# The roles of metabolic pathways in maintaining primary dormancy of Pinus koraiensis seeds

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# Abstract

Background Korean pine seeds have primary dormancy following dispersal, leading to poor seed germination and seedling establishment. Metabolic homeostasis determines whether the seeds are dormant or non-dormant. However, the specific metabolic pathways that maintain the primary dormancy of pine seeds are poorly understood. Results Metabolic analysis was employed on the embryos of PDRS (primary dormancy released seeds) and PDS (primary dormant seeds) on days 0, 5 and 11 after incubation under a germination-inductive temperature. A larger metabolic switch occurred in PDRS embryos from days 0 to 11. Sixty-six metabolites were significantly changed from days 0 to 5, 83% of which (including most sugars, organic acids and amino acids) increased, which appear to reflect biosynthetic metabolism processes are initiated. Seventy-six metabolites showed distinct variations from days 5 to 11, 74% of which (including most organic acids and almost all amino acids) reduced substantially. Most pronounced one was a major 409-, 75-, 58- and 41-fold reduce in the respective levels of fructose 6-phosphate, inositol-3-phosphate, 3-phosphoglyceric and D-glucose-6-phosphate, which appear to reflect the glycolysis and tricarboxylic acid (TCA) cycle are strongly slowed down. The majority of the metabolites in PDS embryos displayed a relatively larger alteration only during from days 0 to 5. Although 69% of metabolites increased from days 0 to 5, their levels were still lower compared with PDRS embryos. Furthermore, most metabolites were not further accumulated from days 5 to 11. Unlike PDRS embryos, almost all amino acids in PDS embryos did not exhibit a substantial decrease from days 5 to 11. Also, there was not a major decrease in the levels of metabolites involved mainly in glycolysis and TCA cycle, while some intermediates even increased. Conclusions The attenuated biosynthetic metabolism processes, the lower utilization rate of amino acids and the higher operation rate of glycolysis and TCA in embryos maintain primary dormancy.


# Background

The mixed broadleaf Korean pine (*Pinus koraiensis* Sieb. et Zucc.) forests (MBKPFs), which were once dominant from Northeast China to the Far East region of Russia, have been decreasing in the past century under the influence of large-scale industrial deforestation[1, 2]. MBKPFs possess high plant diversity and play a vital, but poorly understood, role in ecosystem services (e.g., water conservation) due to a highly complicated structure[3]. Thus, it is becoming urgent to restore the degraded forests to the MBKPFs for forest management. To assist in this restoration, it is critical to promote the successful seed germination of Korean pine, which is the dominant tree species in MBKPFs. Nonetheless, Korean pine seeds have primary dormancy following dispersal and enter secondary dormancy in the first summer after seed dispersal under natural conditions, thus leading to poor seed germination and seedling establishment[4-6]. To provide effective methods for releasing seed dormancy, to promote seed germination or regeneration, and finally to recover the MBKPFs, it is essential to determine the primary dormancy mechanisms of Korean pine seeds.

Seed dormancy is defined as when an intact viable seed cannot complete germination when it is put under favorable conditions for germination[7]. As seed dormancy is gradually released, the range of conditions over which seeds complete germination progressively widens[8]. If the range of environments over which seeds complete germination cannot be increased, then these seeds are nondormant[8, 9]. The germination process includes three phases: phase I is the process of rapid water absorption by the seed (imbibition), phase II is the process of reactivation of metabolism (lag), and phase III is the stage during which some part of the embryo protrudes from the seed coat[10]. By definition, germination sensu stricto start with the uptake of water by the quiescent dry seed and terminate with the protrusion of the radicle and the elongation of the embryonic axis[10].

Metabolism is initiated to produce energy and building blocks for the various cellular processes that drive the germination of seeds[10-12]. The differential proteomic analysis between dormant and nondormant seeds suggests that energy metabolism and protein metabolism play potential roles in promoting the completion of seed germination. For example, there are 25 proteins that exhibited a differential accumulation pattern between dormant and nondormant *Arabidopsis* seeds after 1 day of imbibition[13]. Furthermore, one out of 8 proteins whose abundances are high in nondormant seeds is related to energy metabolism. The abundances of 57 proteins decline after nondormant *Arabidopsis* seeds are treated with ABA, and the majority (90%) of down-regulated proteins is involved mainly in energy and protein metabolism[13].

A block in metabolic pathways may invoke seed dormancy[14-16]. Metabolomics can detect the differential accumulation of various metabolites at the global level[17, 18]. Information is accumulating on the importance of metabolic pathways as a mechanism of seed dormancy of many herbaceous plants. For example, sucrose metabolism[18, 19], energy metabolism[20], lipid metabolism[13] and amino acid metabolism[18, 21] are repressed in the imbibed dormant seeds.

It has been shown that the following alterations in metabolic pathways were suggested to maintain seed dormancy of pine and other trees through the comparative analysis between imbibed dormant seeds and seeds that experienced moist chilling. These altered metabolic pathways included a lower potential to synthesize protein[22-24] and to produce adenosine triphosphate (ATP) [25] for the dormant seeds during germination. However, the results of some studies revealed that the generation of sugar in the imbibed dormant seeds of *Picea glauca*[14] and the production of amino acids in the imbibed dormant seeds of *Julans regia*[26] are not inhibited relative to seeds that undergone moist chilling. In the studies mentioned above, the metabolic changes occurring in nondormant seeds during the period of germination *sensu stricto* were not determined. Therefore, the actual metabolism that leads to the maintenance of seed dormancy might have been obscured because the comparison between dormant and nondormant seeds was not completely conducted under the same conditions.

We are aware of only two studies on the metabolic mechanism of the seed dormancy of Korean pine. It has been reported that the dormancy of Korean pine seeds is related to the lower levels of reducing

sugars[27]. However, it is not clear whether the potential of primary dormant Korean pine seeds to synthesis reducing sugar is still lower during maintenance of primary dormancy. Moreover, the change patterns of other metabolites are still unclear. By comparing the respiration rates of dormant and nondormant seeds during the period of germination *sensu stricto*, Lower respiratory metabolism may maintain the seed dormancy of Korean pine[28]. However, it is still not clear which metabolic pathway is changed to maintain seed dormancy. *Pinus koraiensis* seed has thick and hard seed coat, and the total thickness of the seed coat of *Pinus koraiensis* varies between 1.20 and 1.30 cm[29]. The thickness of the dense stone cell layer of the seed coat is between 0.40 and 0.43 cm, and the apparently thickened stone cell wall is visible[29]. Many seeds have thick seed coats that can impose dormancy by limiting the available of oxygen to the embryo, inhibiting the water uptake, preventing the exit of inhibitors from the embryo, or by applying mechanical restraint[30]. The previous works have showed that the application of inhibitors of respiration such as cyanide and malonate (inhibit terminal oxidation reactions and tricarboxylic acid (TCA) cycle) and fluoride (inhibits glycolysis) can release the seed dormancy of several species, including lettuce, rice, barley and sunflower[30, 31]. When dormant sunflower seeds were imbibed at non-permissive temperatures for germination, TCA cycle and glycolysis were more active[20]. It can be inferred that seed coat interferes gas exchange, and thus imposes the hypoxia condition inside the seed. Concomitantly, the dormant seed consumes a large amount of oxygen through the glycolysis and TCA cycle. Therefore, other aerobic processes cannot be performed normally, resulting in the maintenance of seed dormancy. This may be a reason for the maintenance of dormancy of hard seed. However, to the best of our knowledge, there have been no studies were conducted to document the relationship between the metabolic pathways, especially energy metabolisms, under hypoxia conditions and the maintenance of primary dormancy of pine seeds. Therefore, we test the hypothesis whether the specific alterations in some metabolic pathways maintain the primary dormancy of Korean pine seeds or not. The objective of our work is to fully understand the dormancy mechanism in Korean pine seeds by providing a wide overview of alterations in primary metabolic processes that occur in dormant and nondormant seeds during the period of germination *sensu stricto*. The inferences from this study will advance the understanding of the seed dormancy mechanism of woody plants that will help establish a simple and very efficient means to release seed dormancy.

## Results

# Changes in the respiration rates of intact primary dormant seeds and the seeds with their seed coats were cracked

After seed coats were cracked, the respiratory rate of primary dormant seeds significantly increased to 0.012 μmol $CO_2$ $g^{-1}$$minute^{-1}$ after 4 days of incubation (Fig. 1a). Furthermore, from the sixth day of

incubation, the respiration rate continually increased and reached a maximum (0.022 μmol $CO_2$ $g^{-1}minute^{-1}$) on the fourteenth day of incubation when the germination is completed.

The respiratory rate of intact primary dormant seeds significantly increased to 0.012 μmol $CO_2$ $g^{-1}minute^{-1}$ after 3 days of incubation and then gradually reduced to 0.006 μmol $CO_2$ $g^{-1}minute^{-1}$ after 10 days of incubation (Fig. 1b). During the rest time of incubation period, the respiratory rate varied between 0.004 and 0.012 μmol $CO_2$ $g^{-1}minute^{-1}$.

Figure 1 is here

The masses of intact primary dormant seeds rapidly increased 23% after 5 days of incubation and then slowly increased 28% during the rest time of incubation period (Fig. 2).

