Changes in soluble proteins and polyamines during citrus seed germination

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Abstract — Introduction. Polyamines and proteins synthetized from free amino acids are present in all plant cells. They are actively involved in cell division and differentiation processes in a wide range of species. This study was an attempt to increase the knowledge of the polyamine and protein content changes during germination of citrus seed. **Materials and methods**. For biochemical characterization of germination, whole and without seedcoat seeds of Cleopatra mandarin mature fruits were placed in Petri dishes on moistened cotton. Polyamine – putrescine (Put), spermine (Spm) and spermidine (Spd) – and soluble protein nitrogenous compounds were determined after different times of imbibition. **Results and discussion**. The seedcoat delayed germination for 16 d while fresh weight of cotyledons increased during the process and the soluble protein content decreased. Within the polyamines, putrescine reached the highest levels and spermine the lowest. In peeled mandarin seed, the protein concentration increased during the first 6 h, with a maximum at 24 h. The levels decreased to a minimum at 12 h, 48 h and 96 h of imbibition. Putrescine was the main polyamine, as compared to spermidine and spermine. **Conclusion**. Polyamines and proteins may play a role in the early stages of germination as the changes observed show. This behaviour confirms that proteins and polyamines are metabolically related (© Elsevier, Paris).

***Citrus reshni* / protein metabolism / plant physiology / germination / seeds**

Évolution du taux de polyamines et de protéines solubles dans le pépin d'agrume en germination.

Résumé — Introduction. Dans toutes les cellules des plantes, des polyamines et des protéines sont synthétisées à partir d'acides aminés libres. Chez de nombreuses espèces, ces substances sont activement impliquées dans la division des cellules et dans les processus de différenciation. L'étude présentée cherche à préciser l'évolution de ces substances lors de la germination d'un pépin d'agrume. **Matériel et méthodes**. Pour caractériser les réactions biochimiques qui ont lieu pendant la germination, des pépins de mandarines mûres (variété Cléopâtre), entiers ou débarrassées de leur tégument, ont été placés en boîte de pétri sur du coton humide. Les composés azotés de polyamines – putrescine (Put), spermine (Spm) et spermidine (Spd) – et de protéines solubles ont été dosés après diverses durées d'imbibition. **Résultats et discussion**. La présence du tégument a retardé la germination de 16 d pendant lesquels le poids frais des cotylédons a augmenté alors que leur taux de protéine soluble diminuait ; pour les polyamines, les taux de putrescine ont été les plus hauts et ceux de spermine les plus bas. Dans les pépins sans tégument, la concentration en protéine a augmenté pendant les six premières heures ; elle a été maximale après 24 h d'imbibition et minimale après 12, 48 et 96 h. La putrescine a eu encore le taux le plus élevé des trois polyamines. **Conclusion**. Les résultats obtenus montrent que les polyamines et les protéines pourraient jouer un rôle au cours des premiers stades de la germination. Cela confirme que les métabolismes de ces deux types de substance seraient liés (© Elsevier, Paris).

***Citrus reshni* / métabolisme des protéines / physiologie végétale / germination / graine**

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Received 3 January 1997
Accepted 19 September 1997

Fruits, 1998, vol. 53, p. 27–33

***Resumen Español**, p. 33*

1. introduction

Germination is a significant event that allows to understand the role of chemical composition in growth and development of plants. The cotyledons of citrus seeds contain large amounts of reserve proteins located in specialized organelles called protein bodies [1]. The first phase of the utilization of nitrogenous reserve compounds involves the hydrolysis of protein to free amino acids which are then transported and incorporated into the developing embryos. These amino acids may be used for the synthesis of enzymes in cotyledons, during the growth of the embryos [2].

Polyamines and proteins synthetized from free amino acids are present in all plant cells. They are actively involved in cell division and differentiation processes in a wide range of species [3]. Polyamines might regulate cell division and promote RNA functions through binding to specific regulatory proteins [4, 5] suggested a relation between polyamine binding proteins and mitotic activity in regions of cell division in leaves, internodes and ovaries of tobacco. Changes in polyamine concentrations accompany variations in the rate of cell division and growth. In the early stages of germination, free polyamine levels first increase and then decrease slowly [6].

