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# Changes in Glycerolipid and Sterol Contents in Leaves of *Borago officinalis* L. Plants as Related to Growth and Development

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**Abstract:** Cotyledonary and primary leaves of *Borago officinalis* L. plants showed a decrease in the contents of phospholipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol in the course of growth whereas free fatty acids and triacylglycerol contents increased gradually during the experimental period. Changes in sterol composition during the development of both types of leaves indicated a high proportion of free sterols and esterified sterols in comparison with steryl glycosides and acylated steryl glycosides. However, the amounts of free sterols and esterified sterols in cotyledonary leaves decreased with increasing age while in primary leaves they tended to increase when the organs became senescent. These alterations in glycerolipid and sterol levels in senescing leaves were accompanied by a decrease in chlorophyll content and an increase in carotenoid content. These results indicated that primary leaves are better predisposed to reduce the severe effects of senescence on extrachloroplastic membranes than cotyledonary leaves.

**Key words:** *Borago officinalis* L., cotyledonary and primary leaves, glycerolipids, growth and development, sterols

## INTRODUCTION

Polar glycerolipids (galactolipids, glucolipids and phospholipids) are constuents of plant cell membranes while neutral lipids (e.g., triacylglycerol) are mostly found in oil bodies (Somerville *et al*., 2000; Raison, 1980). Membrane lipids are important in the modulation of many physiological processes such as adaptation to changes in environmental conditions (Simon, 1974; Raison, 1980; Christiansen, 1983; Kuiper, 1985; Navari-Izzo *et al*., 1990; Aghofack-Nguemezi *et al*., 1991; Aghofack-Nguemezi, 1991) and the onset of senescence (Fobel *et al*., 1987; Wanner *et al*., 1991). The nature and content of sterol in the lipid bilayer of membranes may also determine their physical properties (Eichenberger, 1977; Leshem, 1991). Some sterols are also precursors of oxidized steroids acting as growth regulators collectively named brassinosteroids (Fujioka and Yokota, 2003; Crozier *et al*., 2000).

Seeds of *Borago officinalis* L. plants are known to contain high amounts of γ-linolenic acid which is of great medicinal importance. Thus, many authors have focused attention on the biosynthetic mechanisms of γ-linolenic acid in developing seeds (Stymne and Stobart, 1986; Griffiths *et al*., 1988; Galle *et al*., 1993). Moreover, these plants are appropriate for the study of phyllotaxis-related changes since their cotyledons do not disappear when seedlings develop green leaves but are transformed into photosynthetic organs. Aghofack-Nguemezi (2001) reported that there were temporal and spatial variations in short and medium chain fatty acid contents in glycerolipids in leaves of *Borago officinalis* L. He postulated that these alterations may be implicated in the regulation of the physiological functions of membranes. The present investigation should contribute to a better understanding of the roles of both glycerolipids and sterols in the modulation of plant senescence.

## MATERIALS AND METHODS

**Plant materials:** Seeds of *Borago officinalis* L. were sown and germinated, and the seedlings were grown under controlled environment as previously described (Aghofack-Nguemezi, 2001). Cotyledonary and primary leaves were harvested respectively three and four weeks after sowing onwards, weighed fresh, sealed into small vials, frozen in liquid $N_2$ and stored at-20°C. On each harvest day, samples were equally collected for the determination of dry matter weight.

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**Extraction of total lipids and fractionation by column and thin layer chromatography:** The total lipids were extracted using a 5:1.9:0.5 (v/v) mixture of chloroform, methanol (plus 0.025%, w/v, butylhydroxytoluene) and water. Phospholipids, galactolipids, free fatty acids and triacylglycerol were separated on silica gel 60 by thin layer chromatography according to Gardner (1968) and Mangold (1961). Sterols were separated into fraction on 500 mg silica gel Sep-Pak cartridges (Millipore/Waters, Milford, MA, USA). The column was first washed with hexane. Total lipids were applied to the column in hexane solution and eluted sequentially with hexane/diethyl ether (9:1, v/v), diethyl ether and chloroform/ethanol (25:3, v/v) to yield Esterified Sterols (ES), Free Sterols (FS) and Steryl Glycosides (SG) and Acylated Steryl Glycosides (ASG), respectively. SG and ASG fraction was further fractioned on silica gel plates by thin layer chromatography using chloroform/methanol (90:15, v/v).

