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ANTIOXIDANT SYSTEM IN *BOEA HYGROSCOPICA*: CHANGES IN RESPONSE TO DESICCATION AND REHYDRATION

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Key Word Index—*Boea hygroscopica*; Gesneriaceae; desiccation tolerance; resurrection; ascorbate/glutathione cycle; soluble proteins.

Abstract—In leaves of *Boea hygroscopica* subjected to either rapid or slow dehydration and rehydration, the response to H_2O_2 production was studied by monitoring the changes in the amounts of ascorbic and dehydroascorbic acids as well as the amounts of reduced and oxidized glutathione and related enzyme activities. *Boea hygroscopica* is a resurrection plant in which drought tolerance depends on the rate of desiccation. For this reason, excised leaves of *B. hygroscopica* were subjected either to rapid or slow dehydration, by a 144-hr exposure to 0% or 80% relative humidity, respectively. Slowly dried leaves, rehydrated for 36 hr, were able to resume their activities completely, while rapidly dried leaves were not able to revive. After dehydration, H_2O_2 production decreased both in slowly and in rapidly dried leaves, whereas the levels of total ascorbate and glutathione became two and 50 times as high, respectively, as in the control. During slow drying, reduced glutathione was oxidized, since its content in slowly dried leaves was 50% lower than in rapidly dried leaves. The levels of soluble proteins were found to be, respectively, five and six times as high in rapidly and slowly dried leaves as in the control and rehydrated leaves. The electrophoretic patterns of soluble proteins were similar in both dehydrated leaves, but different from control and rehydrated leaves, which also presented similar patterns. The specific activities of dehydroascorbate reductase (EC 1.8.5.1), glutathione reductase (EC 1.6.4.2) and ascorbate peroxidase (EC 1.11.1.11) also had the same values in both slowly and rapidly dried leaves. Following rehydration, oxidative processes were intensified: H_2O_2 levels and the oxidation of ascorbate and glutathione mostly increased during this period in which the functioning of the ascorbate/glutathione cycle was induced.

INTRODUCTION

Most desiccation-tolerant species show definite limits of tolerance beyond which tissue damage or death occurs. In these plants, drought injury has been variously attributed either to excessively rapid dehydration or rehydration, or to exposure to an extremely dry atmosphere or prolonged air dryness [1].

The resurrection of *Boea hygroscopica*, as already observed in *Borya nitida* [2], seems to be linked to the rapidity of the dehydration process. It has been observed that slowly dried leaves (80% relative humidity) are able to survive air dryness in a physiological state called anabiosis; on the contrary rapidly dried leaves are not able to revive [3].

Details of the mechanisms involved both in tolerance and in the initiation of damage are scanty. However, in *Sporobolus stapfianus*, a resurrection plant able to survive air dryness following rapid water loss (0% relative humidity), we found that desiccation injury may mainly occur during rehydration when oxidative processes intensify, while during desiccation, defence enzymes increased or, at least, remained at high levels [4].

Desiccation tolerance may depend on the plant's ability to process species of activated oxygen. It is known that activated forms of oxygen are formed by univalent electron reduction of dioxygen in water deficit conditions, when there is a reduction of energy dissipation through carbon fixation and an increase in the relative allocation of photosynthetic electron transport to O_2 [5].

The possibility that desiccation tolerance may depend on the plant's ability to process species of activated oxygen is particularly interesting in the case of *B. hygroscopica*. The pecularity of this resurrection plant is to acquire drought tolerance depending on the rate of water loss. For this reason, knowledge of the changes of the substrates and the enzymes of the ascorbate/glutathione cycle in both slowly and rapidly dehydrated leaves, in comparison with control and rehydrated leaves, might give us a better comprehension of the molecular aspects of metabolic reactivation following desiccation.

RESULTS

Rapid drying led to extremely low water contents within 48 hr (0.3% RWC) with very little further change,

whereas slow desiccation took 72 hr to determine a constant value of less than 2% RWC (Fig. 1A). Rapidly dried leaves were not able to revive, whereas slowly dried leaves recovered their normal metabolic status within 36 hr (Fig. 1B).

After dehydration, H_2O_2 decreased (Fig. 2) in both slowly and rapidly dried leaves, reaching *ca* half the value of the control leaves. After rehydration, the H_2O_2 level increased by 46% in comparison with the control leaves.

