Safflower (*carthamus tinctorius* l., asteraceae) is an oilseed species with fast seed resource mobilization

O cártamo (*carthamus tinctorius* l., asteraceae) é uma espécie oleaginosa com rápida mobilização de recursos em sementes

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ABSTRACT  
Safflower (Carthamus tinctorius L.) is a crop with wide commercial potential, due to the broad adaptability of cultivation and use conditions, but commercial initiatives are deficient. This scenario extends to academic research, which also focuses little on understanding the involved processes from the germination of its seeds to the correct management of adult plants. In this context, the current study seeks to identify the primary metabolites stored in the safflower seed and to evaluate how the mobilization of these reserves occurs during seed germination and seedling establishment. Safflower seeds of the cultivar S-351 produced in the experimental field of Universidade Federal de Jataí, from the second season of 2017/2018, were used. To obtain the seedlings, safflower seeds were sown in moistened sand with 60% retention capacity and kept at 25 °C. At 1, 2, 3, 4, 5, 8, 15 and 22 days after sowing, the seedlings were subjected to analysis of length and histological characterization of cotyledons, the latter of which also evaluated at 12 h after sowing. Biochemical (carbohydrate, protein, lipid and chlorophyll) and anatomical analyses were performed to assess reserve mobilization, both of which were evaluated at
five germination times (12h, 3, 5, 8, 15 and 22 days after sowing). The data regarding the periods during reserve mobilization were subjected to regression analysis. The main source of reserve in safflower is lipids, followed by carbohydrates and proteins, with reductions of 37% for lipids, 3% for carbohydrates and 0.5% for proteins, during germination and seedling development. The mobilization of reserves of safflower seeds was accompanied by changes of cell features, which became vacuolated and with multiple plastids, and by the synthesis of chlorophyll a, which peaked between 9 and 11 days after sowing, followed by the final reduction caused by senescence.

Keywords: histochemistry, biochemistry, anatomy, oilseed.

1 INTRODUCTION

Safflower (Carthamus tinctorius L., Asteraceae) is an oilseed herb with good adaptability to different climatic conditions (EMONGOR, 2010; KENNEDY et al.,...
2023), and it has become an interesting crop alternative in Brazilian rotation cultivation systems. Safflower has high resistance to lack of water and low relative humidity, adapts to different soil types and tolerates from the highest to the lowest temperatures (BONAMIGO et al., 2013). These characteristics enable safflower cultivation along virtually the whole year and in different regions of Brazil (BONAMIGO et al., 2013), making it an alternative to a well-consolidated system, which is the soybean-corn succession. Despite the high production potential and the high adaptability, safflower is still little relevant in the Brazilian economy, due to the lack of knowledge and scarcity of information about this crop (LIMA et al., 2019; CHAVES NETO et al., 2020). In this context, studies focusing on evaluating the different aspects of germination and production of safflower are promising, which can elucidate questions related to the seed composition, post-embryonic development, quality of its seeds, among others.

The cultivation of safflower occurred by seeds. Therefore, germination is a decisive step towards obtaining a uniform and highly productive crop. At this stage there is a high vulnerability due to the various biotic and abiotic factors that can interfere and influence this process, such as diseases, injuries and stresses (RAIJOU et al., 2012). In the germination process, embryonic cells expand and the seed coat ruptures with the protrusion of the radicle, which is usually the first part to emerge from the seeds in most plant species (MAGALHÃES et al., 2010). Primary metabolites, mainly sugars, proteins and lipids, are usually stored in seeds (BEWLEY, 1997; REN et al., 2007; GALLARDO et al., 2008; SORIANO et al., 2011; AGUIRRE et al., 2018) and assist in the metabolic maintenance of the embryo, besides providing useful energy to the germination process. These reserves normally are synthesized and stored in the cotyledons and/or in the endosperm (BEWLEY, 1997). Seed carbohydrates are mainly represented by oligosaccharides (CIMINI et al., 2015) and polysaccharides (AGUIRRE et al., 2018). Lipids and proteins are stored in seeds in lipid/oleosome and protein bodies (TAN-WILSON and WILSON, 2010; HUANG, 1994). The type of reserve in the seeds and its distribution in the tissues vary depending on the taxon of the plant and the development program of each species.
Seed reserves also promote the development of seedlings after germination (SANTOS and BUCKERIDGE, 2004; MENA-ALÍ and ROCHA, 2005; ERBAS et al., 2016). The carbohydrate reserve mobilization is one of the first biochemical processes after post-imbibition, which proved the amount for respiration (VERMA et al., 2015). Protein and lipid bodies are rapidly consumed for early embryo development too (BEWLEY and BLACK, 2013; GRAHAM, 2008). These reserve mobilization supply energy amount for the seedlings until their total establishment and beginning of the photosynthetic process (SANTOS and BUCKERIDGE, 2004; MENA-ALÍ and ROCHA, 2005; ERBAS et al., 2016). So, the development and establishment of seedlings constitute a step that is directly correlated with the sufficient amount of seed reserves (SANTOS and BUCKERIDGE, 2004; MENA-ALÍ and ROCHA, 2005).