Figure 2 is here

# Germination of PDRS and PDS

After fresh dry seeds were moist cold stratified for 6 months, their germination percentage reached 93% (Fig. 3a). After fresh dry seeds were stored at -20°C for 6 months, the seeds attained a germination percentage of 5% (Fig. 3b).

Figure 3 is here

# Principal component analysis of metabolites in the embryos

Principal component 1 (PC1) explained 40.7% of the total variance, whereas principal component 2 (PC2) explained 31.6% of the total variance (Fig. 4). Six clusters of samples (PDRS, PDRS5, PDRS11, PDS, PDS5 and PDS11) were observed in PCA. Compared with PDS, the progression of PDRS from days 0 to 11 was accompanied by more changes in metabolites. There was a large difference in metabolites in PDS embryos from days 0 to 5. However, from days 5 to 11, the metabolic changes in PDS embryos were minor, i.e., “PDS5” clusters and “PDS11” clusters were situated relatively close to one another.

Figure 4 is here

# Clustering analysis of metabolites in the embryos

The contents of the corresponding metabolites from groups D, E, F and G were higher in PDRS embryos compared to PDS embryos (depicted on the heat map, Fig. 5). Most metabolites in groups A, B and C

exhibited higher contents in PDS embryos compared with PDRS embryos, which contained most polyols and free fatty acids (Fig. 5).

Figure 5 is here

# Fold changes of important metabolites with a VIP value > 1 in PDRS embryos

Sixty-six metabolites with VIP > 1 significantly contributed to the separation of PDRS5 from PDRS (Fig. 6). 83% of the metabolites with VIP >1 (including the majority of sugars, organic acids and amino acids) accumulated from days 0 to 5. Seventy-six metabolites with VIP > 1 significantly contributed to the separation of PDRS11 from PDRS5 (Fig. 6). 74% of the metabolites with VIP >1 (including the majority of organic acids and amino acids) reduced substantially from days 5 to 11. Most pronounced one was a major 409-, 75-, 58- and 41-fold reduce in the respective levels of fructose 6-phosphate, inositol-3-phosphate, 3-phosphoglyceric and D-glucose-6-phosphate (Fig. 6). There was a 2-6 fold increase in the levels of xylose, glucose, glucopyranose, fructose and trehalose from days 0 to 5 and a further 1-3 fold continual increase from days 5 to 11(Fig. 6).

Figure 6 is here

# Fold changes of important metabolites with a VIP value > 1 in PDS embryos

Sixty-five metabolites with VIP > 1 significantly contributed to the separation of PDS5 from PDS (Fig. 7). 69% of the metabolites with VIP >1 (including the majority of sugars, organic acids and amino acids) accumulated from days 0 to 5. The most noticeable one was a 104-, 55-, 30-, 21- and 16-fold increase in the respective levels of D-glucose-6-phosphate, fructose 6-phosphate, 3-phosphoglyceric acid, oxoglutaric acid and fumaric acid (Fig. 7). Fifty-three metabolites with VIP > 1 significantly contributed to the separation of PDS11 from PDS5 (Fig. 7). The levels of these metabolites displayed relatively small changes from days 5 to 11.

Figure 7 is here

# The main altered metabolic pathways in PDRS embryos

Those pathways located on the top right corner of the ‘metabolome view’ are the main altered metabolic pathways (Fig. 8). Amino acid metabolisms (glycine, serine and threonine metabolism, alanine, aspartate

and glutamate metabolism, arginine and proline metabolism and tyrosine metabolism), carbohydrate metabolism (glyoxylate and dicarboxylate metabolism, Pyruvate metabolism, TCA cycle and glycolysis) and the metabolism of cofactors and vitamins (pantothenate and CoA biosynthesis) and glycerophospholipid metabolism were the main altered metabolic pathways in PDRS embryos from days 0 to 5 (Fig. 8a).

From days 5 to 11, the following metabolic pathways were mainly altered. These metabolic pathways included amino acid metabolism (alanine, aspartate and glutamate metabolism, beta-alanine metabolism, glycine, serine and threonine metabolism and arginine and proline metabolism), carbohydrate metabolism (citrate cycle, pyruvate metabolism, glycolysis and pentose phosphate pathway), lipid metabolism (sphingolipid metabolism) and the metabolism of cofactors and vitamins (pantothenate and CoA biosynthesis) (Fig. 8b).

Figure 8 is here

# The main altered metabolic pathways in PDS embryos

With the exception of glycerophospholipid metabolism, those metabolic pathways undergone significant changes in PDRS embryos also displayed significant alternation in PDS embryos from days 0 to 5 (Fig. 8c). In addition, pentose phosphate pathway also showed major alternation from days 0 to 5. The pronounced altered metabolic pathways from days 0 to 5 also significantly changed from days 5 to 11, except glycine, serine and threonine metabolism and pyruvate metabolism (Fig. 8d).

# Comparative metabolic pathway analysis between PDRS and PDS

From days 0 to 5, there was an increased activity of carbohydrate metabolism and amino acid metabolism in PDRS embryos and a similar, although less intense, increase in PDS embryos (Fig. 9). From days 0 to 11, most intermediates of carbohydrate metabolism and amino acid metabolism in PDRS embryos decreased substantially. In contrast, the changes in these metabolites (including sugars, organic acids and amino acids) in PDS embryos were minor.

Figure 9 is here

## Discussion

77% and 64% of metabolites substantially increased in PDRS and PDS embryos from days 0 to 5, respectively. It is therefore concluded that biosynthetic metabolic processes are more predominant in

both PDRS and PDS embryos after 5 days of incubation. Carbohydrate metabolism (i.e., glyoxylate and dicarboxylate metabolism, TCA cycle, pyruvate metabolism and glycolysis) and amino acids metabolisms (i.e., glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism, arginine and proline metabolism and tyrosine metabolism) were the main stimulated metabolic pathways in both PDRS and PDS embryos after 5 days of incubation.Other studies also showed that both dormant and nondormant seeds have upregulated genes after 24 h of water imbibition[32]. Respiration and energy production could proceed in both dormant and nondormant seeds[33]. The activation of metabolic pathways depends on water entry. The increase in the levels of most metabolites in PDS embryos after 5 days of incubation is most likely associated with rapid initial uptake of water by dry seeds. It has also been documented that dramatic water uptake occurred during from days 0 to 5. The levels of carbohydrate metabolic pathways intermediates were significant lower in both PDS5 and PDS11 embryos compared with PDRS5 embryos, implying that the degree of increasing activities of carbohydrate metabolism is much lesser in PDS embryos. The attenuated carbohydrate metabolism could be associated with the prevention of storage reserve degradation. It has also been reported that the expression of genes involved in carbohydrate degradation gradually declines following transfer of dormant *Euphorbia esula* seeds to germination conditions for 21 days[34].

*De novo* protein synthesis is required for germination. However, the contents of free amino acids are too low to support protein synthesis during germination[30], therefore, storage protein is degraded into amino acids. The proteins in embryo are first degraded and then their components are used locally[30]. Although the activities of amino acids metabolisms are increased in both PDS and PDRS embryos from days 0 to 5, the increasing degree is less in PDS embryos. Therefore, it is plausible that amino acids biosynthesis pathways are highly repressed in PDS embryos compared with PDRS embryos. The expression levels of genes encoding protein degradation, and the abundances of important components of amino acid and protein metabolisms progressively declined with increasing imbibition time of dormant seeds[21, 34]. In contrast, there is an interconversion between amino acids once seed dormancy is released. D*e novo* synthesis pathways of amino acids can also be induced.

Korean pine seeds contain greater than 60% fat, and the majority of fat is located in megagametophyte[35]. Oxygen is required for β-oxidation of fatty acids to produce acetyl-CoA. Acetyl-CoA is then converted to sucrose through the glyoxylate cycle and gluconeogenesis[36]. Lactate level was lower in PDRS embryos than that in PDS embryos from days 0 to 11, indicating that the amounts of oxygen are relative sufficient in PDRS embryos compared with PDS embryos. However, after cracking seed coats, the respiration rats of moist chilled seeds were higher compared with intact moist chilled seeds (unpublished data). It can be inferred that oxygen in seed is still insufficient even primary dormancy is already released. Oxygen consumption may be reduced in the embryos of PDRS by diminishing glycolysis and TCA cycle. Therefore, more oxygen may be supplied to megagametophyte for β-oxidation of fatty acids, leading to the accumulation of sucrose. Sugars were then transported throughout the embryo, where it supports growth and development of embryo. The continual accumulation of sugars (xylose, glucose, glucopyranose, trehalose, fructose and sucrose) in the embryos

of PDRS from days 0 to 11 may also support this hypothesis. In *Pinus lamberdaea* seeds released from primary dormancy, sucrose levels also constantly accumulated during germination[37]. The accumulation of osmotically active substances (sugars and amino acids) results in a further increase in water uptake[38]. The substantial increase in the levels of most sugars may cause seed to absorb water more rapidly, leading to testa rupture. However, this conclusion needs to be further demonstrated by other experiments.

