THE INVOLVEMENT OF GUAIACOL PEROXIDASE IN EMBRYO DEVELOPMENT OF *Vigna unguiculata* (L.) Walp. DURING GERMINATION

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Abstract: Guaiacol peroxidase (GPX) is involved in a wide range of vital plant physiological processes primarily development and stress tolerance. However, the precise role of this enzyme in each seed part of the plant and in the various stages of development is still poorly known. Our work consisted in studying the kinetics of the activity of guaiacol peroxidase in cotyledons and embryonic axes (radicle and plumule) during the different phases of *Vigna unguiculata* (L.) Walp. germination. The results obtained showed that the kinetics of GPX during germination was triphasic. Indeed, it was activated after the first 30 minutes of imbibition, then decreased during the germination phase and reactivated after the 8th hour in plumule and cotyledons, but after the 20th hour in the radicle. On the other hand, our results showed that the GPX activity was extremely strong in the plumule compared to the other two seed parts. As for the cytochemical analysis, our study showed that during the germination imbibition phase, there is a slight accumulation of hydrogen peroxide H_2O_2, mainly in the meristematic tissues. The involvement of guaiacol peroxidase during germination of *V. unguiculata* (L.) Walp differs according to the seed part and the germination phase. The activation, the reduction and then the reactivation of the activity of this enzyme during germination could be explained by its multiple regulations, given that the existence of several isoenzymes. We can also conclude that during germination, GPX is mainly involved in development rather than in antioxidant activity.

Keywords: Gaïacol peroxidase; oxygen peroxide; germination; cotyledons; radicle; plumule; *Vigna unguiculata*.

INTRODUCTION

Peroxidases are ubiquitous hemoproteins in eukaryotes and prokaryotes. They present a broad polymorphism and are encoded by a family of genes resulting from the duplication and diversification of a parental gene, followed by the acquisition of new enzymatic capacities [45]. Guaiacol peroxidase (GPX) is a class of glycoproteins (class III peroxidase or secreted peroxidases) that belongs to a large family of multigenic proteins [33]. In addition to isoenzymes that can be generated by post-transcriptional and post-translational changes [45, 47], probably due to this high number of isoforms and the heterogeneous regulation of its expression, guaiacol peroxidase participates in many physiological and biochemical processes such as plant development [18], senescence [21], lignin biosynthesis, seed partogenesis via AIA degradation and ethylene biosynthesis [16, 20-46], cell wall thickening through polysaccharide and protein bridging [18, 27], wound repair [24] and parasite defence [2]. Thanks to its important capacity to eliminate active forms of oxygen, GPX is active under stress conditions [3]. Indeed, this enzyme consumes H_2O_2 using guaiacol or pyrogallol as an electron donor [31]. GPX is located in different cellular compartments (walls, membranes, mitochondria, cytosol and vacuoles); its presence allows water to be formed from hydrogen peroxide in these cellular seed partelles [18]. A large variability in the response of this enzyme is observed in the literature depending on the plant species, the studied seed part [12], the stage of development [18] and environmental conditions [33, 34]. However, although GPX signaling is variable, it appears that it can be refined by isoform measurement [12].

On the other hand, several studies have clearly shown that crop production and the establishment of good agricultural crops depend closely on seed germination, which is a crucial stage in the life cycle of higher plants [13]. Germination is defined as the transitional phase between the dry seed stage and the appearance of the radicle [8]. The germination process is divided into three phases [9]. The imbibition phase which represents a seed hydration process related to passive imbibition of dry tissues associated with water movement first occurring in the apoplastic spaces. This phase is characterized by the activation of mitochondrial enzymes that will produce ATP. The second phase or germination is associated with the re-establishment of metabolic activities and repairing processes at the cell level. It is characterized by the resumption of respiration and metabolic activity as well as by the activation of phytohormones, particularly gibberellins, which contribute to the synthesis of hydrolases (alpha amylase, proteinases, lipases or nucleases) necessary for the degradation of reserves, cell division and elongation. The third phase or post-germinative growth phase is also characterized by water ingress and a significant increase in respiration. During this phase, there will be the initiation of growing processes associated to cell elongation and leading to radicle protrusion. The hyper-consumption of oxygen is linked to neo-synthesized enzymes [4, 29]. Germination is a very complex process based on molecular properties ensuring the progress of the three phases and is influenced by many endogenous and exogenous factors [42]. Reactive oxygen species (ROS) are involved in various aspects of seed physiology. Their generation, which occurs during germination, may lead to oxidative stress and cellular damage, resulting in seed

deterioration. However, cells possess detoxifying enzymes that scavenge ROS and participate in seed survival [5, 39].

