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Low-phytate grains to enhance phosphorus sustainability in agriculture: genetic analysis on the *low phytic acid1-1* maize mutant

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GENERAL INTRODUCTION AND THESIS SUMMARY

Phosphorus is a non-renewable resource

Food production, coming from modern intensive farming systems, depends on the constant supply of inputs, such as nitrogen, phosphorus, and potassium. The phosphorus (P) used in agricultural processes is obtained from rock phosphate, a non-renewable resource that has no substitutes (Cordell et al., 2009; Bennet and Elser, 2011). There are two key reasons why the sustainable use of phosphate is important: the supply is running out, and paradoxically much of what is produced is wasted and results in environmental damage (Baker et al., 2015).

The mineable deposit areas are limited and geographically concentrated in a small number of countries: in fact, only five countries hold the 85% of the world's phosphate rock reserves, with 70% found in Morocco and Western Sahara. In most countries, food systems rely on importing phosphorus fertilizers, making them vulnerable to P supply risks (Cordell and White, 2014). At current mining rates, the worst estimates for reserve longevity are 50 years and the best around 200 years (Baker et al., 2015).

However, geopolitics, policies, fees, taxes, and legislation can affect immediate access to available P reserves (Khabarov and Obersteiner, 2017). This vulnerability was observed in 2008 when the price of phosphate rock increased by 800%, resulting in a rise in fertilizer prices that impacted the livelihoods of many of the world's poorest farmers (Baker et al., 2015; Khabarov and Obersteiner, 2017). Already now, 1 in 7 farmers cannot afford enough fertilizers to maintain fertile soils, impacting their ability to produce food. Without a change, the insufficient use of P fertilizer in Africa will lead to reductions in crop yields of nearly 30% by 2050 (Van der Velde et al., 2014). In contrast, in countries of Europe, North America or Southeast Asia, the heavy application of P-fertilizer results in different problems, such as the rapid depletion of non-renewable P resources, the increased production cost, and the eutrophication of water (Baker et al., 2015).

It was estimated that nearly 50% of the elemental P used in global crop production is accumulated in the seeds in a storage form as phytic acid (PA) (Lott et al., 2000). Seeds are an important component of food and feed, but the capability of monogastric animals to use the P from PA is limited (Raboy, 2009). In industrialized countries, livestock farmers use feed with high P concentrations or add P salts (e.g., dicalcium phosphate) for animal nutrition. In fact, the 5% of globally P demand is for feed additives (Schröder et al., 2011). Taken together, these factors encourage a way toward the phosphorus recovering and reduction in demand and losses for crops and livestock, contributing to a more sustainable agriculture (Cordell et al., 2009).

Phytic acid is an antinutrient

Phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate; $InsP_6$; PA) is ubiquitous in eukaryotic cells and constitutes the most common storage form of phosphorus in plant seeds (from 60% to 85%) (Raboy, 1997). PA is synthesized in the endoplasmic reticulum and during maturation is deposited in the protein storage vacuole inside inclusions called globoids (Raboy, 2002). In mature maize kernels, 80% of PA is localized in the embryo and in the scutellum, while the remaining 20% in the aleurone layer (O'Dell et al., 1972). Due to its negative charge at the physiological pH, PA chelates positively charged cations (such as iron, zinc, potassium, magnesium, calcium), forming poorly bioavailable phytate salts. During germination, these mixed salts are hydrolysed by a group of enzymes called phytases, releasing both myo-inositol and cations, to support seedling growth (Laboure et al., 1993). Only ruminants can degrade PA thanks to the presence of symbiotic bacteria endowed with phytase activity in their digestive systems. Vice versa, PA forming mixed salts is mainly excreted by monogastric animals (including humans) because they do not possess the phytase enzyme: they assimilate about ~10% of the phytate in the feed, while ~90% is excreted, contributing to P pollution, water surface eutrophication and algal proliferation. Therefore, farmers must supply mineral phosphorus to the feed of monogastric animals, also implying an economic problem (Raboy, 2009). Different approaches have been proposed to tackle the problems connected with PA: phytases industrially produced can be added to the feed and the enzymatic activity releases inorganic phosphate for animal use, thus reducing the P excreted (Greiner and Konietzny, 2006); incubate the feed in water for a short time to activate the potential endogenous phytases present in the seeds; use of transgenic animals able to produce fungal phytases in their salivary glands (Golovan et al., 2001); use engineered crops to increase the production of active phytases in seeds; isolate *low phytic acid* mutant plants.

About *low phytic acid* crops

Among the different strategies proposed to overcome the problems related to PA, in the last decades many *low phytic acid* (*lpa*) mutants have been isolated in all major crops (Raboy, 2009). They are characterized by a reduced amount of PA, followed by a proportional increase in inorganic phosphate, without altering the total P content. These mutants are characterized by many potential benefits, mainly in: (i) improving phosphorus management in non-ruminant production; (ii) contributing to enhance the sustainability and reduce animal waste P; (iii) increasing mineral bioavailability as a strategy to tackle mineral deficiencies, as recently reviewed (Raboy, 2020). The increased bioavailability of P and cations in *lpa* seeds was confirmed by nutritional trials on monogastric animals (Mendoza et al., 1998; Hambidge et al., 2004, 2005). In this way, mineral P integration or phytase addition is not required and the presence of P in wastes is drastically reduced (Raboy, 2009).

Any discussion on seed PA should consider the fact that it represents the major bottleneck in P flux through the world agricultural ecosystem (Figure 1). The total amount of PA phosphate produced annually by the main seed crops represents a sum equivalent to almost 65% of annual fertilizer P production (Lott et al., 2000). This bottleneck represents a key target in efforts to reduce the negative environmental impact of agricultural production. Agricultural P runoff contributes to water pollution and water surface eutrophication, which in turn causes oxygen depletion and "dead zones" (Oliveira and Machado, 2013).

Low-phytate grains can help reduce the world-wide problem of eutrophication in two ways: i) primarily through its beneficial change in seed chemistry, in which total P remains unchanged, but substantially more of that P is bioavailable for monogastrics, resulting in lower P in "waste" (Figure 1); ii) via *lpa* alleles that alter the chemistry of the seed and affect reduced seed total P amount. For instance, if one could reduce seed P by 25% without affecting yield, that would be equivalent to increase the "fuel efficiency" of that crop, at least in terms of macronutrient P; the same amount of grain per unit of production would be obtained, but during the harvest remove 25% less of P would be removed from the field, leaving it in the field for following years (Rose et al., 2013).

Low phytic acid mutants have been isolated in major crops by distinct methods: by chemical mutagenesis in barley (Larson et al., 1998; Rasmussen and Hatzack, 1998; Bregitzer and Raboy, 2006), in wheat (Guttieri et al., 2004) and in common bean (Campion et al., 2009; Cominelli et al., 2018); by physical and chemical mutagenesis in rice (Larson et al., 2000; Liu et al., 2007) and in soybean (Wilcox et al., 2000; Hitz et al., 2002; Yuan et al., 2007).

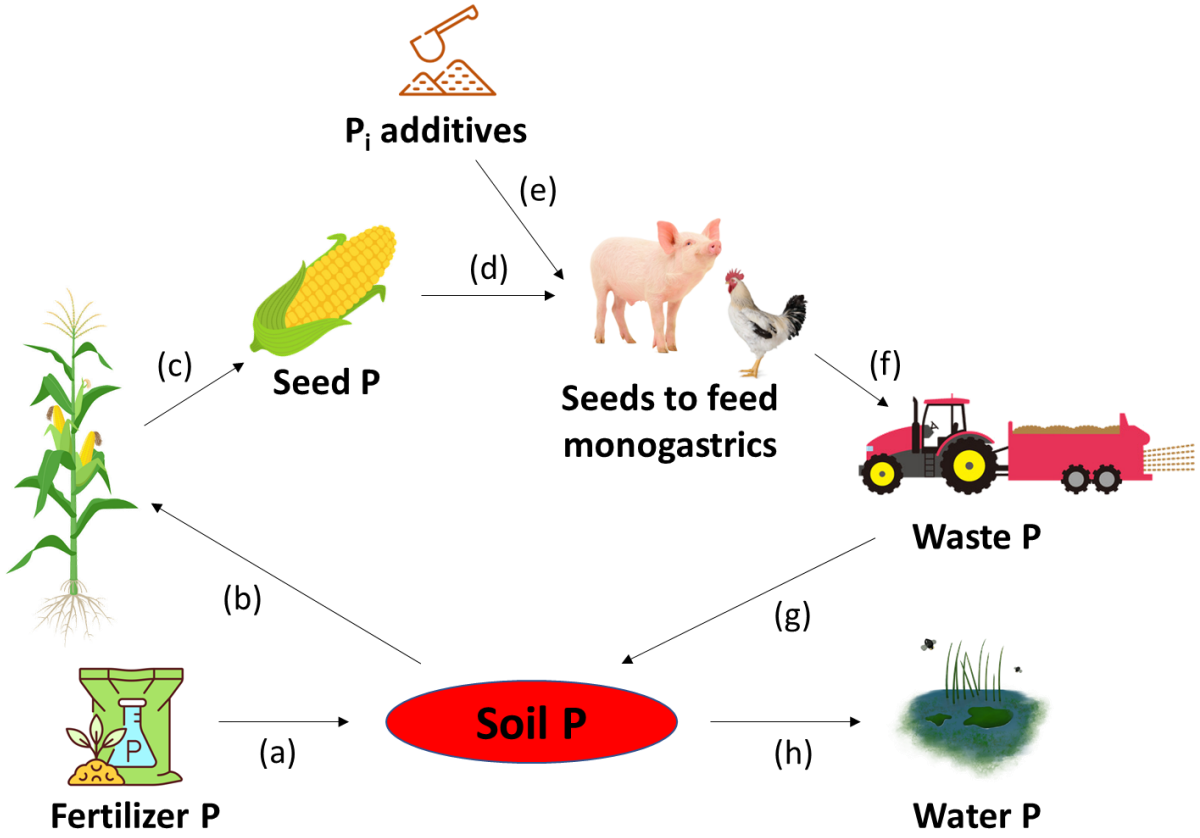

Figure 1. Schematic representation of Phosphorus cycle in agricultural systems.
(a) Use of industrially produced Pi (inorganic phosphate) to fertilize the soil.
(b) P_i is absorbed by roots from the soil and translocated into the plant. Only a small quantity of P_i is available for the plant because P_i is scarcely mobile and remains bound to soil particles.
(c) Phytic acid (PA) is the main storage form of phosphate in seeds.
(d) Seeds are mainly used as feed for livestock.
(e) Pi additives are added to the feed of monogastric animals.
(f) The lack of phytases activity in monogastics causes the presence of large amounts of P in manure as undigested PA.
(g) Manure containing high amounts of undigested PA is used to fertilize the land. Up to 80% of P can be rapidly fixed in forms unavailable to plants.
(h) The increased Pi content could increase the P_i loss to the aquatic environment with risk of eutrophication.

About *low phytic acid* mutants in maize

In maize, three *lpa* mutants have been isolated: *lpa1* (Raboy et al., 2000; Pilu et al., 2003) and *lpa2* (Raboy et al., 2000) by chemical mutagenesis, *lpa3* by transposon tagging (Shi et al., 2005). Compared to the other mutations in maize, *lpa1* exhibited the major reduction of PA in the seed; this decrease is followed by a proportional increase of inorganic or free P, without modifying the total amount of P. *lpa1* mutations are caused by lesions in *ZmMRP4* gene (accession number EF586878), a multidrug-associated-protein (MRP) belonging to the subfamily of ATP-binding cassette (ABC) transmembrane transporters (Shi et al., 2007). MRP proteins are implicated in different roles in the

plant, such as xenobiotic detoxification, regulation of stomatal guard cell movements, and oxidative stress tolerance (Gaedeke et al., 2001; Swarbreck et al., 2003; Klein et al., 2006; Hwang et al., 2016). Four important mutations have been isolated so far in the *ZmMRP4* PA transporter: *lpa1-1*, consisting of a point mutation that determines an A1432V substitution (Shi et al., 2007); *lpa1-241*, a paramutagenic allele described by Pilu and colleagues which causes a series of negative pleiotropic effects depending on its strength (Pilu et al., 2005, 2009); *lpa1-7*, probably determined by a mutation in the coding sequence (Cerino Badone et al., 2012); *lpa1-5525*, a *lpa1* mutant allele obtained by transposon tagging mutagenesis, but not yet fully characterized (Borlini et al., 2019).

In particular, *lpa1-1* is the most promising *lpa1* mutant from an agronomic point of view: it shows a moderate reduction in PA content (66%) and it is the only one viable in its homozygous state (Raboy et al., 2000). On the other hand, in *lpa1-241* and *lpa1-7* mutants, displaying a drop in PA content greater than 80%, germination is suppressed (Pilu et al., 2005; Cerino Badone et al., 2012).

Unfortunately, the reduction of PA in *lpa* mutants causes a series of adverse pleiotropic effects on the seed and on plant performance, such as reduced germination and emergence rate, lower seed filling and weakening in stress resistance (Raboy et al., 2000; Pilu et al., 2005; Doria et al., 2009; Cerino Badone et al., 2012) (Figure 2).

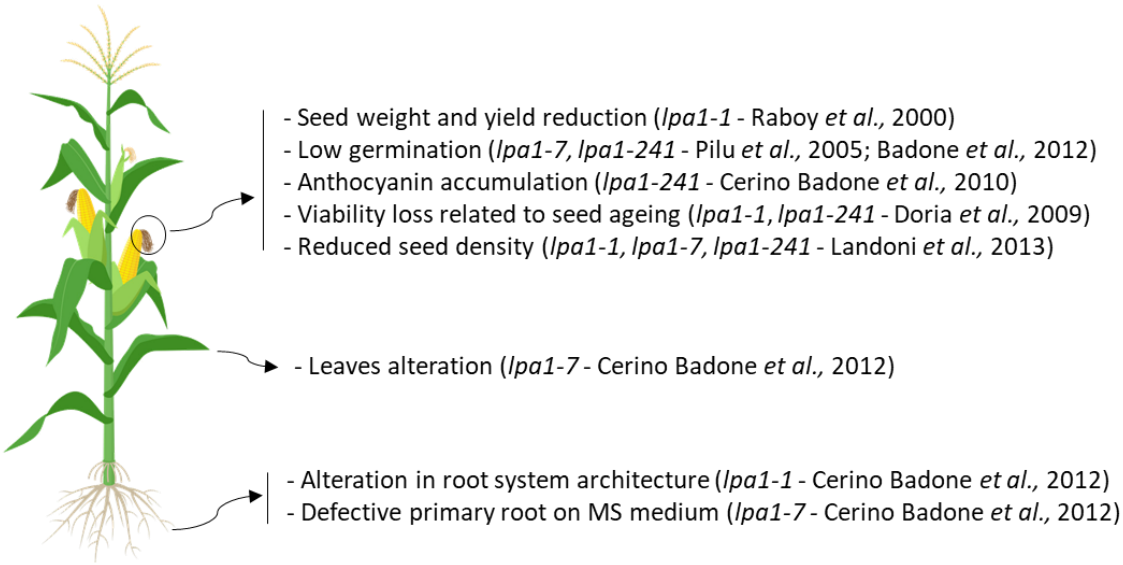

Figure 2. Summary of the main pleiotropic effects characterizing *lpa1* mutants in *Zea mays* L.

Summary of the thesis work

The *low phytic acid* trait can provide several potential benefits to the nutritional quality of foods and feeds and to the environmental P sustainability in agriculture (Sparvoli and Cominelli, 2015). Nevertheless, the reduction of PA in *lpa1* mutants is frequently accompanied by negative pleiotropic

effects leading to agronomic defects which may affect either seed viability and germination or plant development, thus limiting the interest of breeders. Although some significant results have been reached, the isolation of *lpa* mutants improved for their nutritional quality and with a good field performance remains a goal so far not fully achieved for many crops, including maize.
In this PhD thesis we have initially summarized the main pleiotropic effects that have been reported to date in *lpa* mutants in five productive agronomic species, and we have addressed some of the possible challenges to overcome these problems and improve the breeding efforts for *lpa* mutants (**published review; Colombo et al., 2020, first manuscript presented in this PhD thesis**).

Among the negative pleiotropic effects associated with these maize mutants, we focused mainly on two agronomic factors affecting the yield:

1) the increased susceptibility to drought stress in *lpa1-1* is one of the main limitations of this mutant. In a context of climate change, the problem of drought stress will be even more frequent and severe in the next years. In this PhD thesis, *lpa1-1* was compared to wild genotypes using different approaches, spanning from controlled conditions to the field. In all these experiments, the collection of agronomic data was divided into two parts:
    i) hypogeal parameters were collected to study the root system architecture, not yet investigated in this mutant
    ii) epigeal parameters were collected in different growth stages on the above-ground part of the plant

2) the reduced seed germination strongly limits the performance of *lpa1-1* in the field, negatively affecting the yield. To overcome these problems and restore germinability in *lpa1-1* seeds, two possible approaches have been proposed in this work:
    i) a genetic approach, based on classical breeding
    ii) seed priming technology

Regarding the first goal, low water availability is considered a primary limitation to crop productivity all around the world (Lynch, 2007; Lobell et al., 2014) and the severity of drought stress on crops will increase in the future because of climate change (Tebaldi and Lobell, 2008). The greater susceptibility to water scarcity in *lpa1-1* could be caused by an alteration in root system architecture (RSA) or by differences in the aerial part of the plant, such as a reduced photosynthetic activity or an altered stomatal regulation. In this PhD thesis, we have investigated drought stress using three different approaches, including hydroponics, greenhouse, and field.

In hydroponics, the RSA of *lpa1-1* did not appear to be less developed compared to the wild-type; rather, the mutant root system was characterized by a greater development in the first weeks after germination and the measured parameters highlighted an early growth of lateral roots and root hairs. So, RSA did not appear to be a limiting factor in *lpa1-1*, and we thought that the different susceptibility to drought stress between the two genotypes could be caused by a different root depth. To monitor root depth weekly, we set up two experiments in the greenhouse and plants were grown in transparent PVC mesocosms filled with soil. From the results obtained, it emerged that there were no significant differences either in the depth of the embryonic primary root or in that of post-embryonic crown roots. Hence, our research shifted to the epigeal part of the plant: from the data collected it emerged that *lpa1-1* exhibited reduced leaf temperature compared the inbred line B73, probably due to increased water loss from stomata. We also used the fluorimeter, and it was found that the efficiency of photosystem II was lower in mutant plants, probably due to a greater dissipation of energy in the leaves.

These experiments were performed under controlled or semi-controlled conditions, and it emerged that the drought stress in *lpa1-1* seemed to be caused by a reduced photosynthetic efficiency and not by a shallower root system **(published paper; Colombo et al., 2022, second manuscript presented in this PhD thesis).**

Based on the results obtained in controlled or semi-controlled conditions, we set up a dedicated experiment at the experimental field of University of Milan in Landriano (PV, Italy, N 45°18′, E 9°15′). The plants of the inbred line B73 and its relative *lpa1-1* were grown till flowering, roots were sampled and cleaned. The parameters collected on the hypogeal part of the plant confirmed a greater development of lateral roots in the mutant; these roots are essential for the uptake of water and nutrients, particularly in stressful conditions. On the epigeal part of the plant, measurements were carried out in two different conditions: under well-watered conditions and under moderate drought stress. The parameters collected with the CIRAS-2 revealed a greater stomatal opening in the mutant under well-watered conditions: the transpiration rate and the stomatal conductance were significantly higher in *lpa1-1* compared to the wild-type. These results were in line with the previous experiments in controlled conditions – where *lpa1-1* exhibited a lower leaf temperature and a greater stomatal opening compared to the wild-type – but also in accordance with the results obtained by analyzing the carbon stable isotope composition ($\delta^{13}C$) of B73 and *lpa1-1*.

The situation changed completely when water stress occurred: the same parameters did not change in the wild-type, while dropped in the mutant: the net photosynthesis decreased by 58%, the transpiration rate by 63% and the stomatal conductance of the 67%.

This experiment conducted in the field supports once again the hypothesis that the increased

sensitivity to drought stress in the mutant is mainly caused by an altered stomatal regulation and not by a less developed root system **(published paper; Colombo et al., 2022, third manuscript presented in this PhD thesis).**

In the same manuscript, we described a randomized block experiment conducted in the university experimental field with the aim of estimating the yield of the mutant. To our knowledge, after the isolation of this mutant by Raboy and collaborators in 2000, no articles that estimated the yield of *lpa1-1* in the field have been published. The experiment was conducted for two years in two different genetic backgrounds and plants were grown under high-input conditions. We compared *lpa1-1/lpa1-1* vs +/+ control in B73 genetic background and *lpa1-1/lpa1-1* vs +/+ in B73xMo17 genetic background. From the parameters collected it emerged that *lpa1-1* seemed to have comparable seed weight/ear than the relative control; the main problem of this mutant remains the reduced emergence in the field (~40%), which limits the interest of breeders and, at the moment, makes impossible the creation of a competitive commercial variety**.**

Starting from the reduced emergence in the field recorded in the mutant, our efforts have focused on restoring germinability in *lpa1-1* seeds. The study of this pleiotropic effect represented the second goal of this work.

Despite being considered as an antinutritional factor, PA exhibits a strong antioxidant activity, avoiding the formation of reactive oxygen species (ROS), thus preserving the viability of seeds (Doria et al., 2009). The reduction of PA in *lpa* mutants results in the accumulation of free iron cations (and high level of toxic ROS), causing a reduced seed germination and a viability impairment related to seed ageing, previously reported on *lpa1* mutants.

In this phD project, we set up two different experiments to study both natural and artificial ageing: using a historical series of naturally aged seeds, we showed that *lpa1-1* seeds aged faster compared to wild seeds; then, to mimic natural ageing, we set up an accelerated ageing treatment at different temperatures: incubating the seeds at 57 °C for 24 h, the wild-type germinated at 82.4% and *lpa1-1* at 40%, while at 60°C only *lpa1-1* was no longer able to germinate.

We also proposed two possible solutions to overcome the problem of seed ageing and, in general, of reduced seed germination: first, classical breeding was used to constitute synthetic populations carrying *lpa1-1* mutation with genes pushing anthocyanin (natural antioxidant) accumulation in the embryo (R-navajo allele). We thought that in this way it was possible to compensate the loss of antioxidant activity caused by PA reduction in *lpa* mutants. The results showed that the presence of R-navajo in *lpa1-1* genotype was not able to improve the germinability (-20%), but this approach could be useful to improve the germinability in non-mutant genotypes (+17%). The second approach is based on seed priming technology, a pre-sowing treatment that enables seed to germinate more

efficiently and increases stress tolerance. In this work, hydropriming was tested on *lpa1-1* and wild-type seeds subjected to accelerated ageing: it emerged that germinability was improved by 20% in *lpa1-1* seeds, suggesting a possible role of seed priming in restoring germination rates and accelerating germination **(submitted paper; Colombo et al., 2022, fourth manuscript presented in this PhD thesis).**

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Research review

MRP Transporters and Low Phytic Acid Mutants in Major Crops: Main Pleiotropic Effects and Future Perspectives

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Abstract: Phytic acid (PA) represents the major storage form of seed phosphate (P). During seed maturation, it accumulates as phytate salts chelating various mineral cations, therefore reducing their bioavailability. During germination, phytase dephosphorylates PA releasing both P and cations which in turn can be used for the nutrition of the growing seedling. Animals do not possess phytase, thus monogastric animals assimilate only 10% of the phytate ingested with feed, whilst 90% is excreted and may contribute to cause P pollution of the environment. To overcome this double problem, nutritional and environmental, in the last four decades many low phytic acid (*lpa*) mutants (most of which affect the PA-MRP transporters) have been isolated and characterized in all major crops, showing that the *lpa* trait can increase the nutritional quality of foods and feeds and improve P management in agriculture. Nevertheless, these mutations are frequently accompanied by negative pleiotropic effects leading to agronomic defects which may affect either seed viability and germination or plant development or in some cases even increase the resistance to cooking, thus limiting the interest of breeders. Therefore, although some significant results have been reached, the isolation of *lpa* mutants improved for their nutritional quality and with a good field performance remains a goal so far not fully achieved for many crops. Here, we will summarize the main pleiotropic effects that have been reported to date in *lpa* mutants affected in PA-MRP transporters in five productive agronomic species, as well as addressing some of the possible challenges to overcome these hurdles and improve the breeding efforts for *lpa* mutants.

Keywords: *low phytic acid mutants*, phytic acid, *MRP transporters, pleiotropic effects, nutritional and environmental problems*

Introduction

In plants, phytic acid (PA) (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate) represents the major storage form of phosphate (P) in seeds (up to 85%) (Raboy, 1997). PA is synthesized in the endoplasmic reticulum and during maturation it is deposited in the protein storage vacuole inside inclusions named globoids (Raboy, 2002). The location of the PA reserve inside the seed varies depending on the species: in maize it is mainly accumulated in the embryo and in the scutellum, while in rice and wheat, 80% of PA is found in aleurone and maternal teguments, and only small quantities are in the embryo (O'Dell et al., 1972). In legumes, such as soybean and common bean, more than 95% of PA is found in cotyledons (Ariza-Nieto et al., 2007). Due to its high negative charge at the physiological pH, PA chelates cations (such as iron, zinc, potassium, calcium, magnesium), forming poorly bioavailable phytate salts. During germination, phytase and other enzymes degrade PA releasing *myo*-inositol,

orthophosphate and cations, which can be remobilized to support seedling growth (Laboure et al., 1993). Among animals feeding on seeds, only ruminants can degrade PA thanks to the presence in their digestive systems of bacteria endowed with phytase activity. However, monogastric animals (including humans) possess almost no phytase activity in the digestive tract, thus they degrade only about ~10% of the phytate in the feed, while ~90% is excreted. Therefore, farmers breeding pigs, poultry, fish and other monogastric animals must provide supplementary feed with mineral phosphorus and cations. Due to the paucity of the global inorganic P reserves, this in turn implies an economic problem. Moreover, the excreted amount of PA-derived P is high in manure, and consequently in soils, thus contributing to P pollution and to eutrophication of groundwater, a serious environmental problem (Raboy, 2009). For these reasons, PA is considered an anti-nutritional compound and its reduction or elimination in cereal and legume seeds has been and is still an important challenge in plant breeding programs. Among the different strategies used to achieve this result, many low phytic acid (*lpa*) mutants have been isolated in all major crops (Naidoo et al., 2012; Sureshkumar et al., 2014). These mutants may have some advantages, mainly (i) improving phosphorus management in non-ruminant production, (ii) contributing to enhance sustainability and reduce animal waste P, and (iii) increasing mineral bioavailability as a strategy to combat mineral deficiencies, as recently reviewed (Raboy, 2020). According to the step of the PA biosynthetic pathway, *lpa* mutations can be divided into three categories: 1) mutations affecting the first step in which *myo*-inositol 3-phosphate synthase (MIPS), the first enzyme of the biosynthetic pathway, transforms glucose 6-P into *myo*-inositol(3)-monophosphate leading to a relevant decrease in PA accumulation and a simultaneous increase in inorganic phosphate (Pi); 2) mutations in different genes coding for enzymes involved in the successive phosphorylation steps of PA pathway, from *myo*-inositol(3)-monophosphate to PA leading to accumulation of inositol phosphates (InsPs) intermediates which represent a distinctive characteristic only for this second class of mutants; 3) mutations affecting the transport and storage of PA into the vacuole through the MRP transporters (Fig. 1). In the last category of mutants, PA is exposed to the attack of dephosphorylating enzymes, thus strongly decreasing the amount of PA and increasing that of Pi, the same features registered also in the first category of mutants. This similarity between categories 1 and 3 generated a lot of confusion in the first characterization of some mutations in PA transporter genes. In fact, the first experiments carried out to map the maize *lpa1* mutation seemed to reveal a lesion in a member of the gene (located on chromosome 1, coding for *MIPS*). Moreover, in mutants affecting *ZmMRP4*, *ZmMIPS1S* expression is reduced (Raboy et al., 2000; Pilu et al., 2003; Shukla et al., 2004; Shi et al., 2007); later, mapping and expression data found that in maize both *ZmMIPS* and *ZmMRP4* map very closely on chromosome 1S. A few years later, transposon mutagenesis experiments performed by Shi et al.

demonstrated that *ZmMRP4* (accession number EF86878), coding a multidrug resistance-associated-protein (MRP), is the gene responsible for *lpa1* mutation (Shi et al., 2007). All these mutations cause the lack of PA transfer from the cytosol into the storage location of the vacuole. This, in turn, probably exposes PA to a dephosphorylation process carried out by cytosolic phosphatases, thus remarkably decreasing the final amount of phytate and simultaneously increasing that of free Pi and cations, which during maturation accumulate into vacuolar protein bodies in seed storage tissue.