After cracking seed coat, the respiration rates of PDS increased rapidly, indicating that seed coat restricts germination by limiting the uptake of oxygen. The maintenance of higher lactate levels in PDS embryo compared with PDRS embryos further documented that the availability of oxygen is limited in PDS embryos. In addition, the levels of carbohydrate metabolism (i.e., TCA cycle, glycolysis and pentose phosphate pathway) intermediates were significant higher in PDS11 than that in PDRS11. These data indicate that when oxygen is not sufficient, relatively high rates of glycolysis and TCA cycle still occur, consuming large amounts of oxygen in PDS embryo. Thus, the availability of oxygen might be reduced in PDS megagametophytes; leading to retarded β-oxidation of fatty acids. It can also be found that only the levels of xylose, glucose and lactose in PDS embryos increased slightly from days 0 to 11. Furthermore, the levels of 14 out of 16 sugars in PDS embryos were significantly lower compared with PDRS embryos. After transfer of primary dormant *Pinus lamberdaea* seeds to germination conditions, sucrose levels initially increased and then subsequently decreased[37]. The sugar contents in PDS embryos may be too low to cause seed to absorb water and the seed coat to rupture. Therefore, seed primary dormancy was maintained. PDS cannot make corresponding and rapid adjustments from days 5 to 11 (i.e., reducing glycolysis and TCA cycle) to respond to the surrounding hypoxic environment, whereas PDRS can. However, further studies are needed to elucidate the relationship between the distribution of oxygen and sugar in embryo and megagametophyte and the maintenance of primary dormancy in Korean pine seeds.

The levels of those amino acids that participated in alanine, aspartate and glutamate metabolism, beta-alanine metabolism, glycine, serine and threonine metabolism and arginine and proline metabolism exhibited a substantial decrease in PDRS embryos from days 5 to 11. *De novo* protein synthesis occurred during early phase II of *Arabidopsis* seed germination[39]. While, several amino acids exhibited relatively smaller decrease in PDS embryos from days 5 to 11, suggesting that *de novo* synthesis of protein was blocked. The expression levels of genes related to protein synthesis declined in imbibed dormant seeds[40]. Dormant seeds are enriched for genes related to repressed translation capacity[39-41]. Glutamate, aspartate, asparagine and proline were the major amino acids in both PDS and PDRS embryos. These amino acids are also dominant in *Pinus taeda* and *Pinus banksiana* seeds[42, 43]. Glutamine and asparagine have high nitrogen/carbon ratio and are particularly suitable for the storage of N[44], and frequently used as transport compounds transporting nitrogen to the rapidly growing tissues[43, 45]. A 24 fold decrease of asparagine occurred in PDRS embryos from days 5 to 11, but there was a 2 fold increase of asparagine in PDS embryos, implicating that the utilization of amino acids is likely less intense in PDS embryos. Polyamines (putrescine, spermidine and spermine) can activate the occurrence of protein synthesis[24]. Putrescine accumulated rapidly in PDRS embryos but declined gradually in PDS

embryos from days 5 to 11, implicating that the capacity for protein synthesis is lower in PDS embryos. This change in amino acid metabolism in PDRS embryos from days 0 to 11 follows similar patterns as reported previously[18].

## Methods

Two batches of seeds were used in the present study. Seeds collected in October 2013 were used for the determination of gas permeability and water permeability of seed coat. The presence or absence of a gas barrier is identified by determining the difference in respiration rate between the intact primary dormant seeds and the seeds with their seed coats were cracked. By monitoring the changes in the masses of intact primary dormant seeds under germination conditions, we determined whether the seed coat interferences with water uptake. Seeds collected in 2014 were used for metabolomics analysis.

# Seed collection

Fresh Korean pine cones were collected from 30 trees aged 50 years old in a Korean pine plantation in October 2013 and October 2014, respectively, at the Qingyuan Forest CERN, Chinese Academy of Sciences (CAS), Northeast China (41º51.102' N, 124º54.543' E, 456-1116 m a.s.l.). The gymnosperm cones were opened to release the seeds with the help of threshing machine. Then, these fresh seeds are dried indoors until the moisture content of the seeds is around 10%.

# Determination of water permeability and gas permeability of seed coat

Those seeds that collected in October 2013 were immediately used for the determination of gas permeability and water permeability of seed coat. We determined the respiration rates of intact primary dormant seeds and the seeds with their seed coats were cracked. Every 25 seeds (intact primary dormant seeds or the seeds with their seed coats were cracked) were placed in a 9-cm diameter Petri dish. There were eight layers of filter paper in each Petri dish. These filter papers were previously moistened with 12 ml deionized water. Each Petri dish was placed in a 1.3L air-tight plastic box. At the same time, a beaker containing 10 ml NaOH (1 mol $L^{-1}$) solution was also put in plastic box to trap $CO_2$. The plastic box was then incubated in light/dark (14/10 h) at alternating temperature regimes of 25/10°C in a controlled growth chamber (MGC-450HP-2, Bluepard, Shanghai, China). This incubation temperature was selected according to the NFSSTC standards for the *Pinus*[46]. Photosynthetic photon flux density was about 120 μmol $m^{-2}$ $s^{-1}$ located at the top of Petri dishes. $BaCl_2$ (1 mol $L^{-1}$) was used to precipitate $CO_2$ absorbed by NaOH in the beaker. Then the remaining NaOH in the beaker was titrated with 1 mol $L^{-1}$ HCL. The volume of HCL that consumed during the titration is used to calculate the amount of $CO_2$ produced by seed respiration. Deionized water was added at any time during incubation to ensure suitable water

conditions for seed respiration. The respiration rate was measured every day and expressed as μmol $CO_2$ $g^{-1}$ $minute^{-1}$. Three replicates were carried out separately for seed respiration.

Every 25 intact primary dormant dry seeds were placed in a 9-cm diameter Petri dish. Eight layers of filter paper moistened with 12 ml deionized water were placed in each Petri dish. Three Petri dishes were used as three replicates. The Petri dish was covered with plastic wrap to reduce moisture loss and then incubated under germination conditions. Every 24 h, seeds were removed from the filter paper, blotted dry, weighed to the nearest 0.1mg and returned to the Petri dishes. The percentage of increase in seed mass was calculated using the following formula. The percentage of increase in seed mass = [(the masses of seeds incubated under germination conditions – the masses of primary dormant dry seeds)/ the masses of primary dormant dry seeds] × 100.

# Preparation of seeds that were used for metabolomics analysis

The seedlot collected in October 2014 was divided into two portions, one of which was immediately stored at -20°C until the experiment examining the metabolism of primary dormancy. The other portion of the seedlot was moist chilled in early November. The specific procedure of moist chilling was as follows: seeds were first given a 7-day running water soak and then buried in soil at 50 cm depth for 6 months (November 2014 - April 2015) in the Korean pine plantation[28].

After moist chilling, the germination capacity of moist chilled seeds was assessed to ascertain whether they had been released from primary dormancy. The primary dormancy released seeds in the present study refer to those seeds after approximately 6 months of moist chilling. In late April 2015, fresh dry seeds were removed from -20°C storage conditions. The germination experiment was subsequently conducted with these seeds under germination conditions to determine whether the seeds were primary dormant status. The primary dormant seeds in the present study refer to those dry seeds after approximately of 6 months of storage at -20°C. Primary dormancy released seeds and primary dormant seeds were then used for metabolomics analysis.

Each germination test consisted of three replications of 20 seeds each. These 20 seeds were placed in a 9-cm diameter Petri dish with eight pieces of filter paper (Xinxing, Hangzhou, China) moistened with deionized water. Deionized water was added to the Petri dish to ensure appropriate moisture required for seed germination. All dishes were wrapped with plastic film (Miaojie, Shenyang, China) to reduce water loss and then incubated in light/dark (14/10 h) at alternating temperature regimes of 25/10°C in a controlled growth chamber (MGC-450HP-2, Bluepard, Shanghai, China). Photosynthetic photon flux density was about 120 μmol $m^{-2}$ $s^{-1}$ located at the top of Petri dishes. Germination was considered completed and was then recorded when radicle protrusion was greater than 2 mm[47] and was assessed

at two days intervals for 6 weeks. At the end of the germination test, the seeds failing to complete germination were cut to test the seed embryo viability with a tetrazolium method.

# Experimental design of metabolomics analysis

The results on moist chilled seeds germination revealed that the radicle protrusion of the Korean pine seeds started at 11 days of imbibition. Primary dormancy released seeds were incubated under a germination inductive temperature for 11 days. The following seed samples were selected for metabolic analysis: primary dormancy released seeds, 5-day incubated primary dormancy released seeds corresponding to germination *sensu stricto* (i.e. none of the seeds showed visible germination at this stage), and 11-day incubated primary dormancy released seeds corresponding to germination *sensu stricto* (conditions subsequently abbreviated PDRS, PDRS5, and PDRS11, respectively).

Primary dormant seeds were incubated under a germination inductive temperature for 11 days. The following seed samples were selected for metabolic analysis: primary dormant seeds, 5-day incubated primary dormant seeds, and 11-day incubated primary dormant seeds (conditions subsequently abbreviated PDS, PDS5, and PDS11, respectively). After transfer of PDS to germination conditions for incubation, these seeds cannot germinate. Therefore, we recognize that the primary dormancy of seed was maintained during 11 days of incubation.

