyzing enzymes, not all of these enzymes have high catalytic activity. Soil micromycetes, yeast and members of *Bacillus* and *Enterobacter* genera produce some of the most active extracellular phytases known to date. Bacterial phytases from *Bacillus* and *Enterobacter* possess high specific activity, have pH-optimum in a broader range of pH 3.5 to 7.5 and temperature optimum at 37˚C - 70˚C [19] [51]. Many alkaline bacillar phytases are characterized by narrow substrate specificity, which is restricted only to phosphomonoester bonds in phytate molecules [33]. In addition, many bacterial phytases, such as histidine acid phytases from *A. niger* and *E. coli* and *Bacillus* β-propeller phytases, are resistant to proteolytic degradation by pepsin, papain, pancreatin and trypsin [52] [53] [54] [55]. All these features make bacterial phytases a very attractive option for animal feed additives.

5. Microbial Phytases as Molecular Biofertilizers

High agricultural productivity in the future will largely depend on the continued technological advances to reduce fertilizer application rates and the cost of food production. The use of bacterial phytases is thus envisioned as an effective means to improve plant growth and yield. While plants are unable to extract phosphorus from soil phytate, this limitation can potentially be overcome if plants are treated with certain phytate-degrading bacteria, sometimes also called biofertilizers. A substantial number of rhizosphere bacterial species are now known to be beneficial for plant growth as they increase availability of otherwise insoluble phosphorus-containing soil compounds. For

example, the availability of phytate-derived phosphates was improved when wheat seeds were pre-incubated with soil bacterium *Pseudomonas sp*. CCAR59 that is known to have extracellular phytase activity [50]. Other known bacterial biofertilizers include species of *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Azospirillum* and *Burkholderia* [56].

Indeed, soil microorganisms are often viewed as an abundant source of biofertilizers [57]. *Enterobacter* strains selected from the rhizosphere of legumes have a positive effect on plant growth and phosphorus nutrition and are known to produce phytases [58]. Due to the presence of high phytase activity a strain of *Pseudomonas* sp. from Australian agricultural soils was able to release up to 80% of all phosphate from phytate, thus positively affecting plant development [59]. A number of bacteria with phytate-hydrolyzing activity that are able to improve plant phosphorus nutrition was isolated from white lupine (*Lupinus albus*) rhizosphere in Japan [60]. Almost all of these strains were classified as representatives of *Burkholderia* family. In addition, some *Pseudomonas*, *Enterobacter*, and *Pantoea* isolates are also able to release inorganic phosphate from phytate [61]. Overall, it is now becoming increasingly clear that phytase-producing soil bacteria (mostly belonging to gamma-proteobacteria) are widespread in the rhizosphere of different plant species.

In general, bacterial biofertilizers containing live microorganisms are widely used to improve crop yield in India, China, Iran and other countries. Specifically, the use of nodule bacteria, Azotobactor/Azosporillum and Phosphobacteria-based biofertilizers is relatively common [62]. Microbial biofertilizers are typically produced in liquid, powder and granular formats [63]. Biofertilizers as a highly efficient alternative to chemical fertilizers are praised for their relative ease of application, non-toxic and eco-friendly nature, and cost effectiveness [64]. The positive effect of bacterial biofertilizer cells on plant physiology can generally be associated with increased availability of limiting nutrients, such as nitrogen, phosphorus, group B vitamins and amino acids. In addition, several other positive effects of biofertilizers on plant growth have generally been noted: suppression of diseases caused by plant pathogens (possibly through competition with pathogenic microorganisms for root colonization), microbial synthesis of plant growth regulators, and reduction of ethylene levels in root cells [65]. However, the more widespread commercial use of bacterial biofertilizers is to some degree limited by our insufficient knowledge of the ecological, molecular and physiological impact of microbial communities on plant growth [66]. Nevertheless, the use of natural soil bioresources, including soil microorganisms, can serve as a promising alternative to the currently standard application of inorganic fertilizers.

The latest scientific findings are consistent with the notion that microbial phytases play a fundamental role in soil phosphorus life cycle. Indeed, due to their potentially substantial agronomic and ecological value for plant growth during periods of long-term phosphorus deprivation, microbial phytases become an appealing target for industrial use [61]. For example, treatment of seeds with a fungal extracellular phytase promoted plant phosphorus nutrition in soils with high phytate content [67]. Similarly, enrichment of phosphate-limited soils with phytase-producing bacteria, such as *Bacillus mucilaginosus* and *B. amyloliquefaciens*, was shown to improve growth of tobacco and corn, respectively [65] [68]. Finally, bacterial phytases also positively impact plant

nutrition by freeing up important soil microelements typically chelated by phytate. Thus, the use of biofertilizers in the form of either bacterial culture liquid purified microbial phytases or live phytase-producing bacterial strains can be viewed as an efficient and environmentally friendly approach to increase bioavailability of soil phosphorus and reduce the currently widespread use of inorganic phosphate fertilizers.

