Characterisation and Evaluation of Species of the *Boraginaceae* Family as Source of Gamma-Linolenic Acid for Mediterranean Conditions

A. De Haro, M. del Río, B. Alcaide, H. Rapoport
Instituto de Agricultura Sostenible (CSIC)
Alameda del Obispo. 14080 Córdoba, Spain

A. Cabrera
ETSIAM
Universidad de Córdoba, Spain

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Abstract

Gamma linolenic acid (GLA) is an essential fatty acid that is being used for the treatment of health problems related to deficiencies in essential fatty acids and prostaglandins. Borage plant is an important source of GLA, but the shattering habit of the genus limits large scale seed production. Looking for alternatives to the available borage material and for the possibilities of borage and other plant species belonging to the same botanical family as potential crops in Mediterranean conditions, this work describes: i) the evaluation of the seed quality of European borage population, and further selection of entries with high oil and GLA, and low erucic acid content, ii) the isolation of two borage mutants with improved seed production characteristics, iii) the first results of the anatomical and molecular studies in relation with the reproductive system and the cariotype of normal and mutants plants, and iv) the beginning and characterization of a germplasm collection of *Boraginaceae* plant species for its fatty acid composition and adaptability to semi arid environments: *Anchusa azurea* and *Echium boissieri* highlight as potential new crops for GLA production.

INTRODUCTION

Gamma linolenic (all-cis-6,9,12-octadecatrienoic) acid (GLA) is an essential fatty acid in demand for clinical and pharmaceutical applications. This fatty acid is precursor of indispensable compounds in the body such as prostaglandins (PGE_1) and leucotrienes (LT). Many factors, such aging ageing, stress, diabetes, high alcohol intake and nutritional deficiencies, have been shown to interfere the biosynthesis of GLA from linoleic acid *via* Δ-6 desaturase. Health problems related to deficiencies in essential fatty acids and prostaglandins are being treated by supplementation of the diet with vegetable oils containing this fatty acid (Gunstone 1992; Horrobin, 1999).

The most common commercial sources of GLA for pharmaceutical uses are evening primrose (*Oenothera biennis* L.) and borage seed oil. Borage seed appears to be the richest known plant source of GLA, with 16 to 28 percent of GLA from a total seed oil content of 27 to 37 percent. Additional advantages of borage in comparison with evening primrose is the annual life cycle and the larger seed of borage that make harvest and oil extraction easier (Muuse et al., 1988; Janick et al., 1989; Galwey and Shirlin, 1990).

Large scale commercial production of borage is limited because of the non-uniform seed maturation and shattering habit of the plant, factors that cause a large amount of ripened seed fall to the soil before and during harvest. Janick and co-workers (1989) have attempted to find solutions to the seed-shattering challenge with the development of vacuum harvesters and in vitro production of GLA by zygotic and somatic embryos culture. Galwey and Shirlin (1990) initiated a program of selection in borage for GLA, oil content, and seed production, including mutagenesis with sodium azide and with ethyl methane sulfonate (EMS), but no mutant phenotypes were produced in subsequent generations.

In addition to borage, other members of the Boraginaceae family also contain GLA and represent potential sources of GLA (Janick et al., 1989). Among these, are a

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large number of annuals native to Mediterranean members of the Boraginaceae that grow during the winter, taking advantage of the available rains and developing the flowering shoots when climatic conditions are adequate. Therefore, borage and the above mentioned species of Boraginaceae represent potential new crops for Mediterranean and semiarid conditions.

This paper describes the approaches and results obtained by our research group aiming to: 1) test the available European borage germplasm and select genotypes with the best seed quality characteristics, 2) produce new borage lines with improved characteristics for seed and GLA production, 3) progress in the knowledge of the reproductive system of borage, and the modifications produced y the mutants, 4) study the chromosome architecture of borage by developing and applying molecular techniques (fluorescence in situ hybridization, FISH), and 5) collect, characterise, and select other Boraginaceae species that could be used as new sources of GLA in semiarid conditions.