Specifically, every 20 PDRS were placed in a 9-cm diameter Petri dish. Every 20 PDS were also put into a 9-cm diameter Petri dish. There were 12 Petri dishes for PDRS and 12 Petri dishes for PDS, respectively. Those PDRS (PDS) in 12 Petri dishes were then incubated under germination conditions for 11 days in a same manner that described in germination test. Six Petri dishes containing PDRS and six Petri dishes containing PDS were used as replicates both at 5 days after incubation and 11 days after incubation. There were also six replications of 20 PDRS each and six replications of 20 PDS each before incubation. Before incubation, after 5 days of incubation and 11 days of incubation, those seeds in six Petri dishes were removed and washed three times with deionized water and then dried with a filter. The embryos were dissected from the rest of the seed structure, immediately frozen in liquid $N_2$ and pulverized in liquid $N_2$, lyophilized and stored at -20°C until metabolite analysis. Six replicates for each seed treatment (PDRS, PDS) and before sampling and at each sampling time were analyzed separately for metabolite measurements.

# Sample preparation

The 100 mg embryo powder was put into a 2 mL Eppendorf tube and extracted with 1.5 mL of 80% methanol. Tridecanoic acid (0.4 μg $mL^{-1}$) was applied as an internal standard. In order to adequately extract the metabolites, the sample was first vortexed for 5 minutes and then centrifuged at 20598 x g for 10 minutes. After that, 800 μL of the supernatant was removed into another 1.5 mL Eppendorf tube and

lyophilized for 10 h. Then, 100 µL of methoxyamine solution (20 mg $mL^{-1}$) that was prepared with pyridine was added to the Eppendorf tube to dissolve the dry residue. This solution sample was then incubated in a water bath for 90 minutes at 37°C to perform the oximation reaction. Subsequently, 80 µL of MSTFA (N-Methy-N-(trimethyl-silyl) trifluoroacetamide) was added to the sample and incubated in a water bath for 60 minutes at 37°C to conduct the silylation reaction. Then, 200 µL of the supernatant was removed into a sample vial the lid crimped in place and used for gas chromatography-mass spectrometry (GC-MS) analysis.

# GC-MS analysis

A QP 2010 GC-MS equipped with an AOC-20i automatic injector (Shimadzu, Japan) was used in the present study. Chromatographic separation of metabolites was accomplished on a 30 m×0.25 mm×0.25 µm DB-5 MS column (J&W Scientific, Folsom, CA, USA). The injection temperature was 300°C. Helium (99.9995%, ShunTai, ShenYang, China) was used as a carrier gas. The constant flow of the carrier gas was set as 2.4 mL $minute^{-1}$. The injection volume was 1 µL. The split ratio was set as 10:1. In order to achieve ionization of the metabolites, the electron impact model at 70 eV was used. The temperature of the interface was 280°C. The temperature of the ion source was set to 230°C. The mass spectra scan scope ranged from 33 to 500 m $z^{-1}$. The scan speed was 5 scans $second^{-1}$. The solvent cut time was 5.7 minutes. The column temperature was maintained at 70°C for the first 3 minutes and then increased to 310°C at the rate of 5°C $minute^{-1}$. The 310°C was maintained for 5 minutes.

The peak deconvolution and identification analysis were conducted with the AMDIS (automated mass spectral deconvolution and identification system, National Institute of Standards and Technology) software by mass spectra matching. Furthermore, the mass spectra, retention time, and retention index of commercial standards were also used to identify the structures of metabolites. As long as peak area is less than 1000 or signal-to-noise ratio is below 20, the peak will be excluded[48]. In order to reduce the probability of false positives and eliminate artifact peaks, methods described in several studies were also applied in the present study[48-50].

# Statistical analyses

The germination percentage was defined as the number of seeds completing germination/total number of viable seeds × 100%. Principal component analysis (PCA) was performed to visualize the classification of 6 samples (PDS, PDS5, PDS11, PDRS, PDRS5 and PDRS11) using the MetaboAnalyst (http://www.metaboanalyst.ca/). All metabolite data were normalized by using unit-variance (UV) scaling before performing PCA. In UV-scaling, each variable was mean-centered and divided by the standard deviation. In order to visualize the patterns of change in the differential metabolites, hierarchical cluster analysis (HCA) was conducted with MetaboAnalyst on the basis of Pearson correlation coefficients between different metabolites. The HCA plot was then divided into seven groups to clearly show the

relationships and trends among the differential metabolites in the six samples. Partial least squares discriminant analysis (PLS-DA) was then used to identify the differentially expressed metabolites that contributed to the separation of each of the four pairs of samples. Therefore, a total of four partial least squares discriminant analyses were conducted. Specifically, those differentially expressed metabolites were identified by inspecting loadings plots from PLS-DA. Only those metabolites with VIP (variable importance in the projection) > 1 and significant changes ($P < 0.05$) in content were considered to have significant contributions to the classification of each of the four pairs of samples[48, 51, 52]. These four pairs of samples are as follows: PDRS5 vs PDRS, PDRS11 vs PDRS5, PDS5 vs PDS and PDS11 vs PDS5. Fold changes of the metabolites with VIP > 1 between each of the four pairs of samples were also calculated. Fold change was calculated as the ratio between two group means and then $\log_2$ transformed. Specifically, they were calculated by the following formulas: $\log_2^{(PDRS5/PDRS)}$, $\log_2^{(PDRS11/PDRS5)}$, $\log_2^{(PDS5/PDS)}$ and $\log_2^{(PDS11/PDS5)}$. The calculation of fold changes was conducted with MetaboAnalyst. Those metabolites with VIP > 1 were then subsequently subjected to metabolic pathway analysis to identify and visualize the significantly altered metabolic pathways between PDRS5 vs PDRS, PDRS11 vs PDRS5, PDS5 vs PDS and PDS11 vs PDS5. The pathway enrichment analysis and pathway topological analysis in the MetaboAnalyst web tool were used together to perform metabolic pathway analysis[53]. The *P* value calculated with the'Global Test'algorithm in pathway enrichment analysis was used to indicate the significance of enriched metabolic pathways[54]. The impact value calculated with the 'Relative-Betweenness Centrality' algorithm in pathway topological analysis was used to estimate the relative importance of metabolic pathways[53]. The impact-value threshold was set to 0.1 to identify the most relevant metabolic pathways[55, 56]. Finally, the result of the metabolic pathway analysis (metabolome view) was rendered in a graph format. The *P* value of each metabolic pathway was log-transformed and then set as the Y-axis and the pathway impact value was set as the X-axis. Each node in a graph represents a metabolic pathway. The node color indicates the *P* value of each metabolic pathway, while the node radius indicates the impact value of each metabolic pathway[54]. Therefore, dark red, large circles located in the top right corner of the "metabolome view" represent the main altered pathways compared to the yellow, small circles located in the left of the graphs. The software VANTED was then used to visualize the pathway map of the significantly altered metabolites[57].

## Declarations

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# Availability of data and materials

The datasets used and/or analyzed during the current study are available from the authors on reasonable request (Jiaojun Zhu, jiaojunzhu@iae.ac.cn; Yuan Song, songyuan_in2000@yeah.net).

# Competing interests

The authors declare that they have no competing interests.