A high proportion of these *lpa* mutants have been shown to carry mutations in genes coding for Multidrug-Resistance-Proteins (MRPs). These proteins belong to the ABCC cluster of plant ATP-binding cassette (ABC) transporters found in many species which translocate anions of various organic molecules across intra-cellular membranes (Shi et al., 2007; Gillman et al., 2009; Nagy et al., 2009; Xu et al., 2009; Panzeri et al., 2011; Sparvoli and Cominelli, 2014; Cominelli et al., 2020b). Such a class of mutants appears the most interesting one, since it shows the highest drop in PA level together with a concomitant substantial increase of free P and a consequent supposed increase in free cations. Unfortunately, almost all *lpa* mutations described during the last four decades, including the ones affecting the PA-MRP transporters, are associated with poor agronomic performance which is linked to many negative pleiotropic effects regarding mainly (but not exclusively) seed viability and plant development (Raboy et al., 2000; Meis et al., 2003; Guttieri et al., 2004; Pilu et al., 2005; Bregitzer and Raboy, 2006). Pleiotropic effects in *lpa* mutants may be ascribed to the pivotal role of inositol metabolites as signaling molecules in key cellular pathways, such as hormonal perception, epigenetic control of the chromatin landscape, cellular trafficking and calcium homeostasis (Sparvoli and Cominelli, 2015). In plants it is almost accepted that $InsP_6$ instead of $InsP_3$ is involved in signaling. The first evidence was the finding that, in guard cells, $InsP_6$ triggers intracellular Ca^{2+} release after ABA addition with an efficiency ≈100 times higher than that of $InsP_3$ (Lemtiri et al., 2003). However, despite evidences for the signaling pathway canonical $InsP_3$/$InsP_6$ receptors have never been reported in plants. These mutants have received very little interest until now, mainly due to their negative pleiotropic effects. However, a recent analysis suggested that the choice of strategies alternative to the use of *lpa* mutants (such as the addition to animal feed of P or phytase to increase the component of available phosphorus) has been done without calculating the possible long-term money-saving deriving from using the *lpa* crops (Raboy, 2020).

The present review focuses in particular on the pleiotropic effects reported to date in cereals' and legumes' *lpa* mutants affected in PA-MRP transporters, which have disclosed a number of very interesting clues to shed more light on seed physiology and to offer tools suitable to develop biotechnological and sustainable approaches aimed at improving food and feed.

MRP-type ABC transporters and PA transport

ATP-binding cassette (ABC) transporters are plant transmembrane transporters that beside being involved in the transport of molecules necessary for plant growth (hormones, lipids, metabolites and defense compounds) across cell membranes, are involved in different plant processes, such as xenobiotic detoxification, regulation of stomatal guard cell movements and oxidative stress tolerance (Gaedeke et al., 2001; Swarbreck et al., 2003; Klein et al., 2006; Hwang et al., 2016). In most cases the driven transport occurs against electrochemical gradients using the energy supplied by ATP hydrolysis (Wilkens, 2015). ABC transporters are ancient macromolecules widespread in all organisms, and in plants 8 subfamilies have been identified. They are generally characterized by a common structure composed of two soluble nucleotide-binding domains (NBD1, NBD2) and two hydrophobic transmembrane domains (TMD1, TMD2), which contain six transmembrane α-helices (Fig. 2). NBDs contain the Walker A and Walker B motifs separated by around 120 amino acids as well as an ABC "signature". In most cases domains are forward-oriented in the following way: TMD1-NBD1_TMD2-NBD2, however the NBDs and TMDs may be arranged in the opposite fashion: NBD1-TMD1_NBD2-TMD2, and ABC transporters 'made up by half-size' units also exist (Verrier et al., 2008). Multidrug-resistance-associated (MRP) proteins belong to the ABCC cluster of plant ABC transporters. Unlike other ABC transporters, MRP proteins are characterized by an additional extremely hydrophobic N-terminal extension (TMD0) consisting of around 220 amino acids. TMD0 contains five transmembrane α-helices, it is positioned before TMD1 and is connected via a cytosolic loop (CL3) to the rest of the protein (Sparvoli and Cominelli, 2014) (Fig. 2). These proteins share a very high degree of similarity among different species (Cominelli et al., 2020a). The role of TMD0 in plants is not yet defined, while normally the CL3 portion plays a key function in the recognition and transport of the substrate (Gao et al., 1998). In 2007 Shi et al. isolated the maize *lpa1* mutation affecting the *ZmMRP4* gene (accession number EF586878), through the screening of a transposon mutagenized plant collection. These authors demonstrated for the first time that an MRP-type ABC transporter was required for PA transport (Shi et al., 2007). This finding was biochemically confirmed in 2009 by Nagy and co-workers who isolated a mutant in the *Arabidopsis thaliana AtMRP5* gene, ortholog of *ZmMRP4* (Nagy et al., 2009). This gene had been characterized a few years earlier for functions apparently unlinked to PA transport, such as root growth, lateral root formation, stomatal movement regulation, anion transport, water use efficiency and guard cell hormonal signalling (Gaedeke et al., 2001; Klein et al., 2003; Su et al., 2007). As a result of these findings, other *PA-MRP* genes and their corresponding mutants were later characterized in species of agronomic interest such as *Oryza sativa* L. (Liu et al., 2007; Xu et al., 2009), *Glycine max* (L.) Merr. (Gillman et al., 2009, 2013; Saghai Maroof et al., 2009), *Phaseolus vulgaris* L. (Panzeri et al., 2011;

Cominelli et al., 2018), *Triticum aestivum* (RNAi lines in the *ABCC13* genes), (Bhati et al., 2016) (Sup. Mat. Table 1). Although the gene structure (exon-intron arrangement) of PA-MRP transporters is similar in the different crops (Cominelli et al., 2020b), the main difference between cereals (excluding the hexaploid wheat harbouring three different *ABCC13* genes) and legumes is in gene number: maize and rice are characterized by a single gene copy (*ZmMRP4* and *OsMRP5*, respectively), while legumes have two or three paralogues: *PvMRP1* and *PvMRP2* in common bean and *GmMRP3*, *GmMRP13* and *GmMRP19* in soybean (Panzeri et al., 2011; Sparvoli and Cominelli, 2014; Cominelli et al., 2018). Indeed, these two species shared a whole-genome duplication event (Lavin et al., 2005) and later soybean underwent another independent whole-genome duplication (Schmutz et al., 2010). PA-MRP protein sequences are highly conserved, even if it is not well known which amino acid residues are involved in PA transport. A multiple alignment of the amino acid sequences in comparison with the sequence of Arabidopsis ABCCs, highlighted some peculiarities: a conserved stretch of lysine residue (found in the cytosolic loop between NBD1 and TMD2), but also the fact that several amino acid residues (Lys and Arg) located in the two TMD domains, seem to be involved in PA transport (Sparvoli and Cominelli, 2014).

Pleiotropic effects of *lpa* mutations in *PA-MRP* genes

The use of *lpa* mutations, in terms of increasing nutritionally cation bioavailability in the diet, enhancing phosphorus management and reducing environmental impact due to reduced P waste in non-ruminant production, could be an important tool to increase the sustainability of agricultural production.

Unfortunately, *lpa* mutations, including the ones affecting the *PA-MRP* genes, are frequently accompanied by negative pleiotropic effects visible either at the level of seed or plant, thus limiting the interest of breeders (Raboy et al., 2000; Meis et al., 2003; Pilu et al., 2005; Landoni et al., 2013; Raboy et al., 2020).

To study the pleiotropic effects of mutations in the PA-MRP transporters, it is important to take also into consideration the possible variation in the content of inositol pyrophosphates (PP-InsP), caused by the mutation. A small pool of PA present in the cell is further phosphorylated to form PP-InsP, containing one or two diphosphate groups ($InsP_7$ and $InsP_8$, respectively). PP-InsP have important roles in energy metabolism, hormone signaling (mainly jasmonate), and Pi sensing (Freed et al., 2020). A recent review pointed out that different Arabidopsis *lpa* mutations affecting PA biosynthetic genes, also cause a reduction in the content of $InsP_8$ and in some cases of $InsP_7$. Due to the important role of these molecules, a decrease in their content may affect pathogen response and Pi sensing (Freed et al., 2020). On the other hand, the Arabidopsis *mrp5* and the maize *lpa1* mutants show

increased content of both $InsP_7$ and $InsP_8$. Hence, from this point of view, *PA-MRP* genes can be considered an interesting target for the development of *lpa* mutants not compromised in P homeostasis and in jasmonate signaling (Freed et al., 2020).

In the following sections, we will describe the main pleiotropic effects so far reported in *lpa* mutants affected in PA-MRP transporters in five important productive agronomic species: maize, rice, wheat, soybean and common bean (Fig. 3).

Maize

In maize, *lpa1* mutations are caused by lesions in the *ZmMRP4* gene. Four important mutations have been isolated so far in the *ZmMRP4* PA transporter: *lpa1-1*, consisting of a point mutation that determines an A1432V substitution (Shi et al., 2007); *lpa1-241*, a paramutagenic allele described by Pilu et al. which causes a series of negative pleiotropic effects depending on its strength (Pilu et al., 2005, 2009); *lpa1-7*, probably determined by a mutation in the coding sequence, even if the nature of a paramutagenic allele can be discarded due to its stability (Cerino Badone et al., 2012); *lpa1-5525*, a recently found *lpa1* mutant allele obtained by transposon tagging mutagenesis (Borlini et al., 2019), but not yet fully characterized. All these mutations lead to a reduction in the kernel PA content, accompanied by a proportional increase in P_i, even if the total P remains unchanged. In particular, *lpa1-1* allele shows a 66% reduction in PA content and is viable in its homozygous state (Raboy et al., 2000), while in the case of *lpa1-241* and *lpa1-7* mutants, displaying a drop in PA content greater than 80%, germination is suppressed (Pilu et al., 2005; Cerino Badone et al., 2012). Among the negative pleiotropic effects associated with these maize mutants, a seed weight reduction ranging from 8 to 23% characterized *lpa1-1* (Raboy et al., 2000). This decrease appears to be mainly caused by endosperm loss and consequently results in an agronomic yield reduction. Concerning this mutant, it was also observed that under field conditions, *lpa1-1* is more susceptible to drought stress, probably due to an alteration in mature root system development (Cerino Badone et al., 2012).

The *lpa1-241* mutant showed a variety of morphological and physiological changes of which the negative effects appear connected to the "strength" of the mutation. In fact, in the *lpa1-241* mutants the PA content is variable and it was shown that individual seeds with less than 20% of wild type PA content are unable to germinate (Pilu et al., 2005). Such an observation might be explained by the finding that an imperfect alignment between root and shoot primordia occurs, thus introducing an asymmetry in the body plan (Pilu et al., 2005, 2009). An embryo-rescue technique (embryos removed from the seed and transferred to Murashige and Skoog, MS medium) allowed the restoration of high germination capacity in *lpa1-241* seeds, even if many defective seedlings were found and their growth was slower compared to wild type.

The characterization studies carried out on the same maize *lpa1-241* mutant allowed the discovery of a hitherto unknown role of the PA presence in the seed. In fact, Doria and co-workers used this mutant as a tool to study the consequences of the lack of this important reserve substance on seed survival and longevity (Doria et al., 2009). In this study the focus was on iron homeostasis; in the anaerobic cell environment the oxidation of unchelated Fe^{2+} to Fe^{3+} is a potential source of Reactive Oxygen Species, considered the main cause of the viability loss related to seed ageing.

Due its ability to remove cations, PA was hypothesized to be a good candidate for protecting the embryo from such oxidative processes. Consequently, these authors collected data on germinability, free iron level, free radical relative abundance both by EMR (Electronic Magnetic Resonance) and histological evidence, protein carbonylation level, amount of damage to DNA, degree of lipid peroxidation, tocopherol level and antioxidant capacity level of seeds of maize B73 (control) and of an isogenic low phytic acid mutant (*lpa1-241*), either unaged or incubated for 7 days in accelerated ageing conditions (46 C° and 100% relative humidity). Results clearly demonstrated that *lpa1-241* mutant seeds, compared to the wild type ones, show: 1) a lower germination capacity, which decreased further after accelerated ageing; 2) about 50% more free or weakly bound iron; 3) upon accelerated ageing, an higher content of free radicals mainly concentrated in the embryo, a higher extent of carbonylation of seed proteins and of damage (apurinic/apyrimidinic sites) on DNA, whereas lipids did not appear more peroxidated, although□□-tocopherol content was decreased by about 50%, probably because it is consumed just to prevent membrane peroxidation; 4) an higher level of antioxidants such as total glutathione and tocopherol, the synthesis of which is probably induced by the increase of ROS, as well as a higher level of anti-radical power measurable by the DPPH test.

These findings were interpreted in terms of previously reported, but never proven, antioxidant activity of PA through iron complexation. In conclusion, behind the fundamental role of P and cations storage, PA appears to play another important function consisting in the protection of embryo viability from oxidative stress during seed maturation and dormancy.

Another pleiotropic effect in *lpa1-241* concerns the accumulation of anthocyanins in the kernel.

In fact, Goodman *et al.* observed that *ZmMRP4* is expressed in the aleurone layer and is co-regulated with another MRP protein (*ZmMRP3*), expressed in all tissues accumulating anthocyanins, particularly in the husk suggesting that it is somehow involved in anthocyanin transport (Goodman et al., 2004)

These expression data were confirmed in lines carrying both the *lpa1-241* mutation and the alleles of the genes involved in anthocyanin biosynthesis active in the kernel, a change from red to bluish color occurred in the scutellum of the *lpa1-241* mutant kernel, thus suggesting a possible role of

ZmMRP4 in the transport of this pigment: in fact, when anthocyanins are transported in the vacuole, due to the acid pH, they assume the typical reddish color, but if MRP is not functional, they are not transported and accumulate in the less acid environment of the cytosol where they retain the bluish tint (Badone et al., 2010).

As well as the *lpa1-241* mutant, also *lpa1-7* showed several agronomic defects due to the strong reduction (> 80%) in PA content (Cerino Badone et al., 2012). Compatibly with a recessive monogenic behavior, an inability to germinate was observed in both filter paper germination tests and in field conditions. This mutation was lethal in the homozygous state (*lpa1-7*/*lpa1-7*), although the embryo rescue technique could recover the germination capacity. Among other pleiotropic effects, seedlings on MS medium were characterized by slow growth and defective primary roots, partially compensated by the development of secondary roots. Moreover, the leaves of homozygous *lpa1-7* plants showed alterations compared to the wild type and light green stripes between leaves' venation were clearly visible (Cerino Badone et al., 2012). These observations were confirmed by data showing a decrease in chlorophyll, carotenoid and trichome length as well as an increase in trichome density (Cerino Badone et al., 2012). Histological analysis aimed at comparing features of *lpa1-7*/*lpa1-7* and wild type kernels highlighted a reduction in the mutant embryo dimension and a misalignment between the radical primordium and the embryo body in *lpa1-7* (Cerino Badone et al., 2012).

A different seed density between all *lpa1* mutants and the respective wild types was highlighted (Landoni et al., 2013). In the same work, the *lpa1-7* mature kernel was characterized by a clearly visible cavity in the endosperm, that was absent in the wild type.

Rice

The rice ortholog of *ZmMRP4* is *OsMRP5*; the two genes share 83% of nucleotide sequence identity and the two proteins share 91% of amino acid identity (Shi et al., 2007). Different mutants at the *OsMRP5* locus have been identified (Liu et al., 2007; Xu et al., 2009): *Os-XS-lpa2-1* consisting of a point mutation in the sixth exon that causes a P1156S substitution in TMD2 (Xu et al., 2009); *Os-XS-lpa2-2*, a 5 bp deletion in the first exon that leads to a frame shift at amino acid 452, causing the occurrence of a premature stop codon at the amino acid 474 (Xu et al., 2009). *Os-XS-lpa2-1* is characterized by a 20% reduction in PA content and is not lethal when in homozygosity. However, the PA decrease was found to be much higher in the lethal mutant *Os-XS-lpa2-2* (> 90%). Moreover, a T-DNA knock out line (4A- 02500) in which *OsMRP5* was disrupted, showed the same reduction in PA content (~90%) and appeared lethal in the homozygous state (Xu et al., 2009). Comparing *Os-XS-lpa2-1* mutants with their respective wild types, different pleiotropic effects associated with reduced seed viability and plant performance were pointed out (Zhao et al., 2008). The simplified

relative vigor index (a parameter that combines germination rates, seedling height and seedling weight) is reduced by 7.8% in *Os-XS-lpa2-1*, despite a germination rate which is similar to that of wild type. Moreover, a significant decrease in field emergence rate was observed (65% in *Os-XS-lpa2-1*, versus 84% in the wild type), while a 5% reduction in grain weight was measured in the mutant. Conversely, no significant differences were found in grain yield and other yield parameters, such as ripened grain rate, number of grains per panicle and number of productive tillers (Zhao et al., 2008). In the same work, an artificial ageing test was performed on mutant rice seeds (42 °C and 95% relative humidity for 7 and 14 days), but in contrast to what was observed in the maize *lpa 241-1* mutant described previously, no significant difference was found. This discrepancy might be attributed to the different location of the PA deposits, 90% of which in rice seed are in the aleurone tissue, whilst in maize seed they are in the germ, which is obviously a much more critical location in relation to the maintenance of the germination ability. So, according to this theory, rice seed might endure much better than maize the oxidative stress connected with the paucity of PA.

Os-XS-lpa2-2 is an allelic mutant of *Os-XS-lpa2-1* and is characterized by severe agronomic defects. Due to the strong reduction in PA (>90%), this mutant cannot germinate naturally, but seedlings can be produced from immature embryos through *in vitro* culture on MS medium (Xu et al., 2009).

Wheat

In hexaploid wheat three copies of the *TaABCC13* gene are present and the encoded proteins show a high degree of similarity with the other cereal PA-MRP transporters (Cominelli et al., 2020b). The TaABCC13 proteins have been previously described as cadmium transporters (Bhati et al., 2015). In a subsequent publication, the *TaABCC13* genes were silenced through RNA-interference (RNAi) and in the silenced lines, a reduction of 22-34% in seed PA content was observed. Moreover, these lines were characterized by a decrease in grain filling, numbers of spikelets, kernel viability, delayed germination, early emergence of lateral roots, and defects in metal uptake and development of lateral roots in the presence of cadmium stress, compared to the non-transgenic lines. These data show that *TaABCC13* is important for several other aspects of growth, as well as for grain nutritional quality, for root development and detoxification of heavy metals (Bhati et al., 2016).

A common alteration in the maize *lpa1* mutant and in the silenced *TaABCC13* wheat lines refers to defects in root growth and development (Cerino Badone et al., 2012; Bhati et al., 2016). In a previous work, it was shown that the Arabidopsis *mrp5-1* mutant seedlings, grown on standard medium (0.5 x Murashige and Skoog -MS- medium), showed a reduction in primary root elongation, accompanied by an earlier growth of lateral and secondary roots. However, when seedlings were grown on a more complete medium (1x MS medium), a reverse phenotype was obtained. A two-fold increase in auxin

content was also recorded in roots of *mrp5-1* seedlings compared to the wild type ones when grown on standard medium (Gaedeke et al., 2001), indicating that PA transport is important for auxin accumulation and signaling. The phenotypic alterations in root growth and development described in crops are similar to the ones described in Arabidopsis that can be considered as a model system to further study these aspects.

Soybean

Mutants in PA-MRP transporters were found not only in cereals, but also in legumes (Wilcox et al., 2000; Campion et al., 2009; Cominelli et al., 2018). In soybean, chemical mutagenesis was used on the breeding line *CX1515-4* and two independent and non-lethal *lpa* mutants were isolated: *M153* and *M766* (Wilcox et al., 2000). Although the initial analysis of the *M153* line suggested that only a single locus was responsible for the *lpa* phenotype, a few years later it was found that the low phytate trait was controlled by two recessive alleles at two independent loci, initially called *pha1* and *pha2* and subsequently renamed *lpa1* and *lpa2* (Oltmans et al., 2004; Gao et al., 2008). These loci correspond to two genes (*Gm03g32500* and *Gm19g35230*) that code for PA-MPR transporters (GmMRP3 and GmMRP19) which are mutated in the independent soybean line *CX1834* deriving from *M153* (Gillman et al., 2009; Saghai Maroof et al., 2009). It was shown that these transporters are homologous to *ZmMRP4* and *AtMRP5* (Gillman et al., 2009, 2013; Saghai Maroof et al., 2009). A third MRP protein, GmMRP13 (*Gm13g18960*), was identified on chromosome 13 (Panzeri et al., 2011). In the *M153* line, the *lpa1-a* allele carries a nonsense mutation at R893, which results in a truncated protein (Gillman et al., 2009; Saghai Maroof et al., 2009), while the *lpa2-a* allele causes a R1039K change. In *M766*, the *lpa1-b* allele is characterized by a T>A SNP at intron 9, which introduces an alternative splicing site; the *lpa2-b* allele shows a single base change in position 1039 (as in *lpa2-a* allele) that results in an early termination (Gillman et al., 2013).

Both *M153* and *M766* are characterized by a significant decrease in PA content (80% and 76.3% respectively), although the greatest drop in PA (94% less compared to the parental line) was achieved in the double mutant, obtained by combining the *lpa1-a* allele from *M153* and the *lpa2-b* allele from *M766* (Wilcox et al., 2000; Oltmans et al., 2004, 2005; Gillman et al., 2013). As in cereals, this strong PA reduction is often associated with negative pleiotropic effects. The first agronomic trials were conducted with lines derived from *M153*. Comparing these *lpa* mutants with their respective wild types, a ~22% reduction was observed in seedling emergence, as well as a decrease in plant density (Hulke et al., 2004). Anderson and Fehr demonstrated that the growth environment strongly influences the performance of low phytic acid cultivars: data collected in a tropical environment (Puerto Rico) were statistically different from those taken in a temperate environment (Iowa), where

germination and seedling emergence were higher (Anderson and Fehr, 2008). With the aim of overcoming the reduced seedling emergence, Spear and Fehr proposed backcrossing as a strategy to obtain *lpa* progeny with unchanged seedling emergence (Spear and Fehr, 2007). Moreover, they highlighted a greater susceptibility to seedborne fungal infections in *lpa* lines during germination, which could lead to reduced field emergence (Spear and Fehr, 2007).

Common bean

Among the species analyzed so far, common bean was the first characterized by mutations in PA-MRP transporters that did not seem to cause negative pleiotropic effects. Over the years, two mutants have been isolated by chemical mutagenesis: *lpa1* (also known as *lpa280-10*) (Campion et al., 2009) and *lpa1*^{2}, initially identified as 08IS-1281 mutant line (Cominelli et al., 2018). The *lpa1* mutation is caused by the defective *PvMRP1* gene and is characterized by a missense mutation in TMD2, that leads to E1155K amino acid change. In the allelic mutant *lpa1*^{2}, a single base change occurs in TMD1 resulting in a non-sense mutation, and consequently in a truncated protein. In both of these two mutants, PA reduction (90% in *lpa1* and 75% in *lpa1*^{2}) is followed by a proportional increase in free P_i, while the total P remains unchanged. Despite this drop in PA content, the agronomic performance of the *lpa1* mutant was found to be the same as that of the wild type, or even better (Campion et al., 2009, 2013). This seems to be due to the presence of the *PvMRP2* paralogue, which would complement the absence of a functional *PvMRP1* gene in all plant organs except in the seed (Panzeri et al., 2011; Cominelli et al., 2018). *PvMRP2* is a highly conserved orthologous gene of *Gm13g18960*, and the proteins they encode share more than 80% of similarity with *PvMRP1*, while the similarity shared with *AtMRP5* and *ZmMRP4* proteins is lower (Panzeri et al., 2011).

In the agronomic trials carried out by Campion and co-workers, no significant differences were found in the agronomic parameters measured on the seed and on the plant (Campion et al., 2009, 2013). Germination tests carried out under ageing (45 °C and 100% relative humidity for 48 and 96 h) and stressing environmental conditions (0.4 M NaCl treatment) demonstrated that *lpa1* does not show significant differences compared to the wild type. In particular, a lower MGT (mean germination time in hours) value in the mutant pointed out that there was even a germination response which was faster than in the parental genotypes (Campion et al., 2009). In essence, in the different growth environments tested (growth chamber, greenhouse and open field), this common bean *lpa1* mutant was not shown to be associated with any negative pleiotropic effects and to be able to afford the same good results as the wild type as concerns seedling emergence, plant growth and grain yield. It was also shown that this mutant is hypersensitive to abscisic acid at germination (Panzeri et al., 2011).

Moreover, the common bean *lpa1* mutant has a higher drought resistance index (Chiozzotto et al.,

2018). In Arabidopsis and common bean, mutations in *AtMRP5* and *PvMRP1* genes respectively, confer increased tolerance to drought. Interestingly, stomata of the Arabidopsis *mrp5-1* mutant leaves showed reduced sensitivity to light compared to the wild type ones, with the consequence of closer stomata under standard growth conditions (Klein et al., 2003). At a macroscopic level, the guard cell phenotype of the *mrp5-1* mutant confers reduced water loss from detached rosette leaves, reduced transpiration rate, improved water use efficiency, and enhanced drought stress tolerance (Klein et al., 2003). Electrophysiological measurements demonstrated that the Arabidopsis mutation impairs both ABA and cytosolic Ca^{2+} activation of slow (S-type) anion channels and ABA activation of Ca^{2+} permeable channel currents in the plasma membrane of guard cells (Su et al., 2007), suggesting that *AtMRP5* is a central regulator of ion channels of ABA and Ca^{2+} signal transduction in guard cells. In a model proposed by Nagy et al. (2009), PA would induce the release of Ca^{2+} from the vacuole to the cytosol and would block K^+ flux from inward channels. *AtMRP5* is necessary to transport PA from the cytosol to the vacuole, thus avoiding the continuous PA signaling. Mutations in *AtMRP5* would affect PA export into the vacuole, causing an increase of PA concentration in the cytosol. Cytosolic PA might bind to Ca^{2+} and other divalent cations and/or may induce a continuous Ca^{2+} release, thus disturbing the Ca^{2+}-dependent signaling pathway. Moreover, it may reduce K^+ uptake into guard cells by inhibiting K^+ inward rectifying channels. It is not clear why in Arabidopsis and common bean, mutations in *AtMRP5* and *PvMRP1* genes respectively, confer increased tolerance to drought, (although most likely through different mechanisms), while for the mutation in the maize *ZmMRP4* gene the opposite was shown, and further studies are required to understand the reason for this discrepancy. However, a clarification of these aspects may help in defining strategies to develop crop *lpa* mutants.

The above cited positive results reached in common bean prompted researchers to investigate the nutritional potential of *lpa1* through *in vitro* and *in vivo* trials, aimed at verifying whether it may improve the bioavailability of micronutrients, particularly iron. As regards *in vitro* trials, in 2013 Campion and co-workers introduced the *lpa1* trait in common bean lines harbouring the Lf (lectin free) trait and producing white or colored (brown or black) beans. Then they used the Caco-2 (human epithelial colorectal adenocarcinoma cells) model to measure the amount of iron adsorbed by these cultured cells from administered bean extracts. Results showed that the bioavailable iron in *Lf + lpa* white bean seeds is on average twelve times higher than in wild type as well as in *Lf + lpa* colored seeds. These results, although "much an *in vitro* test is worth", seemed to have disclosed a promising key tool to improve iron bioavailability from common bean. Indeed, a prompt confirmation arrived when Petry et al. (2018) published a paper describing an *in vivo* trial carried out on young, non-iron deficient women fed with a porridge made with wild type or *lpa 280-10* beans

and cooked in boiling water for almost two hours. Iron absorption, measured as erythrocyte incorporation of stable iron isotopes (Fe^{57}, Fe^{58}) from the *lpa* line, was found to be 50% higher and the total amount of iron absorbed per test meal was 85% higher than from wild type beans (Petry et al., 2016).

Despite the good agronomic performances of the common bean *lpa* mutants, undesired pleiotropic effects were described regarding their use in human diets. A second study carried on by the same group among Rwandese women proved that, while supplying diet with *lpa* beans is beneficial to iron absorption (as it happens if biofortified beans with increased iron content are used), *lpa* beans also cause adverse gastrointestinal symptoms, due to a hard-to-cook (HTC) phenotype, likely caused by the thermal stability of lectins in these lines (Petry et al., 2016). A recent publication further investigates the origin of the HTC phenotype in *lpa1* lines (Cominelli et al., 2020a). The observed HTC phenotype in *lpa1* was shown to be correlated with the redistribution of calcium cations within the seed, providing evidence for the "phytase-phytate-pectin" hypothesis; according to this idea, the reduction of PA chelating activity (due to increased phytate dephosphorylation or to reduced phytate content) determines a migration of divalent cations to cell-wall-middle lamella, resulting in the formation of insoluble pectate complexes that harden the cell walls. The authors confirmed how *lpa1* mutation also reinforces the thermal stability of seed lectins, in particular homotetramers of the antinutritional phytohemmaglutinin L (PHA-L), but not homotetramers of phytohemmaglutinin E (PHA-E) or heterotetramers made up by PHA-L and PHA-E.