After drying, both slowly and rapidly dried leaves showed increased synthesis of ascorbate (Asc) (Fig. 3): total ascorbate (Asc + DHA) and Asc were, respectively, *ca* two and four times as high as in the control, and the dehydroascorbate (DHA)/Asc ratio was less than half the control value. After rehydration, all the ascorbate forms and the DHA/Asc ratio returned to control values.

After dehydration, the increase in total glutathione (GSSG + GSH) (Fig. 4) was of the same magnitude in rapidly and slowly dried leaves (50 times as high as in the control leaves). However, although reduced glutathione (GSH) increased both in slowly and rapidly dehydrated leaves, the increase after rapid drying was 50% higher than in slowly dehydrated leaves.

In control leaves the oxidized glutathione (GSSG) was present in non-detectable amounts, while it dramatically increased after slow dehydration and even more so after rehydration, which led to an increased GSSG/GSH ratio (Fig. 4).

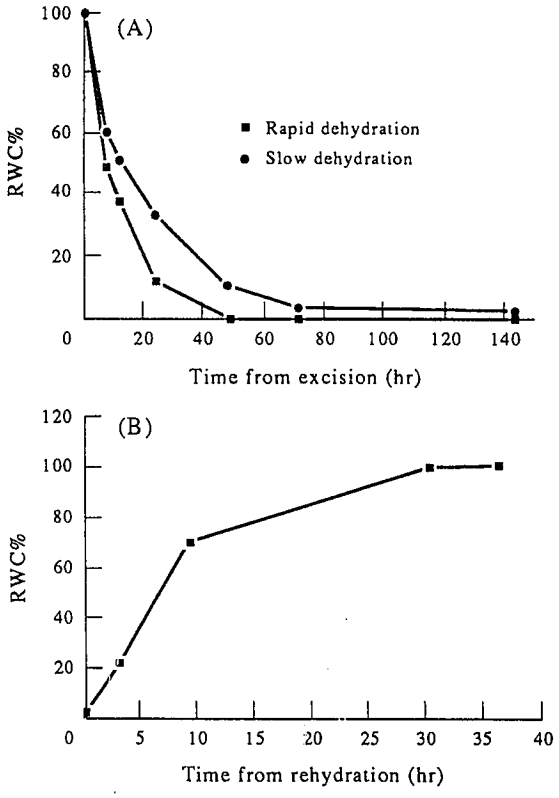

Fig. 1. (A) Dehydration curves of leaves of *B. hygroscopica* subjected to slow (80% relative humidity) or rapid drying (0% relative humidity); (B) rehydration curve of slowly dried leaves.

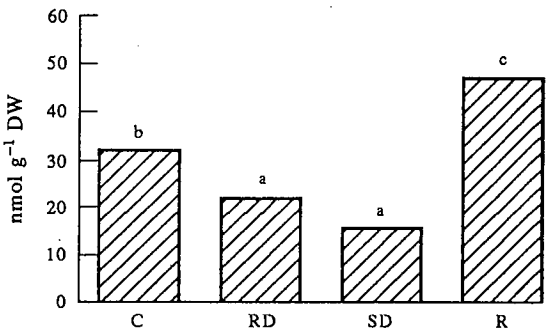

Fig. 2. H_2O_2 contents in leaves of *B. hygroscopica* following slow or rapid drying and rehydration. Results are the means of five replicates of three separate samples. For comparisons among means, analysis of variance was used. Histograms accompanied by different letters are significantly different at $P \leqslant 0.01$. C, control; RD, rapidly dried; SD, slowly dried; R, rehydrated.

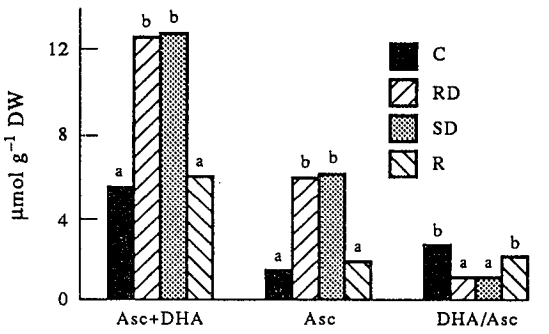

Fig. 3. Ascorbate (Asc) and dehydroascorbate (DHA) contents in leaves of *B. hygroscopica* subjected to slow or rapid drying and rehydration. Results are the means of five replicates of three separate samples. For comparisons among means, analysis of variance was used. Histograms accompanied by different letters are significantly different at $P \leqslant 0.01$. C, control; RD, rapidly dried; SD, slowly dried; R, rehydrated.