The main objective of this work is to study the kinetics of guaiacol peroxidase activity during the different phases of germination, in the three part of the embryo (cotyledons, radicle and plumule) of *Vigna unguiculata* (L.) Walp. Our study attempts to estimate the involvement of GPX in the development of the embryo during the germination, a crucial phase of plant development. According to our knowledge this aspect has never been studied before. In preliminary study, we identified the germination phases of *Vigna unguiculata* (L.) Walp seeds as well as the evolution of the protein content in the cotyledons, the radicle and the plumule. On the other hand, we estimated the accumulation of hydrogen peroxide (H_2O_2) at the embryonic axis at two stages of germination. *Vigna unguiculata* (L.) Walp is one of the main food legumes in Africa [36]. The high protein content of its seeds gives it an important role in the nutritional balance of rural populations [40] and it is an excellent source of vitamin C. It also contains valuable amounts of minerals including iron, zinc and calcium [14].

MATERIAL AND METHODS

Our work had focused on the seeds of the black-eyed bean, *Vigna unguiculata* (L.) Walp a commercial variety, *Lojy Bemaso*, from Madagascar. Healthy seeds, all of the same size, were selected and rinsed with bleach water to decontaminate them.

Kinetics of imbibition of *Vigna unguiculata*

In order to identify the three phases of germination of *Vigna unguiculata* (L.) Walp, ten seeds were put to germinate in a Petri dish lined with absorbent paper at 26°C. These seeds were then weighed every two hours for 26 hours. The imbibition kinetics curve is determined by measuring the percentage of cumulative absorbed water [25].

% cumulative water at time (t) = (Weight (t) - dry weight) * 100 / dry weight

Sampling of cotyledons and embryonic axes

At different stages of imbibition (from 30 min until 24 h), the embryos (without their cotyledons) were removed using pliers. This operation was carried out under low temperature (in crushed ice) to prevent the absorption of water by the embryos and their development to maintain all the seeds at the same physiological stage. Our study was done on the three seed parts separately: the radicle, the plumule and the cotyledons.

Biochemical analyses

Evolution of total water-soluble proteins content

Proteins were measured in the cotyledons, radicle and plumule (separately) during 24 hours of germination with a three hours interval between each measurement. The extraction was carried out cold in Tris-HCl pH 8.1 buffer. Water-soluble proteins were then determined using Bradford's method [11].

The kinetics of guaiacol peroxidase (GPX) activity

The activity of this enzyme was measured in each seed part of the embryo (cotyledons, radicle and plumule) during 24 hours of germination with an interval of three hours between each measurement.

The activity of guaiacol peroxidase was determined according to the method of [31] slightly modified. This technique was based on the increase in the absorbance at 470 nm due to the polymerization of the guaiacol to tetraguaiacol (oxidation) giving an orange coloration in the presence of hydrogen peroxide. The extraction of the enzyme was carried out in potassium phosphate buffer 1 M pH 6.5 in cold condition. After centrifugation for 20 min at 12000 rpm at 4° C, the supernatant (enzyme extract) was recovered. The activity was measured on 700 µL of the enzyme extract to which were added 2.5 mL of the same phosphate buffer used for extraction, 36 mM guaiacol and 10 mM H_2O_2. The reaction begins upon the addition of 100 µL H_2O_2 to the reaction mixture. The activity was monitored as a function of time and expressed in µmoles of oxidized guaiacol per minute and per mg of protein, using the tetraguaiacol molar extinction coefficient ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$).