6. Transgenic Plants as a Promising Alternative to Phosphate Fertilizers

Several new biotechnological advances now make it possible to utilize phytate as an abundant source of phosphorus, especially for farm animals. Many phytases of bacterial or fungal origin are traditionally used as animal feed supplements to improve phosphorus balance in monogastric farm animals, such as pigs, chicken and fish. These include phytases from *Aspergillus ficuum* (niger) (sold as Natuphos), *Aspergillus niger* (All-zyme), *Aspergillus awamori* (Finase and Avizyme), *Aspergillus oryzae* (SP, TP, SF, AMA-FERM, and Phyzyme), and *Peniophora lycii* (Ronozyme, Roxazyme, and Bio-Feed phytase) [69]. Additional technologies include: pre-processing grains to activate endogenous phytases; mutations in phytate metabolism genes that decrease phytate synthesis rate in plant seeds; the use of genetically modified farm animals that produce phytases in saliva or genetic modification of plants to express microbial phytases [19]. The latter is a particularly promising approach as it can serve several purposes. Plants engineered to express phytase genes of microbial origin can secrete extracellular enzymes to rhizosphere where they can break down soil phytate, leading to improved accumulation of phosphorus in plants and increased biomass [23] [70]. In addition, transgenic plants expressing phytases in seeds are expected to have lower seed phytate content and thus represent a more nutritious feed for farm animals.

In several recent studies transgenic plants have been established and evaluated for changes in organic and inorganic phosphorus accumulation in plant tissues and seeds. Histidine acid phytases from *Aspergillus niger*, *A. ficuum*, *A. fumigates* and from other fungi and yeast are widely used in these experiments as they are stable in a broad range of pH and temperature [20] [71]. Bacterial phytase genes, such as 168phyA from *Bacillus subtilis* and appA from *E. coli*, have also been used successfully to generate transgenic plants [19] [72] [73] [74] [75]. In some laboratory experiments these genes have been successfully expressed in transgenic tobacco, soybeans, alfalfa, corn, wheat, sweet potato, canola and *Arabidopsis thaliana*, often indeed improving utilization of phytate as the source of phosphorus [15] [64] [76]-[84].

Biochemical properties of recombinant phytases expressed in plants usually differ very little from the enzymes expressed by their native hosts. Recombinant phytases are often less glycosylated and thus have lower molecular mass, but have otherwise similar enzyme activity, substrate specificity and thermal stability [19]. Transgenic soy roots expressing *A. ficuum* histidine acid phytase (afphyA) were shown to have 6 and 3.5 fold higher catalytic activity and inorganic phosphate content than wild-type control plants, respectively [82]. Transgenic *A. thaliana* plants growing on phytate as the only source of phosphorus showed improved growth associated with overexpression of *A. niger* histidine acid phytase gene *phyA* in roots [79]. Expression of *A. niger* phytase fused

with carrot extensin signal peptide in *A. thaliana* resulted in recombinant phytase secretion into rhizosphere and 20-fold increase in rhizosphere phytase activity [15]. Heterologous expression of *A. niger* phytase in wheat decreased *myo*-inositol hexakisphosphate content in seeds by 86% and had a positive effect on transgenic wheat nutritional properties [78].

Despite the long history of using fungal phytases for transgenic plant research, soil bacteria of the genus *Bacillus* recently emerged as a potentially better source of recombinant phytases. This bacterial genus is characterized by the presence of unique β-propeller phytases that have several key advantages over their fungal counterparts. Bacillar alkaline phytases exhibit narrow substrate preference specific only to phytate, have a remarkably high phytate-hydrolyzing activity at physiological pH values, and are also resistant to both high temperature and proteolytic degradation. Thus, perhaps not surprisingly, more encouraging results in transgenic plant research were recently obtained using bacillar phytases. For instance, transgenic tobacco plants expressing phytase 168phyA from *B. subtilis* showed increased biomass (up to 2-fold) and higher number of flowers and fruits compared to the wild type when grown on phytate as the only source of phosphorus [72]. Similarly, expression of *B. subtilis* 168PhyA phytase in *Arabidopsis thaliana* led to a higher shoot dry weight and an increase in phosphorus content by 100% compared to the wild type [75].