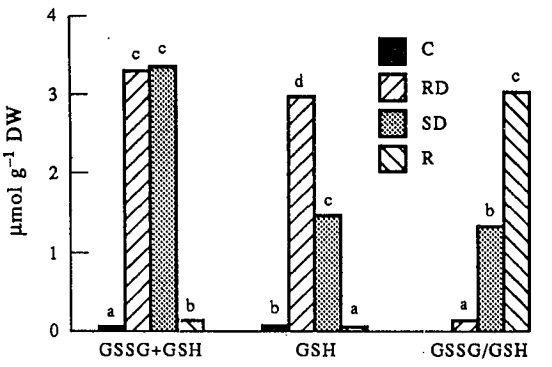

Fig. 4. Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents in leaves of *B. hygroscopica* subjected to slow or rapid drying and rehydration. Results are the means of five replicates of three separate samples. For comparisons among means, analysis of variance was used. Histograms accompanied by different letters are significantly different at $P \leqslant 0.01$. C, control; RD, rapidly dried; SD, slowly dried; R, rehydrated.

Table 1. Effects of slow or rapid desiccation and rehydration on soluble protein contents (mg g^{-1} dry wt) and the specific activities (nkat mg^{-1} protein) of the enzymes of ascorbate/glutathione cycle extracted from *B. hygroscopica*

Treatment	Glutathione reductase	Ascorbate peroxidase	Dehydroascorbate reductase	Soluble proteins
Control	8.3 b	5.5 a	2.5 b	10.3 b
Rapidly dried	2.3 a	5.5 a	0.2 a	51.7 c
Slowly dried	2.8 a	4.7 a	0.3 a	61.7 d
Rehydrated	8.2 b	7.7 b	8.3 c	7.7 a

Values are means of five replicates of three separate samples. For comparisons among means, analysis of variance was used. Means in columns followed by different letters are significantly different at $P \leqslant 0.01$.

The levels of soluble proteins in rapidly and slowly dried leaves were found to be, respectively, five and six times as high as in the control leaves. Differences were also evident between control and rehydrated leaves: soluble proteins were present in a smaller amount in the rehydrated leaves (Table 1). The electrophoretic patterns of soluble proteins were similar in both dehydrated leaves. After rehydration, dried leaves had the same electrophoretic pattern observed in control leaves. The polypeptides more affected by dehydration were the M_r 59.5, 29.8 and 21.0×10^3 bands, which doubled in comparison with the control. The 30.6 and 27.0×10^3 bands were also affected: their amount decreased with water depletion by *ca* 60% (Fig. 5). All of these polypeptides resumed their original values after rehydration.

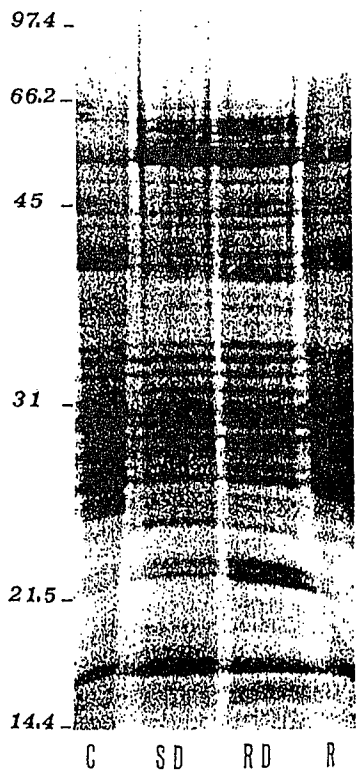

Fig. 5. SDS–PAGE of soluble proteins in leaves of *B. hygroscopica* subjected to slow or rapid dehydration and rehydration. C, control; SD, slowly dried; RD, rapidly dried; R, rehydrated. M_rs of polypeptides are indicated on the left.