Knowledge of the role of proteins and polyamines during germination of citrus seeds is not extensive. This work is an attempt to increase it.

2. materials and methods

2.1. seeds

For biochemical characterization of germination, whole seeds of mature fruits were placed in Petri dishes (15 cm of diameter) on moistened cotton. Groups of 50 seeds and 17 replications were used. One mL of water was added daily to maintain adequate humidity, and germination was carried out under natural light conditions. Nitrogenous compounds were determined at 0, 21, 28 and 35 d of the imbibition. A seed was considered germinated when the radicle emerged.

Another experiment with similar humidity and light conditions, but after removal of the seed-coats, was carried out. Twelve replications of 50 seeds each were used. Here nitrogenous components were analyzed at 0, 3, 6, 12 and 24 h, and then every 24 h until 144 h.

2.2. polyamines

Putrescine (Put), spermine (Spm) and spermidine (Spd) were extracted from 1 g of fresh tissue by the method of Flores and Galston [7]. The polyamines were determined using HPLC (Pharmacia LKB) equipment, a 4 × 250 mm, 5 µm particle size reverse-phase (C-18) column, and detected at 254 nm, run isocratically at 64% MeOH:H_2O at a flow rate of 1 mL·min^{-1} and 20 µm per injection. Polyamine mixtures were used as patterns at 1 nmol·µL^{-1} concentration and 1,7-diaminoheptano (Merck) as internal standard at the same concentration.

2.3. soluble proteins

In a cold mortar with 2.5 mL phosphate buffer solution (pH 6), 0.25 g of cotyledons were ground and then centrifuged at 12 000 *g* for 10 min. Soluble protein was measured according to the Bradford method [8] using BSA as a standard. An aliquot of 20 µL of supernatant was added to 1 mL of Bradford reagent (100 mg of Coomassie G Blue in 50 mL ethanol 96%, adding 100 mL of H_3PO_4 and up to 200 mL of distilled water). The absorbance was read at 595 nm.

3. results and discussion

Figure 1 illustrates changes in fresh weight of the cotyledons during periods of germination and seedling development of unpeeled mandarine seed expressed as percentage increase in fresh weight. There was an increase in fresh weigth of the seed when water was absorbed by the cotyledon tissues, characteristic of an imbibition process during the initial stages of germination. On the other hand, there was a slight increase in the percentage of germination until 21 d of imbibition. García-Agustín and Primo-Millo [9] described some aspects of this process in *Citrus limon*. There was an initial period of rapid non-metabolic water uptake during the first 48 h. Then followed a second phase of slower absorption which extended from 48 h until the sixteenth day after germination. After 16 d the fresh weight of cotyledons decreased.

In our results the fresh weight of the cotyledons decreased during growth and development of the seedlings. This suggested that endogenous substrates were used in respiratory activity, as well as the movement of reserve substances from cotyledons to seedlings.

As shown in *figure 2*, soluble protein content in cotyledons decreased in germinated unpeeled seed during the whole germination process. On the other hand, non-germinated unpeeled seed increased in soluble protein content with notable differences after 21 d when seedling development started. Proteins are recognised as the major form of nitrogen reserves in citrus seed [9]. During germination of Cleopatra mandarin, seed proteins could be transported from reserve sites to sites with high metabolic activity.

Determination of polyamine patterns (Put, Spm and Spd) was achieved by HPLC under the experimental conditions described. High reproducibility retention time was obtained with polyamine standards (4.4 ± 0.15, 6.3 ± 0.21 and 9.0 ± 0.24 min for Put, Spm and Spd, respectively). These data allowed the identification of unknown peacks in seed extracts by chromatography.