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# Figures

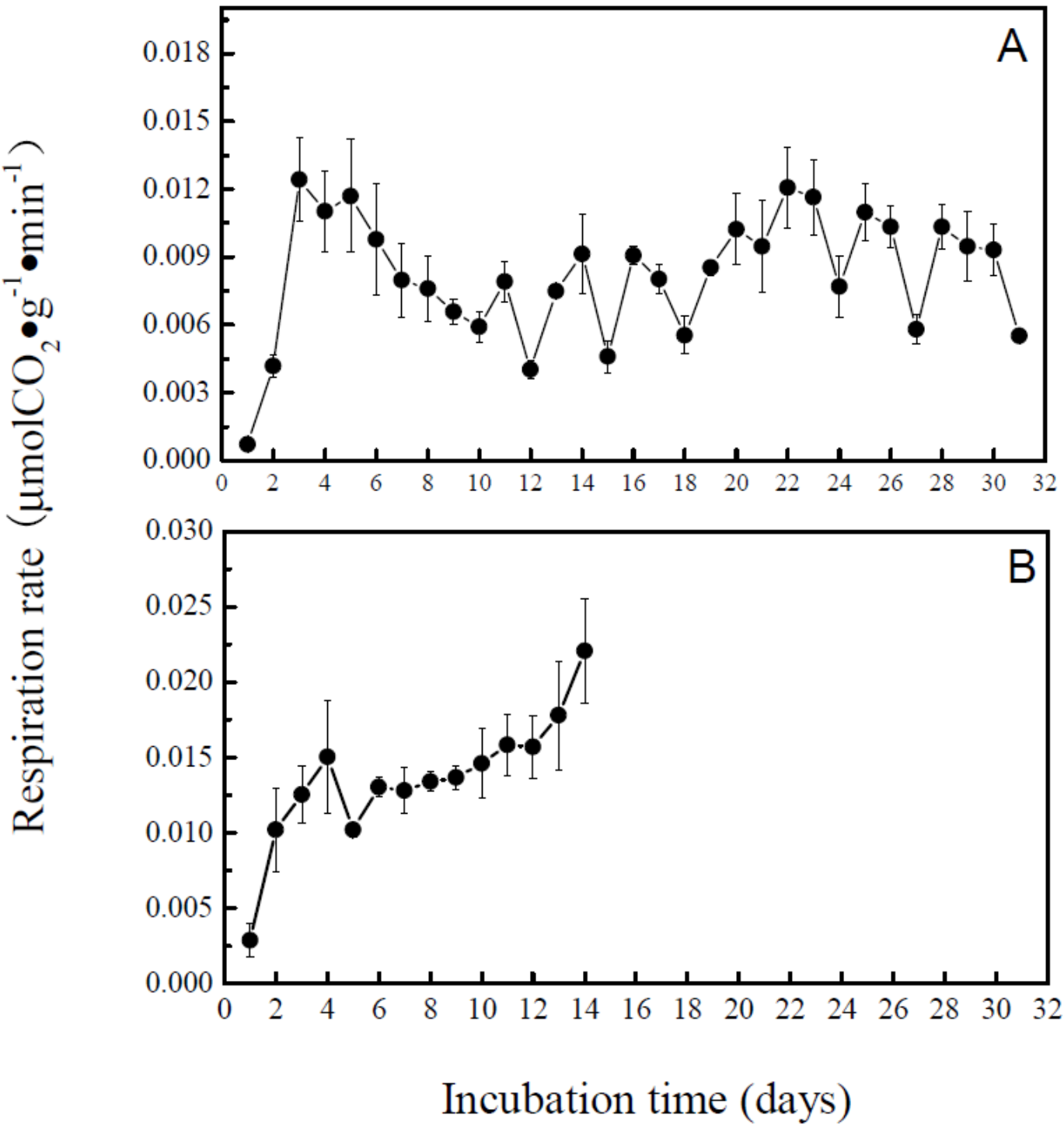



**Figure 1**

a Respiration rates of intact primary dormant seeds with their seed coats were cracked. b Respiration rates of intact primary dormant seeds