The specific activities of the enzymes of the ascorbate/glutathione cycle in slowly and rapidly dried leaves were not different. After the 24 hr rehydration period, glutathione reductase (GR, EC 1.6.4.2) increased up to control value, while ascorbate peroxidase (EC 1.11.1.11) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) were, respectively, 1.4 and 3.3 times as high as in the control (Table 1).

DISCUSSION

After dehydration of *B. hygroscopica*, even if the reduction in H_2O_2 levels is the same in slowly and rapidly dried leaves, the plant's ability to control the increase in the production of activated oxygen may be one of the most important mechanisms in the resurrection of this plant. Chowdhury and Choudhuri [6] found a correlation between H_2O_2 metabolism and water stress tolerance in two species of jute plants. They observed higher activities of catalase (EC 1.11.1.6) and superoxide dismutase (EC 1.15.1.1) and lower H_2O_2 contents in *Corchorus capsularis* in comparison with *Corchorus olitorius*, which is a jute more sensitive to water stress in comparison with the former.

Antioxidants such as Asc and GSH which accumulated during drying (Figs 3 and 4) might constitute a reserve which allows *B. hygroscopica* to tolerate oxidative damage during desiccation, and during rehydration, when the injury caused by desiccation must be repaired. Furthermore, the decreased levels of H_2O_2 after drying are accompanied by a reduced formation of $O_2^{\cdot-}$ by illuminated thylakoid membranes (Navari-Izzo *et al.*, pers. obs.) Both ascorbate and glutathione are water soluble antioxidants involved in the removal of H_2O_2 through the enzymic NADPH/glutathione/ascorbate cycle. Ascorbate may also directly reduce $O_2^{\cdot-}$, quench $^1O_2$ and regenerate reduced α-tocopherol [7].

A resurrection plant can avoid desiccation injury either through induction of synthesis of Asc, as in *B. hygroscopica* (Fig. 3), or through degradation, as in *S. stapfianus* [4]. The capacity of such plants to maintain low DHA/Asc ratios through regeneration of Asc is probably more important than the absolute amount of total ascorbate present during drying.

Glutathione seems to play a very important role in the resurrection of *B. hygroscopica*. During slow drying, glutathione (Fig. 4) may protect enzymes which possess

exposed thiol groups and, through its ability to reduce DHA, it may function in the O_2^- and H_2O_2 scavenging system. The oxidation of GSH during slow drying may be very important also for the protection of plasma membrane from lipid peroxidation. Indeed, *Tortula ruralis* subjected to rapid drying showed visible injury and a greatly enhanced electrolyte efflux in comparison with a slowly drying moss. The damaging effects of rapid drying could be eliminated either by partial desiccation for 1–3 hr before rapid drying, or by placing the dry moss in a 100% relative humidity atmosphere for 1–5 hr before rehydration [1].

Different mechanisms seem to be involved in the resurrection of drought-tolerant plants. One of these is evident in the ability of *B. hygroscopica* to increase the small amount of the constitutive GSH up to 50 times following dehydration. During slow dehydration, inducted synthesis of GSH allows *B. hygroscopica* to reach the same GSH contents previously found in leaves of *S. stapfianus* [4]. Thus, as *B. hygroscopica* is dehydrated, soluble enzymes and membrane components might be protected by solutes that accumulate within the cells, such as ascorbate and glutathione.

Desiccation injury might mainly occur during rehydration, when the plant recovers its catabolic and/or anabolic activities. During this period, oxidative processes intensify and *B. hygroscopica* seems to be more exposed to the attack of activated species of O_2. Indeed the levels of O_2^- (Navari-Izzo, F., 1993, pers. obs.), H_2O_2 (Fig. 2) as well as the oxidation of Asc and GSH (Figs 3 and 4) mostly increased during this period, as we had previously observed in *S. stapfianus* [4].

Also in rapidly dried *T. ruralis*, changes in the ultrastructure of the chloroplast membrane became evident when the tissue was rehydrated. Nevertheless, the ultrastructure returns to normal within 24 hr [1].

The soluble protein accumulation of rapidly and slowly dehydrated leaves in *B. hygroscopica* differs both from non-resurrection plants, which undergo a reduction in protein synthesis and/or an increased protein hydrolysis during dehydration [8], and from the resurrection plant *S. stapfianus*, in which dehydrated leaves show reduced levels of soluble proteins as well as total ascorbate and glutathione [4].

