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The contents are arranged by class of compound within each section, e.g. primary metabolites, terpenoids, phenolics, alkaloids.

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CHANGES OF LOW-MOLECULAR WEIGHT SUBSTANCES IN *BOEA HYGROSCOPICA* IN RESPONSE TO DESICCATION AND REHYDRATION

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Key Word Index—*Boea hygroscopica*; Gesneriaceae; leaves; poikilohydrous; desiccation-tolerant; resurrection; water stress; low-M_r compounds.

Abstract—*Boea hygroscopica* is a poikilohydrous plant. This and similar species are also called desiccation-tolerant or resurrection plants. They have the unique ability to revive from an extreme air-dry condition. Samples of fresh, dried and rehydrated leaves were analysed for the major classes of organic substances like sugars, alditols, inositols, fatty acids, amino acids, betaines, phytosterols and others. The compounds which accumulated in desiccated leaves were sucrose and polyunsaturated C_{18} acids. Abietanes and pimaranes were also detected. Monosaccharides, which are present in fresh leaves, disappeared, whilst phytosterols, stearic and oleic acids decreased to low levels; palmitic acid was unchanged. Rehydration of dried leaves seemed to restore the chemical composition of unstressed leaves. Betaines were not detected in any of the plant samples examined.

INTRODUCTION

Plants that have the ability to survive dehydration down to air-dryness and beyond and that revive on rehydration are termed resurrection plants and are often poikilohydrous, that is their water content closely follows the fluctuation in the dryness of the environment [1–3]. Several cellular processes have been suggested to explain the ability of resurrection plants to survive extreme changes in water content and some interesting information is available regarding the physiological and biochemical changes occurring in numerous desiccation tolerant species. However, only a few papers have appeared reporting detailed studies on the phytochemical changes induced in resurrection plants subjected first to water stress and then allowed to recover. Most recently efforts are being made to identify genes involved in the desiccation tolerance processes [4] and induction of desiccation tolerance during embryogenesis [5].

This study explores the changes of metabolites in fresh, dehydrated and recovered leaves of a resurrection plant species. The amount and composition of a wide range of low-M_r substances present in intracellular solutes of leaves of the desiccation tolerant plant *Boea hygroscopica* is considered in response to fluctuations in the dryness of the environment. The major solutes components considered were monosaccharides, disaccharides, inositol, alditols, free fatty acids and phytol.

RESULTS

General composition of extractives

The amounts and composition of several groups of compounds in fresh, dried and recovered leaves of *B. hygroscopica* are shown in Table 1. The classes of compounds common to all leaf specimens were free fatty acids, amino acids, phytosterols and sugars. The main effect of desiccation is a marked increase of free fatty acids and sugars. Furthermore, phytol, not present in fresh leaves, was found in remarkable amounts in dried and recovered leaves. A trend to restore the original compositional pattern of the low-M_r substances in the investigated tissue was found after leaf recovery from desiccation.

Free fatty acids

The quantity of free fatty acids in fresh leaves corresponded to one-fifth of that of dried material and to two-thirds of that of leaves recovered from desiccation over K_2CO_3 (Table 1). Chain lengths of fatty acid molecules were similar in all samples examined. However, noticeable changes were found in the degree of unsaturation and chain length distribution, particularly for C_{16} and C_{18} acids.

Free fatty acids from neutral ethylacetate extracts (Table 2) have the following major features: (i) the per cent amount of palmitic acid was unaffected by desiccation and remained the same in the recovered leaves; (ii) noticeable changes were found for the C_{18} series of acids: thus, fatty acids from dried and recovered leaves contained only 2% of 18:0 compared to the value of 16% in the fresh material; oleic acid, 10% in fresh leaves, dropped to values as low as 3–4% after desiccation; (iii) linoleic and linolenic acids increased from 18 and 5% to 47–50 and 18–29%, respectively, in the dried and recovered leaves.