Cytochemical detection of hydrogen peroxide (H_2O_2)

Hydrogen peroxide was detected by a cytochemical method using 3,3'diaminobenzidine (DAB). The hydrogen peroxide results in an oxidoreduction with polymerization of the DAB molecule giving a very stable brown precipitate at the reaction site. The embryos were immersed in a 1 $mg.mL^{-1}$ DAB solution in water for 12 hours at room temperature with stirring and in the dark. The DAB solution was prepared before each experiment to avoid self-oxidation [44]. The embryos were then rinsed with 50% ethanol for 10 minutes and then stored in a glycerol-ethanol solution (1/4, v/v) until the photographs were taken under a binocular microscope. This cytological analysis was carried out only at two stages during germination, after 2 h and 8 h of imbibition.

Variation percentage

The percentage change was calculated for each parameter studied according to the following relation: variation percentage (Assay- Control) * 100 / Control

Statistical Test

Experiments were repeated at least five times. The results, presented in the form of curves represent the average values. The bars represent the standard errors of five repetitions. The statistical Student test was carried out using the Excel 2007 software.

RESULTS

Kinetics of imbibition

Results showed that during the germination of *Vigna unguiculata* (L.) Walp seeds, the water absorption took place in three phases: an imbibition phase which lasts about 12 hours, a *sensu stricto* germination phase which took place during 8 hours and

a last phase, the growth phase (or post-germination), which begun after 20 hours of imbibition (Fig. 1).

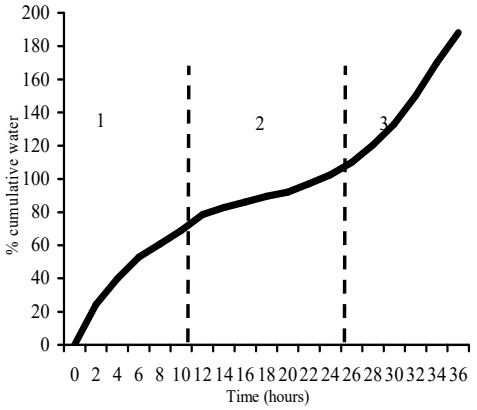

Figure 1. Imbibition kinetic of *Vigna unguiculata* (L.) Walp seeds. (1) Imbibition phase (2) *Sensu stricto* germination phase *"in the strict sense"* (3) Growth phase (irreversible phase)

Evolution of the protein content of embryos

Our curves showed that the evolution of the total protein content of *Vigna unguiculata* (L.) Walp seeds during germination differed according to the seed parts. In cotyledons, the protein content decreased steadily and gradually during germination. On the other hand, in the other parts of the embryo (plumule and radicle), the evolution of the protein content was more or less diphasic. For plumule, the amount of protein was stable during the first 8 hours of germination, corresponding to the imbibition phase. Then, this content increased rapidly to reach 26% of the initial content. For the radicle, the decrease was weak and progressive until the 16th hour of germination. Then, we recorded a slight increased in the protein content which coincided with the beginning of the growth phase (lengthening of the radicle) (Fig. 2).

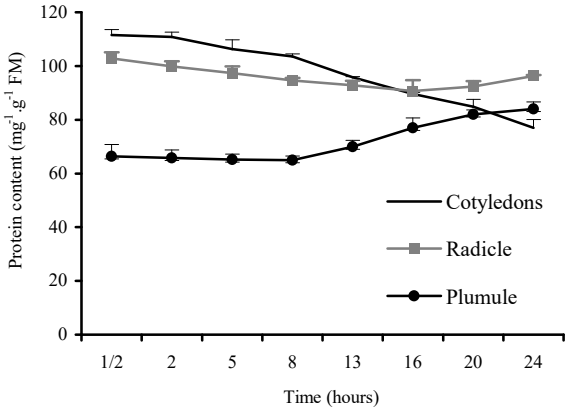

Figure 2. Total protein content evolution during germination of *Vigna unguiculata* (L.) Walp seeds in cotyledons, radicle and plumule. Values are means ± SD

The kinetics of guaiacol peroxidase

It appeared that guaiacol peroxidase activity was extremely high in plumule compared to radicle and cotyledons (3840 times higher). We also noticed that the kinetics of GPX differed from one seed part to another. Evolution curves of the enzymatic activity of GPX in cotyledons, radicle and plumule of *Vigna unguiculata* (L.) Walp seed showed that in the three parts, the activity of GPX was strong during only the first 30 minutes of germination and then it decreased