Despite these encouraging results in laboratory conditions, the situation in actual soils may nevertheless turn out to be less promising. In fact, to date there is limited evidence that such transgenic plants are indeed characterized by improved P acquisition and plant growth in natural soils. One clear example of improved P acquisition from natural agricultural soils was the transgenic expression of phytase and APase genes in alfalfa using a root-specific promoter, though the effect appeared to vary with the type of soil tested [85]. On the other hand, most published reports up to date support the notion that the situation in real soils may be very different from exciting results obtained in laboratory media. For example, experiments with transgenic *Trifolium subterraneum* expressing phytase *phyA* established that improved P uptake and increased plant growth previously observed in agar was compromised when the same plants were grown in real soil [86]. Furthermore, transgenic tobacco plants expressing a fungal phytase gene did not show improved P acquisition when grown in P-deficient soils [87]. Similarly, expression of *B. subtilis* phytase transgene in tobacco resulted in improved phosphorus uptake from phytate only in sterile laboratory conditions but not in real soil [88]. In another report, while constitutive overexpression of AtPAP15 gene in soybeans did result in higher yield, this phenotype was mainly achieved by increased internal P use efficiency rather than by enhanced soil P acquisition [89]. Taken together, these data clearly suggest that effectiveness of plants expressing transgenic phytases might be limited. One potential explanation is that biochemical properties of recombinant phytases, such as stability or optimum pH, could be substantially modified because of local soil conditions. Indeed, microbial community in the rhizosphere may not support physiological and nutrient changes introduced by plants with transgenic phytases [90]. This apparent failure of genetically engineered plants secreting bacterial phytases to demonstrate improved growth in real soil conditions may also stem from

the fact that secreted phytases quickly lose activity in soil possibly due to adsorption of the enzymes (though this process appears slower in the rhizosphere) [87]. This rapid enzyme immobilization may thus quickly limit phytase capacity to interact with phytate in soil and undermine all previously envisioned advantages of such transgenic approach to improving plant phosphorus metabolism.

Nevertheless, with necessary future improvements in phytase choice and growth conditions the use of transgenic plants expressing microbial phytase genes may still be a substantial step towards solving the problem of utilizing soil phytate as the source of phosphorus. As more inorganic soil phosphorus can enter the plants expressing microbial phytases, such plants are expected to have improved nutritional properties for animal or human consumption. Furthermore, transgenic plants expressing microbial phytases are expected to have reduced requirement for external supply of rock phosphate or treatment by biofertilizers, leading to the overall reduction in the price of final agricultural products [72]. Finally, degradation of soil phytate by recombinant phytases could also decrease the extent of soil and ground water pollution caused by organic phosphorus compounds.

7. Towards Future Strategies to Improve Plant Phosphorus Metabolism

To feed the ever-growing world population, modern agriculture will continue to rely on improvements in biotechnology. Two alternative but often overlapping strategies that rely on the use of bacterial phytases should be considered as potentially viable options. Bacterial phytases can either be genetically introduced into crops or supplied to soil as purified enzymes or through application of microbial biofertilizers. In comparison to their eukaryotic counterparts, bacterial phytases are often cheaper to manufacture and easier to express in plants using modern molecular and genetic tools. In addition, bacterial biofertilizers are often easy to cultivate in large volumes and subsequently use to treat plant roots or seeds. When searching for the best way to take advantage of microbial phytases, several factors should first be carefully considered. The first aspect is the need to identify, either bioinformatically or through careful microbiological and biochemical screening of soil microorganisms, producers of highly active and thermo-stable phytases of microbial origin. Towards this goal, our group has recently sampled a number of soils from various ecological habitats (forest, private homesteads, large agricultural complexes and urban landscape) and identified a *Pantoea* sp. 3.5.1 strain producing a novel glucose-1-phosphatase dubbed AgpP with high phytase activity and unusual activation by metal ions [91]. This novel enzyme, while similar in some aspects to several previously characterized fungal phytases, is unique in having a set of biochemical advantages over the well-established industrial enzymes. Specifically, the *Pantoea* sp. 3.5.1 AgpP phytase has pH optimum in the acidic range, suggesting that similar to other phytases, AgpP could function in the upper part of the digestive tract of poultry [92] [93]. However, unlike many fungal phytases, this novel enzyme is most active at 37˚C, much closer to the typical body temperature (37˚C - 42˚C) of most warm-blooded animals [94], thus giving researchers another option in the available arsenal of bacterial enzymes. Finally, in contrast to most other known phytases, activity of AgpP

phytase is stimulated by some metal ions, a desirable feature for an enzyme that breaks down phytate-metal ion complexes [91]. The identification of this and other phytases with improved or more desirable biochemical characteristics can lead to the development of better industrial phosphatases with regulated enzyme activity.