Regarding the *lpa1*^{2} mutant isolated in 2018, preliminary experiments conducted under controlled conditions would suggest that the effects of the mutation are similar to the ones already described for *lpa1* (Cominelli et al., 2018), while no investigation has so far been carried out to verify its nutritional features.

Future perspective and conclusions

It is by now established that there are three main classes of negative pleiotropic effects caused by MRP *lpa* mutations: those affecting seed viability and lowering grain yield in cereals such as maize, rice and wheat, those related to seed emergence in soybean and those affecting important post-harvest qualities of common bean, such as cooking properties and lectin harmlessness.

Which are the strategies so far adopted and currently underway to try to eliminate or at least decrease substantially these effects?

As pointed out above, maize and rice MRP *lpa* mutants are characterized by low seed viability and reduced seedling emergence. As discussed in the previous sections, these defects may be partially or wholly attributable to an anomalous quantity of free iron cations in the seeds of the mutants and to

the consequent high level of toxic ROS originated following the Fenton reaction. A possible and obvious approach to defend any human or plant cell from ROS consists in scavenging these toxic free radicals by means of molecules endowed with antioxidant properties. Thus, aiming at improving the agronomic performance of these mutants it might be sufficient to use classical breeding methods to introgress the ability to synthesize and accumulate natural antioxidants, such as carotenoids or polyphenols in the living tissues of the grain.

In small grain cereals (e.g. wheat, barley, oat, and rye) the problem of the "low yield" associated with the MRP *lpa* genotypes could be simply solved by specific breeding programs. In fact, almost all the work conducted on field performance of *lpa* genotypes present limited data using inbred lines without or with limited breeding activity. Furthermore, these data are often collected in greenhouses/growth chambers or in small experimental fields and frequently with few replications over the years. Last but not least, comparisons are in almost all cases between inbred lines in different genetic backgrounds, where many genetic differences impacting agronomic performance can take place. One of the best ways to compare *lpa* trait and yield (or any other trait, such as nutritional quality) is the comparison between sibling near-isogenic lines (homozygous wild type and *lpa*) obtained by backcrossing and selecting for yield. Nevertheless, several research programs are in progress with the aim to develop *lpa* varieties by conventional breeding and transgenic/genome editing methods and certainly new *lpa* varieties will be released soon.

As concerns soybean, Spear and Fehr (2007) suggested that backcrossing may represent an effective strategy aiming at the development of low-phytate lines devoid of negative pleiotropic effects derived from *CX1834* genetic background. In a recent paper (Boehm et al., 2017), the agronomic performance of two low-phytate lines (*56CX-1273* and *56CX-1283*) obtained through five backcrosses was compared to two high-yielding elite cultivars. As expected, the agronomic trials performed in six locations highlighted that there were no significant differences in field emergence and seed yield.

For common bean, the problems of the MRP *lpa1* trait are not linked to the viability of the seeds or agronomic performances of the plants, but instead to the increase in cooking time and to the risk of lectin poisoning upon multiple consumption of meals prepared with *lpa1* beans (Petry et al., 2016). The former phenotype is caused by the hardening of the cell walls due to redistribution of Ca^{2+} ions, while the latter is linked to the higher thermal stability shown by the PHA-L lectin (Cominelli et al. 2020). Classical breeding approaches are expected to work properly to avoid these problems. In fact, the HTC trait is strongly genotype dependent (Cichy et al., 2020) therefore introducing the *lpa1* trait in a genotype without HTC defect should not affect too much the cooking times, as shown by Cominelli et al. (2020). As to the second problem, it may easily be solved by taking into account the importance of avoiding PHA-L containing genotypes when introgressing the *lpa1* trait in breeding

programs.

Furthermore, the *lpa1* mutant root system has been little taken into consideration. Attention was mainly focused on the aboveground parts of the plant, while the underground part was neglected. However, a greater susceptibility of the mutant plants to water stress has been reported (Cerino Badone et al., 2012). Genetic analyses conducted on maize revealed that some genes (*rtcs*, *rtcl*, *rum1*), involved in auxin signal transduction, are fundamental elements for the development of lateral, seminal and shoot-borne roots (Hochholdinger et al., 2018). Future research should focus more deeply on these genes, modulating their expression in *lpa1* mutants with traditional breeding approaches, or with their specific modulation through genome editing and GMO techniques.

In some cases, an approach different from the classical breeding was used, and *MRP* gene activity was manipulated by transgenic techniques. In particular, seed-specific silencing was conducted on *ZmMRP4* and *OsMRP5* (Shi et al., 2007; Li et al., 2014). In maize, the embryo-specific promoters *Ole16* and *Glb* were used to generate transgenic lines. *Ole::MRP4* constructs were shown to be associated with a 68-87% reduction in PA, while this decrease ranged from 32 to 75% in *Glb::MRP4* transformants. Gene-silencing constructs under the control of *Glb* tended to germinate normally and no significant reduction in seed weight was recorded (Shi et al., 2007). However, no positive agronomic results were obtained in rice with a transgenic approach such as silencing of *OsMRP5* (Li et al., 2014). The *Ole18* promoter, active both in the embryo and in the aleurone, was used for *OsMRP5* silencing and a PA reduction (35.8-71.9%) was observed in the transgenic lines. This decrease was accompanied by a strong increase (up to 7.5 times) in P_i. Comparing these transgenic plants to the respective null siblings, a decrease in seed weight accompanied by reductions in seed germination and seedling emergence was reported (Li et al., 2014). Hence, this strategy does not appear to be effective in rice, unlike that previously reported in maize (Shi et al., 2007), probably due to the different promoters used in the two cereals. In fact, *Ole18* is active not only in the embryo, but also in the aleurone and in the endosperm (Li et al., 2014).

The main conclusion emerging from the above survey of literature is that, with the partial exception for the significant results reported in the case of common bean (Campion et al., 2009, 2013), the goal of achieving MRP *lpa* mutants endowed with no negative pleiotropic effects on a good field performance has not yet been reached for many crops. Moreover, this review highlights that the pleiotropic effects linked to the MRP *lpa* trait not only concern the physiology of seeds and plants, but also affect other aspects connected with the cooking properties and the harmlessness of the grain for consumers.

In conclusion, further breeding work will be necessary to attenuate the negative pleiotropic effects impacting on plant and seed performance before the development of a commercial variety that, to our

knowledge, is only near to be released for common bean.

Supplementary Material

Sup. Mat. Table 1. Main features of MRP-type ABC transporters involved in PA transport.

Species	Gene	Origin of the mutation	Mutant	PA reduction	Pleiotropic effects	Reference
Zea mays	*ZmMRP4*	EMS	*lpa1-1*	66%	Seed weight and density reduction, alteration in roots.	Raboy *et al.,* 2000; Cerino Badone *et al.,* 2012 Landoni *et al.,* 2013
			lpa1-241 *lpa1-7*	>80% >80%	Reduced germination and seed density, susceptibility to oxidative stress, leaves alteration and defective primary root.	Pilu *et al.,* 2005; Doria *et al.,* 2009; Cerino Badone *et al.,* 2012; Landoni *et al.,* 2013
Oryza sativa	*OsMRP5*	γ rays + sodium azide	*lpa2-1*	20%	Reduced vigor, grain weight and field emergence.	Zhao *et al.,* 2008
			lpa2-2	>90%	Lethal	Xu *et al.,* 2009
		T-DNA insertion	4A-02500	90%	Lethal	Xu *et al.,* 2009
Triticum aestivum	*TaABCC13*	Constitutive RNAi	*TaABCC13*	22-34%	Delayed germination, reduced kernel viability, decreased grain filling and early emergence of lateral roots.	Bhati *et al.,* 2016
Glycine max	*GmMRP3* *GmMRP19*	EMS	CX1834	80%	Reduced seedling emergence and decreased plant density. Greater susceptibility to fungi infections.	Hulke *et al.,* 2004; Spear and Fehr, 2007
	GmMRP13	No reported mutant	No reported mutant			Panzeri *et al.,* 2011
Phaseolus vulgaris	*PvMRP1* *PvMRP2*	EMS	*lpa1*	90%	No differences in seedling emergence and grain yield and no effect under stress condition. Faster germination response and higher drought resistance index	Campion *et al.,* 2009; Panzeri *et al.,* 2011; Petry *et al.,* 2016; Chiozzotto *et al.,* 2018; Cominelli *et al.,* 2020
			lpa1^{2}	75%	Preliminary experiments suggest similar effects to *lpa1*	Cominelli *et al.,* 2018

Author contributions

RP proposed and designed the review. FC designed the review, prepared the figures, and wrote the manuscript. DP, EC FS, and EN wrote the manuscript. All authors contributed to the article and

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Figure legend

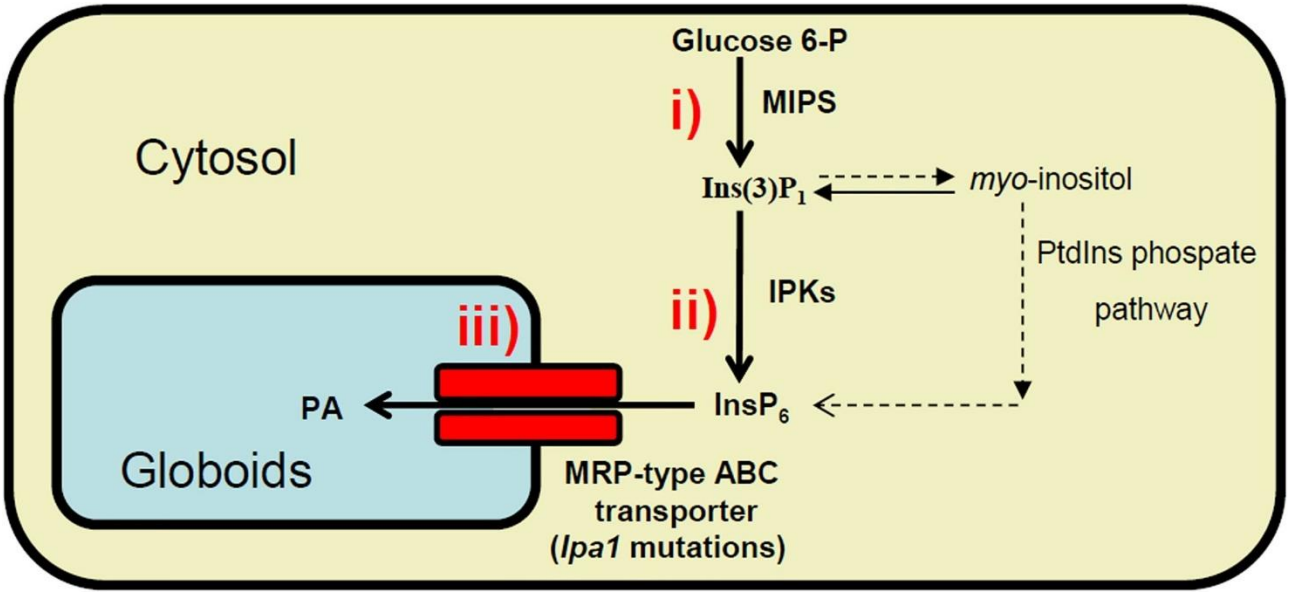

Figure 1. Schematic view of the biosynthetic pathways leading to PA accumulation in globoids (storage vacuoles) in seeds. *lpa* mutations can be divided into three classes: i) mutations affecting the activity of *myo*-inositol 3-phosphate synthase (MIPS); ii) the successive phosphorylation steps of PA pathway, from Ins(3)P1 to the accumulation of PA; iii) transport by MRP transporters and storage of PA into the globoids.

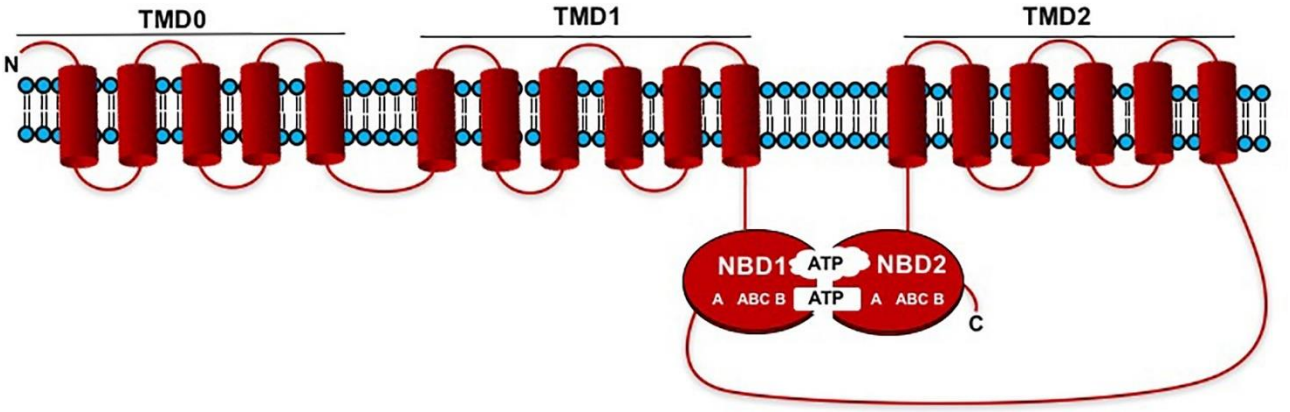

Figure 2. Schematic representation showing the membrane topology models of MRP-type ABCC transporters. The domains are forward oriented in the following way: TMD0_TMD1-NBD1_TMD2-NDB2. NBDs contain the Walker A and Walker B motifs separated by an ABC "signature".

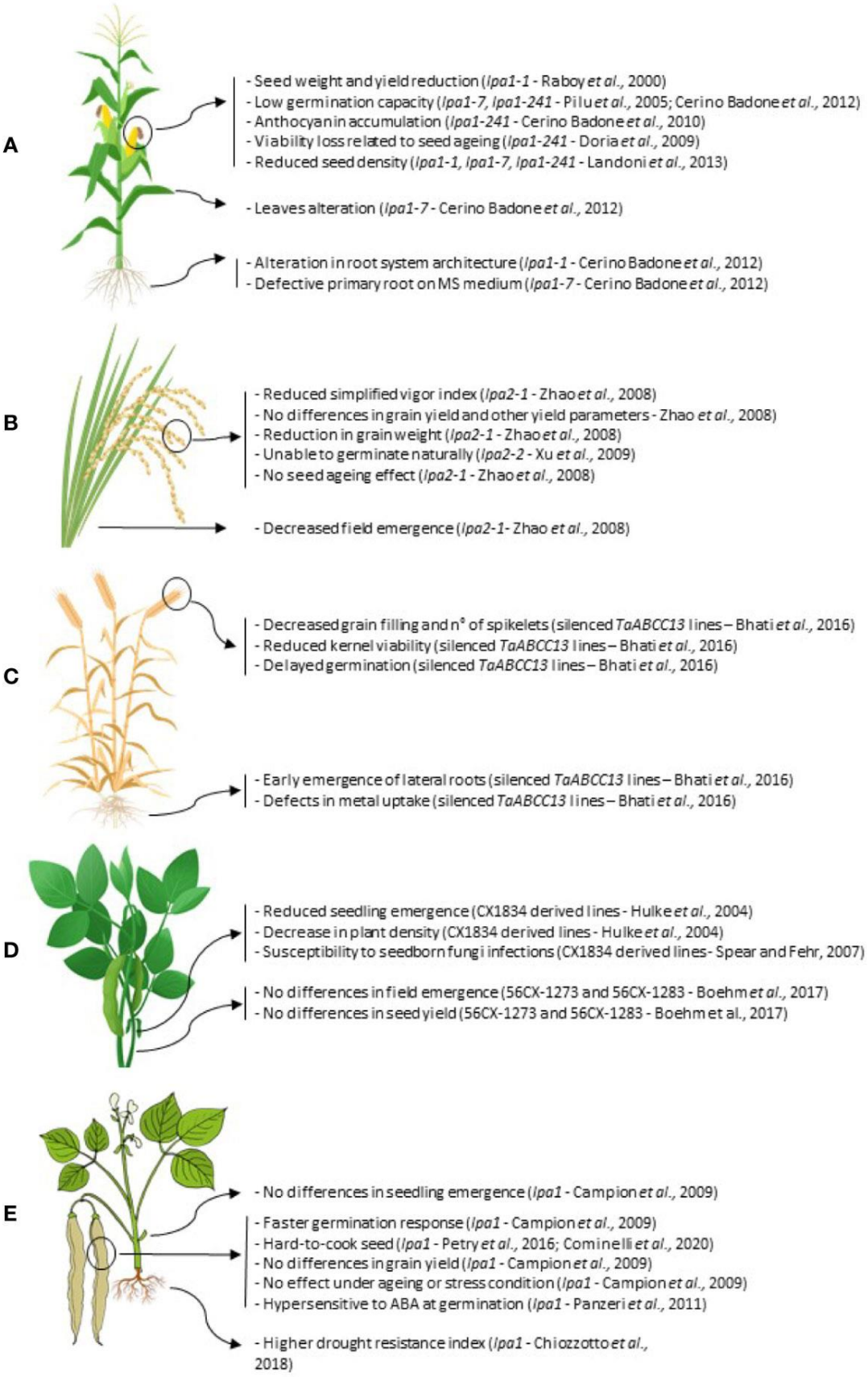

Figure 3. Summary of the main pleiotropic effects in *lpa* mutations in the five agronomic species considered: maize (A); rice (B); hexaploid wheat (C); soybean (D) and common bean (E).

Article

Low-Phytate Grains to Enhance Phosphorus Sustainability in Agriculture: Chasing Drought Stress in *lpa1-1* Mutant

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Abstract: Phytic acid (PA) is an anti-nutritional factor for monogastrics and contributes to phosphorus pollution. The *low phytic acid* (*lpa)* trait can provide several benefits to the nutritional quality of foods/feeds and to environmental sustainability. In maize, four *lpa1* mutants have been isolated, and *lpa1-1* is the most promising. Nevertheless, these mutations are frequently accompanied by many negative pleiotropic effects affecting plant performance. One of these is a greater susceptibility to drought stress, probably caused by an alteration in the root system. In this work, we set up an experiment in hydroponics and two in mesocosms, where pots were built using transparent PVC sheets to better access the roots. The results suggested that neither root architecture nor root depth are limiting factors in mutant plants. In hydroponics, the dry weight of the mutant and the root area per unit of length were twice that of the corresponding wild-type. However, *lpa1-1* exhibited a reduced efficiency of photosystem II (Fv/Fm, 0.810 vs. 0.800) and a reduced leaf temperature (−0.5 °C compared to wild-type), probably due to increased water loss. Furthermore, molecular analysis performed on genes involved in root development (*rtcs*, *rtcl*, *rum1*, and *BIGE1)* revealed the abundance of *rtcs* transcripts in the mutant, suggesting an alteration in auxin polar transport.

Keywords: phosphorus; low phytic acid mutants; phytic acid; drought stress; root system architecture; environmental sustainability; agrobiodiversity

1. Introduction

Phytic acid (PA) (myo-inositol-1,2,3,4,5,6-hexakisphosphate) is the most common storage form of phosphorus (P) in plant seeds [1]. In maize, PA is mainly located in the scutellum and only small quantities are present in the aleurone layer [2]. Phytic acid is accumulated in the protein storage vacuole as phytate mixed salts with different cations (particularly iron and zinc), reducing their bioavailability [3]. During germination, these salts are degraded by the activity of the phytase enzyme, releasing free phosphorus, myo-inositol, and minerals, essential for seedling growth [4]. Only ruminants are able to degrade phytic acid due to the presence of phytases in their digestive system. However, monogastric animals do not possess this enzyme: only the 10% of phytate in the feed is assimilated, while the remainder is excreted, contributing to P pollution and water surface eutrophication. Therefore, farmers must supply mineral phosphorus to the feed of monogastric animals, thus implying an economic problem [5]. Despite being considered an anti-nutritional factor for all these reasons, phytic acid plays a key role as an antioxidant compound. In fact, by chelating iron cations, PA can counteract the formation of reactive oxygen species (ROS), thus preserving the viability of seeds [6,7].

Some breeding programs have analyzed the genetic variability of both phytic acid and inorganic

phosphorus present in a set of fifty inbred lines representing the Iowa lines from B73 to B129 [8,9]. In particular Lorenz and colleagues reported phytic acid values ranging between 2.40–4.09 mg/g for fifty different maize lines [8]. However, the most promising strategy concerns the use of *low phytic acid* (*lpa*) mutants in which the PA content drops to around 1 mg/g [10].

In recent decades, many *lpa* mutants have been isolated in several crops: maize [10–13], wheat [14], barley [15–17], rice [18,19], soybean [20–22], and common bean [23,24]. The *lpa* trait can provide important benefits to the nutritional quality of foods and feeds and can contribute to the environmental sustainability of phosphorus in agriculture.

In maize, three different *low phytic acid* mutants have been isolated and characterized: *lpa1* [10,11], *lpa2* [10], and *lpa3* [25]. Among these, *lpa1* showed the greatest reduction of PA in the seed, followed by a proportional increase of free *P* without altering the total *P* content. Transposon mutagenesis experiments demonstrated that the gene *ZmMRP4* (accession number EF86878) is responsible for the *lpa1* mutation [26]. *ZmMRP4* is a multidrug resistance-associated protein (MRP) that belongs to the subfamily of ATP-binding cassette (ABC) transmembrane transporters [26]. The majority of *lpa* mutants carry mutations in genes that code for MRP proteins, and thus result in a lack of PA transfer from the cytosol to the vacuole. In maize, four *lpa1* mutants have been isolated so far: *lpa1-1* [26], *lpa1-241* [27,28], *lpa1-7* [12], and *lpa1-5525* (not fully characterized) [13]. *Lpa1-241* and *lpa1-7* are not viable in the homozygous state, displaying an 80%–90% decrease in PA [12,27], while *lpa1-1* is the most promising *lpa1* mutant, showing a 66% reduction in phytic acid, followed by a proportional increase in inorganic phosphorus [10]. Unfortunately, the reduction of PA in *lpa1* mutations leads to various negative pleiotropic effects on the seed and, in general, on plant performance, as recently reviewed by Colombo et al. [29]. One of these agronomic defects observed in the field on the mutant *lpa1-1* is an increased susceptibility to drought stress, which might be caused by an alteration in the root system architecture (RSA) [12].

In maize, the root system includes embryonic and post-embryonic roots [30,31]: the former are important for seedling vigor in the early stages of development [32] and include the primary root and a variable number of seminal roots [33], while the post-embryonic root system dominates the RSA of adult plants [34]: it is formed by shoot-borne roots and includes crown roots (at underground nodes of the shoot) and brace roots (at aboveground nodes) [31]. All these types of roots generate postembryonic lateral roots, increasing the absorbing surface of the maize root system [35]. In recent decades, several genes that control maize root development have been isolated and characterized [36–39]. Genetic analyses conducted on maize revealed that many genes (*rtcs*, *rtcl*, *rum1*, and *BIGE1*) involved in auxin signal transduction are fundamental elements for the development of lateral, seminal, and shoot-borne roots [40]. The *rtcs* (rootless concerning crown and seminal roots) mutant

was identified for the first time by Hetz et al. [41] by the complete lack of embryonically seminal roots and post-embryonically shoot-borne roots. The gene *rtcl* (rtcs-like) is the paralog of *rtcs* [36]: the coordinated function of these two paralogous genes in maize root initiation and elongation was reported in Xu et al., 2015 [39]. The *rum1* (rootless with undetectable meristems 1) mutant is defective in seminal roots and lateral roots at the primary root [37], while *BIGE1* (Big embryo 1) identifies a class of genes that regulate lateral organ initiation and results in increased leaf and lateral root number [38].

The aim of this work was to establish the limiting factor in the *lpa1-1* mutant under drought stress, by analyzing and collecting parameters both on the hypogeal and epigeal parts of the plant. We compared *lpa1-1* to a wild phenotype using different approaches, spanning from hydroponics to the greenhouse.

2. Materials and Methods

2.1. Plant Materials and Controlled Growth Conditions

The *lpa1-1* mutation introgressed in B73 inbred line was kindly provided by Dr. Victor Raboy, USDA ARS, Aberdeen, ID, USA. B73 inbred line was provided by the germplasm bank at DISAA, Department of Agricultural and Environmental Sciences—Production Landscape, Agroenergy, University of Milan. All procedures were performed in accordance with the relevant guidelines and regulations.

The first experiment was conducted in controlled conditions. Forty seeds of each genotype (B73 and the relative mutant *lpa1-1*) were sterilized with 5% (v/v) sodium hypochlorite for 15 min and then rinsed in sterile distilled water. Seeds were germinated in a Plexiglas tank covered with sheets of moistened germinating paper in a growth chamber (16 h light/8 h dark) with controlled temperature (22 °C night/28 °C day) and with photon fluence of 270 µmol m^{-2} s^{-1}. At 5 DAS (days after sowing), different parameters were measured: coleoptile length (mm), primary root length (mm), and total number of roots (primary and seminals). After one week, twelve seedlings for each genotype were transferred to hydroponics tanks containing Hoagland nutrient solution [42]. Seedlings were sampled at 16 DAS and several hypogeal and epigeal parameters were collected: shoot length (cm), shoot diameter (mm), dry weight (mg), primary root length (cm), area (cm^2), and area/L (cm^2/cm). In particular, the roots of each sample were scanned (with high-resolution digital scanner) and the images were processed using Adobe Photoshop software: the shadows of the roots and the background of the images were removed, the color of the roots was changed (green) and made uniform. The processed images were analyzed using ImageJ 1.52 [43] and Easy Leaf Area software [44] in order to collect the following data (referring

to each plant): maximum root length and root system area. In addition, the root area/root length ratio was calculated.

2.2. Greenhouse Experiment

Two experiments were conducted successively in the greenhouse at the University of Milan, Italy. The temperature of day/night was 25/18 °C and the relative humidity was 60%–70%. In each experiment, three plants per genotype were grown in mesocosms (13.5 cm × 100 cm, top diameter × height) filled with sandy soil (Green Maxx, VitaFlor) to 10.0 cm from the top. A layer of expanded clay was added to the base. Pots were built using transparent PVC sheets to better access the root system. The cylindrical pots were arranged randomly and three replications for each genotype were performed. To avoid exposure to light, the mesocosms were covered with a cloth. Two days before sowing, each cylinder was irrigated with 4 L of water. Each pot was sown with three seeds and then thinned to a single seedling after 10 DAS. Root growth was measured weekly and three agronomic parameters were collected in both genotypes: plant height, ear height, and culm diameter. Plants were grown until flowering using the same amount of water (1 L every week) and urea (2.5 g per plant). Finally, plants were uprooted, thoroughly washed, and subjected to phenotyping.

2.3. Leaf Temperature, Chlorophyll A Fluorescence, Stomata Opening, and Water Loss

In the mesocosms experiment, thermal images of the fifth fully expanded leaves were taken from 60-day-old *lpa1-1* and wild-type seedlings with a semiautomated long-wave infrared camera system (FLIR T650sc) in the greenhouse. Photos were taken between 11 a.m. and 12 a.m. The temperature of the leaves was then measured using the FLIR ResearchIR Max software.

Chlorophyll a fluorescence was measured using a hand-portable Handy PEA fluorimeter (Hansatech Instruments Ltd., King's Lynn, UK): leaves were dark-adapted for 30 min using the equipped white leaf-clips and fluorescence was induced by three high-intensity light-emitting diodes for one second at the maximal photosynthetic photon flux density (PPFD) of 3500 μmol m^{-2} s^{-1} [45,46]. Among all the parameters measured with the fluorimeter, Fv/Fm is widely considered a sensitive indicator of plant photosynthetic performance and represents the maximum quantum efficiency of photosystem II [47]. The performance index (PI) is essentially an indicator of sample vitality, while Dio/CS represents the energy dissipation of photosystem II.

Using clear nail varnish is a traditional method to measure stomatal density and opening. It is used to take an impression of the leaf which is then viewed under the optical microscope. After preparing an epidermal impression by coating the leaf surface with nail varnish, the dried layer of nail varnish was removed using sellotape and finally applied on to a slide. Representative images of the stomata were

taken on the fifth fully expanded leaf in the wild-type and *lpa1-1* mutant plants after 60 DAS. The ratio between the long side and the short side of the stoma was used as an indication about the shape of the stoma and its opening: the higher the ratio, the more elongated the shape; vice versa, if the ratio was close to 1, the stoma had a circular shape.