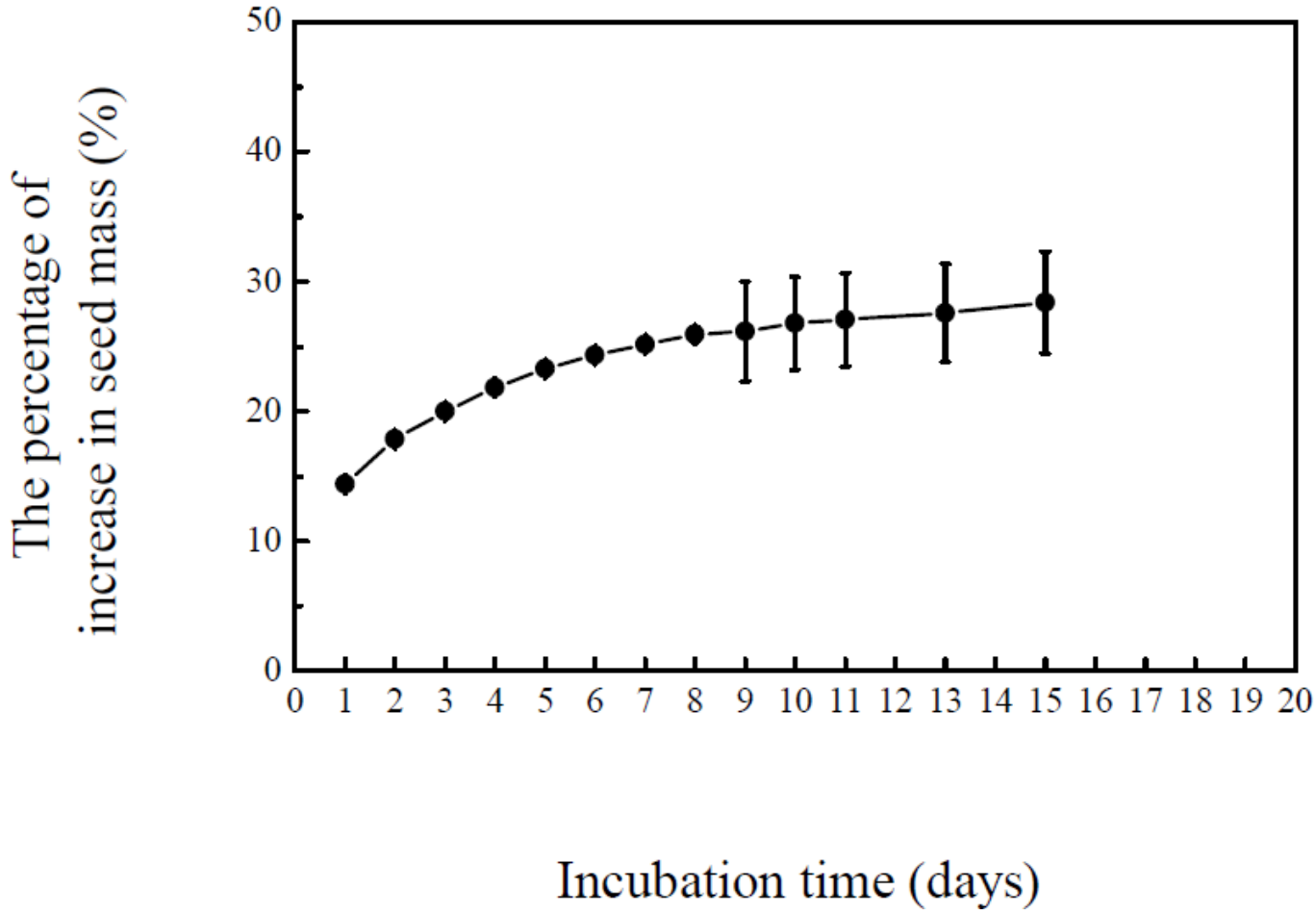



**Figure 2**

Changes in the masses of intact primary dormant seeds

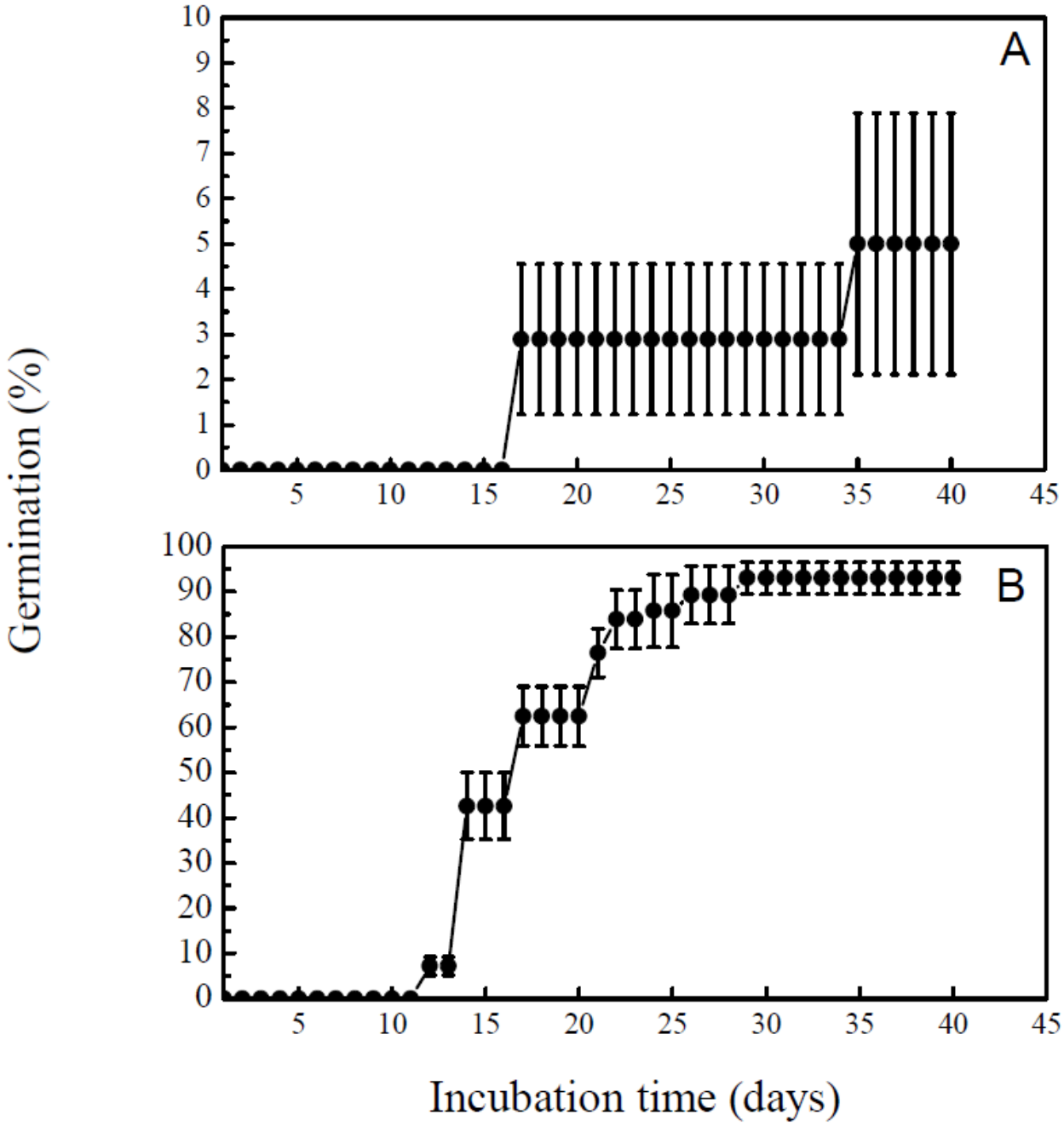
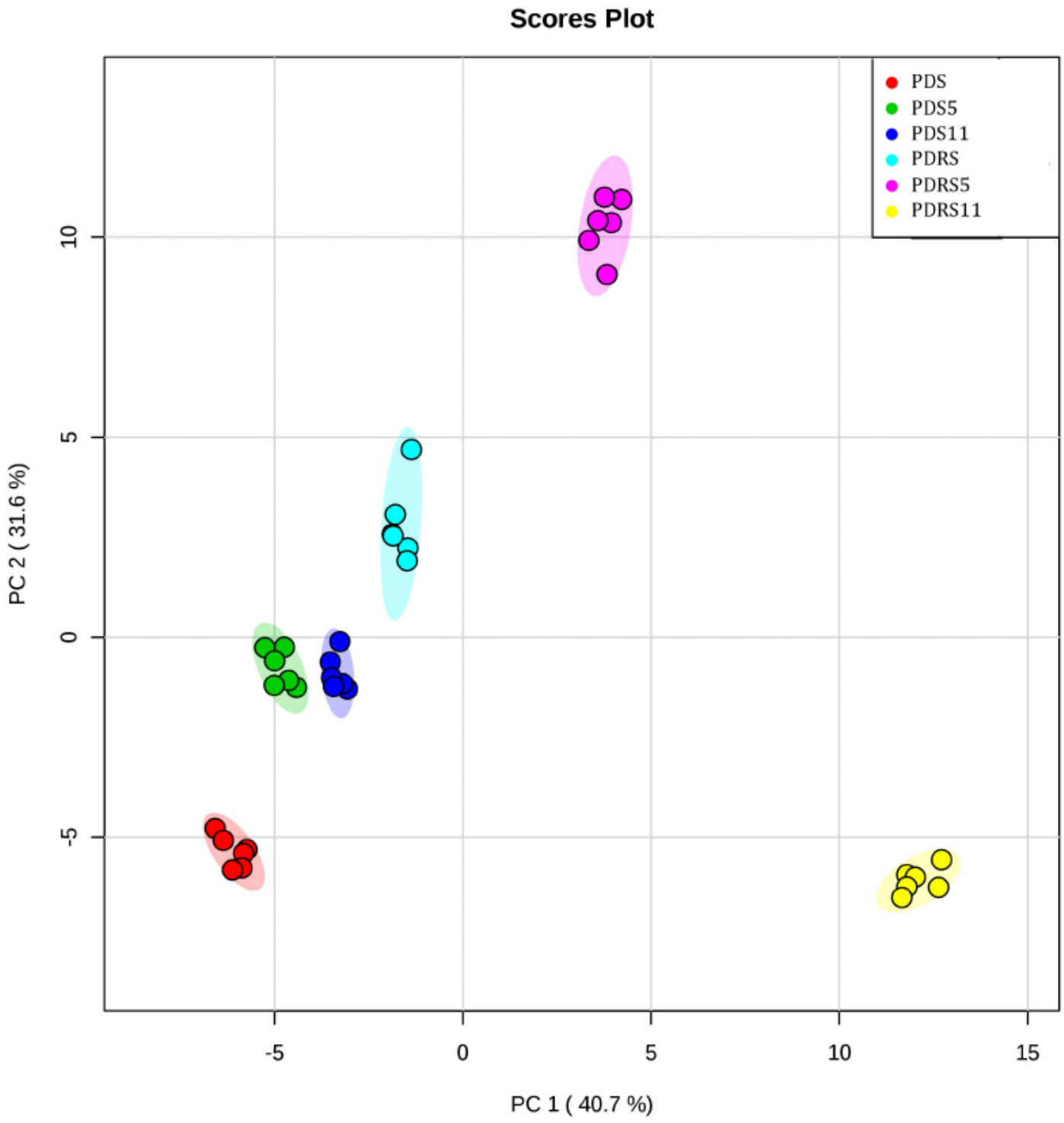


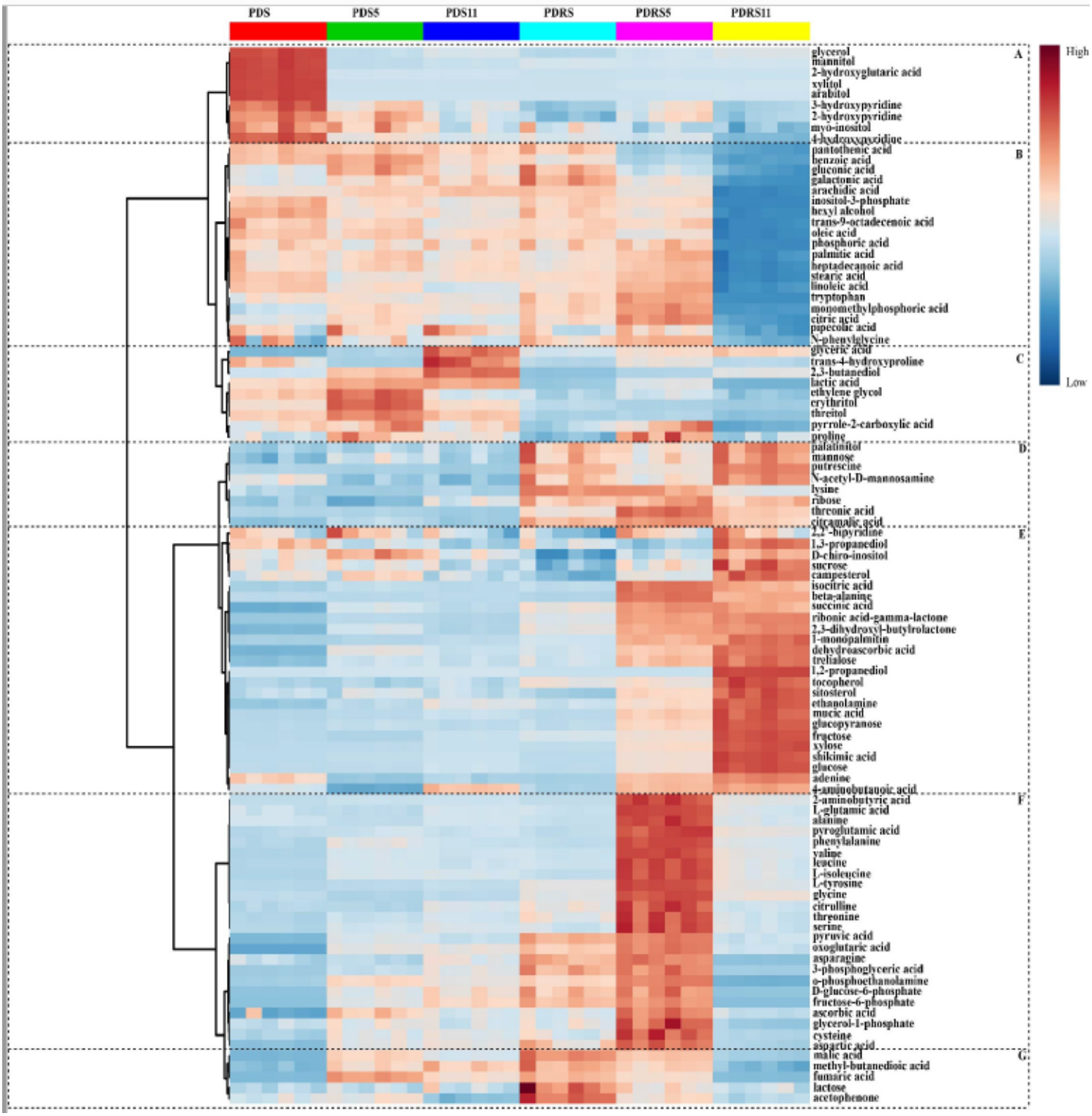

**Figure 3**

a Germination of primary dormancy released seeds. b Germination of primary dormant seeds