Changes in the composition of soluble proteins after dehydration were found in a great number of species, both resurrection and non-resurrection; in *Borya nitida* the induction of desiccation tolerance appeared to be associated with retention or restoration of the pattern of protein bands present in the control [2]. Therefore, the resurrection process in *B. hygroscopica* may also depend on the resumption of the control pattern of soluble protein bands (Fig. 5).

Desiccation tolerance may depend on an alteration of the protein composition, i.e. accumulation of proteins which exert a protective function in different cell compartments. Upon desiccation, the resurrection plant *Craterostigma plantagineum* accumulates proteins with substantial homologies to proteins abundantly expressed during embryogenesis. Many of these proteins are hydrophilic; for this reason the hydroxyl groups on the surface of polypeptides may be substitutes for water [9]. The role of hydrophilic proteins following dehydration is also evident in sunflower seedlings, whose thylakoid membranes seem to be protected by a higher hydrophilic to hydrophobic protein ratio [10].

After desiccation, defence enzyme activities such as DHAR and GR decreased, probably due to the accumulation of GSH and Asc. During rehydration, a higher production of toxic oxygen species and the oxidation of antioxidants stimulated the activity of enzymes of the ascorbate/glutathione cycle (Table 1).

The activation of this defence mechanism may thus play an important role in the resurrection of *B. hygroscopica* as well as in the regeneration of cellular Asc and GSH at the end of the resurrection period.

From the data collected in the present experiment we can conclude that the oxidation of GSH to GSSG during dehydration and the activation of the ascorbate/glutathione cycle during rehydration are two of the principal mechanisms involved in the resurrection of *B. hygroscopica.*

EXPERIMENTAL

Plant material. Boea hygroscopica plants, from seeds originally provided by B. J. Wallace (Royal Botanic Gardens, Sydney), were grown in clay pots on leaf mould in a controlled environment using a light intensity of 120 μmol m^{-2} sec^{-1}, 85–95% rel. hum., constant day/night temperature of 27° and a photoperiod of 16 hr.

Leaves freshly detached from well watered plants were either: (i) used as control leaves; or (ii) subjected to rapid dehydration in sealed 1-l jars over silica gel (0% rel. hum.) or (iii) subjected to slow dehydration in sealed 1 l jars over NH_4NO_3 satd soln (80% rel. hum.), both for 144 hr. Dried leaves were then rehydrated for 36 hr. Rapidly dried leaves were not able to revive, whereas slowly dried leaves appeared green and healthy. The environments with different rel. hum. were obtained as previously reported [4, 11]. The relative water contents (RWC) were calculated as in ref. [4].

Enzyme extractions and assays. All conditions used during extractions assays were as in ref. [4]. Ascorbate peroxidase and DHAR activities were assayed as in ref. [4]. The standard assay for GR by the reduction of GSSG by NADPH, as previously reported for *S. stapfianus* [4], was not suitable for *B. hygroscopica* because of the presence of other NADPH oxidizing enzymes. GR activity was determined by a more specific assay based on the increase in A at 412 nm, when 5,5′-dithio-bis-nitrobenzoic acid is reduced by GSH to produce 2-nitro-5-thio benzoic acid [12]. The assay mixture maintained at 25° contained 0.2 M NaPi buffer pH 7.5, 0.5 mM Na_2EDTA, 0.75 mM DTNB (in 0.01 M NaPi buffer pH 7.5), 0.1 mM NADPH, 0.005 mM GSSG and 50 μl enzyme extract in a 2 ml final vol. The reaction was initiated by GSSG addition.

Proteins were determined according to ref. [13], using bovine serum albumin as a standard.

GSH and GSSG determination. Total glutathione (GSH + GSSG) and GSSG were determined as in ref. [4].

Asc and DHA determination. Asc and total ascorbate (Asc + DHA) were determined as in ref. [4].

Hydrogen peroxide determination. H_2O_2 contents were evaluated following the method of ref. [14], as in ref. [4].