Another experiment in 15 cm pots was set up to determine the time course of the leaf water loss. After 20 days, six plants per genotype were sampled and the third leaf of each seedling was detached and weighed immediately. Leaf weight was then estimated at designated time intervals and water loss was calculated as the percentage of fresh weight based on the initial weight. Significant differences between the wild-type and *lpa1-1* were assessed by Student's t-test.

2.4. ICP-MS Analysis

For the ionomic analysis, seeds of the pure line B73 and the *lpa1-1* mutant were grown on moistened germination paper under controlled conditions, and the coleoptiles and seedlings were sampled at 7 and 14 DAS, respectively, before being dried at 70 °C for two days. Then, 10 mL of HNO_3 (65%) was added to 300 mg of maize dry matter in Teflon tubes. Samples were digested using a microwave digestor system (Anton Paar Multiwave 3000, Austria) in Teflon tubes applying a two-step power ramp (400 W in 5 min, maintained for 10 min; 1000 W in 10 min, maintained for 15 min). The mineralized samples were transferred into polypropylene tubes, diluted 1:40 with Milli-Q water, and element concentrations measured by ICP-MS (Varian 820 ICP- MS, Agilent USA). An aliquot of 2 mg L^{-1} internal standard solution (6LI, 45Sc, 89Y, and 159Tb) was added to the samples and calibration curve to reach a final concentration of 20 μgL^{-1}.

2.5. RNA, cDNA Preparation, and Quantitative Gene Expression Analysis

RNA extraction was performed on *lpa1-1* and wild-type roots at 3 and 8 days after germination, homogenizing 100 mg of roots in liquid nitrogen. Total RNA was extracted using the Gene JET Plant RNA Purification Mini Kit (Thermo Scientific) and treated with Turbo DNA-free Kit (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized with the Maxima First Strand cDNA Synthesis (Thermo Scientific), according to the manufacturer's instructions. First strand cDNA was used as the template for subsequent PCR amplification.

Specific primers for the *orange pericarp-1* (*orp-1*) gene, which encodes the b-subunit of tryptophan synthase, was used to standardize the concentration of the samples [48]. The *orp-1* specific sequences were amplified using the following primers: forward 5′-AAGGACGTGCACACCGC-3′ and reverse 5′-CAGATACAGAACAACAACTC-3′, generating a 207 bp amplicon. A set of specific primers for root genes (*rtcs*, *rtcl*, *rum1*, and *BIGE1*) was selected from previous works [38,39,49]. The gene-

specific primers are listed in Supplemental Table S1. The reaction mix underwent an initial denaturation step at 94 °C for 2 min, 32 cycles of denaturation at 94 °C for 45 s, annealing at the specific primer temperature for 1 min, and extension at 72 °C for 1 min and 30 s. Final extension at 72 °C for 5 min was performed to complete the reaction. Amplification products were visualized on 1% (w/v) agarose gels with ethidium bromide staining.

3. Results

3.1. lpa1-1 Alters RSA in Hydroponics

Under controlled conditions, the *lpa1-1* mutant appeared to grow faster in the early stages after germination compared to the control line (Figure 1). Seeds were germinated on moistened paper, and after 5 days, *lpa1-1* showed higher values than B73 control line both in the coleoptile length (29.53 vs. 18.18 mm) and in the length of the primary root (56.35 vs. 35.13 mm) (Figure 2A,B). Furthermore, the total number of roots (primary and seminals) present in the mutant was statistically higher than in the control (Figure 2C).

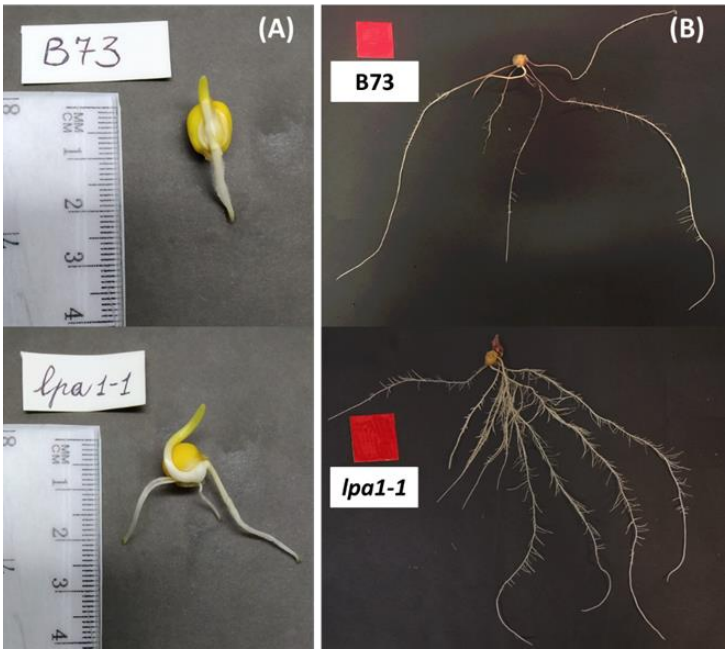

Figure 1. Representative phenotype of wild-type (B73) and *lpa1-1* at 5 DAS (**A**) and at 16 DAS, after growing in hydroponic solution (**B**).

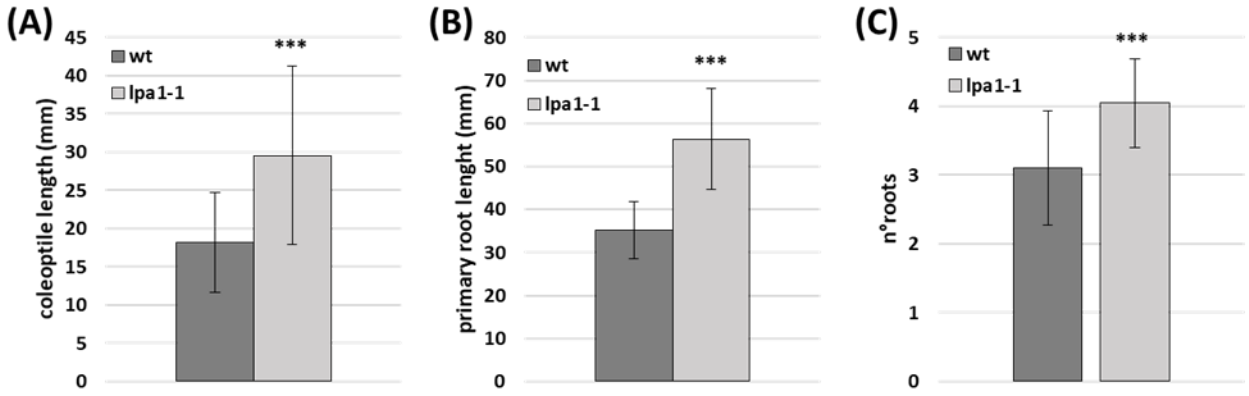

Figure 2. Coleoptile length (**A**), primary root length (**B**), and number of roots (primary and seminals) (**C**) measured at 5 DAS in controlled conditions. The data represent the mean of thirty-eight and twenty-three biological replicates for wild-type and *lpa1-1* homozygous plants, respectively. Significant differences between wild-type and *lpa1-1* were assessed by Student's t-test (*** $p < 0.01$).

After one week, twelve seedlings for each genotype were transferred to hydroponics, and several epigeal and hypogeal parameters were measured at 16 DAS, as shown in Figure 3.

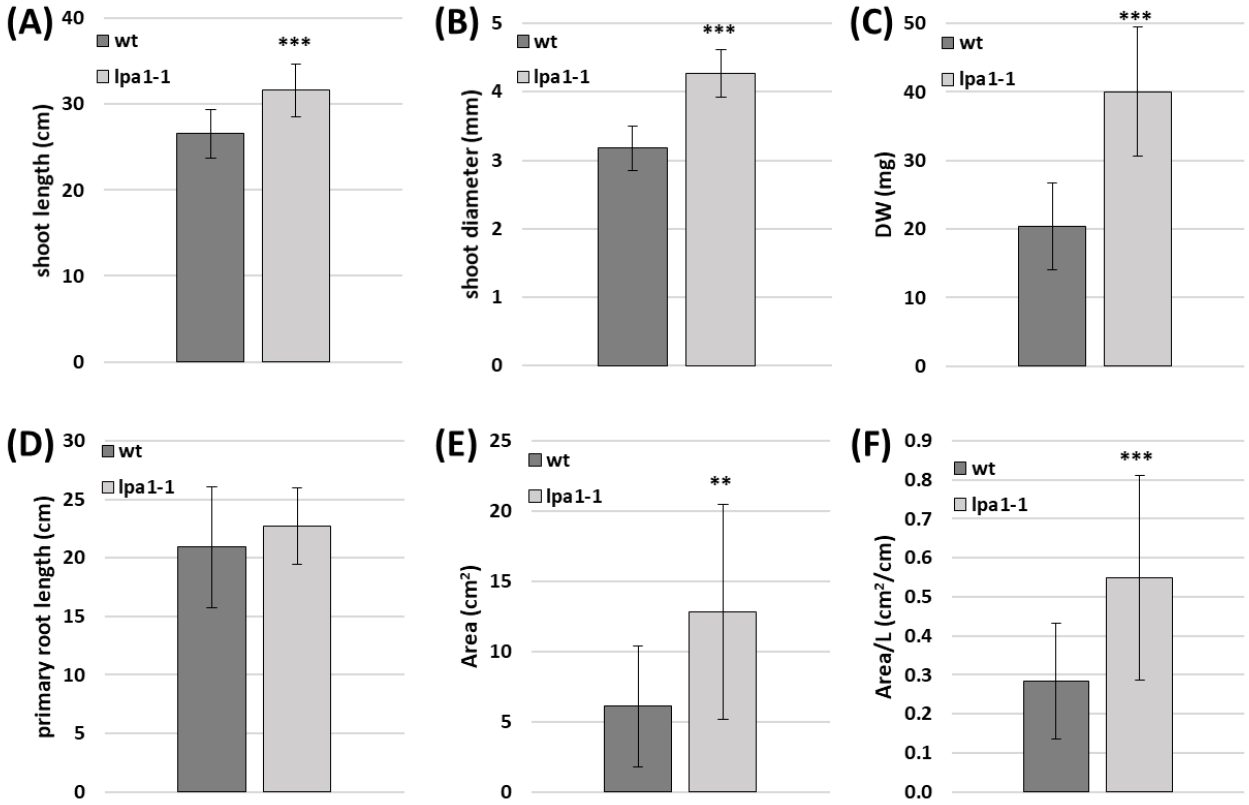

Figure 3. Measurements on the epigeal part of the plant in hydroponics at 16 DAS: shoot length (**A**) and shoot diameter (**B**). Parameters measured on the root system architecture: dry weight, DW (**C**), primary root length (**D**), area occupied by the roots (**E**), and root area per unit of length (**F**). Values represent the mean of twelve biological replicates. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (** $p < 0.05$ and *** $p < 0.01$).

Regarding the epigeal measurements, *lpa1-1* showed statistically higher values both in the length of the coleoptile (Figure 3A) and in the diameter of the culm (Figure 3B). Considering the root system architecture, no significant differences were found in the length of the primary roots between the two genotypes (Figure 3D); in contrast, the dry weight (DW) of the mutant root system was twice that of B73 (Figure 3C) and the area occupied by the entire root system reached 12.8 cm^2 in *lpa1-1* compared to the 6.10 cm^2 of the control line (Figure 3E). Another parameter measured was the root area per unit of length: this ratio was statistically higher in the mutant than in the wild-type (0.55 vs. 0.28 cm^2/cm) (Figure 3F).

In the light of these data, we hypothesized that the greater bioavailability of minerals in *lpa1-1* mutant, in the early stage of development, resulted in quicker development of seedlings than in the wild-type. Hence, the mineral and trace element content were also determined in the early stages of growth by means of ICP-MS analysis. The results showed several differences among the ion compositions of wild-type and *lpa1-1* at two sampling points, 7 and 14 DAS. In the first sampling, phosphorus was statistically more abundant in the mutant (Figure 4). In addition, other cations, such as Na, Mg, Al, K, Ca, and Se, were accumulated in higher amounts in *lpa1-1* compared to the wild phenotype (Table 1). However, at 14 DAS the mutant P content had decreased dramatically and was higher in the wild-type (Figure 4). Similarly, some minerals and trace elements that were previously more abundant in the mutant, at 14 DAS were more available in the control line (Table 1).

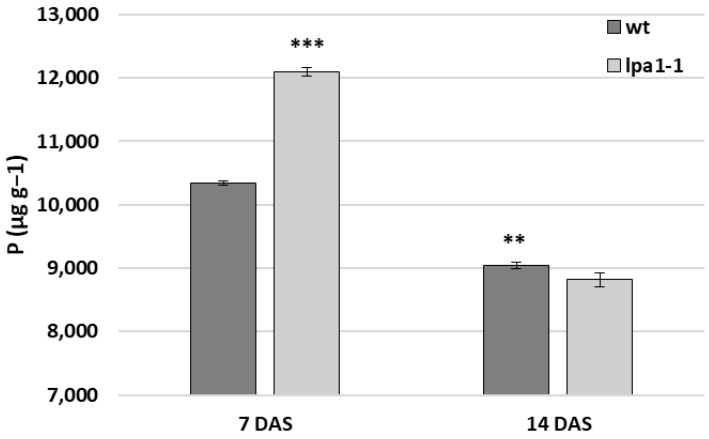

Figure 4. Phosphorus (P) content determined by inductively coupled plasma mass spectrometry (ICP-MS). Analysis performed on maize seedling dry matter of wild-type and *lpa1-1* mutant at 7 and 14 DAS. P content was expressed as micrograms per gram of dry matter. Values represent the mean of three biological replicates. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (** $p < 0.05$ and *** $p < 0.01$).

Table 1. Mineral nutrient and trace element content determined by inductively coupled plasma mass spectrometry (ICP-MS). Analyses were performed on maize seedling dry matter of wild-type and *lpa1-1* mutant at 7 and 14 DAS. The elements were expressed as micrograms per gram of dry matter. SD is shown (*n*

=3, $p < 0.05$).

	7 DAS		14 DAS	
	wt	***lpa1-1***	**wt**	***lpa1-1***
Na	311.77 ± 11.84	650.68 ± 16.21 [a]	135.67 ± 6.66	182.72 ± 22.04 [a]
Mg	2307.66 ± 36.44	2445.65 ± 12.82 [a]	2252.27 ± 57.86	2142.91 ± 26.65 [a]
Al	29.64 ± 8.35	83.25 ± 15.69 [a]	42.22 ± 5.02	47.38 ± 6.14
K	25,511.38 ± 377.77	32,858.76 ± 279.07 [a]	28,154.83 ± 533.08	27,422.72 ± 373.22
Ca	806.69 ± 12.57	881.08 ± 39.03 [a]	3252.48 ± 45.87	3314.33 ± 48.74
Cr	0.46 ± 0.03	0.72 ± 0.14 [a]	2.02 ± 0.26	1.40 ± 0.19 [a]
Mn	9.16 ± 0.31	8.23 ± 0.09 [a]	21.78 ± 1.15	18.44 ± 0.03 [a]
Fe	78.89 ± 6.31	74.63 ± 19.58	128.89 ± 1.86	134.45 ± 24.29
Cu	7.66 ± 1.18	10.15 ± 2.76	7.30 ± 1.03	8.72 ± 1.64
Zn	140.88 ± 3.44	119.18 ± 5.55 [a]	103.96 ± 3.47	93.35 ± 3.81 [a]
Se	0.69 ± 0.02	1.60 ± 0.08 [a]	0.87 ± 0.18	1.60 ± 0.38 [a]

[a] Statistically different from wt, based on t-test.

3.2. Root Genes Involved in Auxin Signal Transduction

The content of minerals and trace elements gives us indications of the rapid development of the mutant in the early stages of growth but does not explain the different root systems between the mutant and the control line. These differences in the mutant RSA could be attributable to an alteration in the polar transport of auxins: it is known that inositol phosphates are involved in the polar transport of auxins, important phytohormones that regulate several plant developmental processes and responses to environmental stresses [50,51]. In this work we selected four genes involved in auxin signal transduction that are key elements for the development of seminal, lateral, and shoot-borne roots in maize: *rtcs1* (accession number Zm00001eb003920), *rtcl1* (Zm00001d048401), *rum1* (Zm00001eb156910), and *BIGE1* (Zm00001eb211050) gene expression levels were analyzed on 3- and 8-day roots of wild-type and *lpa1-1* by reverse transcription polymerase chain reaction (RT-PCR) (see ‘‘Materials and Methods’’ for details). As shown in Figure 5, *rtcs1* was upregulated in *lpa1-1* roots at 8 DAS compared to the wild-type corresponding tissues, but this difference was lower at the 3-day sampling. A similar pattern was observed for its paralog, *rtcl1 (rtcs-like)*, even if the expression at 8 DAS was reduced, and *rum1* showed no differences in expression levels between the two genotypes in either sampling. Moreover, *BIGE1* was upregulated at 8 DAS in the mutant roots than in the wild-type, while these differences were not found at 3 days (Figure 5).

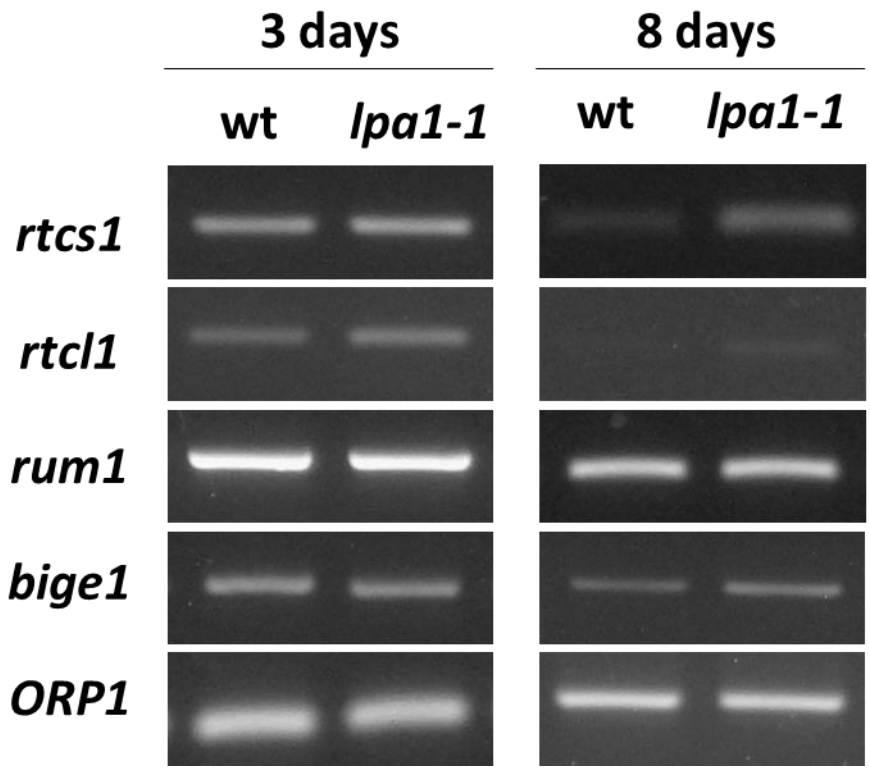

Figure 5. *rtcs1*, *rtcl1*, *rum1* and *bige1* gene expression levels on 3- and 8-day roots of wild-type and *lpa1-1*. *Orp1* gene was used as control.

3.3. Root Depth Is Not Affected in lpa1-1

In the mesocosms experiment, the growth of the embryonic primary root and the post-embryonic crown roots was monitored weekly to understand if a different root depth was the main cause of the drought stress observed in *lpa1-1* mutant. In the first replicate, the primary root of the mutant did not differ significantly from the wild phenotype at any of the four points of detection (Figure 6A). In the second replicate of the experiment, no significant differences were found, except at 36 DAS, in which *lpa1-1* roots were statistically deeper than those of the control (67.83 vs. 54.67 cm, respectively) (Figure 6A). Considering the growth of the post-embryonic crown roots, in the two experiments there were no significant differences in crown root depth, except for the measurement at 64 DAS in the first trial (Figure 6B). Furthermore, the dry weight of the entire root system measured after the final cleaning was statistically the same in both the experiments (data not shown).

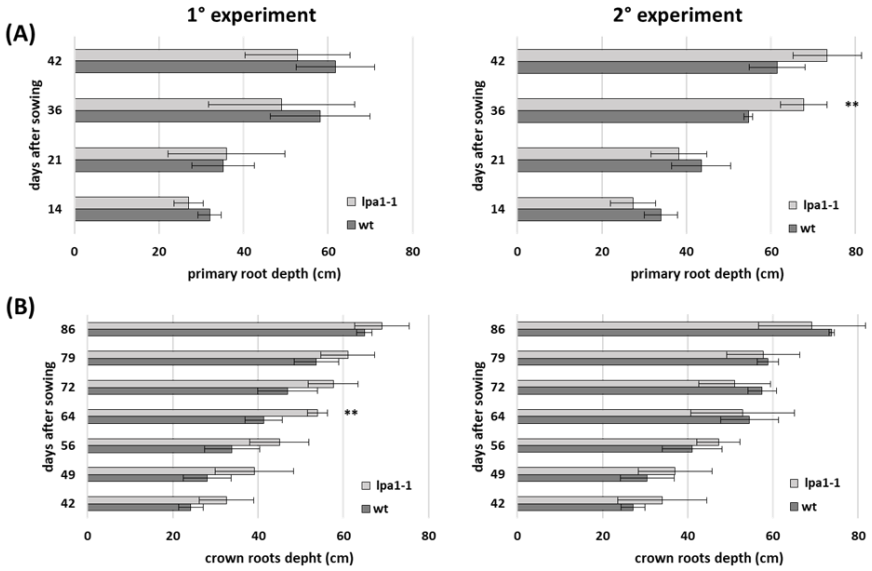

Figure 6. Depth of the embryonic primary root (**A**) and of the post-embryonic crown roots (**B**) measured every week till flowering. The data represent the means of three biological replicates in two different experiments. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (** $p < 0.05$).

Different epigeal parameters were collected on plants grown in mesocosms: the mutant showed a reduced plant and ear height compared to the pure line in most of the samplings (Figure 7A,B). Moreover, the plant culm diameter was measured weekly using an electronic caliper: in both the experiments, culm diameter was higher in the mutant, but the difference was statistically significant at few sampling points (Figure 7C).

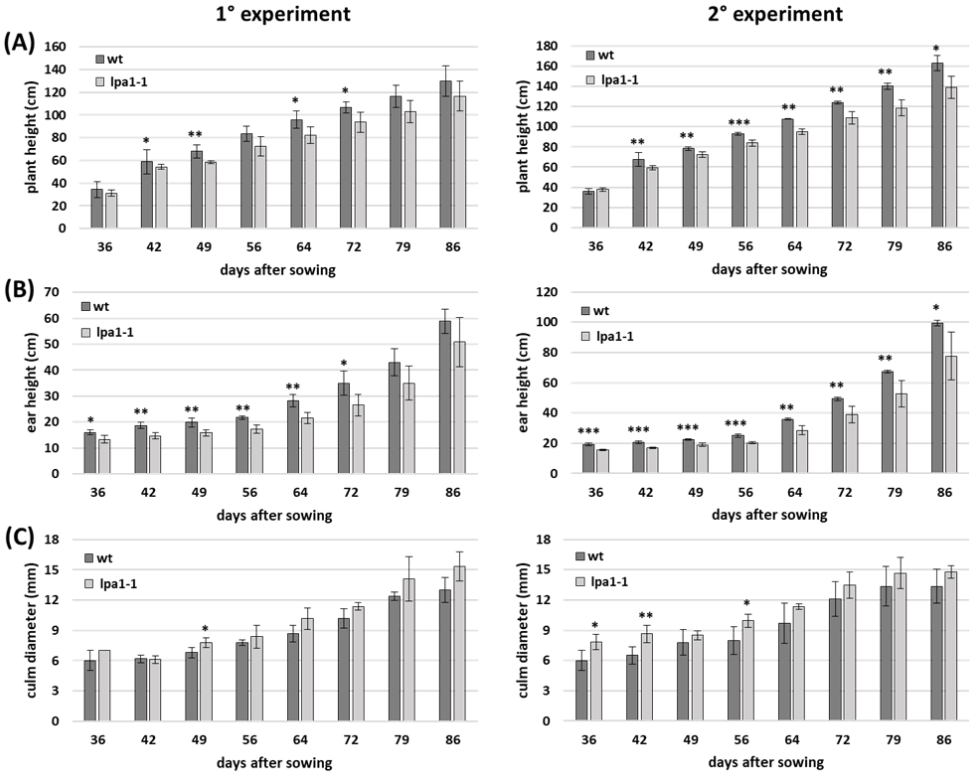

Figure 7. Plant height (**A**), ear height (**B**), and culm diameter (**C**) measured every week till flowering in two different experiments. The data represent the means of three biological replicates. Significant differences between wild-type and *lpa1-1* were assessed by Student's t-test (* $p < 0.1$, ** $p < 0.05$, and *** $p < 0.01$).

3.4. Cuticular Permeability Is Altered in lpa1-1 Mutant

The results obtained so far seem to exclude the root system as the main cause of the drought stress in *lpa1-1*. Hence, our research focused on the aerial part of the plant, and several measurements were carried out.

In order to investigate the impact of drought stress on the mutant, thermography images of the fifth fully expanded leaves of wild-type and *lpa1-1* homozygous mutant were analyzed (Figure 8A). In both the experiments, *lpa1-1* presented a reduced leaf temperature compared to the control (Figure 8B), probably due to a greater water loss from stomata. For this reason, the photos of the stomata were taken with the optical microscope (Figure 8C). The subsequent analysis allowed the calculation of the ratio between the long side and the short side of the stoma. This parameter gave us an indication about the shape of the stoma and its opening: the higher the ratio, the more elongated the shape; conversely, if the ratio was close to 1, the stomata had a circular shape. The stomata of the wild phenotype were characterized by a statistically higher ratio in the two experiments, and, consequently, by a more elongated shape (Figure 8D). In contrast, the stomata of the mutant had a lower ratio and a circular shape, which could cause greater water loss under stressful conditions.

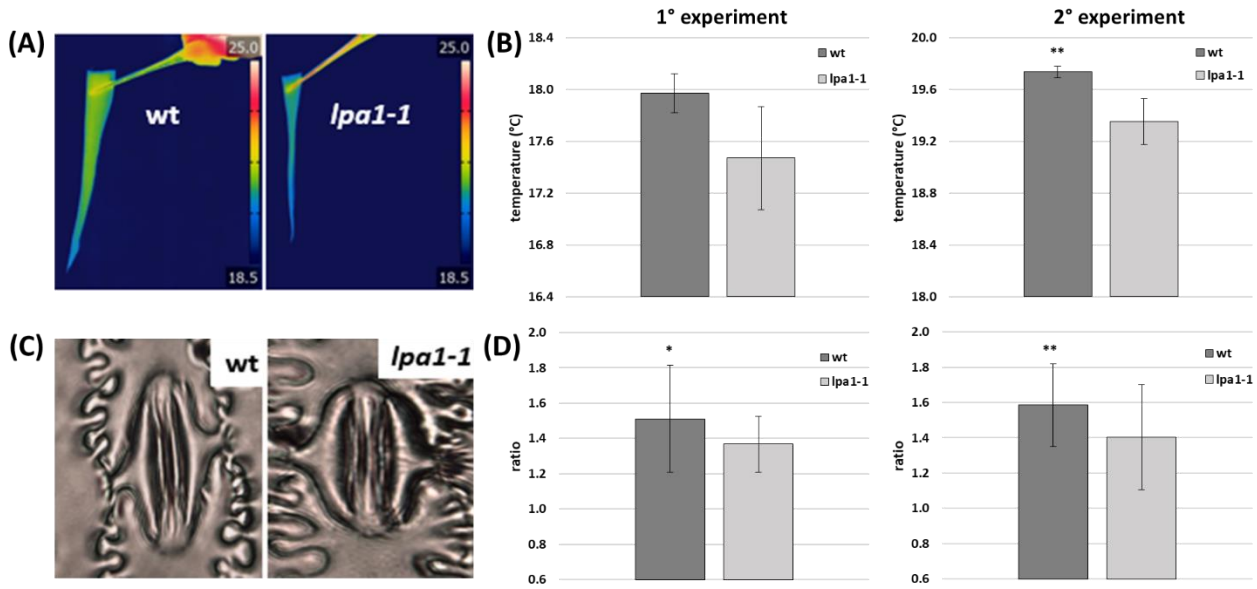

Figure 8. (**A**) Representative images of the leaf temperature acquired with the thermographic camera (FLIR T650sc) in wild-type and homozygous *lpa1-1* plants. (**B**) Temperature of the fifth fully expanded leaf in the wild-type and *lpa1-1* homozygous plants measured at 60 days after sowing (DAS). Values represent the mean of three biological replicates in two different experiments. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (* $p < 0.1$ and ** $p < 0.05$). (**C**) Representative images of the stoma acquired by optical microscope on the fifth fully expanded leaf in wild-type and *lpa1-1* mutant plants at 60 DAS. (**D**) Ratio between the long side and the short side of the stoma. This parameter gives us an indication about the shape of the stoma and its opening: the higher the ratio, the more elongated the shape; if the ratio is close to 1, the stoma has a circular shape. Values represent the mean of eighteen biological replicates. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (* $p < 0.1$ and ** $p < 0.05$).