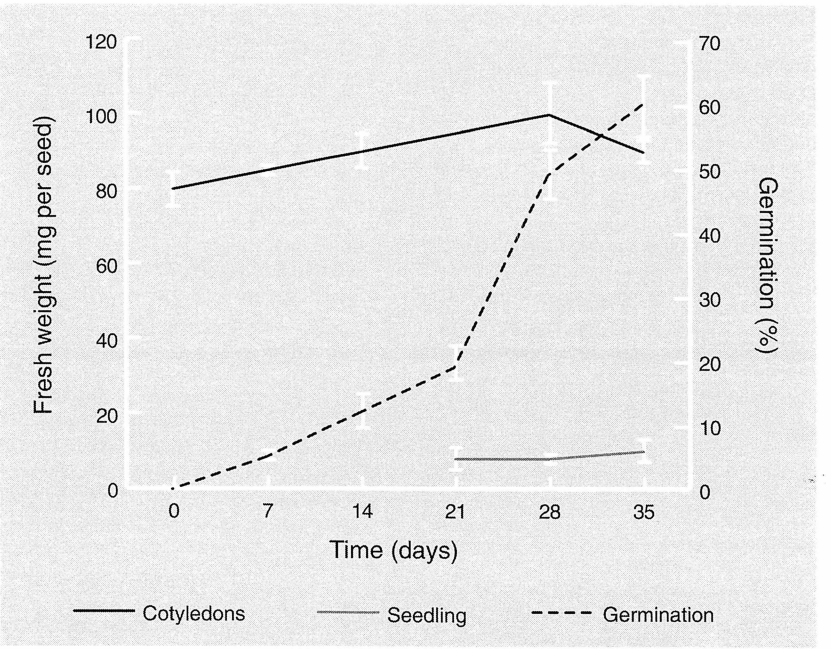

Figure 1.
Changes in fresh weight of cotyledons during germination and seedling development of unpeeled mandarin seed.
Bars represent the standard errors.

During germination, Put and Spd contents decreased in cotyledons of germinated and non-germinated unpeeled seed for 21 d before germination (*figure 3*). Then, in germinated seeds, Put remained at the same concentration until the end of the experiment, and, in non-germinated seeds, it decreased until 28 d, then increased; Spd decreased to a minimum at 28 d and, finally, increa-

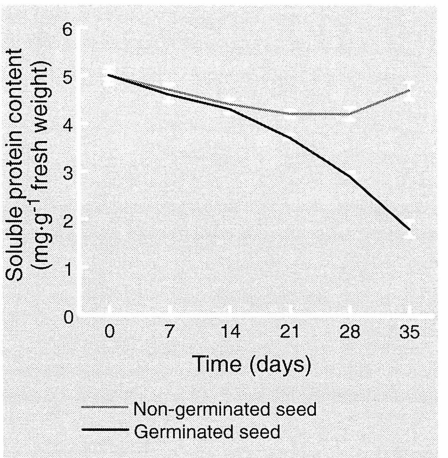

Figure 2.
Soluble protein content in cotyledons of germinated and non-germinated unpeeled mandarin seed.
Bars represent the standard errors.

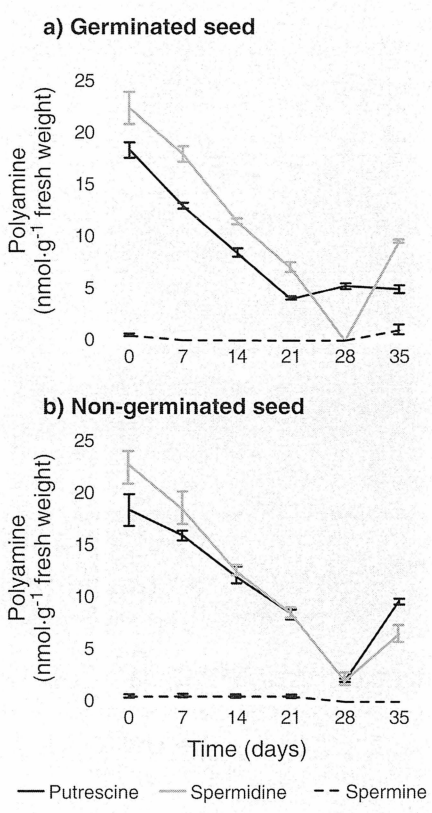

Figure 3.
Polyamine content during germination in cotyledons of germinated (a) and non-germinated (b) unpeeled mandarin seed (Put = putrescine, Spd = spermidine, Spm = spermine). Bars represent the standard errors.