The quantitative data of acids from the aqueous solutions, previously extracted with ethyl acetate at pH 7 then lowered to pH 2 by addition of HCl are reported in

Table 1. Mean yields of classes of compounds found in the methanol–water (7:3) extractives of fresh, dried and recovered leaves of *B. hygroscopica* (mg g^{-1} fr. wt)

Components	Fresh leaves	Dried leaves*	Rehydrated leaves from desiccation over K_2CO_3	Rehydrated leaves from desiccation over LiCl
Free acids	0.43	2.14†	0.65	0.43
Phytosterols	0.29	0.04	0.04	0.06
Phytol	—	0.21	0.04	0.03
Sugars and polyols	6.43	30.70	12.80	2.00
Free amino acids	0.27	0.18	0.13	0.01

*Values refer to the weight of original fresh leaves.
†This fraction included abietane and pimarane diterpenes (see text).

Table 2. Composition (%) of free fatty acids from neutral ethyl acetate extraction of the *B. hygroscopica* samples

Fatty acid	Fresh leaves	Dried leaves	Rehydrated leaves from desiccation over K_2CO_3	Rehydrated leaves from desiccation over LiCl
12:0	1±0.2	tr	tr	tr
14:0	4±0.3	tr	1±0	1±0
16:0	21±2.0	19±4.0	22±1.5	25±3.0
18:0	16±1.0	2±0.3	2±0.5	2±1.4
18:1	10±0.6	3±0.5	3±1.0	4±1.5
18:2	18±2.0	47±3.0	50±3.0	48±4.0
18:3	5±1.0	29±2.0	21±1.7	18±2.0
20:0	14±0.5	tr	tr	tr
22:0	6±0.3	tr	tr	tr
24:0	2±0.4	tr	1±0	2±0
26:0	tr	tr	tr	tr
28:0	2±0.4	tr	tr	tr
30:0	1±0.2	tr	tr	tr

Table 3. Composition (%) of free fatty acids from acidic (pH 2) ethyl acetate extraction of *B. hygroscopica* samples

Fatty acid	Fresh leaves	Dried leaves	Rehydrated leaves from desiccation over K_2CO_3	Rehydrated leaves from desiccation over LiCl
12:0	1±0.2	tr	tr	1±0
14:0	1±0.1	tr	2±0.5	2±0.7
16:0	18±2.0	49±3.0	32±3.5	51±2.5
18:0	42±3.0	7±0.5	3±1.0	16±2.0
18:1	6±0.5	8±0.8	3±0.9	10±1.0
18:2	24±0	24±2.0	44±2.5	6±0.3
18:3	3±0.7	9±1.0	16±0.6	7±0
20:0	1±0.2	1±0.2	tr	1±0.4
22:0	3±0.5	1±0.1	tr	3±1.0
22:1	tr	1±0.5	tr	3±0.4
24:0	tr	tr	tr	tr

Table 3. The chain length range was similar to that of the acids of the neutral extract, whereas relevant compositional changes were observed in the C_{16} and C_{18} acid series. Palmitic acid (16:0), 18% in the fresh leave sample, increased to 32–51% in the dried and recovered leaves. Stearic acid (18:0), the dominant component in the control leaves, was found, under the same conditions, to be greatly reduced in percentage. Oleic acid (18:1) levels

apparently did not show significant variations. while the changes observed in rehydrated leaves for linoleic acid (18:2) were marked. As regards linolenic acid (18:3), the variations found were in line with the data found for the neutral extract shown in Table 2.

Phytosterols

β-Sitosterol, stigmasterol, campesterol and cholesterol were found as free triterpenols in all the extractives. They were present in much larger amount in fresh than in dried leaves. Rehydration did not restore the original levels of concentration of these substances. The proportions of each phytosterol in the various extracts are shown in Table 4; β-sitosterol and cholesterol were present in similar percentages in both fresh, dried leaves and recovered leaves

Phytol

This diterpene was not present in the free state in fresh leaves but became a prominent component in the dried leaves. Phytol appeared to decrease in the recovered leaves (Table 1). The following abietane and pimarane diterpenes were also detected in the dried plants: dehydroabietic acid, sandaracopimaric acid and secodehydroabietane acid [6].