Hence, it seems that the mutant plant suffers more from drought stress compared to the control line. Consequently, the efficiency of photosystem II measured with the fluorometer should also decrease. The parameter Fv/Fm represents the maximum quantum efficiency of PSII and gives information on the potential photosynthetic ability of the plant. In both the experiments, the mean was around 0.810 in the wild phenotype, while it was lower, at 0.800, in *lpa1-1* mutant (Figure 9A). The performance index (PI) had a similar trend to Fv/Fm ratio: the graphs show a statistically significant difference ($p < 0.05$) with higher values in the control line compared to the mutant (Figure 9B). In particular, the values of P.I. in the control line were higher than those of the mutant by 22.5% and 20.7% in the two experiments. On the other hand, the parameter Dio/CS measured with the same fluorimeter represents the energy dissipation of photosystem II. The results showed that *lpa1-1* lost more energy than the wild-type, supporting the hypothesis that the mutant is characterized by a lower photosynthetic efficiency (Figure 9C).

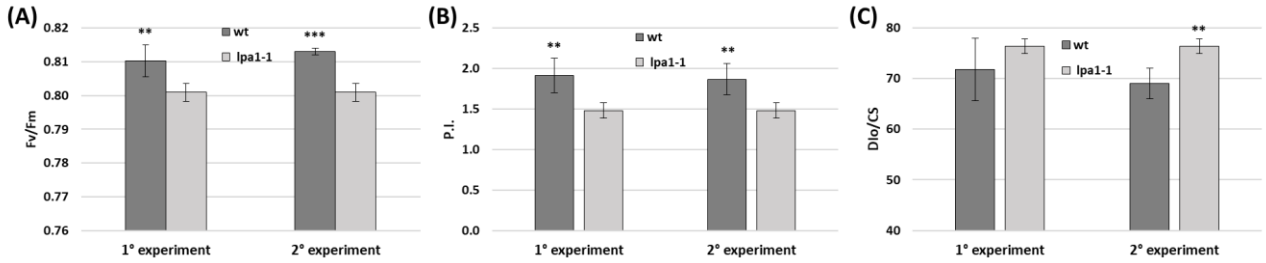

Figure 9. The fluorimeter Handy PEA+ was used to measure Fv/Fm (**A**) and other important photosynthetic parameters, such as the performance index (PI) (**B**) and the energy dissipation of photosystem II (Dio/CS) (**C**). Values represent the mean of three biological replicates. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (** $p < 0.05$ and *** $p < 0.01$).

Moreover, to better understand the impact of the mutation *lpa1-1* on leaf surface permeability, a water loss time course experiment was carried out on the third detached leaf by estimating the loss of weight with respect to the initial leaf fresh weight. The resulting profiles showed that *lpa1-1* was characterized by a higher water loss rate compared to wild-type plants (Figure 10).

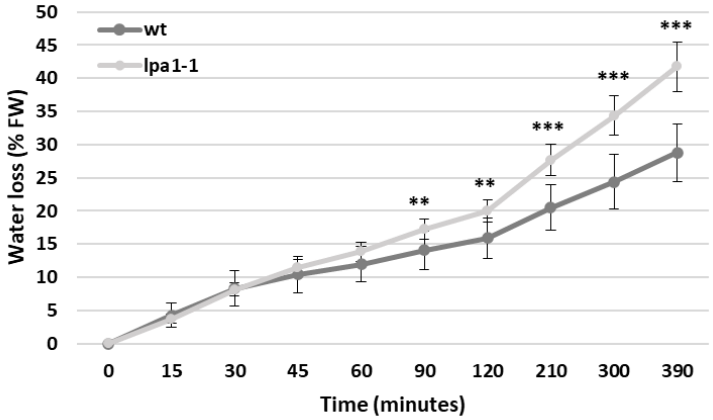

Figure 10. Percentage of water loss in the third detached leaf of homozygous *lpa1-1* and wild-type. Values represent the mean of six biological replicates. Significant differences between wild-type and *lpa1-1* were assessed by Student's *t*-test (** $p < 0.05$ and *** $p < 0.01$).

4. Discussion

Phytic acid is considered an anti-nutritional factor for human and monogastric animals and it is also involved in environmental problems of phosphorus pollution. In underdeveloped countries, the lack of important cations such as iron and zinc in the diet represents a serious problem for human health. On the other hand, in rich countries the problem is not nutritional, but related to feed: farmers must supply mineral phosphorus to the feed of monogastric animals, thus increasing the cost [5].

In the past few years, some programs have analyzed the genetic variability of both phytic acid and inorganic phosphorus present in different accession collected in the germplasm bank [8,9]. Lorenz and colleagues reported that the phosphorus fraction of wild maize grains could be modified by

breeding and selection, demonstrating that the phytic acid–phosphorus and, consequently, the mineral nutrients' accumulations are controlled by several QTLs [9]. However, in this context, the most promising strategy concerns the use of *low phytic acid* (*lpa*) mutants. The *lpa* trait can provide several potential benefits to the nutritional quality of foods and feeds and to the environmental P sustainability in agriculture [52].

In maize, four different *lpa1* mutants have been isolated and characterized so far, and *lpa1-1* is the most promising. However, reducing PA content in seeds to improve nutritional quality affects seed quality and plant performance, as recently reviewed by Colombo and coworkers [29]. Among these pleiotropic effects, a greater susceptibility to drought stress was observed in the field on *lpa1-1*, and our recent research focused on this agronomic defect.

In fact, in recent decades, low water availability is considered a primary limitation to crop productivity all around the world [53,54]. The frequency and the severity of drought stress on crops will increase in the future because of climate change [55]. The greater water loss observed in *lpa1-1* could be caused by an alteration in root system architecture or by differences in the aerial part of the plant. The aim of this work was to establish which was the limiting factor in the mutant *lpa1-1*, analyzing and collecting parameters on both the hypogeal and epigeal parts of the plant.

Regarding the root system architecture, many differences were found between the mutant and the wild-type phenotype (Figure 1). From the data measured after 5 days on wet paper, a greater development of the mutant was observed compared to the pure line B73 (Figures 1A and 2B,C). The fast growth of *lpa1-1* in the first weeks after germination was also confirmed at 16 DAS, after the transfer of the seedlings to hydroponics (Figure 1B). Once again, the mutant's root system appeared more developed: both the dry weight and the root area per unit of length were twice that of B73 (Figure 3C,F). These differences in the mutant root system could be attributable to an alteration in the polar transport of auxins. In fact, several studies carried out on different species have shown that inositol phosphates are involved in the polar transport of auxins, important phytohormones that regulate numerous plant developmental processes and responses to environmental stress [50,51]. For instance, a previous study on Arabidopsis reported that null mutations in the inositol–phosphate synthase *MIPS1* gene had dramatic impacts on plant development, including impaired embryogenesis and altered cotyledon development, root agravitropism, reduced transport of auxin, and altered root cap organization [56].

Furthermore, another factor that could influence the faster growth of *lpa1-1* is the higher bioavailability of phosphorus and minerals in the early stages of development (Table 1, Figure 4). Similar measurements at ICP-MS were previously carried out on seeds by Landoni et al. [57]. In this work, these analyses were conducted for the first time on seedlings. These results confirmed a strong

chelating effect of PA on mineral cations in the control line, while the *lpa1-1* mutant was characterized by more bioavailable cations that do not need to be released gradually by the phytase enzyme.

At this point, since the root architecture did not appear to be a limiting factor, we thought that drought stress in the field could be caused by different root depths. Root depth plays a central role in plant resistance to water stress [58,59]. In order to analyze this trait, plants were grown in transparent mesocosms. The use of mesocosms allowed the control of different conditions, such as soil type and moisture, temperature, light intensity, pot sizes, and nutrient and water inputs [60]. From the data collected weekly, it emerged that in the two experiments there were no significant differences (except at a few sampling points) either in the depth of the embryonic primary root (Figure 6A) or in that of post-embryonic crown roots (Figure 6B).

Therefore, the experiments conducted in this work showed for the first time that root depth did not appear to be the main cause of the drought stress observed on *lpa1-1* mutant plants in the field, even if the RSA showed many differences in the two genotypes. In particular, the mutant root system was characterized by a greater development in the first weeks after germination: the higher dry weight and the larger root surface recorded in hydroponics seemed to be determined by early lateral root and root hair development.

For this reason, from the literature we selected many root genes involved in auxin signal transduction that are fundamental for the development of lateral, seminal, and shoot-borne roots [40]. Expression analysis carried out on 3- and 8-day roots of wild-type and *lpa1-1* revealed the high abundance of *rtcs* transcripts in the mutant, in line with the role of this gene. The upregulation of *rtcs* in mutant plants suggested an alteration in the polar transport of auxins. In the same way, expression analysis on *BIGE1* (involved in lateral organ initiation) confirmed the upregulation of *BIGE1* transcripts at 8 days.

Despite this possible alteration in the auxin polar transport, the root system was excluded as the main cause of the drought stress, and so our research focused on the aerial parts of the plant. Epigeal data collected at 5 and 16 DAS in the experiment performed under controlled conditions confirmed the faster development of *lpa1-1* in the early stages (Figures 1A and 2A,B). In fact, due to the reduction of phytic acid in *lpa* mutants, the cations are more bioavailable, while in the wild phenotype they tend to form phytate salts. The situation changed completely after two weeks: the seedling phosphorus content decreased dramatically in the mutant (Figure 4), and several minerals (previously more abundant in *lpa1-1*) were more abundant in the control line (Table 1). The measurements carried out in the mesocosms experiment on the aerial parts of the plant confirmed this hypothesis: after a few weeks, the agronomic performance of B73 improved significantly and some important parameters

(such as plant height and ear height) were statistically higher at most of the sampling points in both the experiments (Figure 7A,B). The shortening of the internodes found in *lpa1-1* plants and the increased culm diameter (Figure 7C) could be caused by an alteration in the polar transport of the auxins. In fact, some known maize mutants, such as *brachytic 2* (*br2*) and *brevis plant1* (*bv1*), are characterized by shortened internodes and are deficient in auxin transport [61–63].

In the same experiment conducted in the greenhouse, several epigeal parameters were measured in both genotypes in order to understand the causes of drought stress. *Lpa1-1* exhibited reduced leaf temperature compared to its control (Figure 8A,B), possibly due to increased water loss from leaves (Figure 10). It was found that the efficiency of photosystem II measured with the Handy PEA fluorimeter was lower in mutant plants (Figure 9A,B), probably due to a greater dissipation of energy in the leaves (Figure 9C). In this context, we cannot exclude that the water loss observed in the *lpa1-1* mutant is also associated with alterations in the cuticle and/or cell wall composition. In fact, both cuticular waxes and lignin constitute a natural protection against damage caused by biotic and abiotic factors, as well as a waterproof barrier that regulates water loss and leaf gas exchange [64,65].

In conclusion, further breeding work will be necessary to clarify the causes of water loss in this mutant and to attenuate the negative pleiotropic effects impacting on plant performance. The aim is to overcome these agronomic defects and exploit the potential of the *lpa* trait, especially for underdeveloped countries, where diet is based on staple crops.

In this work, the experiments were performed under controlled or semi-controlled conditions. Field tests are underway to confirm the hypothesis that drought stress is caused by a reduced photosynthetic efficiency in the *lpa1-1* mutant, and not by a shallower root system, as previously reported. In this work, the comparisons and measurements were carried out using the pure line B73. The introgression of the *lpa1-1* mutation into a new genetic background (such as commercial hybrids) could represent a solution and could improve the performance of the plant, contributing to overcome the effects of drought stress described here. Several breeding programs are in progress in order to develop new *lpa* varieties by conventional breeding and transgenic/genome editing methods. In this way it will be possible to exploit the nutritional properties of this mutant and improve the P management in agriculture.

Supplementary Material

Name	Sequence
rtcs-fw	5’ GGTACCAAGACTGCGAGGAC 3’
rtcs-rv	5’ CCCCAGGCTAAAGTCCAAA 3’
rtcl-fw	5’ GAACACCTTCGACTTCGAG 3’
rtcl-rv	5’ CAGGTAAGCATAAGCCACG 3’
rum1-fw	5´ CCTGCATCCAAGGAAGACAT 3´

rum1-rv	5´ CTTGACATCACGAACCATCG 3´
BIGE1-fw	5’ GGGCTGGAGCTCACCAACGACGCCGAG 3’
BIGE1-rv	5’ GCCAAGCCACTCTAGCTATGGTACTAG 3’

Supplementary Table 1. Gene specific primers used in this study

Author Contributions: Conceptualization, R.P. and F.C.; Methodology, F.C.; Software, L.G.; Validation, G.B., M.G. and M.P.; Formal Analysis, F.C.; Investigation, F.C.; Resources, R.P.; Data Curation, F.C.; Writing—Original Draft Preparation, F.C.; Writing—Review and Editing, G.B.; Visualization, M.G.; Supervision, R.P.; Project Administration, R.P.; Funding Acquisition, R.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Original Research

The Potential of *Low Phytic Acid1-1* Mutant in Maize (*Zea mays* L.): A Sustainable Solution to Non-Renewable Phosphorus

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Abstract

Background: Phosphorus is an essential component of fertilizers and feed and in recent decades has become one of the main sustainability issues as a non-renewable resource. In plant seeds, the main reserve of phosphorus is phytic acid, a strong anti-nutritional factor for monogastrics and a pollutant of cultivated lands. The reduction of phytic acid in cereal seeds has become a major challenge in breeding programs to increase the nutritional quality of foods and feeds and to improve the environmental phosphorus sustainability in agriculture. In maize (*Zea mays* L.), four *low phytic acid* (*lpa*) mutations have been isolated and *lpa1-1* is the most promising. However, the reduction of phytic acid in *lpa1-1* leads to many adverse pleiotropic effects on the seed and in general on plant performance. A seed weight reduction and a consequent yield loss were previously described in this mutant.

Method: In this work, a field experiment to study seed weight and yield was conducted for two years in two different genetic backgrounds (B73 and B73/Mo17). Furthermore, the greater susceptibility of *lpa1-1* to drought stress was also investigated: a dedicated field experiment was set up and measurements were carried out under optimal water conditions and moderate drought stress.

Results: From the first experiment it emerges that under high-input conditions, *lpa1-1* seems to have comparable or even better seed weight/ear than the relative control. The main problem of this mutant remains the reduced field emergence (~40%). In the study of drought stress it was found that the increased sensitivity in the mutant is mainly caused by an altered stomatal regulation, but not by a less developed root system, as previously reported. When the stress occurred, the parameters measured did not significantly change in the wild-type, while they dropped in the mutant: the net photosynthesis decreased by 58%, the transpiration rate by 63% and the stomatal conductance by 67%.

Conclusions: Some possible solutions have been proposed, with the aim of developing a commercial variety, which remains the main goal to exploit the nutritional benefits of *low phytic acid* mutants.

Keywords:

low phytic acid mutants; *lpa*; drought stress; root system architecture; stomatal conductance; environmental sustainability; seed quality; carbon isotope discrimination

1. Introduction

In recent decades, phosphorus (P) has become one of the main sustainability issues as a non-renewable resource. P is a crucial element for animal and plant production and is involved in many

physiological and biochemical processes. It was the first element to be recognized as an essential mineral nutrient for plants, and its functions cannot be replaced by any other mineral nutrient [1]. Being an essential component of fertilizers and feed, the increasing production of food has increased the rate of mobilization of reserves, and the price of this mineral is continuously rising. It is estimated that in the world, there will be reserves accessible with current technology for another 90 years at current consumption rates. However, its consumption will increase as the population grows [1].

In plant seeds, the main reserve form of P is phytic acid (PA, myo-inositol-1,2,3,4,5,6-hexakisphosphate) [2], an insoluble phosphate compound considered a strong anti-nutritional factor, the degradation of which occurs during germination by a group of enzymes called phytases [3].

Only ruminants can degrade phytic acid thanks to the presence of phytases in their digestive tract. Phytic acid is poorly digested by monogastrics: as it is not assimilated, PA is expelled with animal excrement, becoming a pollutant of cultivated land and contributing to water surface eutrophication. Since it is a compound poorly assimilated by monogastrics, farmers must add mineral phosphate to the animal feed, thus leading to an increase in costs [4]. Hence, the reduction of PA in cereal seeds has become a significant challenge in breeding programs to increase the bioavailability of micronutrients and improve seed nutritional quality, mainlyfor the benefit of populations whose diet is based on these staple crops. In the last decades, many *low phytic acid* (*lpa*) mutants have been isolated in several important crops, such as maize [5–8], wheat [9], barley [10–12], rice [13,14], soybean [15–17] and common bean [18,19]. In maize (*Zea mays* L.), there are three different *lpa* mutations (*lpa1*, *lpa2,* and *lpa3*) depending on the step of the biosynthetic pathway they affect, with *lpa1* showing the lowest PA content in the seed, followed by a proportional increase in inorganic P [5, 6].

In maize (*Zea mays* L.), *lpa1* mutations are caused by lesions in the *ZmMRP4* gene. Until now, four *low phytic acid* mutations have been isolated in the *ZmMRP4* PA transporter: *lpa1-241* [20, 21], *lpa1-7* [7], *lpa1-1* [22] and *lpa1-5525* (not fully characterized) [8]. *lpa1-241* and *lpa1-7* are lethal in the homozygous state, displaying an 80%–90% decrease in PA, while *lpa1-1* is the only one viable in homozygosity, showing a lower reduction in phytic acid (66%). Although it is not lethal, reducing PA in *lpa1-1* leads to many adverse pleiotropic effects on the seed and, in general, on plant performance [23]. Among these agronomic defects, a seed weight reduction ranging from 8 to 23% was found in this mutant [5]. This decrease appeared to be mainly caused by endosperm loss, resulting in an agronomic yield reduction. Moreover, it was also observed that *lpa1-1* was more susceptible to drought stress, probably due to an alteration in mature root system development [7]. Colombo and coworkers recently excluded this hypothesis: in recent work conducted in controlled or semi-controlled conditions, it emerged that the drought stress in the mutant *lpa1-1* seemed to be caused by

a reduced photosynthetic efficiency and not by a shallower root system [24].

Maize's root system architecture is composed by embryonic and post-embryonic roots [25, 26]. The former, which includes the primary root and a variable number of seminal roots, is important for seedling vigor in the early stages of development [27, 28]; the post-embryonic roots dominate the mature root system and are formed by crown roots (at underground nodes of the shoot) and one or more whorls of brace roots [26, 29]. All these roots generate post-embryonic lateral roots, mediating the absorption of water and nutrients from the soil [30].

In maize, a hypothetical root system ideotype for optimizing water and nitrogen uptake was presented by Lynch and is called the SCD ("steep, cheap and deep") ideotype [31]. It was reported that one whorl of brace roots is preferable to multiple whorls: in fact, the brace roots from younger whorls appear later in the development and are less useful for soil resource acquisition. Furthermore, it was reported that the first above-ground node should have high occupancy and should be entirely occupied by brace roots that reach the soil, thus giving stability to the plant [31].

Based on these data, the present work aimed to study two of the main pleiotropic effects that characterize the mutant *lpa1-1* in maize (*Zea mays* L.), i.e., the seed weight reduction and the greater susceptibility to drought stress. Here we report the results of a field evaluation performed for two years in two different genetic backgrounds, highlighting a good seed weight/ear of *lpa1-1* under high-input conditions and limiting the problem of this mutant to the field emergence. Furthermore, in another trial, we collected different epigeal and hypogeal measurements to study drought stress and to confirm in the field what was previously reported by Colombo and coworkers under controlled conditions.

2. Materials and Methods

2.1 Genetic Materials

In this work, we used four different genetic materials which were compared pairwise. The first pair was *lpa1-1/lpa1-1* vs. +/+ control in "B73" genetic background kindly provided by Dr. Victor Raboy, USDA ARS, Aberdeen, ID, USA. B73 is an inbred line used in the 80's for the hybrid production, now widely used as a benchmark. The second pair was *lpa1-1/lpa1-1* vs. +/+ control in "B73/Mo17" genetic background. These two genotypes were obtained by crossing *lpa1-1/lpa1-1* B73 with "Mo17" inbred line provided by the germplasm bank at DISAA, Department of Agricultural and Environmental Sciences—Production Landscape, Agroenergy, University of Milan. The F1 obtained was selfed, generating an F2 population scored by Chen's assay and genotyped to isolate *lpa1-1/lpa1-1* and +/+ genotypes (as described in the following sections). Following three cycles of sib crossing,

we obtained two synthetic populations differing only for the presence of the *lpa1-1* in homozygous status (Fig. 1).

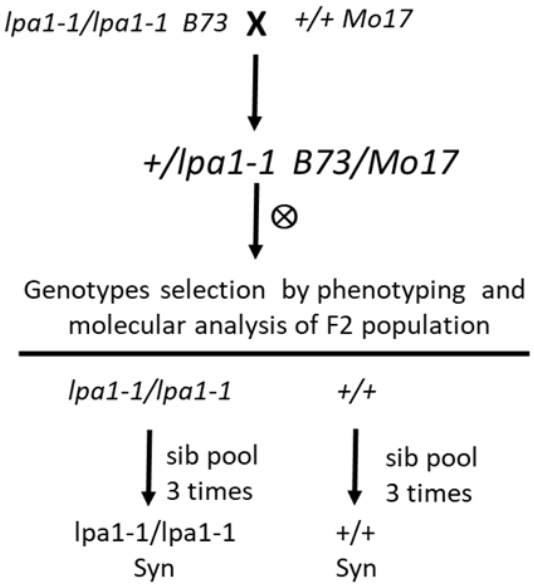

Fig. 1. Pedigree scheme used to obtain the two synthetic populations in B73/Mo17 genetic background. The two synthetic populations differ only for the presence of the *lpa1-1* in homozygous status.

2.2 Chemical and Molecular Validation of the Genetic Material

The Chen assay is a colorimetric method used to distinguish the HIP (high inorganic phosphate) phenotype. The HIP phenotype is diagnostic for the presence of the *lpa1* mutation. We used the qualitative Chen assay with some modifications [32]. The seeds were ground in a mortar using a pestle, and 100 mg of the flour obtained was placed in a microtiter plate with 1 mL of 0.4 M HCl solution. After 1 h at room temperature, 100 µL of the extract was transferred to another microtiter plate, adding 900 µL of Chen's reagent (6 NH_2SO_4, 2.5% ammonium molybdate, 10% ascorbic acid, H_2O [1:1:1:2, *v*/*v*/*v*/*v*]). The blue-colored phosphomolybdate complex was visible after 1 h: the intensity of the blue color is directly proportional to the free phosphate content.

DNA was extracted from wild-type and mutant leaves as described by Dellaporta *et al*. [33]. PCR was performed with the primers 13L (5'-CTTCATGATCTGCGGTCACG-3') (forward primer, position +5293) and 51R (5'-AAGCATCAGCTTCGGGTAATGT-3') (reverse primer, position +6460) and 2 µL of DNA aliquots were used as a template. The reaction mix underwent an initial denaturation step at 94 °C for 2 min, 37 cycles of denaturation at 94 °C for 15 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s. Extension at 72 °C for 5 min was performed to complete the reaction. Amplicon length was verified on 1% (w/v) agarose gels, and the presence of the mutation in *lpa1-1* was confirmed by Sanger sequencing.

2.3 Experimental Design and Agronomic Analysis

Experiments were carried out in 2020 and 2021 in the experimental field of the University of Milan located in Landriano (PV), Italy (N45°18′, E9°15′). The seeds of each genotype were sown at the end of April in both years. The experiment was laid out in randomized blocks, and each variety (B73, B73/Mo17, and the respective *lpa1-1* mutants) was cultivated in three plots, for a total of 12 plots. The size of each plot was about 10 m^2 (5 m × 2.1 m), sown in three rows, with a density of 60 plants per plot. The experimental fields were in maize–maize rotation with standard soil fertilization (about 220 kg/ha of nitrogen). The maize plants were grown by conventional farming methods (pre-emergence herbicide was applied). Water was applied by sprinkler irrigation as needed.

The following agronomic data were collected in the two backgrounds in both years:

Plant height: the distance between the base of the male inflorescence (panicle) and the ground level was measured considering more than 30 plants chosen randomly in the three plots. Plant height was measured in mid-July during full flowering using a measuring rod.

Ear height: insertion height of the first ear measured considering the distance between the ground level and the insertion point of the first ear in the stem. These data were collected from the same plants used for plant height.

Moreover, five representative ears for each plot (15 ears for each genotype) were randomly selected for grain yield estimation in the two backgrounds in both years. The ears were harvested on September 12th, and the seeds were immediately dried to 12–13% of relative humidity. Later, the following parameters were collected:

Ear length: measured with a ruler on 15 ears for each genotype.

Average single seed weight: single representative seeds were selected from each ear and were measured using an analytical weight scale (Precisa XB 220A, 0.1 mg). The test was performed in triplicate.

The number of seeds per ear: evaluated by counting all the seeds of each sampled ear.

Seeds weight/ear: measured by weighing all the seeds that make up an ear.

2.4 Drought Stress: Root Sampling and the Estimation of Photosynthesis, Transpiration Rate and Stomatal Conductance

In parallel, 200 seeds of the inbred line B73 and 200 seeds of the relative *lpa1-1* mutant were sown in a non-irrigated part of the field to study drought stress in field conditions.

In particular, root sampling was carried out in the season 2021, and the root system architecture of the wild-type was compared with the relative *lpa1-1*. Only two days prior to sampling, the fields

were irrigated with 12 mm of water to facilitate the excavation of roots. At flowering, ten roots per genotype were excavated in the first 30 cm using standard shovels. The excavated roots were shaken to remove the main fraction of soil adhering to the roots, and the remaining soil particles were removed by vigorous rinsing at low pressure, according to Trachsel *et al*. [34]. On the cleaned roots, different parameters were collected: number of above-ground whorls occupied with brace roots (BW); brace root number (BO); angle of the first arm of brace roots originating from the first whorl in relation to horizontal (BA1); culm diameter at the base of the sampled plant (CD).

Later, a crown root and a brace root were detached from each root system to estimate the root density. The roots of each sample were scanned (using a high-resolution digital scanner). The images were processed with Adobe Photoshop software: the shadows of the roots and the background of the images were removed, and the color of the roots was changed to green and made uniform. The processed images were analyzed using ImageJ 1.52 [35] and Easy Leaf Area software (University of California, USA) [36] to calculate the root area/root length ratio.

Different parameters in the epigeal part of the plant were collected: net photosynthesis (P_n), transpiration rate (E) and stomatal conductance (GS) were measured on B73 and its relative *lpa1-1* with a portable CIRAS-2 photosynthesis system (PP-Systems, UK) (CO_2 350-360 µmol mol^{-1}; PAR 1700 µmol m^{-2} s^{-1}; 75% humidity). Measurements were carried out at two different moments: the 6th of July under optimal water conditions (no stress) and the 16th of July under moderate drought stress. All the measurements were taken between 11 and 12 am on the third last leaf, the temperature was 30 ± 1 °C and the humidity 75%.

2.5 Stable Carbon Isotope Analysis: $\delta^{13}C$ Determination

Stable carbon isotope analysis was carried out on flag leaves of both B73 and *lpa1-1*/*lpa1-1* plants grown in the field and sampling was done on the 14th of July. Harvested leaves were preventively dried at 80 °C and then ground to a fine powder by using a grinder. Samples were prepared by adding 1 mg of dry powdered plant tissues into 5 × 9 mm tin capsules. Capsules were carefully closed by folding them with cleaned tweezers and were then transferred to an auto-sampler.

The $\delta^{13}C$ values of samples were measured using a Flash 2000 HT elemental analyzer coupled, *via* a ConFLo IV Interface, with a Delta V Advantage isotope ratio mass spectrometer (IRMS), interconnected to the software Isodat 3.0 (Thermo), according to Bononi *et al*. [37]. Briefly, the combustion/reducing reactors, combined in a single quartz tube, were heated at 1020 °C. The He gas flow was 120 mL min^{-1} and 100 mL min^{-1} for carrier and reference, respectively. The O_2 purge for flash combustion was 3 s at a flow rate of 175 mL min^{-1} per sample. The GC separation column was maintained at 45 °C. The CO_2 reference gas pulse was introduced three times (20 s each) at the

beginning of each run. The run time of the analysis was 600 s for a single run. The analysis of each sample (6 flag leaves for each genotype) was performed five times.