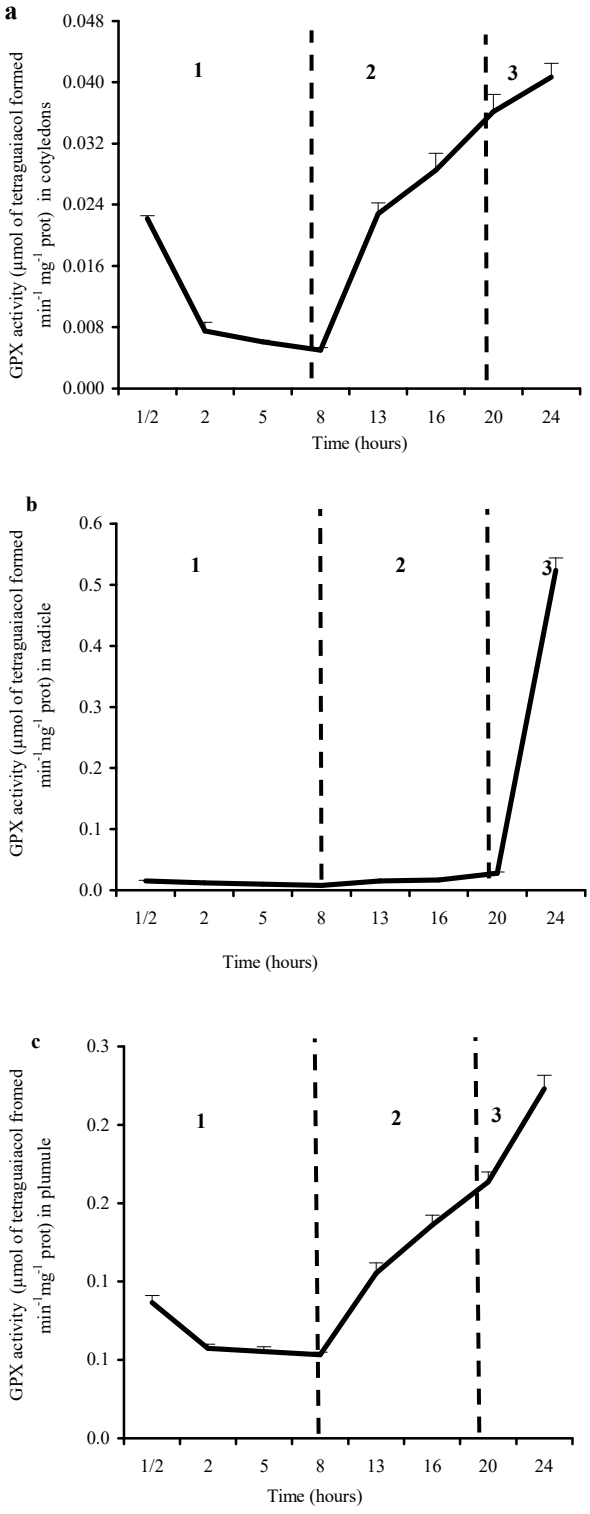

Figure 3. Guaiacol peroxidase activity evolution in cotyledons (a), radicle (b) and plumule (c) during germination of *Vigna unguiculata* (L.) Walp seeds. (1) Imbibition phase (2) *Sensu stricto* germination phase (3) Growth phase (irreversible phase). Values are means ± SD