Electrophoretic analysis. SDS–PAGE was performed according to ref. [15] using 12% acrylamide. The gels were run at 20° with a constant current of 35 mA gel^{-1}. Gels were stained with 0.2% Coomassie Blue R-250 in 50% MeOH and 7% HOAc. The amount of protein loaded on each lane was 80 μg, and molecular mass standards were: phosphorylase b (97.4×10^3), bovine serum albumin (66.2×10^3), ovalbumin (45×10^3), carbonic anhydrase (31×10^3), soybean trypsin inhibitor (21.5×10^3) and lysozyme (14.4×10^3). Densitometric analyses of gels were performed with a Vernon P.I.6 apparatus.

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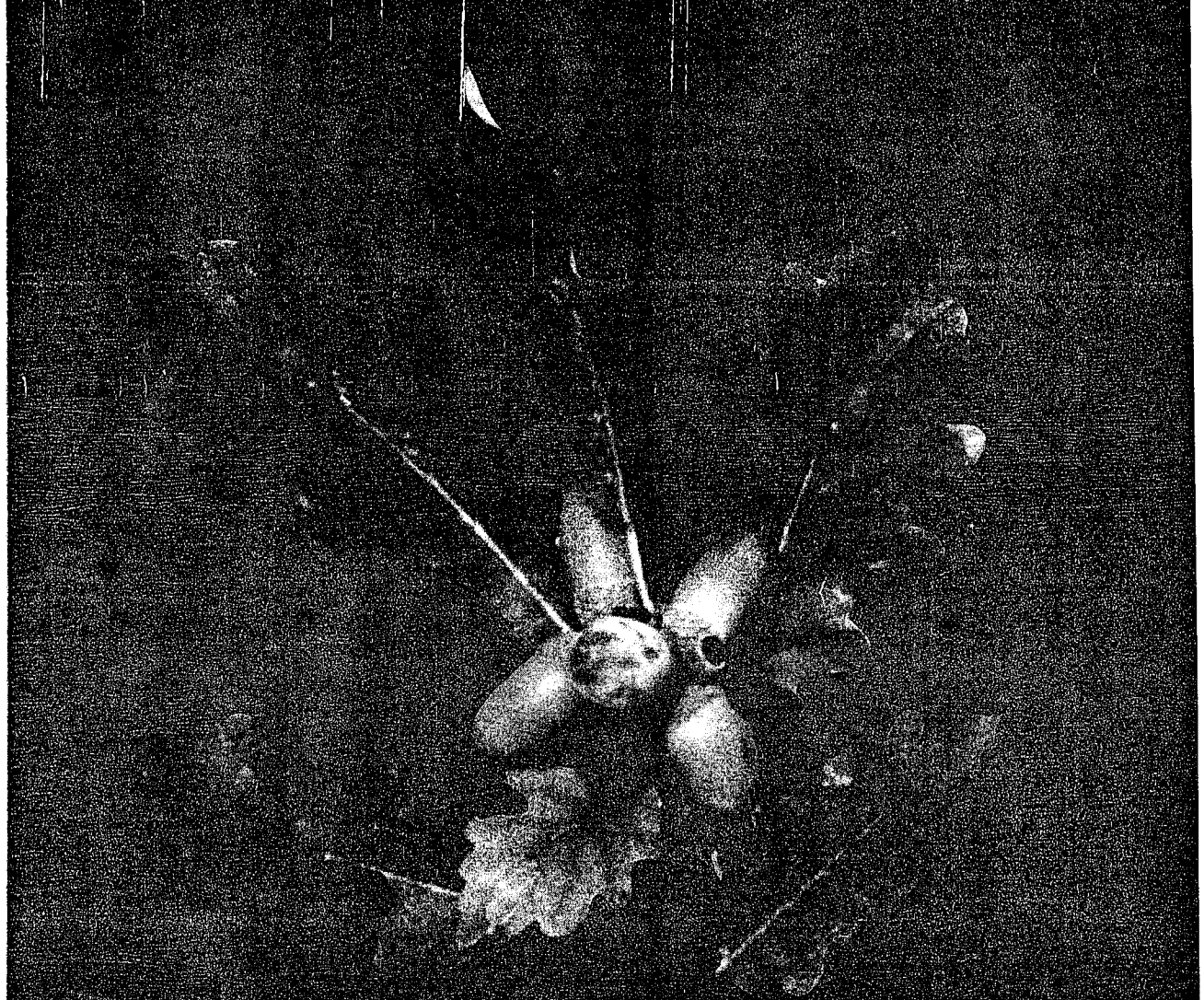

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