Calibration was performed using three secondary reference materials provided by IAEA: NBS18 ($\delta^{13}C = -5.014 \pm 0.035$ ‰); IAEA-600 ($\delta^{13}C = -27.771 \pm 0.043$ ‰); IAEA-612 ($\delta^{13}C = -36.722 \pm 0.006$ ‰). Two in-house solid standards, sulfanilamide ($\delta^{13}C = -27.23 \pm 0.06$ ‰) and methionine ($\delta^{13}C = -30.01 \pm 0.05$ ‰), were used for normalization and quality assurance.

The isotope ratio $^{13}C/^{12}C$ was expressed using the standard $\delta^{13}C$ notation:

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{VPDR} - 1] \times 1000$$

which expresses the part per thousand deviation of the isotope ratio $^{13}C/^{12}C$ of a sample relative to an international standard, the Vienna Pee Dee Belemnite [38].

2.6 Determination and Quantification of Abscisic Acid

For the quantification of abscisic acid (ABA), 100 seeds of the two genotypes (B73 and the relative *lpa1-1* mutant) were germinated under controlled conditions. After 2 weeks, the plant leaves were sampled, and the following method was applied for the quantification of ABA.

Sample preparation and extraction method: samples were ground by an electric blender and an amount of 5.0 ± 0.1 g of sample was weighed in a 40 mL glass tube and 5.0 ± 0.1 g of anhydrous sodium sulfate and 10 mL of methanol were added. The sample was vortexed for 10 min and after 5 min was vortexed for more 5 min; then, the mixture was centrifuged for 2 min at 3500 rpm. The supernatant passed through a 0.45 µm membrane and was directly analyzed by HPLC-DAD (20 µL).

Reagents and standard: the reagents used for the determination and quantification of ABA were analytical-grade water, methanol and acetic acid (Carlo Erba – Italy). Standard abscisic acid (purity ≥98%, lot number BCCG6453) was from Supelco (Milan, Italy).

Apparatus: high performance liquid chromatography analysis of ABA was performed on a Shimadzu instrument (Milan, Italy) equipped with two pumps (10AD vp), a diode array detector (SPD-M10A vp), a system controller (SCL-10A vp) and a Rheodyne 20 mL injection loop (Cotati, CA, USA). The HPLC pumps and diode array system were controlled by computer using a LC Solution version 1.25 SP5 workstation program (Shimadzu, Milan, Italy). The analytical column employed was a Kinetex XB-C18 (150 mm × 3.0 mm, particle size 5 µm) by Phenomenex (Bologna, Italy).

Chromatographic conditions: the mobile phase consisted of (A) water/acetic acid (99:1 v/v) and (B) methanol. The operating conditions were as follows: 0–30 min linear gradient from 10% B to 100% B, 30–50 min isocratic elution 100% B, 50–70 min linear gradient from 100% B to 10% B, and 70–80 min isocratic elution 10% B. The flow rate was 0.4 mL min^{-1}. The injection volume was 20 µL

and the wavelength used for the detection was 265 nm.

Analytical quality assurance: the abscisic acid content was quantified using the calibration curve. A stock standard solution of ABA was prepared dissolving the compound in methanol (concentration 1.0 mg mL^{-1}). Working standard solution of 0.25, 0.50, 1.00, 2.00, 2.50, 4.00, 5.00, 8.00, 10.00, 12.50, and 20.00 μg mL^{-1} were prepared by diluting the stock solution with methanol from 0.25 to 20.00 g mL^{-1}. The calibration curve was linear ($R^2 = 0.9987$) in the range 0.25–20.00 μg mL^{-1}. The standard deviation was 0.05 (derived from 10 replicated analyses). ABA content was expressed as mg kg^{-1} of fresh vegetable matrix. The limit of detection (LOD) was 0.01mg kg^{-1} and the limit of quantification (LOQ) was 0.1 mg kg^{-1}. The recovery was 98 ± 3% for concentration between 0.50 and 5.00 mg kg^{-1}, and 102 ± 3% for concentration between 5.00 and 20.00 mg kg^{-1}.

3. Results

3.1 Constitution of the Genetic Material by Chemical and Molecular Analysis

In this work, we used four different genetic materials compared pairwise. The first pair was *lpa1-1/lpa1-1* vs. +/+ control in B73 genetic background. The second pair was obtained by crossing *lpa1-1* B73 with Mo17 inbred line provided by our germplasm bank. The F1 obtained was selfed, generating an F2 population scored by Chen's assay and genotyped to isolate *lpa1-1/lpa1-1* and +/+ genotypes (as described in the following sections). Following three cycles of sib crossing, we obtained two synthetic populations differing only for the presence of the *lpa1-1* in homozygous status (Fig. 1).

The presence of the *lpa* trait in the material used in the following experiments was confirmed through chemical and molecular methods. The qualitative Chen assay for free phosphate allowed us to rapidly distinguish the *lpa1-1* mutants with respect to the wild phenotype: the former were colored blue in contact with the Chen reagent, while the wild-types remained white due to the low content of free phosphate (Fig. 2A). Later, Sanger sequencing was used to molecularly confirm the presence of the mutation (Fig. 2B): in comparison with the wild-type allele, the coding sequence of the *lpa1-1* allele is characterized by the presence of a Single Nucleotide Polymorphism (SNP), C to T, in position 5759 with respect to the starting codon on the genomic sequence (Fig. 2C).

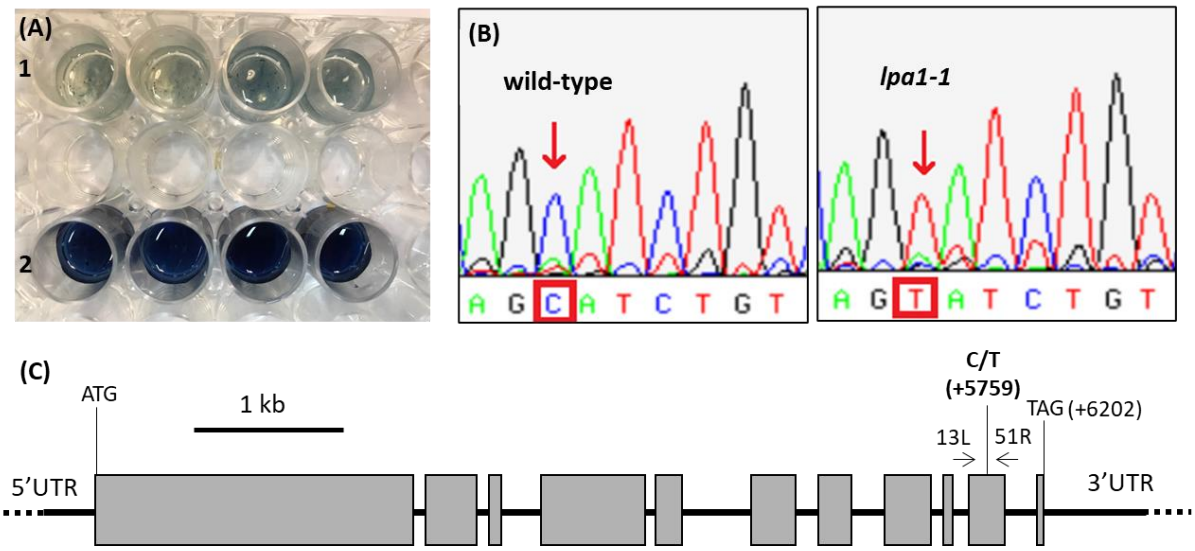

Fig. 2. Chen assay for free phosphate. (A) microtiter row 1 showed the results of the assay performed on four wild-type seeds; in row 2, the blue-colored phosphomolybdate complex was visible and represented four mutant seeds. (B) The presence of the mutation (C to T) was molecularly confirmed by Sanger sequencing. (C) Structure of the *ZmMRP4* gene and position of the Single Nucleotide Polymorphism (SNP), C to T.

3.2 Agronomic Performance of lpa1-1 in Two Backgrounds over Two Years

The field experiment was organized in randomized blocks; plants were grown under high-input conditions, and water was not a limiting factor during the season. Field emergence percentage was calculated by counting the number of plants present between the V2 and V4 stages of development and dividing by the total number of seeds. This parameter was measured in both the backgrounds and years and appeared to be reduced in *lpa1-1* compared to the wild phenotype, as reported in Table 1. This value was much lower than the germination of the same seeds under controlled conditions at the optimal temperature and humidity (data not shown).

Table 1. Field emergence percentage measured in the randomized blocks experiment by counting the number of plants present between the V2 and V4 stages and dividing by the total number of seeds. The data were collected in 2020 and 2021.

Genotype	Field emergence (%)	95% Fiducial Limit	Field emergence (%)	95% Fiducial Limit
	2020		2021	
B73	100	0.00	100	0.00
lpa1-1 (B73)	37	6.67	45	6.91
B73/Mo17	100	0.00	100	0.00
lpa1-1 (B73/Mo17)	36	6.71	43	6.88

Furthermore, the plants of *lpa1-1* grown under field conditions were characterized by a lower plant and ear height compared to the relative wild-types in both the years analyzed and in both the backgrounds (Fig. 3A,B). In the control line B73, the plant height reached an average of 189.6 cm in

2020 and 198.3 cm in 2021, while the mutant *lpa1-1* introgressed in B73 was 184.3 and 181.1 cm in height, respectively (Fig. 3A). On the same plants used for plant height, ear height was also measured. Ear height was statistically higher in B73 than *lpa1-1* in both the years (127.1 vs. 93.4 cm in 2020 and 134.8 vs. 94.3 cm in 2021) (Fig. 3B).

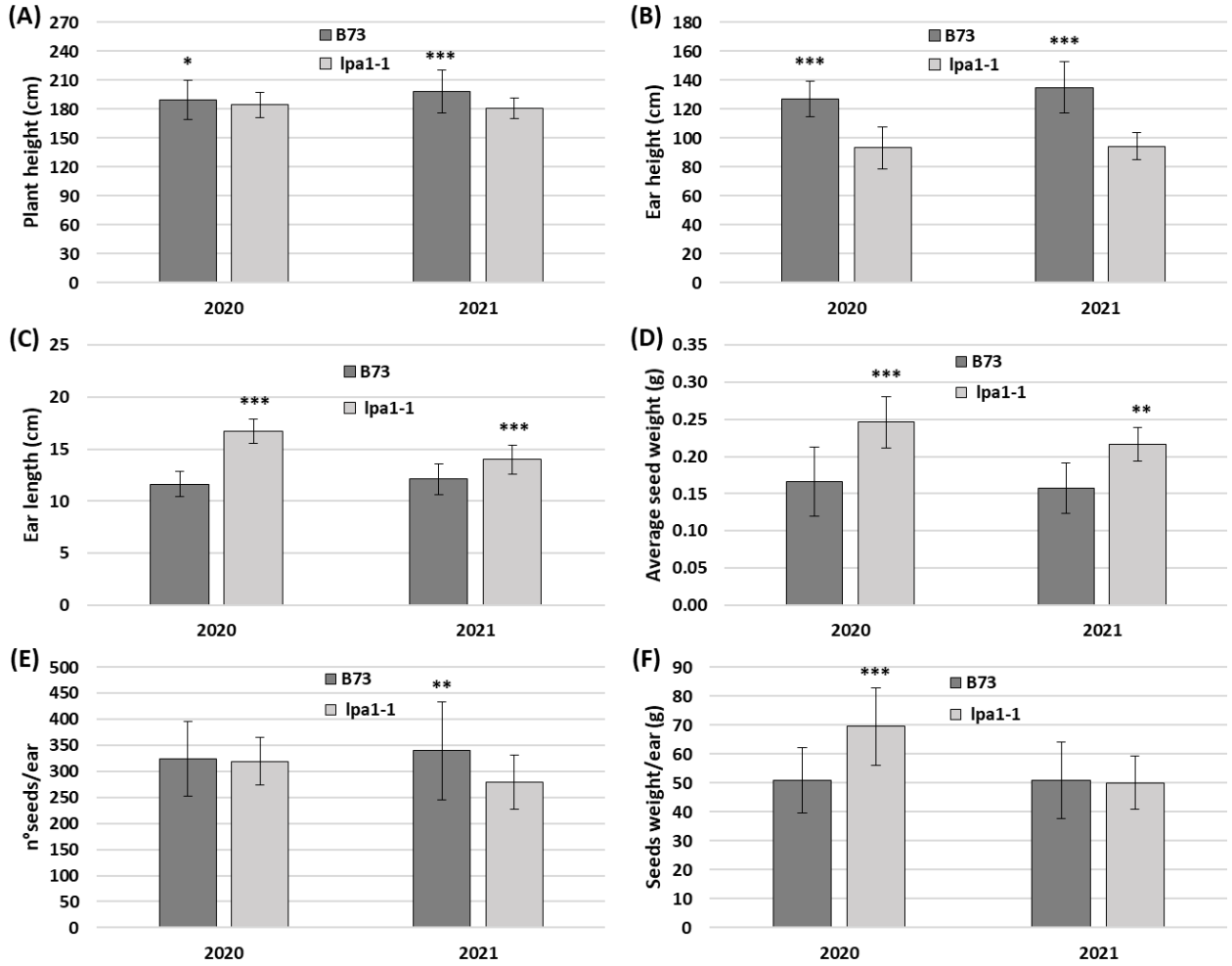

Fig. 3. Agronomic parameters collected in the field experiment on the first genetic background (B73). Plant height (A) and ear height (B) measured at flowering in B73 and in its relative *lpa1-1*. The data represent the means of n ≥30 biological replicates. Ear length (C), average single seed weight (D), number of seeds/ear (E) and seeds weight/ear (F). The data were collected in 2020 and 2021 and represent the means of 15 biological replicates. Significant differences between the wild-type and *lpa1-1* were assessed by Student's *t* test (*$p < 0.1$, **$p < 0.05$ and ***$p < 0.01$).

The same trend was recorded in the synthetic population B73/Mo17, although, in general, these plants were taller than in the first background due to the hybrid vigor. The height of B73/Mo17 plants reached 234.6 cm in 2020 and 243.2 cm in 2021, while the mutant *lpa1-1* introgressed in B73/Mo17 was 189.6 and 190.1 cm high, respectively (Fig. 4A). Also, ear height was statistically higher in the wild-type than *lpa1-1* in both the years (142.9 vs. 103.7 cm in 2020 and 147.8 vs. 105.3 cm in 2021) (Fig. 4B).

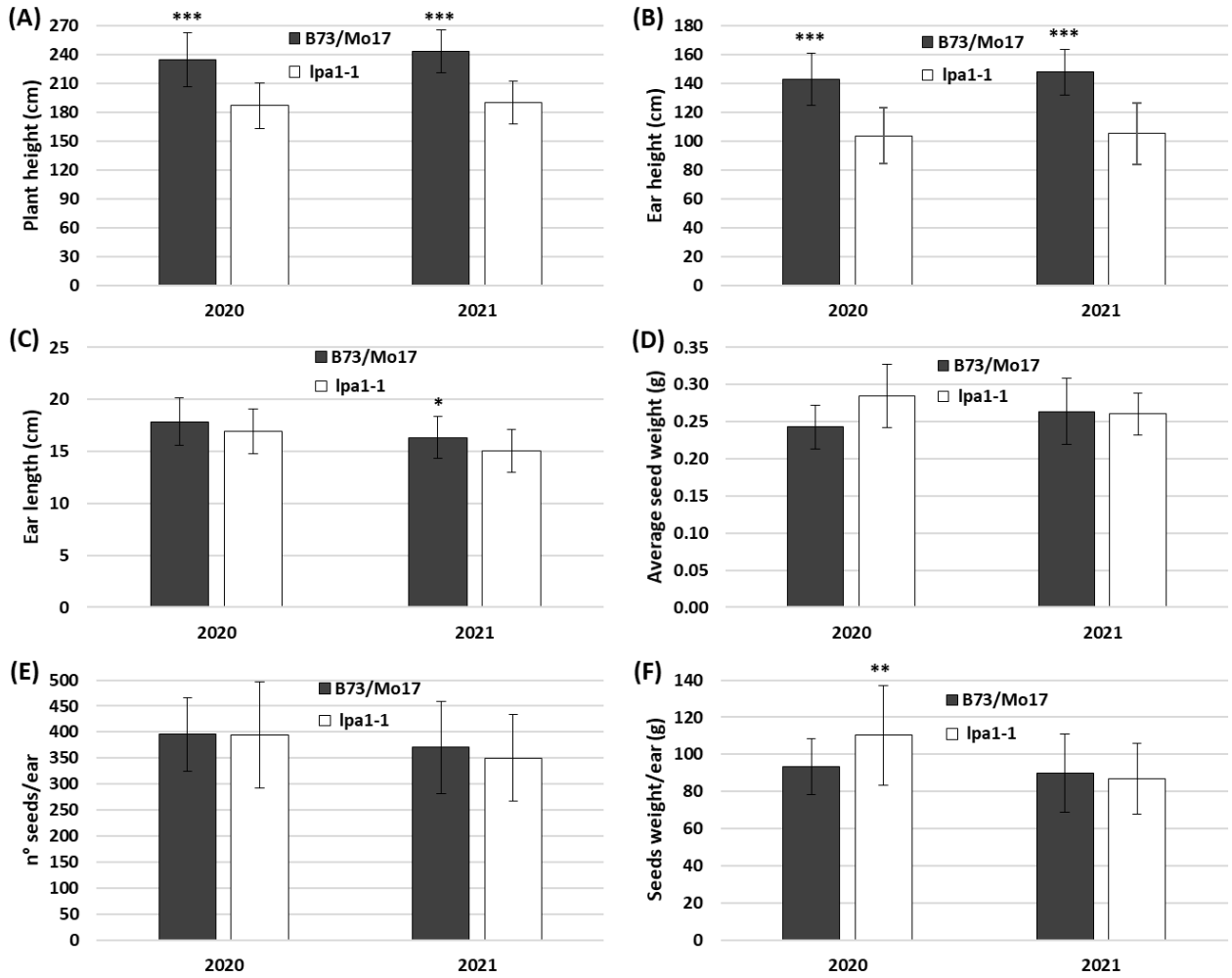

Fig. 4. **Agronomic parameters collected in the field experiment on the second genetic background (B73/Mo17).** Plant height (A) and ear height (B) measured at flowering in B73/Mo17 and in its relative *lpa1-1*. The data represent the means of n ≥30 biological replicates. Ear length (C), average single seed weight (D), number of seeds/ear (E) and seeds weight/ear (F). The data were collected in 2020 and 2021 and represent the means of 15 biological replicates. Significant differences between the wild-type and *lpa1-1* were assessed by Student's *t* test (*$p < 0.1$, **$p < 0.05$ and ***$p < 0.01$).

After collecting these agronomic data at flowering, other parameters (ear length, average single seed weight, number of seeds/ear, and seeds weight/ear) were measured at harvesting for grain yield estimation, collecting n ≥5 ears for each plot (in total, 15 ears per genotype).

Under optimal growth and water conditions, *lpa1-1* showed equal or superior performance compared to the inbred line B73. In particular, the ear length and the average seed weight were statistically higher in the mutant in both the years (Fig. 3C,D). In contrast, the number of seeds per ear was statistically the same in 2020 and slightly higher in B73 in the following year (Fig. 3E). An important parameter in grain yield estimation is the seed weight per ear, which was statistically superior in *lpa1-1* in 2020 and the same between the two genotypes in 2021 (Fig. 3F).

These results were confirmed in the other genetic background, the synthetic population B73/Mo17. Most of the parameters measured were statistically the same in the wild-type and in the

relative mutant, and even the seed weight/ear of *lpa1-1* in 2020 was statistically higher than in the control (110.24 vs. 93.38 g).

3.3 The Root System Is not a Limiting Factor in lpa1-1 under Drought Stress

Another experiment on *lpa1-1* was conducted in the field in order to study its response to drought stress conditions. In this case, the plants of B73 and *lpa1-1* were grown in a non-irrigated part of the field. Ten roots per genotype were sampled at flowering and then cleaned by vigorous rinsing (Fig. 5A). Photos of the two genotypes were taken (Fig. 5B), and among the parameters collected, no significant differences were found regarding the brace roots, as indicated in Table 2. Also, the diameter of the culm, an epigeal parameter measured at the base of the sampled plant, was statistically the same between *lpa1-1* and the wild-type (Table 2).

Table 2. Parameters collected after cleaning the roots: number of above-ground whorls occupied with brace roots (BW); brace root number in the 1st whorl (BN); angle of the 1st arm of brace roots originating from the first whorl in relation to horizontal (BA1); culm diameter at the base of the sampled plant (CD).

Genotype	Brace whorls (BW)	Brace number (BN)	Brace angle (BA1)	Culm diameter (CD)
B73	1.40 ± 0.52^{ns}	15.44 ± 1.88^{ns}	49.00 ± 5.61^{ns}	27.95 ± 3.35^{ns}
lpa1-1	1.70 ± 0.48^{ns}	16.60 ± 1.35^{ns}	50.39 ± 5.89^{ns}	26.74 ± 2.70^{ns}

Values represent the mean and the standard deviation of ten biological replicates. For each parameter, comparisons were made in column. Significant differences between the wild-type and *lpa1-1* were assessed by Student's *t* test (**p* < 0.1, ***p* < 0.05, ****p* < 0.01 and *ns*, not significant).

Furthermore, a crown root and a brace root were detached from each root system to estimate root density (Fig. 5C). Images were processed, and the area occupied per unit of length was calculated, as indicated in Fig. 5D. This ratio was statistically higher in the mutant than in the wild-type in both types of post-embryonic roots: in the case of crown roots, the area reached 1.70 cm^2/cm in *lpa1-1,* while it was 0.89 cm^2/cm in the inbred line B73. The same trend occurred with brace roots: 0.90 vs. 0.60 cm^2/cm, respectively. Considering these data, the root system architecture of *lpa1-1* did not appear to be a limiting factor under water limitation.

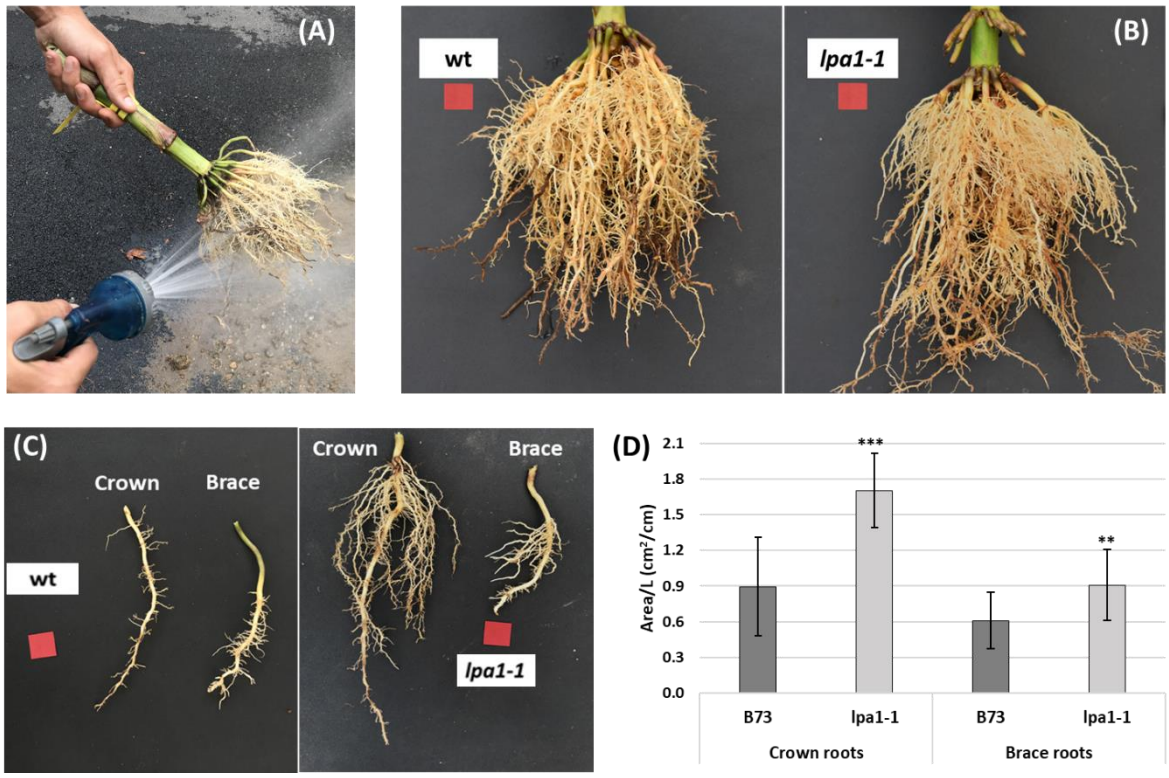

Fig. 5. Schematic representation of the root sampling and collected data. Ten roots per genotype were sampled in the field through a shovel and the soil was removed with a vigorous rinsing (A). An example of B73 and *lpa1-1* root system architecture (B). A representative crown root and a brace root were detached from each root system (C) and the area occupied per unit of length was calculated with Easy Leaf Area software (D). Significant differences between the wild-type and *lpa1-1* were assessed by Student's *t* test (*$p < 0.1$, **$p < 0.05$ and ***$p < 0.01$).

3.4 Leaf Gas Exchange Was Altered in lpa1-1 in Drought Stress Conditions

The results obtained so far seem to exclude the root system as the leading cause of the drought stress in *lpa1-1*. Therefore, our research focused on the epigeal part of the plant, and several measurements were made at two different moments: under optimal water conditions (16% of volumetric soil moisture) and under moderate drought stress (6% of volumetric soil moisture). In Fig. 6A it is possible to observe how the leaves of the two genotypes appeared when the measurements under moderate drought stress were carried out: the leaves of the mutant appeared altered, stressed, and curled upwards. The three parameters collected with the CIRAS-2 Portable Photosynthesis System highlighted that under optimal water conditions the transpiration rate and stomatal conductance were significantly higher (by 31% in both cases) in *lpa1-1* compared to the control line, revealing a greater stomatal opening in the mutant (Fig. 6C,D). Pn did not significantly differ in the two genotypes under non-stressful conditions, although the mean was higher in *lpa1-1* than in the wild-type (29.70 vs. 24.70 µmol m^{-2} s^{-1}) (Fig. 6B). When water stress occurred, these three parameters did not significantly change in the wild-type, while they dropped in the mutant: the net photosynthesis decreased by 58% (Fig. 6B), the transpiration rate by 63% (Fig. 6C) and the stomatal conductance of

the 67% (Fig. 6D), revealing *lpa1-1* as alterated in stomatal regulation under more stressful water conditions.

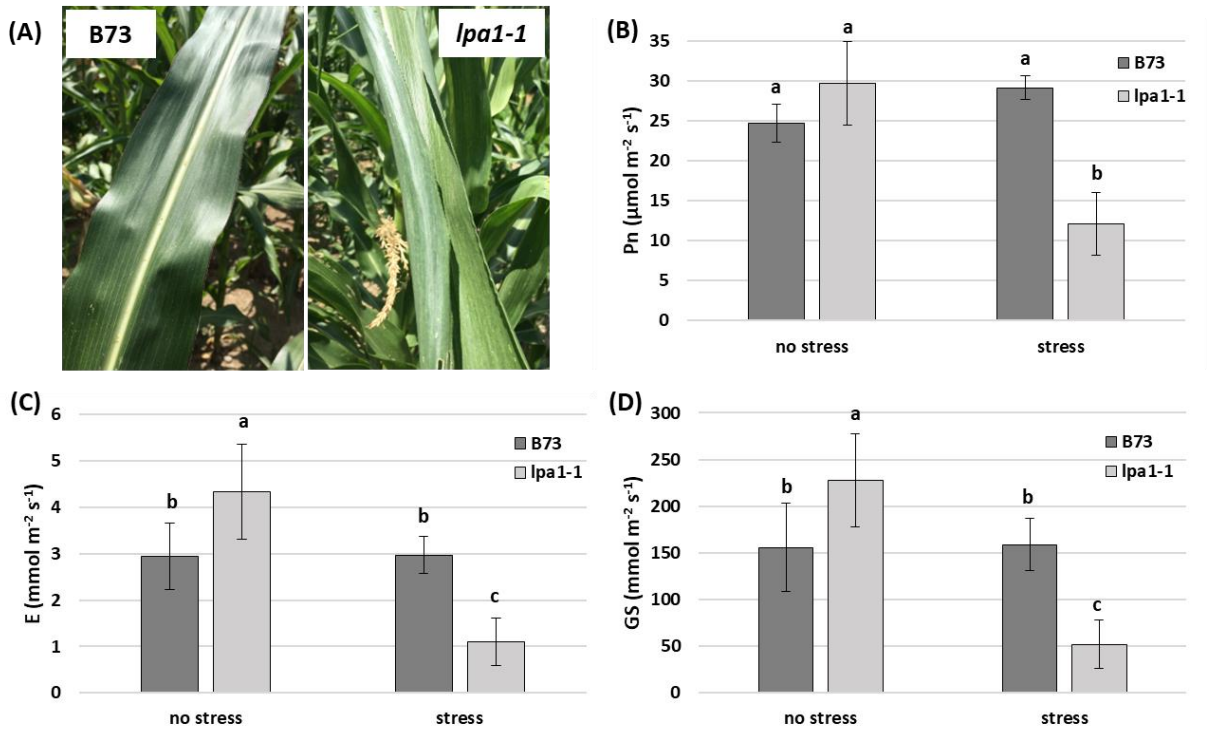

Fig. 6. Measurements carried out in the field at two different moments, under optimal water conditions (no stress) and under moderate drought stress. (A) Under moderate drought stress, the leaves of *lpa1-1* were stressed and curled upwards compared to the wild phenotype. The photos were taken the same day. The CIRAS-2 Portable Photosynthesis System was used in the field to measure the net photosynthetic CO_2 rate (Pn) (B) the transpiration rate (E) (C) and the stomatal conductance (GS) (D). Values represent the means of eight biological replicates. Data were analyzed with one-way ANOVA and post-hoc Tukey test considering four values (B73/*lpa1-1*, no stress/stress).