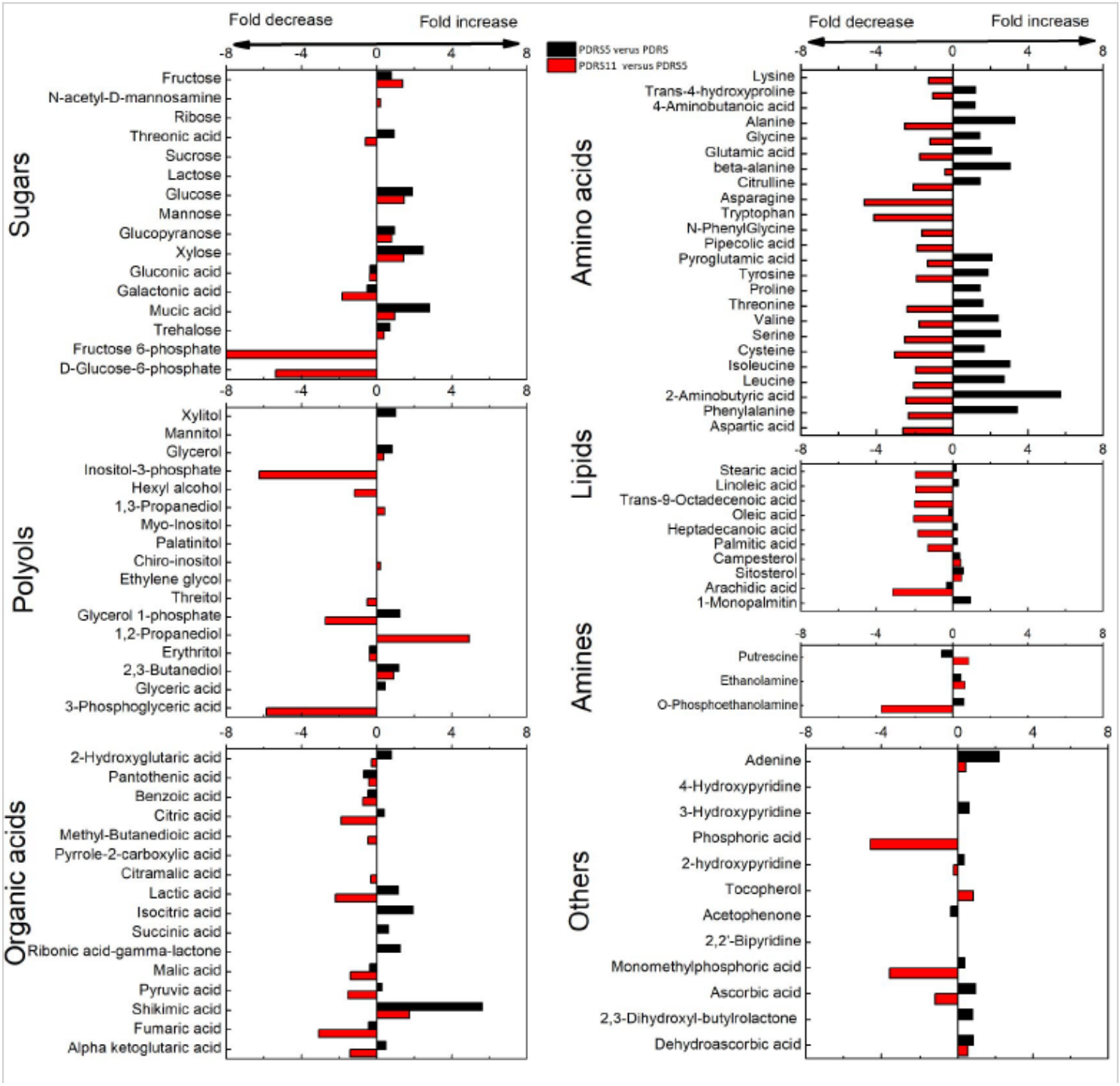

## Figure 4

Principal Component Analysis (PCA) based on identified metabolite levels in the embryos of six different types of seeds. PDRS: primary dormancy released seeds, PDRS5: 5-day incubated primary dormancy released seeds, PDRS11: 11-day incubated primary dormancy released seeds, PDS: primary dormant seeds, PDS5: 5-day incubated primary dormant seeds and PDS11: 11-day incubated primary dormant seeds



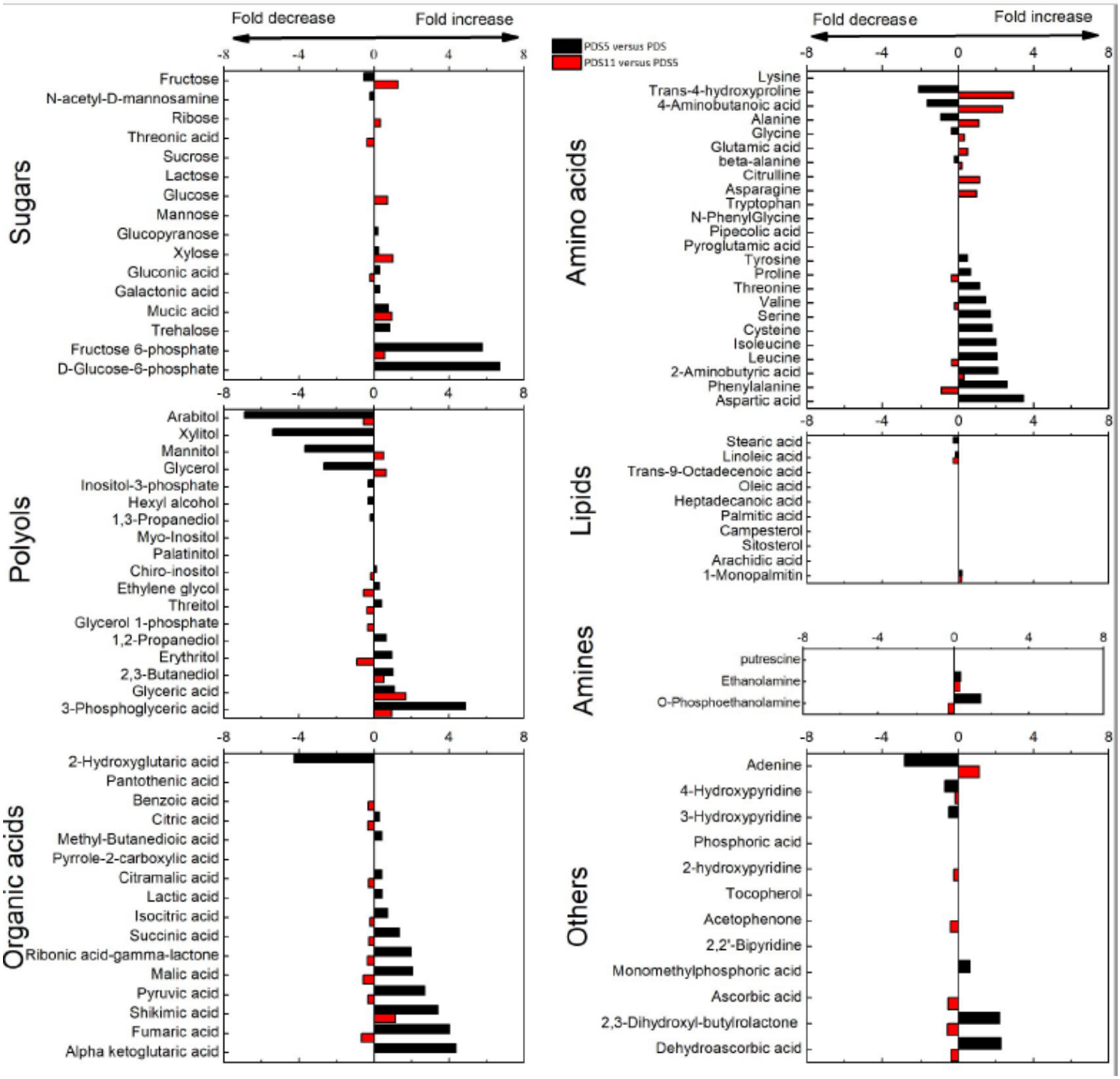

## Figure 5

Principal Component Analysis (PCA) based on identified metabolite levels in the embryos of six different types of seeds. PDRS: primary dormancy released seeds, PDRS5: 5-day incubated primary dormancy released seeds, PDRS11: 11-day incubated primary dormancy released seeds, PDS: primary dormant seeds, PDS5: 5-day incubated primary dormant seeds and PDS11: 11-day incubated primary dormant seeds