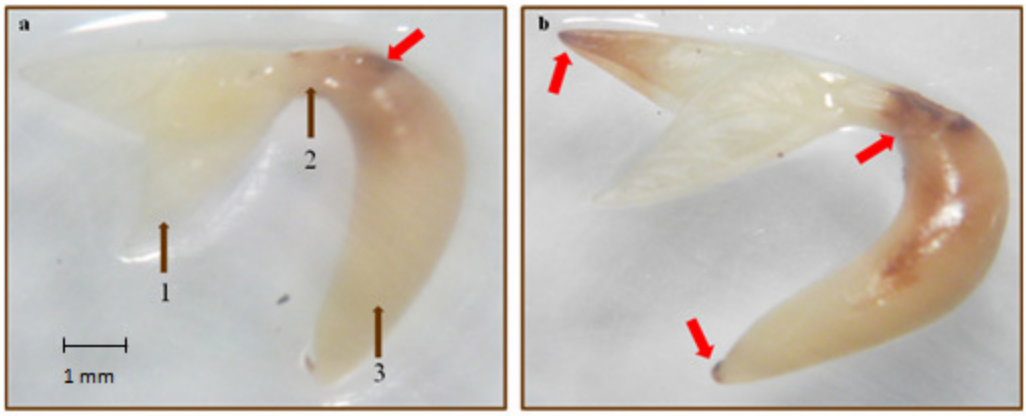

Figure 4. Photographs showing the detection of hydrogen peroxide (H_2O_2) by DAB, taken under binocular magnifying glass with 8x magnification, on a representative embryo after 2 hours (a) and 8 hours of i mbibition (b) of *Vigna unguiculata* (L.) Walp seeds. The presence of hydrogen peroxide (H_2O_2) was indicated by browning due to the polymerization of diaminobenzidine (DAB). (1) Radicle (2) junction (3) plumule.

gradually during the imbibition phase and more particularly in cotyledons (-77%). On the other hand, at the 8th hour of germination, this activity rapidly increased continuously, particularly in cotyledons (+356%) and plumule (98%). But in the radicle, after eight hours of germination the activity of GPX resumed very slowly until the 20th hour of germination (beginning of the growth phase). This activity increased markedly with a rate of variation of +2755% (Fig. 3).

Cytological detection of hydrogen peroxide (H_2O_2)

DAB results showed that after 2 hours of soaking *Vigna uguiculata* seeds in distilled water, there was a low accumulation of hydrogen peroxide and particularly at the plumule level. Our results also indicated that H_2O_2 production intensified after 8 hours of imbibition (beginning of the *sensu stricto* germination phase). This accumulation was essentially marked at the radicle tip, the radicle-plumule junction and the plumule (Fig. 4).

DISCUSSION

Results showed that during the germination of *Vigna unguiculata* (L.) Walp seeds, the seed underwent several metabolic and biochemical modifications. These changes, and particularly the activity of GPX, differ depending on the part and germination phase.

Identification of the three germination phases of Vigna unguiculata

According to the curves obtained we can deduce that during the germination of *Vigna unguiculata* (L.) Walp seeds, water absorption is characterized by a triphasic model, which is similar to the configuration of most seeds [9, 29]. The first phase or imbibition phase corresponded to a rapid water intake. The second phase was a plateau phase which ends with the breakthrough of the radicle and the last phase (post-germination or growth) was characterized by the resumption of imbibition. Until the end of the germination phase *stricto sensu*, the seed can be dehydrated without being affected, but when the radicle has begun its growth, dehydration is fatal [7]. The beginning of growth thus marks the transition from a reversible physiological state to an irreversible state.

Evolution of protein content

On the physiological level, our results showed that during germination, there is a decrease in cotyledon reserve proteins. This explains why these proteins are gradually hydrolyzed by proteases that lyse protein reserves (aleurone grain) by providing the energy and amino acids necessary for metabolism [9, 23] and by promoting the formation of phytohormones such as auxin, responsible for cell elongation [4, 29]. In plumule, an increase in protein content was observed at the end of the imbibition phase and corresponds to a neosynthesis of proteins (notably enzymes) necessary for embryo growth [38]. At the radicle level, just before its lengthening, we recorded a decrease in the protein content which indicated that there was a hydrolysis of proteins (probably reserves) whose products (osmolytes) will cause an increase in osmotic pressure which will allow a turgidity necessary for cell extension [9]. However, at the beginning of the growth phase, results showed a neosynthesis of root proteins.