To further support this hypothesis, we measured the carbon stable isotope composition of the maize flag leaves of the two genotypes grown under optimal water conditions - i.e., when both transpiration rate and stomatal conductance were higher in *lpa1-1* than in the wild-type - since stomatal conductance may significantly alter $^{13}C/^{12}C$ discrimination during CO_2 fixation [39]. $\delta^{13}C$ analysis revealed that B73 and *lpa1-1* differently discriminate the carbon stable isotopes under optimal water conditions (–13,41 ± 0.02 ‰ vs. –12.58 ± 0.09 ‰, respectively) ($p < 0.01$). Hence, the difference between the two values of $\delta^{13}C$ was $\Delta_{(lpa1\text{-}1\ -\ B73)} = +0.83 \pm 0.11$ ‰, suggesting that a different regulation of stomatal conductance occurred during the growth. Indeed, the $\delta^{13}C$ values of the organic matter accumulated during the growth mirrors the carbon stable isotope separations occurring under different stomatal conductance, thus maintaining the memory of all the activities that determined them.

4. Discussion

Phytic acid represents the main P reserve in mature seeds and is degraded during germination by the activity of a group of enzymes called phytases [2, 3]. It is well known that phytic acid is characterized by remarkable chelating properties, and besides making P unavailable, it binds different minerals (such as Fe and Zn), making them unavailable for monogastric animals due to the lack of phytases in their digestive systems. Despite being an anti-nutritional factor for all these reasons, PA is considered an important antioxidant compound: by chelating iron cations, phytic acid can counteract the formation of reactive oxygen species (ROS), thus preserving seed vitality [40–42]. In recent decades, phosphorus has also become a topic of primary importance because it is a non-renewable resource. In fact, phosphorus is widely used in agriculture and the increased food production has increased the rate of reserve mobilization. The price of the mineral is continuously rising and at current consumption rates it has been estimated that phosphorus reserves will run out in 90 years [8].

In this context, the reduction of PA in cereal seeds has become a major challenge in breeding programs to increase the bioavailability of micronutrients and improve seed nutritional quality. In the last decades, many *low phytic acid* (*lpa*) mutants have been isolated and characterized in all major crops and *lpa1-1* is the most promising in maize. However, the reduction of PA in seeds can affect the vitality of the seeds and in general the agronomic performance of the plants [23]. Among these adverse pleiotropic effects, a seed weight reduction was previously described [5] and a major susceptibility to drought stress was observed in *lpa1-1* [7].

In the first part of this work, we report the results obtained in a field study conducted for two years in two different genetic backgrounds. In this way it was possible to investigate the first of the two agronomic defects previously reported, i.e. the reduction in seed weight and the consequent yield loss that characterize *lpa1-1*. To our knowledge, after the isolation of this mutant by Raboy and collaborators in 2000 [5], no trials in field conditions have been conducted in recent years to evaluate its yield in comparison to the wild phenotype. In this experiment, plants were grown under high-input conditions and water was not a limiting factor during the season.

In both the backgrounds, *lpa1-1* showed a reduced stature compared to the wild-type (Figs. 3,4A,B), confirming that the shortening of the internodes could be caused by an alteration in the auxin polar transport, as occurs in other known maize mutants, such as *brachytic 2* (*br2*) and *brevis plant1* (*bv1*) [43–45]. For grain yield estimation, different parameters were measured at harvesting and *lpa1-1* surprisingly showed equal or superior performance to the inbred line B73 (Fig. 3C,D,E,F). To confirm what was measured in the first background, the same parameters were collected in the synthetic population B73/Mo17 and also here *lpa1-1* showed a good seed weight/ear (Fig. 4F). This

was not the first case in which a *low phytic acid* mutant had a similar performance to its respective wild-types: in barley, the majority of *lpa* mutations appeared to have little or no effect on yield [12, 46], although the cultivation in more stressful environments remains problematic [47]. In the same way, the *lpa1* mutants in common bean do not exibit negative agronomic effects, described in mutants affected by the orthologous gene [42], thanks to the presence of the *PvMRP2* paralog gene, which is able to complement the function of *PvMRP1* in organs different from the seed [19]. From the data collected here, it emerged that the biggest problem connected with this mutant remains the reduced field emergence, which limits the interest of breeders. In fact, starting from the seed weight/ear, we can estimate the yield considering 80.000 plants/ha and an average germination of 40% in *lpa1-1* in the field: these low germination rates that characterize the mutant cause a drop in yield compared to the wild phenotype (data not shown). Future work should focus on restoring seed germination, a crucial point in increasing potential yields in the mutant.

As discussed above, the agronomic defects may be caused by an increased amount of free iron cations present in the mutant seeds and the consequent higher level of toxic ROS [41]. A possible approach to defend any plant cell from reactive oxygen species consists in scavenging these toxic radicals by means of antioxidant molecules. Thus, with the aim of improving the agronomic performance of these mutants, classical breeding could be used to introgress the ability to synthesize and accumulate natural antioxidants (such as polyphenols) in the living tissues of the seed.

In the second part of this work, we focused on the increased susceptibility to drought stress observed in *lpa1-1*. This pleiotropic effect was previously studied under controlled and semi-controlled conditions by Colombo *et al*. [24] and it was hypothesized that the drought stress appeared to be caused by a reduced photosynthetic efficiency and not by a shallower root system, as initially hypothesized by Cerino Badone *et al*. [7]. To confirm this hypothesis in non-controlled conditions, in this work different epigeal and hypogeal measurements were collected in the field. In this experiment, the plants of B73 and its relative *lpa1-1* were grown in a non-irrigated part of the field. At flowering, ten roots per genotype were sampled and then cleaned by vigorous rinsing (Fig. 5A). Among the parameters collected, the root area occupied per unit of length was statistically higher in *lpa1-1* than in the wild phenotype (Fig. 5D). Therefore, the root density was higher in the mutant due to a greater development of the lateral roots, which are essential for the uptake of water and nutrients, particularly in stressful conditions. Thus, the mutant root system did not appear to be the main cause of drought stress in *lpa1-1* and for this reason our research shifted to the epigeal part of the plant, where measurements were carried out in two different conditions: under optimal water conditions and under moderate drought stress. In the latter case, the leaves of the mutant appeared stressed and curled upwards compared to the wild phenotype, as shown in Fig. 6A.

The three parameters collected with the CIRAS-2 Portable Photosynthesis System revealed a greater stomatal opening in the mutant than in the wild-type under optimal water conditions: in fact, the transpiration rate and the stomatal conductance were significantly higher in *lpa1-1* compared to the wild-type (Fig. 6C,D). These results were in line with what was reported by Colombo *et al*. [24] in controlled conditions – where *lpa1-1* exhibited a lower leaf temperature and a greater stomatal opening compared to the wild-type – but also in accordance with the results presented here, obtained by analyzing the carbon stable isotope composition ($\delta^{13}C$) of the flag leaves of both B73 and *lpa1-1* grown under optimal water conditions. In fact, the discrimination of the carbon stable isotopes occurring during C4 photosynthesis, which is inversely related to stomatal conductance [39], was higher in B73 (–13.41 ± 0.02 ‰) than in *lpa1-1* (–12.58 ± 0.09 ‰), indicating a differential capacity of the two genotype to regulate the stomatal conductance.

Conversely, when water stress occurred, the net photosynthetic transpiration rates, as well as the stomatal conductance did not significantly change in the wild-type, while they dropped dramatically in the mutant: the net photosynthesis decreased by 58%, the transpiration rate by 63% and the stomatal conductance by 67%, revealing an alteration of stomatal regulation in *lpa1-1* under drought stress. This experiment conducted in open field conditions strongly supports the hypothesis that the increased sensitivity to drought stress in the mutant is mainly caused by an altered stomatal regulation efficiency, not by a less developed root system.

Phytic acid not only plays a central role in phosphate storage but is also involved in several plant processes such as biotic and abiotic stress response, hormonal responses, P homeostasis and signal transduction [48,49]. The interaction of inositol phosphates with auxins seems to explain the shortening of the internodes that occurs in our mutant. Another plant hormone, abscisic acid (ABA), appears to interact with the inositol phosphate pathway and seems to have an important role in the plant's response to drought stress. In fact, a typical effect of ABA is to reduce the leaf water loss by closing the stomata and at the same time defending itself from microbes by limiting their entry through the stomatal pores [50]. In this work, a quantification of ABA was conducted on seedlings grown for 14 days in controlled conditions. This preliminary experiment gave us an unexpected result, since the mutant showed a significantly higher level of ABA compared to the wild-type (9.8 ± 0.49 vs. 2.3 ± 0.11 µg/g) (**Supplementary Fig.1**). In light of these results, we hypothesized that the mutant already starts with high levels of ABA in the seed and consequently is less sensitive to it when the stress occurs. Another hypothesis is that the high amount of ABA recorded in maize *lpa1-1* compared to the wild-type could be accompanied by lower levels of gibberellic acid (GA), a plant hormone with a central role in seed germination. In fact, it is well reported that GA and ABA are in antagonistic regulation and play opposite roles in plant growth and development processes [51].

Regarding other crops, Arabidopsis *mrp5* mutant and common bean *lpa1* mutants, affected in the *AtMRP5* and *PvMRP1* orthologous genes, showed opposite responses to ABA during germination: germination was strongly inhibited in common bean *lpa1*, while it was unaffected in *mrp5* seeds [52, 53]. Despite these differences in ABA-sensitivity, both these mutants were characterized by a better drought tolerance compared to the wild phenotype. Arabidopsis *mrp5* mutant was characterized by closer stomata to prevent water loss, reduced transpiration rate and improved water use efficiency [52]. Nagy and coworkers reported that *AtMRP5* plays a central role in regulating stomatal aperture and in controlling the anion channels of the plasma membrane of guard cells [54].

It is not clear why the mutations in *AtMRP5* and *PvMRP1* confer increased drought tolerance (most likely through different mechanisms), while for the mutations in maize *ZmMRP4* gene the opposite was shown [7].

Further studies both in controlled and field conditions are required to understand the role of ABA in our mutant and the clarification of these aspects may help in defining strategies to develop crop *lpa* mutants.

5. Conclusions

In conclusion, this work highlights the possible advantages of *low phytic acid1-1* mutant in increasing the nutritional quality of foods and feeds and in improving the environmental sustainability of phosphorus in agriculture.

From the data collected in the field, it emerged that the biggest problem of this mutant remains the reduced field emergence, which consequently limits the yield. Furthermore, it was found that the increased sensitivity to drought in the mutant is caused by an altered stomatal regulation, but not by a less developed root system, as previously reported. Several studies are now under way with the aim of reducing the negative pleiotropic effects in *lpa* seeds. Among the possible solutions, traditional breeding could be used to introgress the *lpa1-1* allele into a new genetic background (e.g., a commercial hybrid) with the aim to select plants with a higher field performance and a better stress response. Furthermore, another possible strategy is represented by seed priming, a pre-sowing treatment that allows the seeds to germinate with a higher efficiency, also increasing the tolerance to abiotic and biotic stresses and individual plant performance.

Overall, the study of these possible solutions is now under way, but more work is still necessary before the development of a commercial variety, which always remains the main goal to exploit the nutritional benefits of *low phytic acid* mutants.

Author Contributions

FC and RP designed the research study. FC performed the research. SS, AA, SKS, MB, FT, FFN provided help and advice in the experiments conducted. FC, MB, FT, FFN analyzed the data. FC, FFN, RP wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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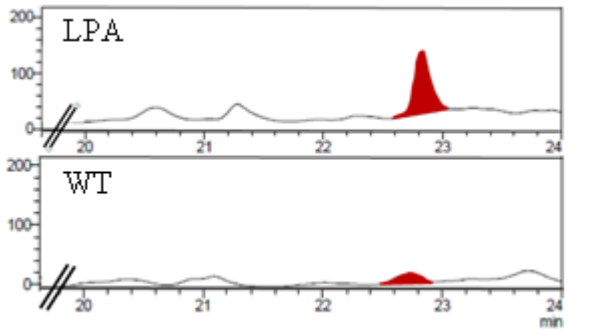
Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material



Supplementary Fig. 1. HPLC-DAD trace for detection of absiscic acid in samples *lpa1-1* and wild-type. The concentration of ABA in the extracts produced with the method described in the text are 5.1 µg mL^{-1} and 1.2 µg mL^{-1} respectively.

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Article

Study of seed ageing in *lpa1-1* maize mutant and two possible approaches to restore seed germination

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Abstract: Phytic acid (PA) is a strong anti-nutritional factor with a key antioxidant role in countering ROS accumulation. Despite the potential benefits of *low phytic acid* mutants, the reduction of PA causes pleiotropic effects, e.g. reduced seed germination and a viability loss related to seed ageing. In this work, using a historical series of naturally aged seeds, we show that *lpa1-1* seeds aged faster compared to wild-type seeds. To mimic natural ageing, we set up an accelerated ageing treatment at different temperatures: incubating the seeds at 57 °C for 24 h, the wild-type germinated at 82.4% and *lpa1-1* at 40%. We also propose two possible solutions to overcome these problems: i) we used classical breeding to constitute synthetic populations carrying the *lpa1-1* mutation with genes encouraging anthocyanin accumulation in the embryo (R-navajo allele). The results showed that the presence of R-navajo gene in *lpa1-1* genotype was not able to improve the germinability (-20%), but this approach could be useful to improve the germinability in non-mutant genotypes (+17%); ii) hydropriming was tested on *lpa1-1* and wild-type seeds subjected to accelerated ageing: it emerged that germination was improved by 20% in *lpa1-1,* suggesting a possible role of seed priming in restoring germination, with promising outcomes toward the improvement relevant maize cultivars.

Keywords: phosphorus; phosphate rock; *low phytic acid* mutants; plant breeding; aging; seed quality; seed priming; hydropriming; environmental sustainability; antioxidant

1. Introduction

Iron (Fe) and zinc (Zn) deficiencies are common in humans, particularly in those countries whose diet is mainly based on one staple crop. The lack of these and other minerals in the diet can severely limit the intellectual and physical capacity of people, adversely affecting their health [1,2]. Different approaches to increase Fe and Zn levels and to improve seed nutritional quality in crops have been proposed, including the lowering of phytic acid (PA) (myo-inositol-1,2,3,4,5,6-hexakisphosphate, InsP6) in seeds. PA represents the main phosphorus (P) storage form in the seed and is one of the major constraints to micronutrient bioavailability [3]: in fact, due to its negative charge at the physiological pH, PA tends to chelate positively charged cations (such as Fe^{2+}, Zn^{2+}, K^{+}, Mg^{2+} and Ca^{2+}) forming phytate mixed salts which accumulate in protein storage vacuoles [4]. During germination, phytate salts are degraded by the activity of the phytase enzyme, releasing free phosphorus and minerals, essential for seedlings growth [5]. These salts are largely excreted by non-ruminant animals (including humans) as the phytase activity in their digestive system is absent or limited: only the 10% of phytate in animal feed is assimilated, while the remainder is excreted, thus contributing to P pollution and water eutrophication [1].

Despite being considered an antinutrient because of these environmental problems and for its chelating effect on minerals, PA plays a key role as an antioxidant compound: by chelating free iron cations, PA is an inhibitor of the iron-driven formation of reactive oxygen species (ROS) and of lipid peroxidation *in vitro*, thus preserving the viability of seeds [6–8]. The prevention from oxidative events is an important antioxidant function of PA within plant seeds and can help to explain why seeds belonging to many plant species are viable for a long time, even if they contain a potentially dangerous mixture of iron, oxygen, and unsaturated fatty acids [7,8].

In recent decades, mutations that significantly reduce the levels of PA in the seed have been isolated in the major crops, such as maize [9–12], barley [13–15], rice [16,17], wheat [18], soybean [19–21] and common bean [22,23]. The development of *low phytic acid* (*lpa*) crops is considered an important goal in plant breeding programs aimed at improving the nutritional quality of food and feed, as well as supporting a sustainable phosphate management in agriculture [24].

In maize, among the three *lpa* mutations isolated so far (*lpa1*, *lpa2* and *lpa3*), *lpa1* showed the lowest PA content in the seed, leading to a proportional increase in free phosphate, without altering the total P content [9,10]. Transposon mutagenesis experiments demonstrated that the *lpa1* gene encodes a multidrug-associated-protein (MRP) named *ZmMRP4* (accession number EF586878) [25]. MRP proteins are transmembrane transporters involved in different functions in plants, such as organic ions transport, xenobiotic detoxification, transpiration control and oxidative stress tolerance [26,27]. The majority of *low phytic acid* mutants carry mutations in genes that code for MRP proteins, resulting in a lack of transfer of PA from the cytosol to the vacuole.

In the case of maize, four *lpa1* mutants have been isolated and characterized: *lpa1-1* [25], *lpa1-241* [28,29], *lpa1-7* [11], and *lpa1-5525* (not completely characterized) [12]. *lpa1-241* and *lpa1-7* mutants are characterized by high reductions of PA (up to 80-90%), and therefore are not viable in the homozygous state; *lpa1-1* is characterized by a moderate reduction of PA (about 66%) and is the most promising *lpa1* mutant [9]. Unfortunately, the reduction of PA in *lpa1* mutations is associated with various adverse pleiotropic effects on the seed and in general on plant performance [30]. Among these pleiotropic effects, reduced seed germination in the field and viability impairment related to seed ageing were previously reported in *lpa* mutants [31], but no experimental data on natural ageing were presented.

Seed ageing is one of the major issues in the storage of seeds. It is defined as deteriorative changes occurring in the seed over time which increase its vulnerability to external challenges and decrease its ability to survive. The loss of seed viability greatly depends on environmental factors, mainly seed moisture, storage temperature and relative humidity (RH) [32,33]. The process of seed ageing is normally accompanied by an accumulation of ROS, high concentrations of H_2O_2, malondialdehyde,

and end-products of lipid peroxidation [34–36]. An accelerated ageing test was initially developed to estimate the longevity of seeds in commercial storage [37]. A few years later, the test was evaluated as an indicator of seed vigour in a wide range of crops [38] and was successfully related to field emergence [39].

Based on these findings, in this work we showed, for the first time to our knowledge, that *lpa1-1* seeds aged faster under room conditions, compared to their related wild-type seeds, leading to a progressive decrease in seed germination and a lower tolerance to adverse storage conditions. We also proposed two possible solutions to overcome the problems related to seed germination: i) first, we used classical breeding to constitute maize synthetic populations carrying the *lpa1-1* mutation together with regulatory genes pushing the anthocyanin accumulation in the embryo. We thought that in this way it might be possible to compensate the loss of antioxidant activity caused by PA reduction in *lpa* mutants; ii) the second approach was based on seed priming technology, a pre-sowing treatment which leads to a physiological state that enables seed to germinate more efficiently. In both cases, the goal is to find a solution to these pleiotropic effects in *lpa1-1* mutant, thus improving seed germination and the consequent seedling growth.

2. Results

2.1. Accelerated ageing mimics natural ageing

In this work, we used seeds of the B73 inbred line and its related *lpa1-1* mutant to study the effect of natural ageing on seed germinability. The experiment was carried out in 2021, so the seeds of 2020 were aged by one year, those of 2019 by two, etc. Seeds of the two genotypes, which had been stored at room temperature for several years, were germinated in controlled conditions on germination trays filled with soil and the germination percentage (%) was noted after 7 days (Figure 1A). It emerged that the mutant was more susceptible to natural seed ageing compared to the wild-type. In fact, if from the first (2020) sampling point the germination of the two genotypes was statistically identical, the trend changed with the seeds of 2013, where the wild-type germinated at 60% while the mutant dropped to 4%. Good germination percentages of the inbred line B73 were recorded also with the seeds of 2009, in which half of the seeds sown were able to germinate (50%) (Figure 1A). Although the germination in controlled conditions remained high in the first years of natural ageing, what changed was the vigour and development of the seedlings in the early stages of growth. To highlight these differences, the heights of 10 randomly selected plants of each genotype were measured at 13 days after sowing (DAS) (Figure 1B): it emerged that the height of the inbreed line B73 remained quite stable despite the natural ageing (15.44 ± 1.76 cm in 2020 vs 13.93 ± 1.52 cm in 2014), but the opposite occurred in *lpa1-1*, which lost much of its post-germinative vigour over the years: the

seedlings of 2020 reached 20.27 cm, a higher value compared to the wild-type. In fact, in the mutant the phosphate and the other micronutrients are not chelated, and therefore are more bioavailable in the first stages after germination compared to B73. However, from only one year more of storage, the mutant plants measured 4 cm less than from 2020, while the same parameter dropped to 11.41 cm for 2017 and 7.93 cm for 2014 storage (Figure 1B).

To mimic natural ageing, an accelerated ageing treatment was carried out on seeds of both genotypes. After some preliminary tests, a 24-hour heat treatment at different temperatures (50, 55, 57, 60 °C) and 100% RH was chosen. The treatment was followed by a germination test in controlled conditions: the graph clearly shows that the inbred line continued to have a high germination rate despite the heat treatment at 55 °C, while the germination dropped significantly in *lpa1-1* (96.9 vs 46.6%, respectively). The same trend was maintained at 57 °C (82.4 vs 40%), while at 60 °C the mutant was no longer able to germinate (Figure 1C). For plant height, this parameter was statistically similar in the inbred line between 20 and 55 °C. In our mutant, the trend was similar to that seen previously in Figure 1B: at room temperature the height reached 18.74 cm, while it dropped to 11.1 cm with a treatment at 57 °C (Figure 1D).

The correlation analysis between years of natural ageing, germination percentage and height of the plants highlighted the significant negative correlations of between years of ageing with both germination percentage and plant height, as well as a significant positive correlation between germination percentage and plant height (Figure 1E). The correlation analysis performed between Celsius degrees applied for the accelerated ageing, germination percentage and height of the plants revealed a significant negative correlation between the temperature used for artificial ageing and plant height, as well as a significant positive correlation between germination percentage and plant height (Figure 1F). Overall, the correlation patterns observed for natural and artificial ageing displayed similar trends.

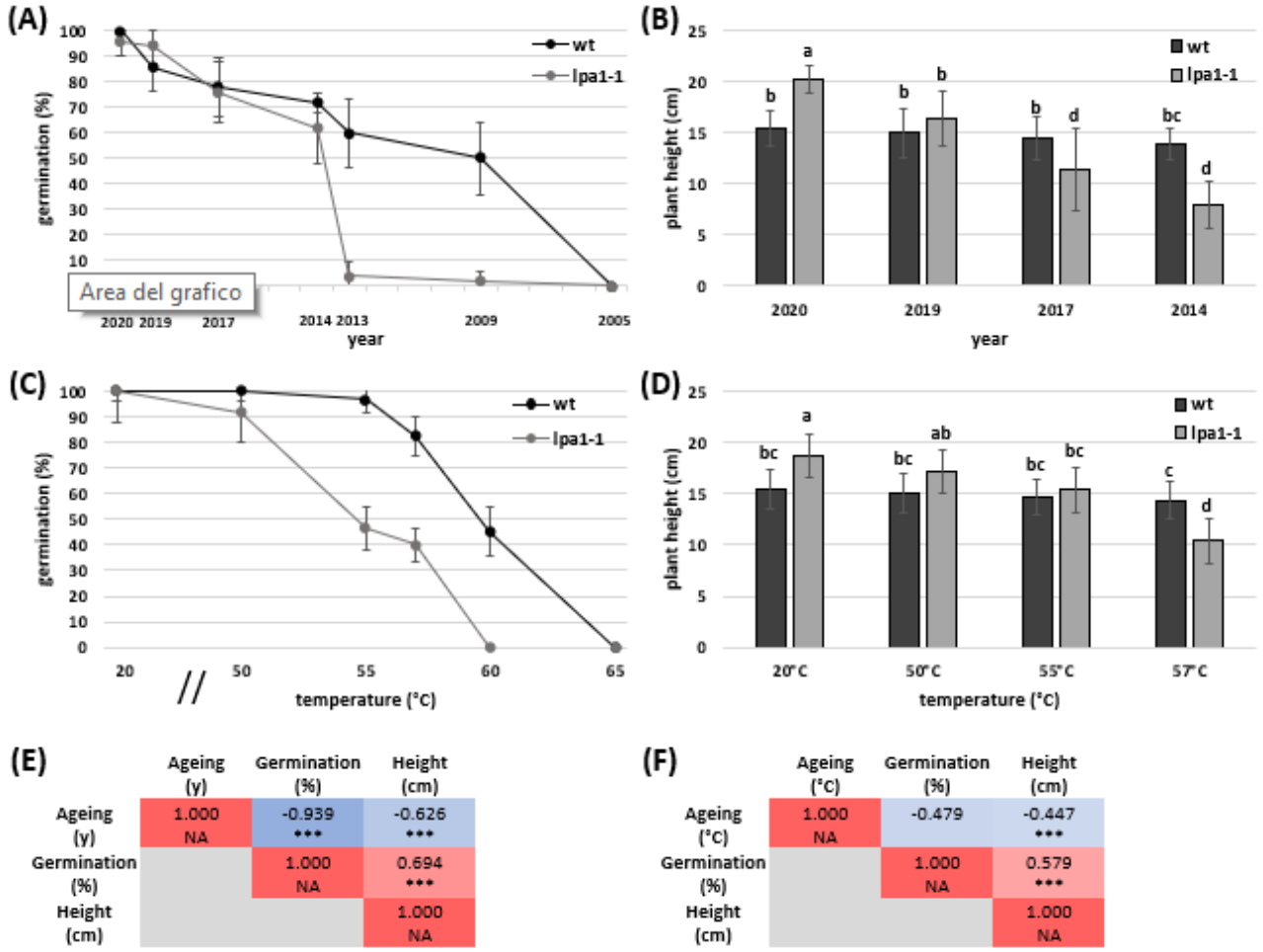

Figure 1. Germination percentage of B73 and *lpa1-1* seeds from different years **(A)** and relative seedlings height measured at 13 DAS **(B)**. Accelerated seed ageing was performed for 24 hours at 50, 55, 57, 60 and 65 °C with 100% RH **(C)** and plant height was measured at 13 DAS **(D)**. Different letters indicate statistically significant differences (Tukey test, $p<0.05$). Heat map of the correlation analysis performed between years of natural ageing, germination percentage and height of the plants **(E)**. Heat map of the correlation analysis performed between Celsius degrees applied for accelerated ageing, germination percentage and height of the plants **(F)**. Pearson's correlation coefficients are reported. Statistically significant correlations are indicated by asterisks (*** $p<0.001$).

2.2. Genetic approach to counteract seed ageing

PA plays an important antioxidant function within plant seeds and the defects that characterize *lpa* mutants may be attributable to an anomalous quantity of free iron cations in mutant seeds and to the consequent high level of ROS. A possible approach to defend plant cells from ROS consists in scavenging these toxic free radicals by means of molecules with antioxidant properties accumulated in the embryo: among them, anthocyanins exhibit a strong effect in counteracting ROS formation. In particular, the R-navajo allele is able to drive the biosynthesis of anthocyanins in the embryo, where PA is stored. In this work, we compared four synthetic populations that differed only for the presence of the *lpa1-1* and R-nj allele in homozygous status. The seeds of each genotype were subjected to accelerated ageing (57 °C, 24 h, 100% RH) and were compared to the relative unaged control. The

plots were arranged randomly on the germination tray, and three independent replicates were set up. In parallel, five unaged seeds for each genotype were weighed (before and after a heat treatment) to determine the seed moisture. These data suggested that seed moisture was statistically identical between the four synthetic populations: colorless and non-mutant seeds had 7.41 ± 0.33% of water, while the relative colored and non-mutant seeds had 7.26 ± 0.19%; colourless seeds carrying *lpa1-1* mutation had 7.47 ± 0.19% of water and coloured mutants had 7.40 ± 0.19%.