Sugars and polyols

Sugars, inositol and alditols are by far the major components in all the four extractives studied. A consistent pattern could be seen in the variation of glucose, fructose, sucrose, inositol and alditols concentration as a result of water content of the leaf (Table 5). Sucrose was the only sugar found in dry leaves, monosaccharides, alditols and inositol having disappeared completely. Rehydration restored the levels of glucose and fructose and reduced the content of sucrose, whilst the amounts of inositol and alditols remained low. Mannose, galactose and other sugars present in lesser amounts reappeared in rehydrated leaves at the same concentration level as in the fresh material.

Free amino acids

Free amino acids represented *ca* 0.25% in fresh material and did not accumulate or vary to any appreciable degree in dry or rehydrated leaves (Table 1). The ^{1}H NMR spectra of the four samples, showed a simple splitting pattern of a double doublet ($J = 6.2$ Hz) in the region δ0.8–1.0 that collapsed to two singlets on irradiation at δ2.1. These signals were tentatively assigned to the isopropyl moiety of valine. No consistent variations were found for any single free amino acid in the four samples analysed (Table 6), whose structures were confirmed by GC-MS of their *N*-heptafluorobutyrylpropylesters [7, 8].

DISCUSSION

This study describes analytical investigations of a wide range of low-M_r organic components in fresh, desiccated and recovered leaves of the resurrection plant

Table 4. Composition (%) of phytosterols from neutral ethyl acetate extraction of *B. hygroscopica* samples

Components	Fresh leaves	Dried leaves	Rehydrated leaves from desiccation over K_2CO_3	Rehydrated leaves from desiccation over LiCl
Cholesterol	2	2	2	2
Campesterol	12	7	4	2
Stigmasterol	3	4	5	12
β-Sitosterol	83	87	89	84

Table 5. Sugar and polyol composition (%) from *B. hygroscopica* samples

Components	Fresh leaves	Dried leaves	Rehydrated leaves from desiccation over K_2CO_3	Rehydrated leaves from desiccation over LiCl
Glucose	8±2.0	tr	18±1.0	15±0.5
Fructose	10±1.0	tr	16±4.0	14±1.0
Sucrose	14±0.0	90±2	40±3.0	43±1.5
Inositol	31±3.0	tr	1±0.5	tr
Alditols	5±0.5	tr	1±0.0	1±0.0
Mannose, galactose	10±1.0	—	10±1.0	6±1.0
Aldonic acids	3±0.2	—	1±0.0	1±0.1
Fatty acids	2±0.1		tr	—
Unidentified	17±2.0	10±1	11±0.8	12±0.5

Table 6. Composition (%) of free amino acids from *B. hygroscopica* samples

Components	Fresh leaves	Dried leaves	Rehydrated leaves from desiccation over K_2CO_3	Rehydrated leaves from desiccation over LiCl
Ala	2	2	4	9
Gly	13	6	3	6
Val	10	7	8	4
Ser	10	16	13	8
IsoLeu	12	3	14	3
Pro	3	2	18	9
Lys	8	5	6	2
Glu-NH_2	tr	2	tr	7
Asp-NH_2	18	18	5	15
Phe	6	4	6	16
Tyr	2	1	3	2
Glu	13	30	15	12

B. hygroscopica. The following qualitative and quantitative changes, probably induced by desiccation were observed.

The formation of phytol in dried leaves is noteworthy. Phytol is derived from the mevalonic acid pathway and is the lipophilic component of chlorophylls. Although its concentration 0.2 μmol g^{-1} as a free alcohol in dried leaves is lower than reported values of 2.7–3.7 μmol g^{-1} of phytol bound in chlorophyll for *Laminium album*, *Stellaria specie* and *Triticum vulgare*, [9] it is possible that it might originate from the hydrolysis of chlorophyll. Alternatively, and although very little is known, to our knowledge, on the effect of drought on free and bound phytol in plants [10], it is tempting to speculate upon the possibility that phytol and possibly abietanes and pimaranes might replace ABA in the expression of the peculiar physiological properties of resurrection plants [11, 12].