The separated components were visualized under UV light after spraying with primuline/acetone/water (10:80:20, w/v/v). A semi destructive coloration of the spots was also done. The thin layer plates were sprayed with ammonium sulfate/water/sulfuric acid (20:100:4, w/v/v) and incubated for 20 h at 105°C. In each case the components were identified in comparison with co-chromatographied authentic standards.

Obtention of free sterols from esterified sterols, steryl glycosides and acylated steryl glycosides.

Esterified sterols were saponified using a solution of 10% (w/v) KOH in 95% ethanol for 17 h at 60°C. The hydrolysis of SG and ASG was performed with a 1% $H_2SO_4$ methanolic solution for 17 h at 60°C. After hydrolysis, hexane/diethyl ether/water (3:2:1, v/v) was added to ES, SG and ASG solutions. The mixture was homogenized, the hexane/diethyl ether phase was recovered and evaporated to dryness under $N_2$.

**Esterification of fatty acids and preparation of free sterol derivatives:** Transesterification of fatty acids in lipid classes was done with borontrifluorid methanol as in Metcalfe *et al.* (1966). Free sterol derivatives were obtained singtrimethylchlorosilane/hexa methyldisilane/ pyridine (1:3:9, v/v) for 30 min at 60°C. Hexane/diethyl ether/water (4:1:4, v/v) was added to the FS derivative solution. The mixture was homogenized, the hexane/diethyl ether phase was recovered and evaporated to dryness under $N_2$.

**Gas chromatography analysis of free sterol and fatty acid derivatives:** A chrompack CP 9000 chromatograph equipped with a flame ionization detector, an automatic sampler and a PC was used. A 20 m×0.32 mm CP Wax 52CB fused silica column and a 30 m×0.32 mm CP-Sil-5CB fused silica column were used for the analysis of fatty acid methylesters and trimethylsilylesters of sterols, respectively. Fatty acid and sterylesters were identified on chromatograms in comparison with authentic standards. Fatty acid content of each lipid class and individual sterol content were determined using heptadecanoic acid and cholestane as internal standards respectively. Chemical standards and fused silica column were purchased from Sigma Chemie AG and Chrompack (Germany), respectively.

**Determination of pigment content:** Leaf materials were extracted with 80%. Chlorophyll a, chlorophyll b and carotenoid contents were determined from the extracts absorbance values at 470, 646.8 and 663.2. The equations of Lichtenthaler (1987) were used to calculated the concentration of pigments, expressed on leaf weight basis.

**Statistical analysis:** Analysis of variances were carried out to test if there were significant differences in glycerolipid, sterol and pigment contents between the periods. Where significant differences were obtained, the Duncan's Multiple Range Test was performed to separate the means.

## RESULTS

Cotyledonary and primary leaves of *Borago officinalis* L. showed an increase in the amounts phospholipids (PLs), digalactosyldiacylglycerol (DGDG) and monogala ctosyldiacylglycerol (MGDG) at the early stage of growth. The highest values of MGDG and PLs contents in cotyledonary leaves were obtained at the stage of five weeks after sowing whereas the optimum value for DGDG content was reached on the sixth week after sowing. In primary leaves, the optimum value for MGDG content was obtained at the stage of seven weeks after sowing while the highest values of DGDG and PLs contents were obtained at the stage of eight weeks after sowing. There was a continuous increase in the level of free fatty acids (FFA) and triacylglycerol (TG) during the experimental period (Table 1).