Chlorophyll is responsible for the green color of plants, and determines their photosynthetic capacity, supporting plant development (LI et al., 2018). Usually, total chlorophyll content rapidly increases throughout germination and early seedling development, as reported for Delonix regia (Fabaceae) (BARBOSA et al., 2023). Under seedling development, usually, the chlorophyll a/b ratio is high at the beginning of pigment formation, indicating a faster production of chlorophyll a compared to chlorophyll b (OLIVEIRA et al., 2003). This ratio changes when seedlings are subjected to water stress, and the rate of formation of the chlorophyll a/b-protein complex is also reduced (OLIVEIRA et al., 2003). In this sense, the chlorophyll synthesis monitoring from germination to the establishment of seedlings lets us mark developmental stages, or even demonstrate the influence of stressors.

Safflower is a crop with wide agronomic potential, however, there is little knowledge about germination and seedling development to support large commercial initiatives. In this context, the present study seeks to identify the primary metabolites stored in the safflower seed and to evaluate how the mobilization of these reserves occurs during seed germination and seedling establishment. So, we intend to answer the following questions: (i) What are the main reserves of seeds of the safflower, and how their mobilization is associated with seedling development? And (ii) What is the relationship between reserve mobilization and chlorophyll production?
2 METHODOLOGY

2.1 PLANT MATERIAL AND SEED GERMINATION

Safflower seeds (cultivar S-351) produced in an experimental field of “Universidade Federal de Jataí” - UFJ, placed in Jataí municipally, Goiás state, Brazil. The seed lot was homogenized three times using a soil divider (BRASIL, 2009). The seeds were packed in Kraft paper bags and placed in closed low-density polyethylene plastic bags (45.0 x 35.0 cm) to prevent water loss. They were then placed in an air-conditioned environment with a temperature of 8 ºC and average humidity of 80% until the time of the tests. The test run period was approximately one week after harvest.

2.2 MORPHO-ANATOMICAL ANALYSES DURING THE ESTABLISHMENT OF SAFFFLOWER SEEDLINGS

Subsamples of 25 seeds per treatment were established in sand moistened up to 60% of its retention capacity (BRASIL, 2009) in a transparent plastic box (11.0 x 11.0 x 3.5 cm). These seeds were placed in plastic bags and taken to germination chambers at 25 ºC. Seedlings were collected in the lots from the 1st, 2nd, 3rd, 4th, 5th, 8th, 15th and 22nd day after sowing. Photos from each period were taken and, subsequently, their lengths were measured from the root cap to the insertion of the cotyledons, with the aid of the ImageJ program.

Samples of cotyledons were fixed in FAA70 [formaldehyde, glacial acetic acid, ethyl alcohol 70%] for 24 hours (JOHANSEN, 1940), dehydrated in increasing ethyl series and embedded in methacrylate resin [Leica®, Heidelberg, Germany]. Transverse and longitudinal sections with 5 μm thickness were obtained in rotating microtome [Leica, RM2235] and stained with toluidine blue at pH 6.3 (O’BRIEN et al., 1964). The sections were observed under an optical microscope [Leica, DM750] and documented with digital camera [Leica, ICC50HD].