The variation of fructose, glucose, sucrose, inositol and alditols follows a well defined pattern. These compounds are present in fresh leaves in variable percentages with inositol dominant, all but sucrose disappear during desiccation and the disaccharide becomes the only sugar in dried leaves where its level reaches a value of 30.7 mg g^{-1}. In agreement with a previous report [13], rehydration restores roughly the compositional pattern of sugars and polyols of the unstressed leaves.

The clear compositional picture of these classes of compounds indicates a specific function of sucrose in osmotic regulation even though it is reasonable to suppose that the disaccharide might play more than one role in the survival of plants through desiccation and rehydration. The disappearance of glucose, fructose and the other minor monosaccharides under drought stress with the formation of sucrose may simply be the result of their metabolic interconversion. The sharp alteration of inositol level as a consequence of desiccation is of interest considering the involvement of inositol phosphates as second messengers in communication systems between cells [14].

Free amino acids were quantitatively moderate components in *B. hygroscopica* leaves and as the per cent amount of this class of compounds in dried leaves is lower than in fresh material, they can hardly be related to the water potential in this species. This is in contrast to findings for several other species in which proline accumulates during water stress [15, 16].

All efforts to detect the presence of betaines either colorimetrically (Dragendorfs' test) or by NMR failed on all the extracts. Hence, at least in *B. hygroscopica*, these classes of compounds seem not to be involved in the physiological process related to desiccation.

The quantitatively important organic acids present in all four samples investigated were 16:0, 18:0, 18:1, 18:2 and 18:3. The changes in the level of these three metabolically linked C_{18} acids with water-stress and after rehydration were remarkable and, apparently, unequivocal. On desiccation the level of stearic and oleic decreased sharply while a large increase in the polyunsaturated acids linoleic and linolenic was observed. Perhaps, the observed increase in dried leaves of di- and tri-unsaturated fatty acids, is the result of a decrease of both enzymatic and spontaneous lipid oxidation reactions involving, on one side probably superoxide ($O_2^{\cdot -}$) and hydroxyl ($OH^{\cdot}$) radicals and, on the other side, mainly polyunsaturated fatty acids. On rehydration, polyunsaturated fatty acid oxidation rises again and the fatty acid composition tends to return to that of the undesiccated leaves.

The report by Matile [17] that the catabolism of chlorophylls and particularly that bleaching of chlorophylls is strictly dependent on the presence of linolenic acid as well as other unsaturated long chain fatty acids, appears a highly interesting and meaningful hypothesis, even though there are many data in the literature that chlorophyll content of this desiccation-tolerant plant is little affected by drying [1–3]. At this stage, it is clear that further studies are required to obtain a better understanding of the roles played by the major classes of substance described in this paper for the resurrection process in *B. hygroscopica*. A major concern will be also the detection and the significance of already recognized and new chemical messengers.

EXPERIMENTAL

Boea hygroscopica plants, from seeds originally provided by D. F. Gaff, were grown in clay pots on leaf mould in a controlled environment using a light intensity of 5000 lux and 80–90% rel.

hum., constant day/night temperature of 25° and a photoperiod of 14 hr. Plants were kept fully watered. The following four samples of leaves were used: (i) a sample of leaves just detached from plants in February 1989. (ii) Leaves dried in an environment with a rel. hum. of 78% for 9 days, collected in June 1989. (iii) 24 hr rehydrated leaves (from leaves dried in an environment with 60% rel. hum. in June 1989). (iv) 24 hr rehydrated leaves (from leaves dried in an environment with 15% rel. hum. in June 1989).