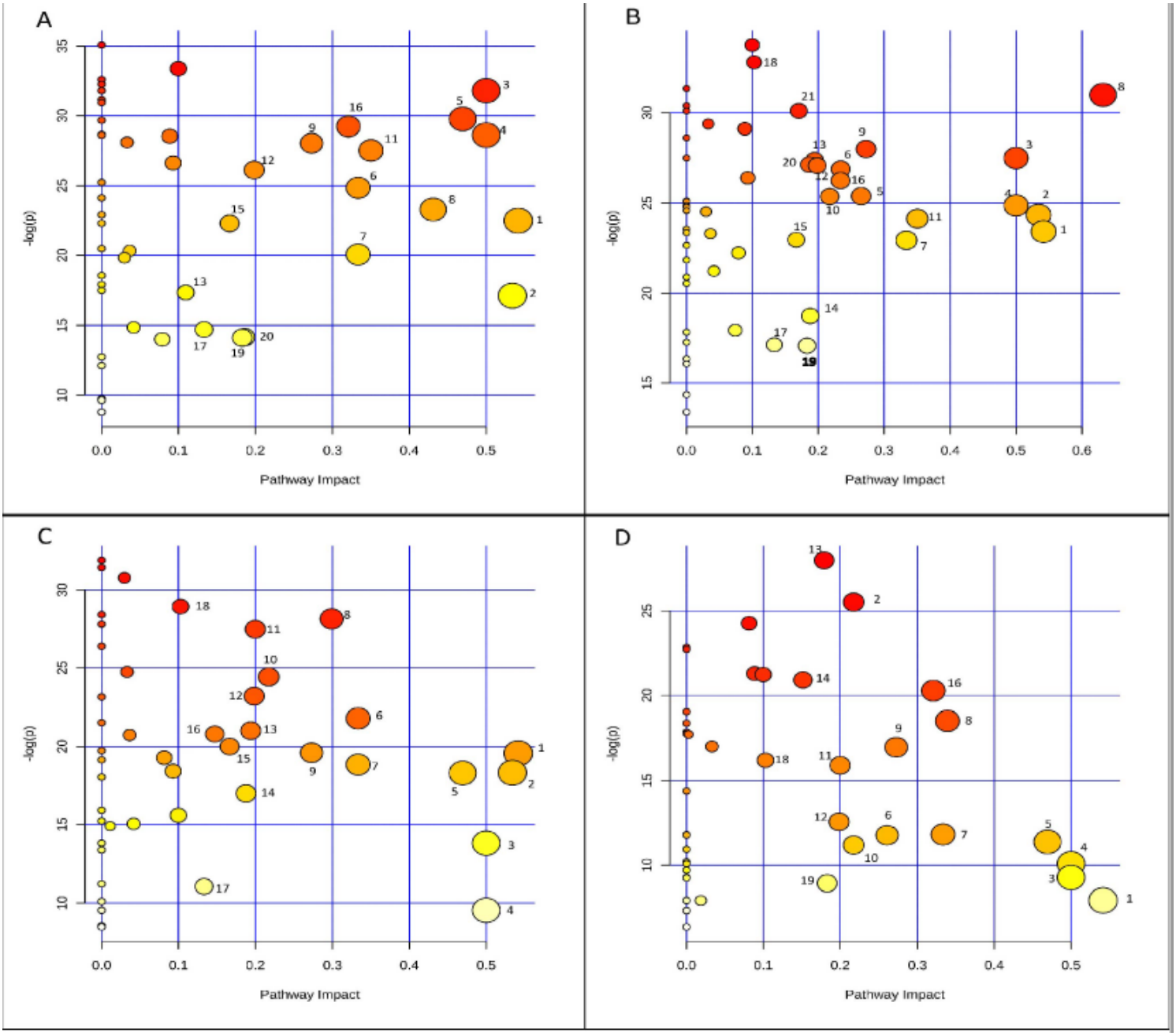

## Figure 6

Changes in the contents of important metabolites with VIP value > 1 in the embryos of PDRS. PDRS: primary dormancy released seeds, PDRS5: 5-day incubated primary dormancy released seeds, and PDRS11: 11-day incubated primary dormancy released seeds. Value bars facing the right of each section indicate the fold-increased content. Value bars facing the left of each section indicate the fold-decreased content




## Figure 7

Changes in the contents of important metabolites with VIP value > 1 in the embryos of PDS. PDS: primary dormant seeds, PDS5: 5-day incubated primary dormant seeds, and PDS11: 11-day incubated primary dormant seeds. Value bars facing the right of each section indicate the fold-increased contents. Value bars facing the left of each section indicate the fold-decreased contents




## Figure 8

Metabolome view of the altered metabolic pathways in the embryos between (A) PDRS5 and PDRS, (B) PDRS11 and PDRS5, (C) PDS5 and PDS, (D) PDS11 and PDS5. PDRS: primary dormancy released seeds, PDRS5: 5-day incubated primary dormancy released seeds, PDRS11: 11-day incubated primary dormancy released seeds, PDS: primary dormant seeds, PDS5: 5-day incubated primary dormant seeds, and PDS11: 11-day incubated primary dormant seeds. Carbohydrate metabolisms (5, Glyoxylate and dicarboxylate metabolism; 6, Citrate cycle; 10, Fructose and mannose metabolism; 12, Pyruvate metabolism; 13, Glycolysis or Gluconeogenesis, 18, Amino sugar and nucleotide sugar metabolism; 14, Pentose phosphate pathway; 19, Ascorbate and aldarate metabolism). Amino acid metabolisms (1, beta-Alanine metabolism; 2, Glycine, serine and threonine metabolism; 3, Phenylalanine metabolism; 8, Alanine, aspartate and glutamate metabolism; 9, Tyrosine metabolism; 16, Arginine and proline metabolism; 21,

Tryptophan metabolism). Energy metabolisms (15, Methane metabolism; 17, Sulfur metabolism). Lipid metabolism (7, Sphingolipid metabolism; 20, Glycerophospholipid metabolism). Metabolisms of cofactors and vitamins (11, Pantothenate and CoA biosynthesis). Biosynthesis of other secondary metbaolites (4, Isoquinoline alkaloid biosynthesis)

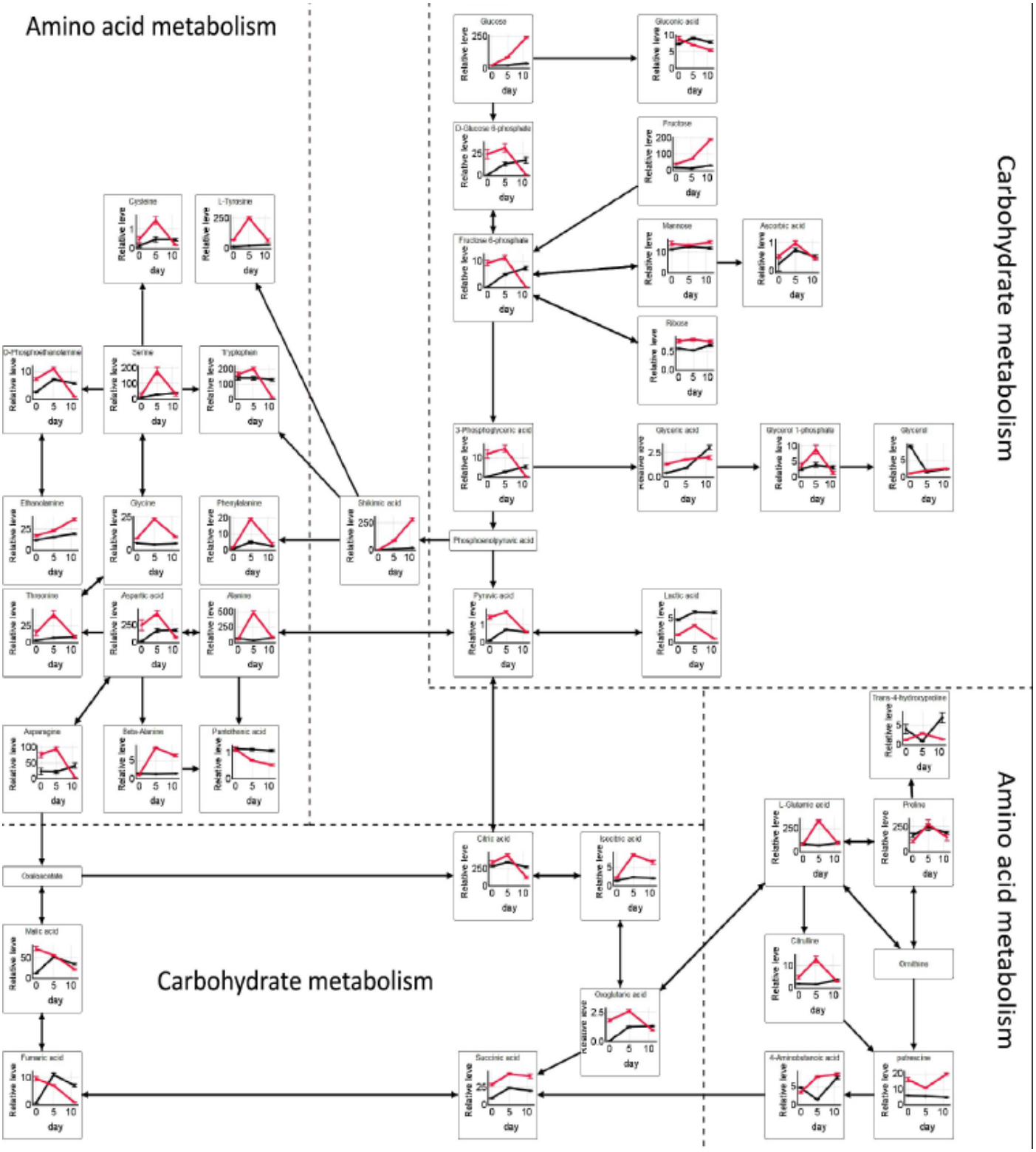



**Figure 9**

Integrative overview of the major metabolic changes in the embryos of primary dormancy released seeds (PDRS) and primary dormant seeds (PDS) under germination conditions. Red and Black indicate PDRS and PDS, respectively