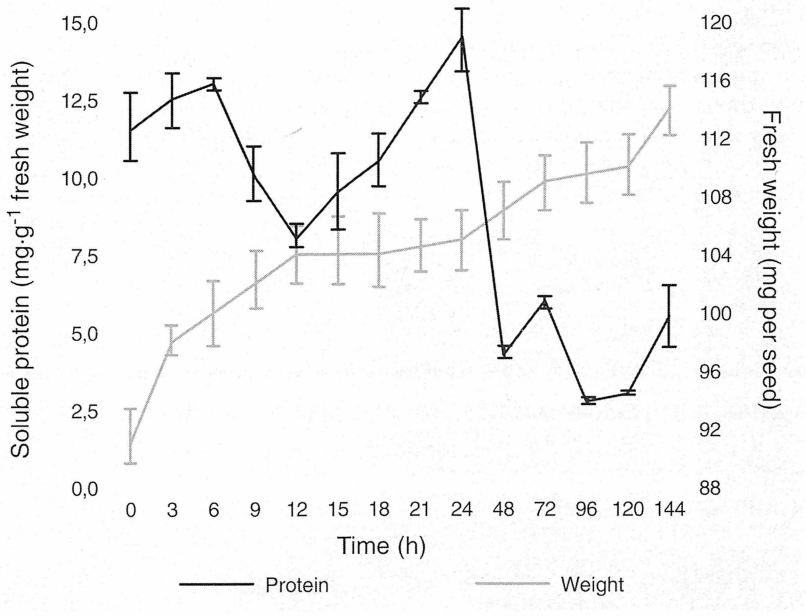

Figure 4.
Changes in soluble protein and seed fresh weight after removal of seed-coat. Bars represent the standard errors.

sed in both germinated and non-germinated seeds. The very low levels of Spm showed no changes during the observation period.

Polyamines increased in the early stages of germination and this was shown to be a critical element of the initiation process in *Phaseolus mungo* [6]. However, the metabolic changes in Cleopatra mandarin that take place during the early stages of germination and the role of the seedcoat in delay of germination are not clear. Therefore, a study at the first moment of germination with mandarine peeled seed was done.

After removal of the seedcoat, protein concentration increased during the first 6 h (*figure 4*), then these levels decreased to a minimum at 12 h of imbibition, which may be associated with the increase of water uptake to seed. Pronounced increases in protein concentrations occurred between 12 h to 24 h. These levels decreased markedly at 48 h and, then, rose slightly after 72 h.

Germination of citrus seeds is characterized by a rapid uptake of water during the first 48 h, which helps in removal of reserve material and the use of these reserves for growth of the axis during germination. The second phase of slower absorption of water by the cotyledons was probably used for the increase in vacuolation when reserves were depleted [1].

There was a continuous increase in water uptake by cotyledons (expressed as fresh weight in mg·seed^{-1}) during all 144 h for peeled seed. The proteins, meanwhile, changed during germination which began at 96 h and reached 56% after 144 h (*figure 5*). Although the experimental conditions were different, germination of unpeeled mandarin seed was similar. Therefore, it is evident that the seedcoat has a retarding effect on citrus seed germination which began at 21 d in unpeeled seed (*figure 1*).

The concentration of polyamines varied during all stages of peeled seed

germination (*figure 6*). Put was present in large amounts during all 144 h of observation. The pattern of changes was different for the three polyamines. Put was the main polyamine, as compared to Spd and Spm, but decreased slightly during the first 15 h. After 6 h, Spd and Spm started to increase reaching a maximum at 24 h, when all three polyamines were at their highest level. All three polyamines decreased during the following 24 h, and, at 96 h, they rose again, Spd and Spm to 5 nmol·g^{-1} fresh weight, while Put reached 14 nmol·g^{-1} f.w.