Second, if a bacterial phytase is chosen for the purpose of generating transgenic plants, one important factor to consider is the choice of efficient expression system in plants. Often transgenic phytases need to be delivered into rhizosphere to help promote degradation of soil phytate and increase soil phosphorus bioavailability. In this case the phytase transgene is often fused with a root-specific promoter and a signal peptide, such as carrot extensin, for efficient enzyme translocation over the membrane [19] [51]. Finally, if the purpose of generating transgenic plants is to reduce phytate content in seeds or other plant tissues, it is often necessary to knock out genes of inositol phosphate biosynthesis pathway [95] or to engineer plants expressing recombinant microbial phytase under the control of constitutive 35S promoter or seed-specific promoters [19] [72] [73] [75]-[80] [83] [84] [96] [97]. Such genetic manipulations are expected to result in improvements in feed nutritional quality, especially for animals with single chamber stomachs (pigs, chicken, fish, etc.), which are unable to extract phosphorus from phytate.

Overall, the development of transgenic plants expressing recombinant phytases (perhaps, induced by phosphate starvation) is often viewed as a promising route to solving problems of soil phosphorus availability and increasing the efficiency of phosphate nutrition in plants [24]. However, potentially encouraging breakthroughs in this area are often limited or stymied due to mistrust of consumers, prohibitive legislation in many countries, as well as still substantial technological limitations. Thus, any future efforts to capitalize on recent scientific discoveries and achievements in generating promising transgenic organisms will need to go hand-in-hand with overall policy change and careful consideration of public concerns towards new biotechnologies.

As an alternative to transgenic plants, phytase-producing microorganisms (biofertilizers) can serve as an efficient path to improving soil phosphorus availability to plants [69] [98]. Richardson emphasized two ways by how soil microorganisms can contribute to phosphorus bioavailability in plants: directly through expression of soil phytate-hydrolyzing enzymes [99]; and through the production of other organic compounds that solubilize or modify phytate thus making it more accessible to other organisms. Secretion of organic acids by rhizosphere bacteria is an especially important characteristic of sustainable phosphorus management in the soil as some evidence suggests that roots of agroforestry species are unable to secrete such acidic compounds [100]. The most common compounds secreted by microorganisms that contribute to organic phosphorus mobilization in soil are organic acids, such as malate, citrate and oxalate [101] [102]. In addition to organic acids, bacteria can produce indole acetic acid (IAA), siderophores, vitamins, amino acids, ammonia and cyanide. Furthermore, microorganisms provided in the context of biofertilizers can compete with other microbes for colonization of plant roots, reduce ethylene production and suppress diseases caused by pathogenic bacteria and fungi [56].

While biofertilizers clearly represent a promising route for modern agriculture, many

obstacles still exist to their successful application in the field. Despite the well-documented positive effects of bacterial biofertilizers on plant growth in some settings, a giant gap needs to be bridged between these successful greenhouse experiments and field studies, where many biofertilizers often fail to substantially improve plant growth [103] [104]. This discrepancy may be due to several factors, such as unfavorable interaction with other rhizosphere organisms, adverse physical and chemical soil properties (e.g., low pH), poor ability of strains to colonize plant roots and other environmental factors, such as high ambient temperature and low rainfall during the growing season. Many of these factors can negatively affect the outcome of biofertilizer application. One possible strategy to overcome these limitations is the use of microorganisms adapted to the particular climatic conditions of agricultural region [105].

8. Conclusion

In the last few decades our reliance on non-renewable rock phosphate fertilizers has become a major limiting factor affecting environmental, political, and economic aspects of modern agriculture. To sustain current and future agricultural needs, several novel approaches to phosphorus management in the field have been proposed, including the use of biofertilizers and genetically engineered plants. A particularly useful synergistic effect could potentially be achieved by the combined use of genetically-modified plants secreting efficient bacterial phytases into rhizosphere and simultaneous application of biofertilizers that contain microorganisms adapted for local environmental conditions. While the ultimate goal of many researchers is to create the best conditions for efficient phosphorus nutrition, increased biomass and yield, more studies are clearly necessary to chart the best strategies and to develop advanced biotechnologies that rely on microbial or plant-produced phytases. Overall, better mechanistic understanding of the relationship between phytase properties, phytate availability and roles in plant physiology will be required to improve plant nutrition.

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Conflict of Interest

No conflict of interest declared.

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