MATERIALS AND METHODS

Evaluation of Seed Quality of European Borage Populations

The germplasm evaluated consisted of: i) white flowered material cultivated as vegetable in the North of Spain (Southern Europe), ii) wild blue flowered roadside populations collected in the South of Spain, and iii) wild and cultivated populations of the North of Europe.

Seed weight was determined by weighing 1000 seeds. Oil content was determined by nuclear magnetic resonance. The fatty acid composition of the oil was determined on a bulk of 10 seeds/entry by simultaneous extraction and methylation, followed by gas-liquid chromatography (Garcés and Mancha, 1993).

Isolation of Two Chemically Induced Mutants of Borage

Due to the lack of variation in seed shattering observed in the germplasm collection, a trial to increase the natural genetic variability by inducing chemical mutagenesis with EMS was done. Approximately 15,000 seeds of white flowered borage belonging to a Spanish accession with high self-fertility (RG-001) were exposed to a solution of 1 % (v/v) of EMS for 16 h under continuous stirring. Plants from the M1 generation were bagged to force self-pollination. Seeds of each bagged plant were sown in a single row to produce the M2 generation and, before flowering, five plants of each row were bagged and self-pollinated to obtain M3 seeds.

In the M3 generation, 50 pollinated flowers from each mutant type were chosen at random to calculate the variability in the number of petals, sepals, ovules and seeds per flower, and to apply statistical analysis (De Haro and Del Río, 1998). Significant differences between means were determined by Duncan's Multiple Range Test.

Anatomical Studies of Mutants with Retention of Petals and Seeds

Fruits in different developing stage of both wild type and mutant type showing petals and seed retention were fixed in formalin, embedded in paraffin, sectioned at 12 μm and stained with tannic acid, iron chloride, safranin, and fast green (Jensen, 1962; Rapoport et al., 2001).

Cytogenetic Characterisation of Cultivated Borage by Fluorescence in situ Hybridization (FISH)

Seeds were germinated for 3 days at 24 ºC. The roots were treated with colchicine in darkness and then fixed in acetic acid/ethanol. The samples was then treated with HCl, placed on acid-carmine solution, squashed and mounted. The selected slides were used for FISH, using the 18/25S rDNA repeat sequence from soybean. The in situ hybridization protocol used was according Cabrera et al. (1999).

Collection and Characterisation of a Boraginaceae Germplasm Collection

A germplasm collection of Boraginaceae species has been constituted at the IAS (Córdoba, Spain) by collecting in situ wild Spanish populations and by exchange with European botanical gardens. The fatty acid composition of the seeds for each accession was determined as previously described for borage accessions.

RESULTS AND DISCUSSION

Evaluation of Seed Quality of European Borage Populations

For seed weight, oil content and GLA and erucic acid tested, an important range of variation was observed. The ranges were wider than those published in previous studies on this species (Muuse et al., 1988; Janick et al., 1989; Galwey and Shirlin, 1990). Entries with oil content higher than 35 %, GLA around 27 % and low erucic acid content (lower than 2.5 %) were selected for further use in breeding programs.

Isolation of Two Chemically Induced Mutants of Borage

Two different types of mutants were obtained in the M2 generation of plants and reproduced in the M3 generation: i) type B: plants with modified flowers: larger than normal and a higher number of petals, sepals, and ovules (almost double than those in the original plants), and ii) type C: plants with closed (C1 type) or partially opened (C2 type) flowers. In these two type C mutants the petals did not follow the general rule of falling two days after opening, but instead remained on the flower, dried, and stayed on the plant to the end of the life-cycle. This modification seems to affect the shattering of the seeds. Type C2 plants show high fertility rate and can be considered as partially indehiscent plants because the mature seeds were retained by partially opened petals and remained on the plant until the end of its life-cycle (Table 2).