Villanueva et al. [6] reported the most rapid increase in the content of these particular polyamines with the maximal rates of RNA and proteins synthesis in fast growing cells during the germination process of *Zea mays*, *Phaseoulus vulgaris*, *Tragopogon porrifolius* and *Triticum aestivum*. This could be explained by the stimulatory effect of polyamines on DNA-dependent RNA polymerase activity, mRNA synthesis, rate of amino acid incorporation and peptide elongation. Polyamines could also play a role in a mechanism for the protection of RNA against RNA-ase activity.

Soluble protein content increased during the first 6 h of germination (*figure 7*). Then it decreased to a lower level at 12 h. Then both total polyamines and soluble proteins increased, to their maximum at 24 h. During the next 24 h, their level decreased. Then both compounds increased, with peaks at 72 h and 144 h for soluble proteins, and at 96 h for total polyamines.

In relation to the decrease in protein levels, Nieves et al. [10] found a remarkable change in electrophoresis patterns during Cleopatra mandarin seed germination, with a concomitant increase in growth of the seedlings. Proteins are a main source of nitrogen reserve compound in citrus seed. They appear as protein bodies located in subcellular entities in the cotyledons [9]. The rapid hydrolysis of the protein bodies coincides with protease activity in an autolytic process during reserve removal [11, 12].

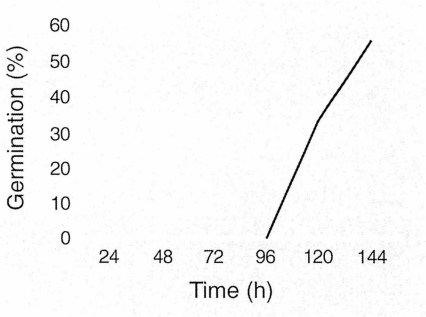

Figure 5.
Behaviour of germination in peeled seed.

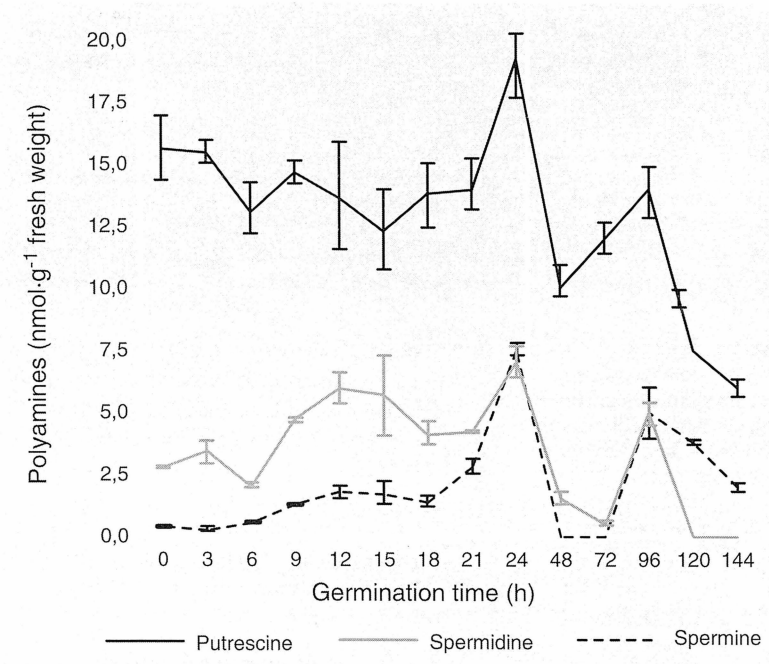

Figure 6.
Changes in concentration of three polyamines (Put = putrescine, Spd = spermidine, Spm = spermine) during germination of mandarin peeled seed. Bars represent the standard errors.

The decrease of three polyamines and soluble protein from 24 h of imbibition evidences the role of this nitrogenous compound in early stages before germination.