The environments with different rel. hum. were obtained in 1 dm^3 airtight closed vials, using a saturated soln of NH_4NO_3 (rel. hum. of 78%) or of K_2CO_3 (rel. hum. of 60%) or a sludge of LiCl (rel. hum. of 15%). The values of rel. hum. inside the vials were measured by an hair igrometer and were a little different from the ones reported in ref. [18].

Leaves dried by equilibration in environments with rel. hum. of 78, 60 and 15% were found to lose water to a moisture content (on a dry wt basis) of 15, 9 and 3% respectively (unpublished data). Leaves dried by equilibration in environments with rel. hum. of 60 or 15% were rehydrated for 24 hr in the same growth conditions. They were laid in a Petri dish containing H_2O. After 24 hr the leaves appeared green and healthy.

Extraction and solvent partitioning. Varying numbers of leaves (10–30) were used for each of the three determinations carried out on the four plant samples (see the preceding paragraph). A typical procedure for a 20 leaf sample was as follows. The on-dry-ice frozen leaf sample was homogenized and extracted (×3) with MeOH–H_2O (7:3, 400 ml) at 5°. After 24 hr the homogenates were filtered and the solid residues were reextracted as above. The filtrates were combined and the MeOH evapd under red. pres. at 35° to yield an aq. residue that was partitioned (×3) against EtOAc. The combined neutral EtOAc extracts were taken to dryness to obtain the organic residue. The aq. solns were set apart for analysis of sugars, inositol, alditols, amino acids and other substances soluble in H_2O.

A portion of the aq. soln was adjusted to pH 2.0 by adding dil HCl and extracted (×3) with EtOAc (40, 15, 15 ml) to obtain the acidic EtOAc extracts that were, in turn, extracted (×2) with $NaHCO_3$ saturated H_2O (40, 20 ml). The latter aq. extract was acidified (HCl) and finally extracted with EtOAc (40, 20, 20 ml). Both the neutral and acidic EtOAc extracts were dried over Na_2SO_4, filtered and evapd to give the neutral and the acidic EtOAc-soluble fractions, respectively.

General procedure for the separation of classes of compounds present in the neutral and acidic EtOAc *fractions.* The frs were dissolved in a small amount of $CHCl_3$ and loaded onto 20 × 20 cm glass plates coated with a 0.25 mm layer of silica gel, type GF_{254} (Merck), activated at 120°. $CHCl_3$ was generally used as the solvent and known esters, alcohols, acids, phytol, phytosterols as the reference standards. The spots were detected, by spraying the plates with H_2SO_4 (1:1) containing 3% CrO_3 followed by heating at 120°. Phytosterols were also visualized by spraying the TLC plates with either Liebermann–Burchard reagent (Ac_2O, $CHCl_3$, H_2SO_4) or carbazole reagent (1% carbazole in EtOH, H_2SO_4). Sugars were visualized with anisaldehyde (EtOH, H_2SO_4, $MeCO_2H$), amino acids by spraying with ninhydrin soln. The material for each class was scraped from the plate and extracted with $CHCl_3$ containing EtOH in 1% (5–10 ml). After filtration and subsequent evapn, the yield for each class of components was determined gravimetrically. Each band sample was checked for purity using TLC.

Free fatty acids were converted into their methyl esters by treating the sample either with an ethereal soln of CH_2N_2 or with MeOH·BF_3.

Sugars and related compounds were converted to trimethylsilyl ethers with a silylation reagent made up of pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) (in the ratio 2:1:1). The derivatized sugars were kept in *iso*-octane for GC and GC-MS analyses.