Plants from both B and C mutant types have been crossed with normal plants to study the genetic changes caused by the mutagenic treatment. F1 plants have been obtained and self-pollinated to produce F2 seeds. Simultaneously F1 plants have been reciprocally backcrossed to both parents to obtain BC1F1 seeds. F2 and BC1F1 generations will be grow and study during the next season.

Anatomical Studies of Mutants with Retention of Petals and Seeds

Longitudinal sections of both normal wild type and mutant type C2 are presented in Fig. 1 and Fig. 2 respectively. In the normal type the detachment of the nutlet occurs when an abscission zone is formed between the elaiosome and the receptacle. In the mutant type C2 the petals and petal accessory structures remain present throughout fruit maturation, pressing and retaining the seeds on the fruit receptacle. Additionally, in all type C mutants the elaiosomes are embedded in the fruit receptacle contributing to the seed retention.

Cytogenetic Characterisation of Cultivated Borage by Fluorescence in Situ Hybridization (FISH)

For the first time, fluorescence in situ hybridization on mitotic chromosomes of borage has been shown. FISH with the 18/25S rDNA probe used in this work has revealed signals on several chromosomes pairs, and can be used to localise nucleolar organizing regions (NORs) and to the cytogenetic characterisation of *Borago officinalis*. FISH on mitotic chromosomes with other DNA probes and the application of this technique to the borage mutants previously described are under study.

Collection and Characterisation of a Boraginaceae Germplasm Collection

At present, this collection includes 130 accessions belonging to 14 species. These species represent a wide range of growth habit and fatty acid composition (Table 3). *Cynoglosum creticum* and *Heliotropium europaeum* contain no GLA in their seed, but high oleic and high linoleic acid, respectively. In contrast, *Symphytum officinale* shows

the highest GLA content, but requires well-watered conditions during seed formation. *Anchusa azurea* and *Echium boissieri* are the species combining adequate fatty acid composition and adaptability to semiarid Mediterranean climates and highlight as potential new crops for GLA production.

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Tables

Table 1. Mean and range of 1000 seed weight (g), oil content (% dry matter) and fatty acid content (% of the oil) of wild and cultivated borage populations.

Borage Populations	N	1000-seed weight (g)	Oil content (%)	Fatty acid content	
				GLA	Erucic
WFNS	130	15.2 (10.8-20.1)	34.5 (31.8-38.0)	23.1 (8.7-28.6)	1.8 (1-3.3)
BFSS	55	14.1 (9.3-18.2)	30.7 (26.7-35.4)	18.9 (12.6-26.5)	1.8 (0.6-2.9)
BFNE	21	23.1 (19.2-26.3)	29.6 (27.6-32.5)	20.4 (13.1-27.2)	3.7 (1.9-7.8)

N = number of entries, WFNS = cultivated white flowered (Northern Spain), BFSS = wild blue flowered (Southern Spain), BFNE = blue flowered (Northern Europe).

Table 2. Variability in flowers and seeds in untreated and mutant borage plants[a.]

		Untreated Plants	Mutant plants		
			Type B[b]	Type C1[c]	Type C2[d]
Petals	Mean[e]	5b	8a	5b	5b
	Range	5	7-9	5	5
Sepals	Mean	5b	8a	5b	5b
	Range	5	7-9	5	5
Ovules	Mean	4b	18.3a	4b	4b
	Range	4	16-25	4	4
Seeds per flower	Mean	2.8a	2.2b	1.2c	2.4a,b
	Range	0-4	0-8	0-4	0-4

[a] Number of flowers inspected in each mutant type and in untreated plants: 50.
[b] Type B : plants with flowers larger than normal and, often, with the style and stigma malformed.
[c] Type C1 : plants with flowers closed and seed retention.
[d] Type C2 : plants with flowers partially opened and seed retention.
[e] Means within the same row with the same letter are not significantly different. Significance was calculated by the Duncan's Multiple Range Test (α=0.05 level).
(Adapted from De Haro and Del Rio, 1998)

Table 3. Mean and range of fatty acids content in Mediterranean Boraginaceae.