Kinetics of guaiacol peroxidase activity

Guaiacol peroxidase allows the elimination of hydrogen peroxide which induces the polymerization of guaiacol into tetragaïacol. Thus, GPX is involved in a wide range of vital physiological processes, essentially plant development and resistance to different stresses [17, 20, 33, 34, 35-43]. Obtained curve showed that the evolution of GPX activity during germination followed a triphasic curve in the cotyledons and embryonic axes. We can explain the activation of GPX at the embryonic level during the first 30 minutes of imbibition by the pre-existence of this enzyme in seeds during their maturation. However, our results did show a sharp decline in this activity in all the seed parts (cotyledons, radicle and plumule) during the imbibition phase just after the first 30 minutes. But this activity resumed strongly in the cotyledons and plumule after the 8th of imbibition. This was similar to our results from the evolution of the protein content at the plumule level which showed an

increase in protein content at the end of the imbibition phase. This corresponds to a neosynthesis of proteins (notably enzymes) necessary for embryo growth [38]. As for the radicle, our kinetics showed that GPX activity resumed less strongly after 8 hours of imbibition, but it accelerated after 20 hours of imbibition, which corresponds to the beginning of the growth phase. This also coincides with the neosynthesis of root proteins. This shows that GPX is largely involved in triggering the germination and growth process of the embryo. Several authors have suggested that this enzyme intervenes on several cellular levels and its roles are multiple, so it must obey several regulatory or signaling pathways. In addition, it has already been shown by [33, 34] that there are four isoforms of membrane guaiacol peroxidase (soluble and microsomal) hence multiple regulations. Thus, some isoenzymes may be positively regulated while others are negatively regulated [34]. Moreover, according to our study we find that the activity of guaiacol peroxidase was extremely strong in plumule compared to radicle and cotyledons, this could be explained by the richness of plumule (future leaf) in protoplasts that will differentiate into chloroplasts during development and growth.

Hydrogene peroxide production

Our study showed that during the germination imbibition phase there is a slight accumulation of hydrogen peroxide H_2O_2, mainly in the meristematic areas. According to [6], ROS, provided that their level of accumulation is finely regulated by a balance between production and elimination, are beneficial for triggering the germination process. Thus, for [6], ROS act as a positive signal capable of lifting seed dormancy by facilitating the transition from a dormant to an active state (oxidative window theory for germination). Apart from their deleterious effects, ROS play a pivotal role in the signal transduction system of seed. Therefore, seed germination probably only occurs when the level of ROS is sustained under a critical threshold, which then triggers ROS-mediated signaling pathways [1]. Recently, [15] summarized new discoveries involving ROS and their interaction with growth regulating hormones in the regulation of seed germination. Several hypotheses would explain this phenomenon of ROS accumulation during germination by their participation in cell wall relaxation, endosperm weakening, programmed cell death of the aleurone layer as well as protection of the seed and incipient seedling against pathogens [10, 22, 26, 28, 30-37]. In dry seed, water uptake is required to activate antioxidant enzymes and for germination to take place. However, this uptake reactivates various metabolic processes, thus contributing to the production and deposition of ROS, which may inhibit germination [48]. On the other hand, the production of ROS is directly associated with germination [41]. Therefore, successful germination strongly depends on effective anti-oxidative processes that can maintain sufficiently low limits of ROS [32]. Indeed, [19] observed a SOD activity peak during early imbibition of soybean seeds, followed by a CAT and POD activity peak. During the germination of several plant species, superoxide dismutase activity plays a critical role in balancing ROS at non-toxic levels [49]. Moreover, genes located in the cotyledon and embryo axes of germinating seeds are highly expressed.

From these results, we can observe that during germination, guaiacol peroxidase was mainly linked to development and not to antioxidant activity, as proof, that it was instantly activated following imbibition since it was pre-existing in the seed and then the activity decreased despite having had an accumulation of ROS at the embryonic level. It was also reactivated in parallel with the phase of metabolic activities and of growing processes.

At the end of this study we tried to study a new aspect of guaiacol peroxidase, which is the evolution of its activity at the level of cotyledons and embryonic axes, during the different phases of *Vigna unguiculata* (L.) Walp seeds germination. Our results lead to conclude that the kinetics of GPX follow a triphasic curve (activation, decrease and reactivation), which shows that this enzyme is very sensitive to germination stages given its strict involvement in embryo development during seed germination. On the other hand, we find that GPX activity is extremely strong in plumule compared to other seed parts. In order to better explain the involvement of GPX during germination and its regulation, it would be interesting to carry out a molecular and genetic study of isoforms.

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