2.3 HISTOCHEMISTRY

For histochemical characterization, transverse sections of the cotyledons collected during the establishment of safflower seedlings, and included in methacrylate, as
described above, were subjected to lugol, PAS/Schiff reagent (MCMANUS, 1948) and Xylidine Ponceau (VIDAL, 1970) to determine starch, neutral polysaccharides and proteins, respectively. Fresh samples of cotyledons were subjected to Sudan III for the detection of total lipids (JOHANSEN, 1940).

2.4 BIOCHEMICAL ANALYSES

For biochemical characterization, the cotyledon samples were collected in each replicate, from one lot, with 12 hours and on the 3rd, 5th, 8th, 15th and 22nd day after sowing. For each day after sowing, 10 seedling samples were collected. These samples were macerated in liquid nitrogen and stored at -20 °C until quantitative analysis of total soluble sugars (TSS), total lipids, soluble proteins and chlorophyll content, and each assay was performed with three replicates.

Determination of total soluble sugars (TSS) - Fresh sample were submitted to phenol sulfuric method aiming quantify soluble sugar (DUBOIS et al., 1956). Total soluble sugars were quantified at 490 nm using glucose as standard curve.

Determination of total lipids – Using approximately 400 mg of fresh samples, repeated extraction with ethers (petroleum ether and ethyl ether) were performed, according to Roese-Gottlieb method (AOAC, 1990) adapted to plant samples. The lipid content obtained in the extraction was measured by gravimetry.

Determination of chlorophyll content – Fresh samples were submitted to hot ethanol extraction (GIBON et al., 2006). Chlorophyll a and b were determined through the absorbances values at wavelengths of 665 nm and 645 nm, respectively. Chlorophylls content were quantified using equations established by Wellburn (1994). The total chlorophyll was obtained from the sum of chlorophyll a and chlorophyll b concentrations.

Determination of soluble proteins - Soluble protein content was obtained using the insoluble fraction (pellet) of hot ethanol extraction (GIBON et al., 2006). The pellet was submitted to an extraction using KOH 0.2 M and consecutively quantified using Bradford reagent (BRANDFORD, 1976). Spectrophotometer measures were performed at 595 nm, and bovine serum albumin (BSA) was used for standard curve.
2.5 STATISTICAL ANALYSIS

Statistical analysis of the data was performed using AgroEstat statistical program. Initially, the data were subjected to the homogeneity and normality tests. The data regarding the periods during reserve mobilization were subjected to regression analysis. Regression equations up to the second order were fitted and those that were significant and with the highest coefficient of determination ($R^2$) were chosen.

3 RESULTS

3.1 EARLY SEEDLING DEVELOPMENT

Safflower showed a rapid seedling development (Fig. 1). In the period of 22 days, the safflower seedlings were about 30 cm long (Fig. 1A). One day after sowing, radicle protrusion was observed and, at the 3rd day, the cotyledons were already exposed and photosynthetic active (Fig 1B-D). Safflower seedlings showed an accentuated growth in the first four days after sowing, and after 10 days the seedlings already presented 65% of the total length expected (Fig. 1A, E), evidencing the rapid seedling development, mainly in the first week after sowing (Fig 1).
Figure 1. Seedling length (A) and comparison between morphology of safflower seedlings (B-G), highlighting mobilization from 12 hours to the 22nd day after sowing. Bars: B and C = 0.5 cm; D, E, F and G = 1.0 cm.
3.2 CYTOLOGICAL AND HISTOCHEMICAL CHANGES OF COTYLEDONS DURING EARLY SEEDLING ESTABLISHMENT

The safflower cotyledon cells showed a dense cytoplasm filled with reserve compounds (Fig. 2A). This characteristic was maintained 1 day after sowing (Fig. 2B), although hyaline regions were observed in the cytoplasm, evidencing the mobilization of reserves. At this initial stage, the cotyledon cells showed completely blushed by Sudan IV, evidencing the presence of high amount of lipid compounds (Fig. 2C). Large and numerous protein bodies were also identified, by Xylidine ponceau histochemical test (Fig. 2D). Starch grains were not detected by the Lugol test during the entire evaluated period of initial seedling development.