Phytosterols and phytol were transformed into the corresponding acetates by treatment with excess Ac_2O and a drop of pyridine in C_6H_6 overnight at room temp. Abietanes and pimaranes were found in the fatty acid fraction and analysed as methyl esters.

Free amino acids were transformed in *N*-heptafluorobutyryl-propylesters according to a procedure similar to those described in the literature [19]. To weighed samples of dried extracts (≈50 mg) from the aq. soln, 5 ml of propanol–HCl (8 M) was added in a vial that was sealed with a Teflon coated cap and then heated under reflux in a heating block at 100° for 30 min. After cooling, excess propanol–HCl was evapd off and 250 μl heptafluorobutyryl anhydride (HFBA) was added. The vial was sealed and heated again at 130° for 40 min. After cooling the samples were evapd to dryness and dissolved in 1 ml of $CHCl_3$ and washed an equal vol. of H_2O to remove excess HFBA and heptafluorobutyric acid. The $CHCl_3$ solns were evapd to dryness and the samples dissolved in 1 ml EtOAc. Appropriate μl aliquots of this soln were utilized for GC and GC-MS determinations.

The amino acids were determined also using an automatic amino acid analyser and ninhydrin detection. The chromatogram of this analysis presented major peaks (*ca* 40%) of unidentified substances. Lack of sufficient plant material precluded further analyses. Sugars and amino acids were quantitated by NMR using the signals of their appropriate protons.

Analysis of classes of compounds by GC. The homologue compositions of free fatty acids as methylesters were determined on a Carlo Erba Model 5160 FID-GC with a capillary column (Supelcowax 10 of 30 m length, 0.25 mm i.d. and 0.25 μm film thickness). All other classes of compounds were analysed on a Carlo Erba Model 4160 GC by using either an OV-1 or an SE-52 capillary column (15 m length, 0.32 mm i.d. and 0.1–0.15 μm film thickness). Programmed chromatograms were run, using appropriate column temps. The carrier gas was H_2 at 0.3 kg cm^{-2}. Typical conditions for the classes of compounds were: fatty acid methyl esters: 90° start temp. programmed at 5° min^{-1} to 250; 10° min^{-1} 270° and then held 30 min at 270°; phytosterol acetates: start temp. 90°; 35° min^{-1} to 180°; 5° min^{-1} to 280°, then held at 280° for 15 min; sugars (as TMSi-derivatives): start temp. 100°; 8° min^{-1} to 250°, 10° min^{-1} to 300°, then held at 300° for 10 min; amino acids (as heptafluorobutyrylpropylesters): start temp. 45°; 5° min^{-1} to 280°; 10° min^{-1} to 310° and then held at 310° for 5 min.

GC-MS. A Hewlett-Packard Model 5890A capillary GC, equipped with a HP 5970 B mass selective detector was used for identification of the compounds by comparison of their mass spectra with ref. spectra. The capillary column used was directly introduced into the ion source operating in the electron impact mode (EI). The chromatographic conditions were as follows: injector 250°, interface 280°, column oven programs as most appropriate. He was the carrier gas with a head pressure of 2.5–3.5 psi, 25 cm sec^{-1} linear velocity. The mass detector was optimized with the disk software under auto-tune condition with perfluorotributylamine calibration.

Mass spectra were acquired over 40–800 mass unit range at 1 scan sec^{-1} with ionizing electron energy 70 eV, electron current 0.3 mA, ion source 200°; the vacuum was 10^{-5} Torr. The samples were dissolved in suitable solvents and injected (1 μl) in a splitless mode.

Detection of free amino acids by selected ion monitoring (SIM). Detection of free amino acids was performed by means of reconstructed ion chromatograms of stored data, using the most

suitable ions. The selected ions were monitored at any time in the chromatogram with a scan of the masses every 100 msec at 2.8 cycles sec^{-1} using an electron energy of 70 eV.

Statistical methods. All values presented in this work are means of three independent experiments ±s.e.

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