Table 2 shows changes in the contents of different sterol classes in cotyledonary and primary leaves in the course of growth and development. In cotyledonary leaves the levels of Free Sterols (FS) and Esterified Sterols (ES) decreased while the amounts of Steryl Glycosides (SG) and Aacylated Steryl Glycosides (ASG) increased during the experimental period. Thus, the contents of FS and ES were significantly higher in cotyledonary leaves at the stage of three weeks after sowing than the other leaves. Contrarily to FS and ES, the amounts of SG and ASG were significantly higher in cotyledonary leaves at the stage of seven weeks after sowing than younger leaves. In the primary leaves the relative values of FS and ES decreased at the early stage of growth and then

Table 1: Glycerolipid composition in cotyledonary (cl) and primary (pl) leaves of *Borago officinalis* L. in the course of growth. Values are means±SD (n = 5)

| Type of leaves | No. of weeks after sowing | Glycerolipid content (μmol/gDW) | | | | |
|---|---|---|---|---|---|---|
| | | MGDG | DGDG | Pls | FFA | TG |
| cl | 3 | 9.23±0.83a | 5.72±0.22a | 10.55±0.62a | 0.73±0.02a | 3.32±0.05a |
| | 4 | 13.75±1.03b | 7.25±0.33b | 15.03±0.93b | 2.45±0.06b | 5.15±0.23b |
| | 5 | 16.32±1.15c | 11.15±0.63c | 18.75±1.05c | 3.15±0.05c | 6.25±0.28c |
| | 6 | 10.25±0.95d | 8.91±0.32d | 16.42±0.85d | 4.08±0.15d | 8.85±0.31d |
| | 7 | 6.25±0.37e | 5.35±0.15e | 13.15±0.51e | 5.78±0.23e | 9.86±0.17e |
| pl | 4 | 7.01±0.35a | 4.25±0.15a | 8.07±0.53a | 1.13±0.03a | 2.16±0.07a |
| | 5 | 13.22±1.25b | 6.33±0.21b | 11.22±0.91b | 2.75±0.05b | 3.75±0.15b |
| | 6 | 17.81±1.56c | 9.42±0.85c | 14.85±1.05c | 3.16±0.11c | 4.93±0.25c |
| | 7 | 21.55±1.15d | 16.35±0.75d | 17.52±1.18d | 3.85±0.15d | 6.08±0.33d |
| | 8 | 19.15±0.82e | 14.08±0.62e | 22.72±1.25e | 4.23±0.12e | 7.55±0.51e |
| | 9 | 17.12±0.55f | 11.5±0.25f | 19.02±0.73f | 4.82±0.23f | 8.92±0.58f |
| | 10 | 15.03±0.95g | 9.15±0.55g | 16.53±0.88g | 5.91±0.32g | 10.37±0.63g |

a, b, c, d, e, f, g: Values from each plant organ followed by different scripts within the column differ significantly a $p<0.01$, DW: Dry matter weight

Table 2: Changes in the levels of sterols in cotyledonary (cl) and primary (pl) leaves of *Borago officinalis* L. in the course of growth. Values are means±SD (n = 5)

| Types of leaves | No. of weeks after sowing | Sterol content (% total sterols) | | | |
|---|---|---|---|---|---|
| | | FS | ES | SG | ASG |
| cl | 3 | 62.97±1.05a | 35.65± 0.82a | 1.05±0.02a | 2.57±0.05a |
| | 4 | 59.25±1.55b | 33.21±1.02b | 3.18±0.01b | 5.83±0.15b |
| | 5 | 55.36±0.75c | 29.45±0.65c | 7.85±0.05c | 9.42 ±0.25c |
| | 6 | 51.73± 0.42d | 25.93±1.15d* | 10.91± 0.75d | 14.73±0.56d |
| | 7 | 46.83±2.05e | 23.85±1.06* | 13.65±0.35e | 18.63±1.01e |
| pl | 4 | 53.85±0.98a | 41.33±1.35* | 1.62±0.02a | 5.84±0.33a |
| | 5 | 50.41±1.05b | 38.64±2.15a* | 4.96±0.15b | 8.62±0.28b |
| | 6 | 46.33±1.75c | 32.83±0.75b | 9.53±0.93c | 13.82±0.72c |
| | 7 | 41.72±0.85d | 26.92±0.37c | 13.25±0.35d | 20.75±1.22d |
| | 8 | 47.62±0.08e | 30.54±0.65d | 8.73±0.42e | 15.35±1.08e |
| | 9 | 54.81±1.25f | 35.95±1.25e | 3.29±0.01f | 8.45±0.53f |
| | 10 | 59.92±1.80g | 37.48±0.83f | 1.52±0.01g | 3.68± 0.11g |