Species	Fatty acid content (% of oil)						
	N	Oleic	Linoleic	α-Linolenic	GLA	Eicosenoic	Erucic
Anchusa azurea	54	27.1 (15.9-38.6)	41.2 (31.1-51.3)	--	10.6 (4.4-23.2)	4.3 (3.7-5.3)	6.5 (2.1-8.5)
Anchusa officinalis	10	21.7 (15.6-29.2)	29.3 (23.5-33.8)	13.5 (8.4-16.9)	16.1 (12.6-21.5)	3.4 (3.1-3.8)	3.2 (1.4-5.6)
Anchusa undulata	6	23.1 (17.6-27.2)	28.3 (28.1-28.5)	12.4 (10.6-15.3)	13.1 (11.3-15)	3.8 (3.6-4.9)	3.5 (3.1-3.8)
Cynoglossum creticum	1	63.5	1.93	8.64	--	7.07	10.66
Echium albicans	6	12.3 (10.6-13.3)	14.2 (14.1-14.6)	42.4 (39.7-45.9)	8.1 (7.4-8.7)	0.7 (0.4-0.81)	--
Echium boissieri	16	13.1 (9.1-18)	10.1 (8.1-12.8)	47.5 (44.2-49.5)	5.6 (4.5-5.9)	0.8 (0.5-0.9)	0.2 (0-0.4)
Echium gaditanum	8	20.3 (15.6-31)	27.1 (23.6-33.1)	20.4 (11.9-28.2)	12.1 (11.4-13.1)	1.8 (0.6-4.3)	1.2 (0-4.1)
Echium plantagineum	1	17.1	19.6	33.1	9.6	0.7	--
Heliotropium europaeum	5	22.4 (16.6-25.4)	66.4 (62.4-69.5)	10.5 (8.6-13.5)	--	--	--
Lithospermum sp.	4	34.6 (33.2-36.2)	37.1 (36.1-37.8)	0 0	9.95 (9.7-9.10.6)	4.4 (4.2-4.6)	5.2 (5.1-5.4)
Nonea vesicaria	5	27.6 (26.6-29.1)	26.3 (25.5-27.2)	9.8 (8.2-10.9)	12.3 (11.4-14.6)	3.7 (2.5-6.4)	1.7 (1.1-3.3)
Omphalodes commutata	3	40.6 (38.6-43.2)	13.5 (11.5-16.2)	8.6 (6.2-10.2)	6.1 (5.5-7.2)	5.6 (5.2-6.2)	10.3 (8.2-12.4)
Onosma tricerosperma	1	20.1	19.93	35.48	5.58	1.01	0.38
Symphytum officinale	10	15.5 (12.4-16.7)	43.1 (40.8-44.8)	1.1 (0.8-1.4)	26.8 (25.5-27.9)	2.1 (2-2.5)	1.2 (1.2-1.6)

Figures

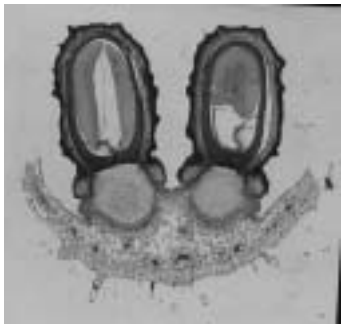

Fig. 1. Longitudinal section of normal-type borage fruit showing two nutlets attached by an elaiosome to the fruit. No petal tissues are present.
(Adapted from Rapoport et al. 2001)

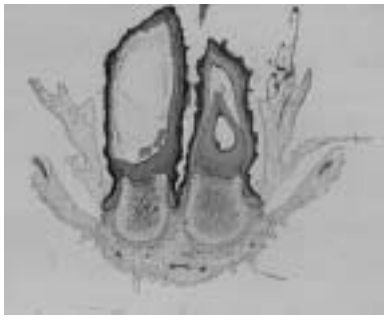

Fig. 2. Longitudinal section of type C2 mutant fruit. The petals are present and press the nutlets.
(Adapted from Rapoport et al. 2001)