4. conclusion

The seedcoat delayed the beginning of the Cleopatra mandarin seed germination by 16 d; this may be associated with the presence of inhibitors in the coat as has been shown before in other

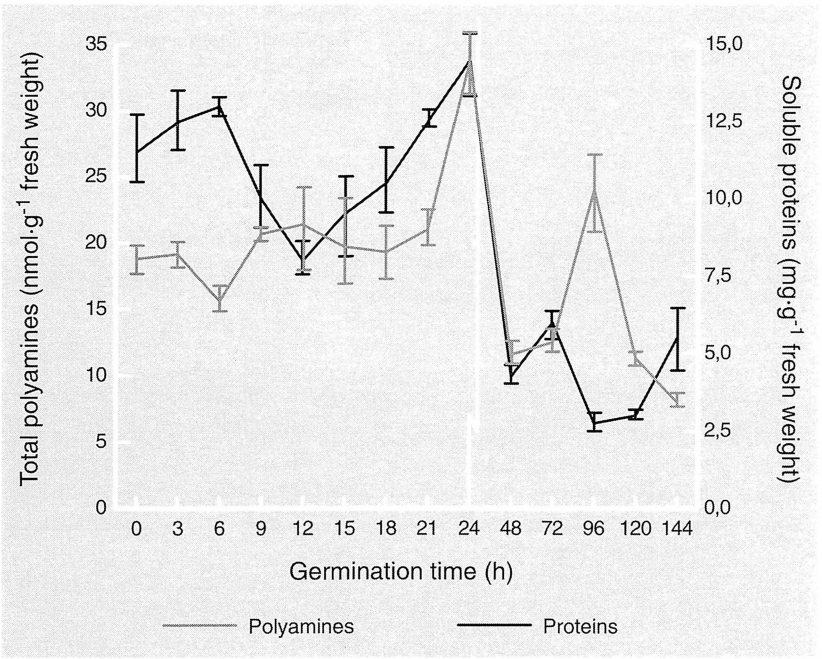

Figure 7.
Changes in total polyamine and soluble protein concentrations during germination of mandarin peeled seed. Bars represent the standard errors.

seeds. The fresh weight of seed increased because of imbibition of the cotyledons, but later decreased when the seedlings began to growth. In contrast to other seeds, the putrescine is the main polyamine during Cleopatra mandarin seed germination. Spermidine and spermine also play a role in the early stages of germination as the pattern of changes shows.

Their joint maximum concentration at 24 h shows that free polyamines and soluble proteins are metabolically related.

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Evolución de la tasa de poliaminas y proteínas solubles en la pepita de cítrico en germinación.

Resumen — Introducción. En todas las células de las plantas hay poliaminas y proteínas que se sintetizan a partir de aminoácidos libres. En muchas especies estas sustancias intervienen de forma significativa en la división celular y en los procesos de diferenciación. Este estudio intenta precisar la evolución de dichas sustancias en la germinación de una pepita de cítrico. **Material y métodos**. Para caracterizar las reacciones bioquímicas que tienen lugar durante la germinación se colocaron unas pepitas de mandarinas maduras (variedad Cleopatra), enteras o sin tegumento, sobre algodón húmedo en caja de Petri. Los compuestos nitrogenados de poliaminas – putrescine (Put), spermine (Spm) y spermidine (Spd) – y de proteínas solubles se dosificaron tras imbibiciones de diversa duración. **Resultados y discusión**. La presencia del tegumento retrasó 16 días la germinación, en ese periodo el peso fresco de los cotiledones aumentó mientras que su tasa de proteína soluble disminuía; en poliaminas cabe señalar que las tasas de Put fueron las más altas y las de Spm las más bajas. En las pepitas sin tegumento, la concentración de proteínas aumentó durante las primeras 6 h, siendo máxima tras 24 h de imbibición y mínima tras 12 h, 48 h y 96 h. La putrescina obtuvo de nuevo la tasa más alta de las tres poliaminas. **Conclusión**. Los resultados obtenidos muestran que las poliaminas y las proteínas podrían desempeñar un papel durante las primeras fases de la germinación. Esto vendría a confirmar la interrelación entre los metabolismos de ambas sustancias (© Elsevier, Paris).

Citrus reshni **/ metabolismo proteico / fisiología vegetal / germinación / semilla**