a, b, c, d, e, f, g: values from each plant organ followed by different scripts within the column differ significantly at $p<0.01$, *: significantly different at $p<0.05$

Table 3: Changes in chlorophyll a (chl a), chlorophyll b (chl b), carotenoids (car) contents in cotyledonary (cl) and primary (pl) leaves of *Borago officinalis* L. during growth. Values are means±SD (n = 5)

| Types of leaves | No. of weeks after sowing | Pigment content (μg/g FW) | | | |
|---|---|---|---|---|---|
| | | Chl a | Chl b | Chl a/Chl b | Car |
| cl | 3 | 76.45±6.40a | 22.12±1.43a | 3.45±0.09a | 30.45±1.03a |
| | 4 | 127.23±7.65b | 37.45±2.15b | 3.39±0.03a | 50.82±3.51b |
| | 5 | 152.95±2.75c | 47.58±2.35c | 3.21±0.03a | 65.22±2.45c |
| | 6 | 121.55±3.12d | 35.25±0.80d | 3.44±0.25a | 90.50±5.33d |
| | 7 | 92.25±1.23e | 25.58±0.51e | 3.60±0.15a | 130.75±8.20e |
| pl | 4 | 102.37±4.52a | 32.15±7.52a | 3.18±0.07a | 40.35±2.65a |
| | 5 | 189.35±2.58b | 47.75±0.8b | 3.96±0.07a | 70.24±5.22b |
| | 6 | 240.05±3.42c | 70.48±1.15c | 3.40±0.01a | 85.12±7.32c |
| | 7 | 274.15±10.55d | 77.83±2.92d | 3.52±0.02a | 103.75±8.15d |
| | 8 | 276.65±8.33e | 76.65±3.12e | 3.60±0.07a | 130.43±9.22e |
| | 9 | 239.32±7.55f | 69.92±0.7f | 3.42±0.05a | 150.75±9.30f |
| | 10 | 182.75±8.81g | 51.83±2.55g | 3.52±0.08a | 180.25±5.32g |

a, b, c, d, e, f, g: Values from each plant organ followed by different scripts within the column differ significantly at $p<0.01$, FW: Fresh matter weight

increased with increasing age. The lowest values of FS and ES contents were obtained at the stage of seven weeks after sowing. Changes in the levels of SG and ASG in primary leaves followed an opposite trend to changes in the levels of FS and ES during the experimental period. The highest values of SG and ASG contents were obtained at the stage of seven weeks after sowing. At each stage, sitosterol, campesterol, cholesterol and stigmasterol were the free sterols found in considerable amounts in both types of leaves.

The levels of chlorophyll a (chl a) and chlorophyll b (chl b) increased and then decreased when cotyledonary and primary leaves tended to become senescent. The highest values of chl a and chl b contents in cotyledonary leaves were obtained at the stage of five weeks after sowing while in primary leaves these values were obtained at the stage of seven weeks after sowing. The chl a/chl b ratio remained nearly constant and the carotenoid (car) levels increased gradually during the experimental period (Table 3).