On the second day after sowing, the reserve components begin to show a granular aspect, in addition to the formation of vacuoles that, apparently, were associated with the mobilization of these compounds (Fig. 2E). Three days after sowing, exposure of cotyledons (Fig. 1D) and higher consumption of the reserve substances were noted. At this stage, the cells showed a large vacuole (Fig. 2F). Interesting, lipid bodies coalesced and were concentrated in a single large body (Fig 2G). On the other hand, protein bodies showed completely degraded (Fig 2H).

From the fourth day after sowing, there was an expressive consumption of the reserve content, and a single vacuole that occupied virtually all cell volume (Fig. 2I-O). At eight days after sowing, the safflower seedlings were already robust, with well-developed shoots and root system (Fig. 1E). At this stage, the cotyledon cells show hyaline cytoplasm (Fig. 2M), with small and few lipid bodies (Fig. 2N). Protein bodies were not identified at this stage (Fig. 2O), evidencing that the mobilization of seed reserve components in the first eight days of germination is directly related to seedling development.
3.3 BIOCHEMICAL ANALYSES

The contents of carbohydrates, lipids and proteins significantly reduced during the germination and initial establishment of the seedling. Safflower seeds showed lipid content higher than carbohydrate and protein. At the beginning, the lipid content was
0.3793 mg/mg of fresh matter of cotyledon at the initial germination stage (Fig. 2A), that is, 37.93% in the initial period, classifying the species as oilseed, due to the proportion relative to the other components found. However, the consumption of lipids is quite fast, and this percentage decreased to 14.27% on the 3rd day after sowing and to 0.59% on the 22nd day after sowing, showing a clear reduction during the 22 days of germination (Fig. 3A).

The carbohydrate level was 49.02 mg/g of cotyledon fresh matter (Fig. 3B), which corresponds to 4.90% of the total chemical composition. At 22 days, the carbohydrate content was 17.90 mg/g of fresh matter of cotyledon, corresponding to 1.79%, which indicates that more than 3% of the carbohydrate reserve was consumed (Fig. 3B).
For proteins (Fig. 3C), as for the other reserve components present in the cotyledons, there were significant reductions during germination. Initially, the cotyledons had 6.53 mg/g of fresh matter, corresponding to 0.65%, and it was degraded to 0.21% (2.07 mg/g of fresh matter) on the 22nd day after sowing.

For total chlorophyll, a small amount of this pigment was observed in the initial moments after sowing, with significant increase when the cotyledons open (Fig. 3D). The maximum pigment content was observed 10 days (167.46 μg/mg of fresh matter) after sowing (Fig. 3D). After that, the chlorophyll content drastically reduced, reaching 84.21 μg/mg of fresh matter, after 22 days of sowing (Fig. 3D).

4 DISCUSSION

The results of this research can be a useful step towards understanding the primary stages of germination and seedling formation of safflower. Rapid development in these initial stages is preponderant for the initial stand, since the more uniform it will contribute to the development and management of the crop (CARVALHO and NAKAGAWA, 2012; GAMA et al., 2019; COELHO et al., 2021), as well as studies of tolerance to abiotic stresses (ALASVANDYARI et al., 2017; ZHOU et al., 2022). In the present study, it was observed that germination occurred one day after sowing, which is in agreement with Gama et al. (2019) better evaluating substrates for germination and even in a vigor test with seedling emergence on the third day in the field (COELHO et al., 2021).

Lipids, carbohydrates and proteins have been found as primary reserve compounds in the safflower cotyledons. These are the main constituents of seed reserve (SUDA and GIORGINI 2000; LIMA et al. 2008), and these substances are quickly consumed during the germination process (BEWLEY and BLACK, 2013), which provides the source of energy and substrate for the embryonic axis in the formation of cellular structures, especially in germination, which is the crucial point of seedling establishment (CAMARGOS et al., 2013), as reported here. The resource mobilization is mediated by enzymatic activity that breaks down complex compounds into simple molecular structures, which can then be translocated to developing organs where they turn into energy for tissue formation (CARVALHO and NAKAGAWA, 2012).
The mobilization of reserves in safflower cotyledons is associated with rapid degradation of lipid and protein bodies up to three days after sowing, which change of cell features to vacuolated and with multiple plastids. The formation of a central compartment, which steadily becomes a large vacuole, was reported for cotyledon cells in the differentiation of olive (*Olea europaea*), after continuous mobilization of protein (ZIENKIEWICZ et al. 2011). The large vacuole formation seems to be a natural process during seed germination that may be associated with the incidence of an intense proteolytic activity addressed to protein body mobilization (ZIENKIEWICZ et al. 2011), as well as a usual phenomenon reported for plant cell maturation (DE, 2000). The vacuole formation and expansion can be a labeling of the typical change of cotyledon role, from stock to photosynthesis, as normally reported for seeds (OSMOND et al., 1980), once there is a chloroplast production parallel to that process.