Aged and unaged seeds were germinated on germination trays and the germination percentage was noted after 7 days (Figure 2A). As reported in Figure 2A, the accelerated ageing treatment had a different effect on the four genotypes: considering only the *lpa* material (R/R lpa/lpa vs. r/r lpa/lpa), the germinability dropped by 60% in coloured seeds and by 40% in colourless seeds, suggesting that the presence of R-nj allele was not able to improve germinability (-20%). On the other hand, considering the wild genotypes (R/R Lpa/Lpa vs. r/r Lpa/Lpa), the germinability dropped by 8% in coloured seeds and by 25% in colourless seeds: the presence of the R-nj allele in this background improved germinability by 17% (Figure 2A).

The same trend was recorded by measuring plant height and weight after 13 days: after the ageing treatment at 57 °C: in R/R Lpa/Lpa the average height of the seedlings decreased only by 12% and the weight by 10%; on the other hand, in coloured and mutant seeds (R/R lpa/lpa), the same parameters dropped by 38% and 50% respectively (Figure 2B), suggesting a possible interference between *lpa* mutation and anthocyanin accumulation (discussed below).

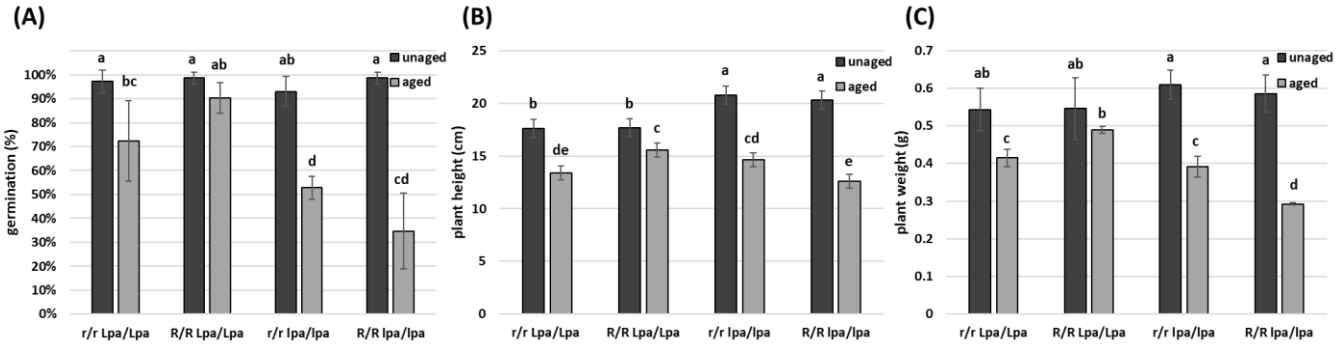

Figure 2. Comparison among the four synthetic populations that differ for the presence of *lpa1-1* and R-nj allele. Samples subjected to accelerated ageing (57 °C, 24 h) were compared to the respective unaged control. Three independent replicates were performed. Germination percentage was noted at 7 days **(A)**; plant height **(B)** and plant weight **(C)** were measured at 13 days. Different letters indicate statistically significant differences (Tukey test, $p<0.05$).

2.3. Hydropriming improves germination in aged seeds

To tackle seed ageing and improve seed germination in *lpa1-1* mutant, the second approach proposed here is based on seed priming technology. Hydropriming duration was established at 12 h based on a

literature review [40]. To assess the effects of 12 h-hydropriming on germination performances in artificially aged wild-type and *lpa1-1* seeds, an experimental system was established, as detailed in Figure 3A. The beneficial effects of hydropriming in comparison with unprimed controls were highlighted using three different germination parameters. In unprimed seeds, as expected from previous results (Figure 3B), a decreased germinability was observed in artificially aged *lpa1-1* seeds compared to wild type seeds (26.67% lower, Figure 3B), whereas hydropriming significantly improved germinability in artificially aged *lpa1-1* seeds (20.00% increase, Figure 3B). Germination speed, expressed as T_{50}, significantly improved in artificially aged wild type and *lpa1-1* seeds in response to hydropriming, compared to their respective unprimed controls, with an average decrease of 11.33 h and 6.02 h in germination time, respectively (Figure 3C). Finally, a significant increase in germination consistency in response to hydropriming, expressed as a higher synchronization index, was observed in artificially aged wild-type seeds, in comparison with their unprimed controls (Figure 3D).

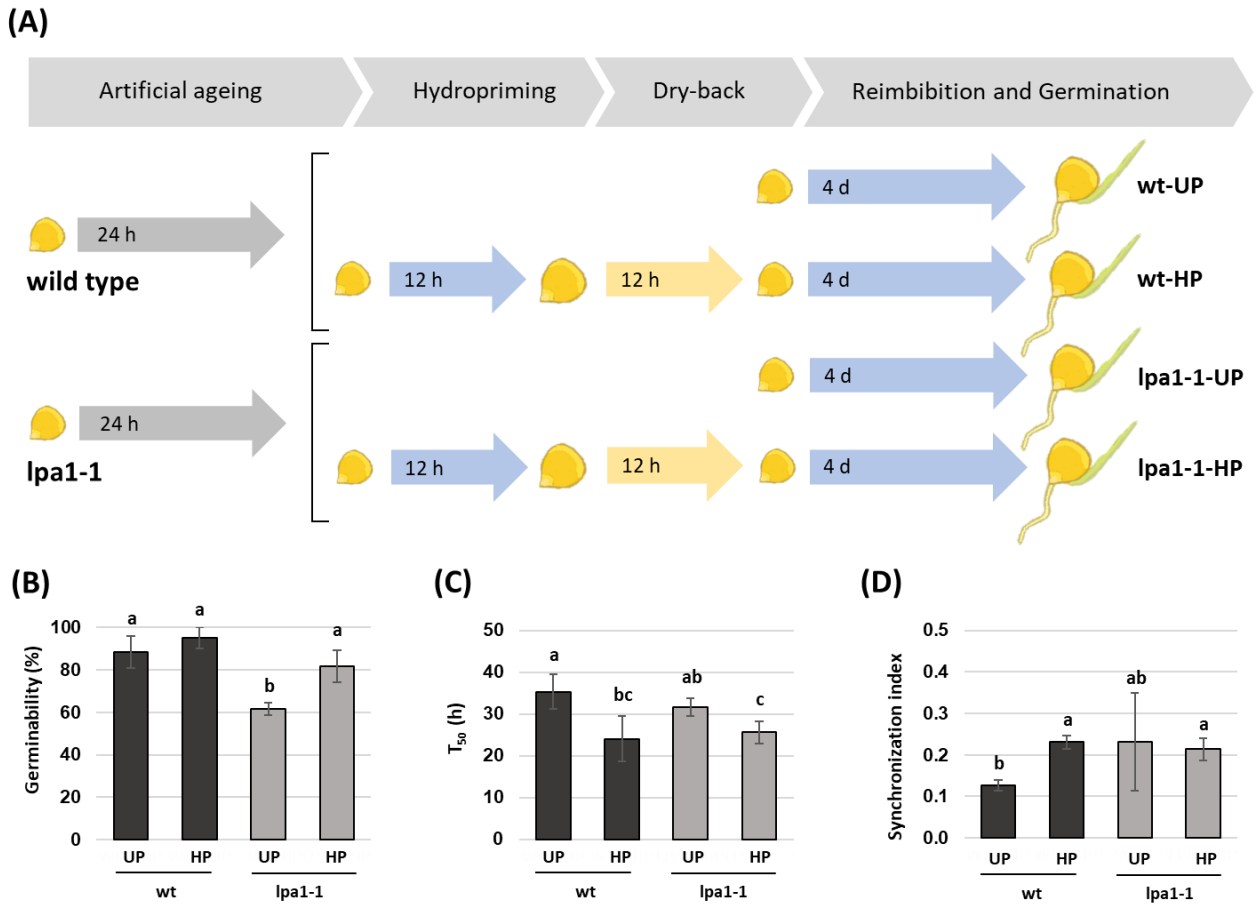

Figure 3. Effects of 12h-hydropriming on wild-type and *lpa1-1 Zea mays* seeds subjected to accelerated ageing. Overview of the experimental system to compare the effects of hydropriming on wild type and *lpa1-1 Zea mays* seeds after artificial ageing **(A)**. Steps including artificial ageing are indicated with gray arrows, steps involving imbibition are indicated with blue arrows, dry-back steps are indicated with yellow arrows. Germinability **(B)**. T_{50} **(C)**. Synchronization index **(D)**. UP, unprimed seeds; HP, hydroprimed seeds. Different

letters indicate statistically significant differences (Tukey test, $p<0.05$) as analyzed with two-way ANOVA and Tukey tests.

3. Discussion

The bioavailability of minerals could be a critical factor in human diet, as their absorption from plant foods is often very low. One of the major constraints to micronutrient bioavailability is phytic acid, a strong anti-nutritional factor that binds positively charged cations forming phytate mixed salts [3]. In this context, *low phytic acid* mutants have been isolated in all major crops and are characterized by a reduced amount of PA, followed by a proportional increase in available phosphate [9,10]. Despite the potential benefits of these mutants, the reduction of PA causes a series of negative pleiotropic effects on the seed and in general on plant performance [30]. Among the different *lpa1* mutants in maize, here we used *lpa1-1* as it is the most promising from an agronomic point of view and we compared it with the inbred line B73.

To our knowledge, in the present work we show for the first time that *lpa1-1* seeds aged faster compared to their relative wild-type, thus causing a progressive decrease in seed germination and a lower tolerance to adverse storage conditions. We built a natural ageing curve using the seeds of the two genotypes which had been stored at room temperature in our department (Figure 1A): it emerged that seed germination decreased over the years, but this parameter dropped to 4% in 2013 only in the *lpa1-1* genotype.

Although germination remained stable in the two genotypes in the first years of ageing, we found that the mutant seedlings lost vigour much faster compared to B73. In fact, *lpa1-1* seedlings of 2020 were taller than the control (20.27 vs 15.44 cm, respectively), while in 2014 the plant height dropped to 7.93 cm in the mutant and remained stable in the wild-type (Figure 1B). The faster development of *lpa1-1* in the early stages of development was previously reported in other studies: due to PA reduction, minerals are more bioavailable in *lpa* mutants, while they remain chelated in the wild phenotype [24,41].

Although in the first years of natural ageing the germination of *lpa1-1* remains comparable to the wild-type under controlled conditions, we know that (unaged) mutant seeds struggle to germinate in open field conditions, thus impairing the yield [42].

After the study of natural ageing, we set up an accelerated ageing treatment that allowed us to study seed ageing without having to wait several years. The accelerated ageing test exposed seeds for a short time to two environmental variables, high temperature and high relative humidity, which cause rapid seed deterioration. High-vigour seed lots will withstand these stressful conditions, deteriorate at a slower rate and have high germination after ageing, compared to low-vigour seed lots [43].

Compared to the normal ageing protocols used to evaluate the characteristics of a seed lot, in this work we chose higher temperatures and for shorter times to maximize the differences in seed germination between the two genotypes. By incubating the seeds at 57 °C for 24 h, the inbred line germinated at 82.4% and *lpa1-1* at 40%, while when the temperature of the treatment was 60 °C, only the mutant was no longer able to germinate (Figure 1C).

Given the relevance of artificial ageing approaches to investigate the effects of natural ageing and long-term storage [44], the correlation analyses displayed in Figure 1E allowed a targeted comparison between the natural and artificial ageing as evaluated in this study. The correlation patterns observed for natural and artificial ageing with germination percentage and plant height (Figure 1E, F) displayed similar trends, with positive correlations between germination percentage and plant height and negative correlation of ageing (quantified in years for natural ageing and in degrees Celsius for accelerated ageing) with germination percentage and plant height.

To tackle the problems related to seed ageing, and in general the reduced seed germination in the mutant, in this work we proposed two possible solutions: first, we used classical breeding to constitute inbred lines carrying the *lpa1-1* mutation together with genes pushing the anthocyanin accumulation in the embryo. In fact, despite being considered an antinutritional factor, PA is a good candidate for protecting the embryo from oxidative processes. For this reason, *low phytic acid* mutants are more exposed to the iron-driven formation of ROS, because of their reduced PA content. In our study, we constituted four synthetic populations that differed only for the presence of the *lpa1-1* and R-nj allele in homozygous status. The R-navajo allele confers the ability to synthesize and accumulate natural antioxidants in the embryo. The seeds of each genotype were subjected to accelerated ageing (a 24 h treatment at 57 °C was chosen) and were compared to the relative unaged control.

We initially hypothesized that the presence of anthocyanins (R/R lpa/lpa and R/R Lpa/Lpa genotypes) was able to maintain high levels of germination after accelerated ageing thanks to the strong antioxidant activity of these pigments [45].

Considering only the *lpa* material (R/R lpa/lpa vs. r/r lpa/lpa), the germinability dropped by 60% in coloured seeds and by 40% in colourless seeds, suggesting that the presence of R-nj allele was not able to improve germinability (-20%) in the mutant line. On the other hand, considering the wild genotypes (R/R Lpa/Lpa vs. r/r Lpa/Lpa), the germinability dropped by 8% in coloured seeds and by 25% in colourless seeds: the presence of R-nj allele improved germinability by 17% in this genetic background (Figure 2A).

In light of these results, it seemed that the presence of R-navajo gene in the *lpa1-1* genotype was not able to improve the germinability, indeed it decreased it.

We can explain these results by considering the biosynthesis and compartmentalization of anthocyanins in maize: anthocyanins are cytoplasmically synthesized and transported in the vacuole by *ZmMRP3* activity; however, *ZmMRP3* does not seem to be the only protein involved in this process [46]. Cerino Badone and coworkers suggested a possible role of *ZmMRP4* in the transport of this pigment: in fact, when anthocyanins are transported into the vacuole, due to the acid pH, they assume the typical red color, while if MRP is not functional, they are not transported and accumulate in the less acid environment of the cytosol, where they retain the bluish colour [47]. This alteration was attributed to a defect in the transport of anthocyanins in the vacuole, causing a mislocalized accumulation of this molecule in the cytosol, suggesting that *ZmMRP4* could have an important role in anthocyanin transport. In this scenario, the low germination of R/R lpa/lpa genotype was caused not only by the damage caused by seed ageing, but also by the phytotoxicity of anthocyanins in the cytosol. Therefore, the use of anthocyanins as natural antioxidants to overcome seed ageing was not functional in improving germination, but had a negative effect in the *lpa1-1* mutant. In future work, the antioxidant properties of other molecules, such as carotenoids or tocopherols, could be investigated.

However, the results obtained showed that the genotype R/R Lpa/Lpa was the one that germinated best after the ageing treatment (Figure 2A). Therefore, the accumulation of anthocyanins did not have a good effect on *lpa1-1* due to the mislocalization of this pigment in the cytosol, but this approach could be useful to improve the germinability in other non-mutant genotypes. In particular, the use of seeds that accumulate natural antioxidants could be a good tool, for instance in underdeveloped countries, where frequently there is a lack of adequate storage facilities and seeds are more exposed to oxidative damage. Further studies on different genetic materials are needed to validate this hypothesis.

The second approach used in this work to overcome the reduced seed germination was based on seed priming technology: a 12h-hydropriming approach was tested on *lpa1-1* and wild-type maize seeds which had been subjected to accelerated ageing. It improved germination performances in both wild-type and *lpa1-1* seeds in terms of germinability percentage and germination speed (Figure 3). In agreement with these results, the positive effects of seed priming in improving seed vigour and alleviate the effects of ageing and storage have long been reported in different crop species and explained in term of enhanced repair and antioxidant responses [48,49]. These results underline the need to explore the roles of PA in seed metabolism along with its implications on seed ageing and the response to priming technology.

4. Materials and Methods

4.1 Genetic material

The *lpa1-1* mutation introgressed in B73 inbred line was kindly provided by Dr. Victor Raboy, USDA ARS, Aberdeen, ID, USA. The inbred line B73 was provided by the germplasm bank at DISAA, Department of Agricultural and Environmental Sciences—Production Landscape, Agroenergy, University of Milan. Seeds of B73 and its relative *lpa1-1* mutant were used to study the effect of natural and accelerated ageing on seed germinability.

4.2 Ageing conditions and germination tests

The seeds used here had been stored at room temperature (20-25°C) in airtight plastic containers for several years in the germplasm bank at DISAA, Department of Agricultural and Environmental Sciences—Production Landscape, Agroenergy, University of Milan.

For each point of the natural ageing curve, 100 seeds of B73 and its relative mutant *lpa1-1* were germinated as described below. To mimic natural ageing, the accelerated ageing involves the exposure of samples of seeds to adverse conditions for a specific period [37]. Accelerated ageing was carried out in a thermostatic oven in which the desired conditions were maintained. For each point of the accelerated ageing curve, 100 seeds of B73 and *lpa1-1* were treated for 24 h at different temperatures (50, 55, 57, 60, 65 °C) in sealed glass boxes with 100% RH. An unaged seed sample stored at room temperature (20 °C) was used as a control.

The accelerated ageing treatment was followed by a germination test in controlled conditions to determine the percentage of surviving seeds. For both natural and accelerated ageing, seeds were germinated in seed germination tray filled with S.Q.10 substrate (peat, sand, compost; Vigorplant) and plants were grown under a long-day photoperiod (16 h light/8 h dark) in a growth chamber with controlled temperature (25 °C night/30 °C day) and with photon fluence of 270 mmol m^{-2} s^{-1}. At 7 DAS (days after sowing) the seed germination percentage was noted and the height of 10 randomly selected seedlings for each sampling point was measured at 13 DAS.

The Pearson's correlation coefficient and the p-values of the correlations were calculated using MetaboAnalyst 5.0 [50] to assess the correlations between ageing (quantified in years for natural ageing and in Celsius degrees for artificial ageing), germination percentage and height of the plants. Statistically significant correlations are indicated by asterisks (*** p<0.001).

4.3 Constitution of the new genetic material and plot experiment

We used classical breeding to constitute four synthetic populations. The genetic materials here were obtained crossing the mutant *lpa1-1/lpa1-1* (in B73) with the line carrying pigmented seeds R-navajo

(R-nj) provided by the germplasm bank at DISAA, University of Milan. R-nj allele can synthesize and accumulate natural antioxidants (anthocyanins) in the embryo, where PA is stored. The F1 obtained was selfed, generating an F2 population scored by Chen's assay and genotyped for all the possible genotypes in homozygosity (as described in the following sections). Following three cycles of sib crossing, we obtained four synthetic populations differing for the presence of the *lpa1-1* and R-nj in homozygosity (Figure 4).

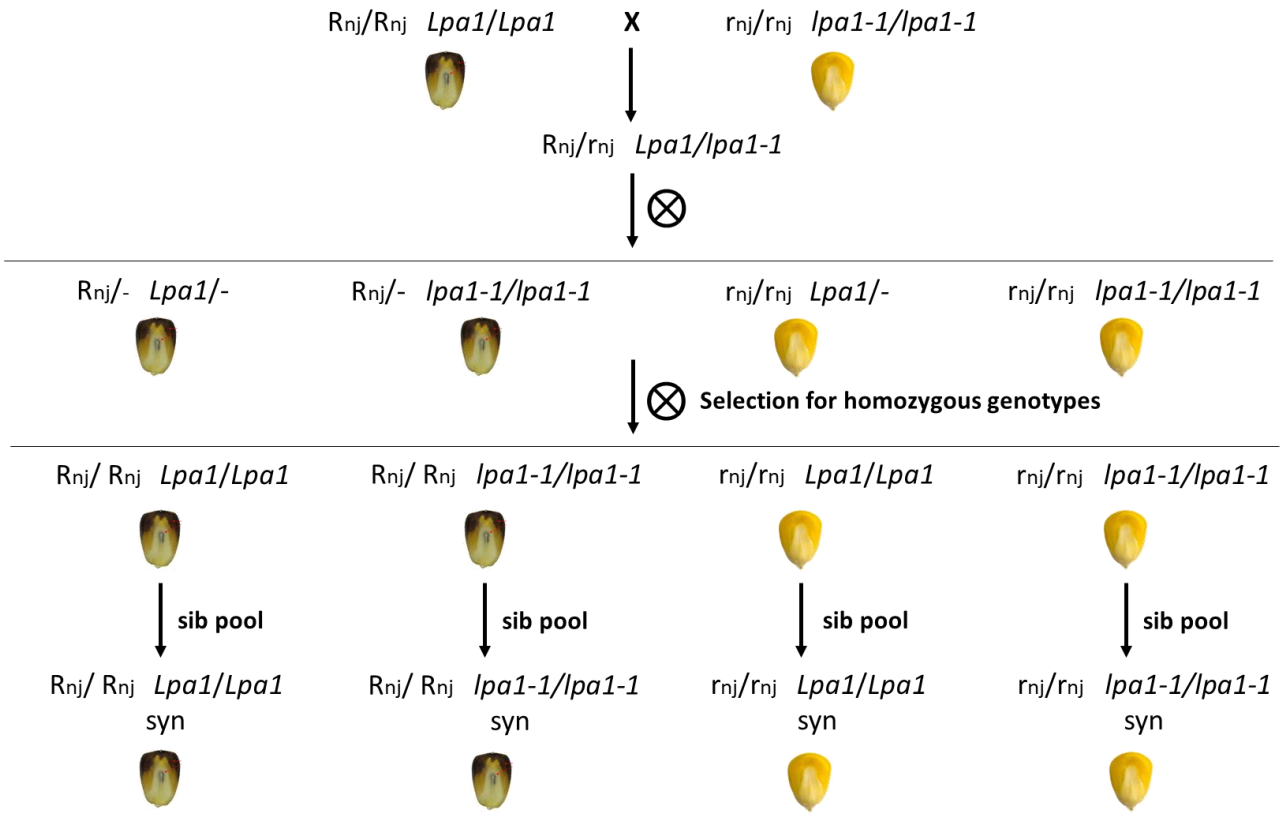

Figure 4. Pedigree scheme used to obtain the four synthetic populations that differ only for the presence of the *lpa1-1* and R-nj allele in homozygosity. The symbol "-" means either dominant or recessive alleles. R-nj is the abbreviation of R-navajo allele.

In every cycle, the presence of the *low phytic acid* trait in the material selected was confirmed through chemical and molecular methods, according to Colombo et al. [42]**.**

Seeds of the four synthetic populations obtained were subjected to accelerated ageing for 24 h and the temperature of 57 °C was chosen for the treatment. For each genotype, three independent replicates with 25 seeds per replicate were germinated together with the respective unaged control (4 aged, 4 unaged x 3 rep). Seed germination was carried out in the growth chamber, as described in the previous paragraph. At 7 DAS the seed germination percentage was noted and at 13 DAS all the seedlings germinated were cut at the base and the height and weight of these seedlings were measured.

To calculate seed moisture, five representative seeds for each genotype were weighed before and after a heat treatment at 80 °C in the thermostatic oven until a constant weight was achieved.

4.4 Hydropriming protocol and germination test

To assess the effects of hydropriming on artificially aged wild-type and *lpa1-1* seeds, accelerated ageing was carried out as previously indicated (57 °C for 24 h). For hydropriming treatments, seeds were transferred in sealed trays (15 x 20 cm) containing a layer of filter paper moistened with 20 ml of distilled water and left soaking for 12 h. After imbibition, hydroprimed seeds were distributed on a layer of absorbing paper and air-dried (dry-back) for 12 h.

For germination tests, artificially aged wild-type and *lpa1-1* seeds subjected to hydropriming (wt-HP and lpa1-1-HP, respectively) and unprimed controls consisting of artificially aged wild-type and *lpa1-1* seeds not subjected to hydropriming (wt-UP and lpa1-1-UP, respectively) were distributed into trays (10 x 15 cm) containing a layer of filter paper moistened with 10 ml of distilled water. Trays were sealed with transparent film to prevent water evaporation. Germination was assessed every 12 h for four days starting from the imbibition of unprimed seeds and the re-imbibition of hydroprimed seeds. Seeds with visible protrusion of the primary root were considered germinated. The following parameters were calculated: G (germinability, final germination percentage), T_{50} (time required to reach 50% of the final germination percentage) and Z (synchronization index) [51]. For each treatment, three independent replicates with 20 seeds per replicate were analyzed. Statistical analyses were carried out by means of two-way ANOVA and Tukey-Kramer tests, using the software developed by Assaad and colleagues [52].

5. Conclusions

In conclusion, seed ageing leads to a reduction in seed quality, storage duration and germinability, causing loss of vigour and making the seeds less viable in the field. This work highlighted for the first time that *lpa1-1* seeds aged faster compared to the wild-type and confirmed the suitability of accelerated ageing treatment as an informative approach to highlight the pleiotropic effects associated with low PA content. Although the introgression R-navajo allele did not give the desired result with the low PA mutant, the use of seeds that accumulate natural antioxidants could be a good tool to improve the germinability in other wild-type material. This approach should consider the introgression of other antioxidant molecules accumulated in the embryo, e.g. carotenoids or tocopherols. The other approach used in this work was seed priming: hydropriming seemed to be a promising solution for restoring germination rates and improving seed viability. Considering the good

results achieved with hydropriming, future work should focus on hormonal seed priming, using molecules such as gibberellic acid (GA_3) to further increase the germination of our mutant. These experiments should be accompanied by appropriate field trials in order to overcome problem of the reduced seed germination of *lpa1-1* in the open field, which remains the major issue for breeders.

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General Conclusion and future perspectives

In this PhD project the main pleiotropic effect that limit the application of *low phytic acid* mutants in maize have been analysed. Among the different *lpa1* maize mutants that have been isolated and characterized in past years, our work was mainly focused on *lpa1-1*, whose moderate reduction of phytic acid (-66%) resulted in non-lethal agronomic defects.

In this PhD thesis, the data were collected under different conditions, spanning from the controlled conditions to the field. The use of such different systems allowed us to have an overview of the potentials but also of the main limits of *lpa1-1*. It emerged that *lpa1-1* seemed to have comparable (or even better) seed weight/ear than the relative control; the main problem of this mutant was the reduced field emergence, which consequently led to lower yields than the wild-type. Overcoming this pleiotropic effect is crucial to increase the interest of breeders towards this mutant, and the promising results obtained in the hydropriming tests opened a way not yet considered to restore seed germination in *lpa1-1*. In addition, the hydropriming treatment accelerated germination and significantly reduced the time to seedling emergence, making the seeds less vulnerable to stress. Considering the positive effect of hydropriming on *lpa1-1* seeds, future works should consider the use of plant hormones to maximize the benefits of seed priming: different molecules can be used, such as gibberellic acid (GA_3), an essential tetracyclic diterpenoids plant hormone, which play an important role in promoting seed germination, stem elongation and seedling growth performance under stressful conditions. These seed priming tests under controlled conditions must be followed by appropriate field tests to evaluate the germination of "primed" seeds compared to the "unprimed" ones. In fact, field tests remain rare and frequently research stops at controlled conditions, even if field trials remains essential.

In the same manuscript it also emerged that, although the introgression of R-navajo allele in *lpa1-1* genotype was not able to compensate the loss of antioxidant activity, this approach paved the way to improve the storage and conservation of non-mutant seeds over the years. This means that seeds that the accumulation of natural antioxidants in seeds could be a useful tool where adequate storage facilities are lacking, and seeds are more exposed to oxidative damage. Further studies on different genetic materials are needed to validate this hypothesis.

Beside these strategies, classical breeding programs continue to be important and are focusing on the selection of genotypes with superior agronomic performance. In this context, the introgression of *lpa1-1* into a new genetic background (e.g. commercial hybrid) is in progress and could improve not only seed germination, but the overall performance of the plant. Breeding programs take several years to develop a new variety, while genome editing approaches are faster and enable the manipulation of

more than one gene involved in PA biosynthesis pathway through multiplex editing. Moreover, scope exists to deploy such tools in developing varieties with optimal PA levels; in fact, the level of PA in the seed determines the severity of the mutation and chemical mutagenesis or genome editing could be useful tools to search for new and less "severe" *lpa1* mutations: finding the right balance in PA reduction could be a solution to mitigate the negative pleiotropic effects.

In conclusion, further works will be necessary to overcome these pleiotropic effects and the release of an improved *low phytic acid* variety could lead to numerous nutritional benefits, as well as a more efficient use of phosphates in agriculture, a fundamental step towards a more sustainable agriculture.