## DISCUSSION

The results showed that at the later stage of growth of leaves of *Borago officinalis* L. there was a decrease in the contents of MGDG, DGDG and PLs whereas the levels of FFA and TG increased. MGDG and DGDG are the major lipid components of chloroplast membranes while PLs are mainly constituents of extrachloroplastic membranes (Dörmann and Benning, 2002; Somerville *et al*., 2000; Quinn and Williams, 1983; Gounaris *et al*., 1986). Matile *et al*. (1989) reported that the breakdown of chloroplastic membranes is the major event during senescence. However, the decrease in the contents of MGDG, DGDG and PLs observed in the present study may be due to the overall destruction of cell membranes. The observed decrease in polar lipid content and corresponding increase in neutral lipid content may be due to the incorporation into the latter lipid class of fatty acids liberated from the former. A similar rearrangement of fatty acids in polar and neutral lipid classes has been observed under a variety of stress conditions (Frosch *et al*., 1990; Hubac *et al*., 1989). The enzyme diacylglycerol acyltransferase plays a role in senescence by sequestering fatty acids de-esterified from galactolipids into triacylglycerol molecules that would appear to be intermediates in the conversion of thylakoid fatty acids to phloem-mobile sucrose during leaf senescence (Kaup *et al*., 2002).

Primary and cotyledonary leaves showed different patterns of changes in the levels of sterols. Although the amounts of FS and ES decreased gradually in cotyledonary leaves, they increased from seven weeks after sowing and more in primary leaves. These results indicated that the effects of cotyledon senescence on the composition of membrane sterols as reported elsewhere (McKersie *et al*., 1978) can't be taken into account for primary leaves. Apparently membrane mechanisms modulating senescence are not similar in both types of leaves. Senescing leaves are characterized by a loss of membrane integrity accompanied by an increase in their permeability which could result to the death of cells (Leshem, 1991; Simon, 1974). Free sterols, SG and ASG are usually found in the endoplasmic reticulum, Golgi vesicles and the plasmalemma while the chloroplast and mitochondria membranes contain only minor quantities of FS, SG and ASG; ES is usually not a component of cell membranes (Eichenberger, 1977). Although FS, SG and ASG are all constituents of membranes, only FS may be implicated in the reduction of permeability of membranes (Eichenberger, 1977; Navari-Izzo *et al*., 1990). Certain free sterols are precursors of steroid hormones (Fujioka and Yokota, 2003; Crozier *et al*., 2000) that have been implicated in many processes including the stimulation of ethylene production (Opik *et al*., 2005; Schaller, 2003; He *et al*., 2003). Thus senescence of primary leaves of *Borago officinalis* plants, contrary to cotyledonary leaves, is possibly controlled by the level of free sterols.

The constancy of chla /chlb ratio when chla and chlb levels declined strongly indicated a breakdown of both types of pigments at almost the same rate. Since chla is predominantly found in the reaction Centre while chlb is exclusively an antenna pigment of leaf photosystems (Rüdiger and Schoch, 1989; Lawlor, 1990), a specific destruction of one zone of the photosystems would have led to differential decrease in chla and chlb levels and hence to changes in chla/chlb ratio. Furthermore, degreening of *Borago officinalis* cotyledonary and primary leaves does not seem to be associated with the conversion of chlb to chla prior to chlorophyll degradation as previously reported (Aghofack and Yambou, 2005; Porra and Scheer, 2000; Matile and Hörtensteiner, 1999). It is not well known why carotenoids are not also destroyed during senescence of leaves. Obviously, senescence of leaves triggers the hydrolysis of certain components like chlorophylls and lipids and the transport of the resulting important elements e.g., Mg, P and S to other growing organs in order to ensure the perpetuation of the species or maximal yield. Moreover, plant destroy chlorophyll and other porphyrins because the senescent cells must be detoxified with regard to the dangerous photodynamic molecules which are liberated upon mobilization of the corresponding apoproteins (Matile and Hörtensteiner, 1999; Matile *et al*., 1989).

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