The safflower may be classified as oilseed species, similar to Euphorbia heterophylla (Euphorbiaceae) that presents lipids as the most abundant reserve in its seeds, comprising 60% of the content in dry seed (SUDA and GIORGINI 2000), as well as sunflower (*Helianthus annuus*), soybean (*Glycine max*), and rapeseed (*Brassica napus*) (REALE et al. 2012). In oilseeds, the triacylglycerols are the main source of energy during germination and development of seedlings (GRAHAM, 2008), losing their oil content almost completely during germination and initial growth of seedlings. For safflower seeds, Abud et al. (2010) reported lipids as an important constituent, as demonstrated in the current study. These reserves usually occur in the triglyceride form, however, they may also be present in the glycolipid and phospholipid forms (ABUD et al., 2010). During germination, the lipids are hydrolyzed and converted to sucrose by β-oxidation and glyoxalate cycle, thus providing energy and carbon skeletons to the seedling formation (THEOUDOULOU and EASTMOND, 2012).

The carbohydrates were the second most abundant reserve in the safflower cotyledon, which indicates its less involvement in seed reserve mobilization and seedling establishment, as also reported for *Cereus jamacaru* (Cactaceae) (ALENCAR et al., 2011). Starch grains are the most reported type of carbohydrate for seeds, but they were not found here. This may indicate the presence mainly of soluble sugars in the safflower
seeds, as shown for E. heterophylla, which presented soluble sugars that comprised approximately 4% of the seed dry mass, with also no detection of starch (SUDA and GIORGINI, 2000). The presence of starch is often associated with cell differentiation and accumulation of reserves (WEBER et al. 1997). Therefore, the absence of this compound in the period studied is compatible with the rapid growth and initial development of safflower seedlings. There was a gradual reduction of carbohydrates in the safflower cotyledons throughout germination and seedling development, a similar reduction was observed in the germination of seeds of jatropha, another oilseed crop (LOPES et al., 2013).

The proteins were the less abundant reserve compounds in the safflower seed, represented by protein bodies mainly visualized inside the cells at the beginning of the reserve mobilization process. A similar result was detected by Ataíde et al. (2017), in Melanoxylon brauna seeds, which showed a trend of mobilization of proteins. The more intense mobilization of proteins in the post-germination period in Eudicotyledons, such as safflower, can be explained mainly by the reduction in pH in the vacuoles due to the introduction of H+ ions after germination, leaving the medium favorable to protease activity (BEWLEY et al., 2013). Proteins stored in seeds are hydrolyzed to free amino acids during germination, which may be a product for the synthesis of new chemical compounds necessary for seedling development (TAN-WILSON and WILSON, 2012).

For safflower cotyledon, its exposure to light was essential for new chloroplast formation and the changing in function. The well-developed chloroplasts in cotyledons at the time of germination may be capable of producing energy (ATP and NADPH), which may accelerate the synthesis of new macromolecules after seed germination (VOZNESENSKAYA et al., 2003). So, the chlorophyll increased after germination in safflower cotyledon, supporting carbohydrate synthesis and energy generation until the cotyledon started senescent, and, then, the concentration of this pigment fell. The chlorophyll decomposition during senescence is part of plant development and leads to the accumulation of colorless catabolites, considered the final products of chlorophyll degradation (STREIT et al., 2005).
5 CONCLUSIONS

The main source of reserve in safflower is lipids, followed by carbohydrates and proteins, with reductions of 37% for lipids, 3% for carbohydrates and 0.5% for proteins, during germination and seedling development. The mobilization of reserves of safflower seeds was accompanied by changes of cell features, which became vacuolated and with multiple plastids, and by the synthesis of chlorophyll a, which peaked between 9 and 11 days after sowing, followed by the final reduction caused by senescence.

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