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Journal of Chromatography A, 922 (2001) 347–354

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Separation of plant membrane lipids by multiple solid-phase extraction

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Received 14 July 2000; received in revised form 18 April 2001; accepted 19 April 2001

Abstract

Plant membrane lipids were separated by multiple solid-phase extraction (SPE) in a single run. Elution was performed continuously through the modulated stationary phase employing only non-aqueous solvent systems. At the different stages of the glycerolipid separation the SPE manifold combined aminopropyl, aminopropyl/silica gel and silica gel/aminopropyl weak anion exchanger columns. The glycerolipid extract of pigment-containing plant tissues was cleared from the pigments onto the aminopropyl column. The aminopropyl column with the glycerolipid extract was then connected to a silica gel column from which monogalactosyldiacylglycerol, phosphatidylethanolamine, phosphatidylglycerol and digalactosyldiacylglycerol were eluted as individual fractions. The elution was performed under polarity, pH and temperature gradient conditions. To continue the separation, the aminopropyl column was discarded and the silica gel column containing the remaining glycerolipid extract was connected to an aminopropyl anion exchanger column. Individual fractions of sulfoquinovosyldiacylglycerol, phosphatidylcholine and phosphatidylinositol were now eluted. The separation process was supported by ammonium counter ions and by the polarity gradient of the elution systems used. The membrane lipids were isolated from pigment-containing (rice and maize leaves and rice leafy stems) and pigment-free (rice roots) tissues. The repeatability for a standard glycerolipid mixture was 2–6% ($n=7$), and for rice leaf lipid extracts, 3–7% ($n=5$). Glycerolipid recovery was 87–95%.

Keywords: Solid-phase extraction; Plant tissue; Lipids

1. Introduction

For semi-preparative isolation of lipid classes, solid-phase extraction (SPE) is a rapid and simple alternative to thin-layer chromatography (TLC) [1–3] and to high-performance liquid chromatography (HPLC) [4,5]. HPLC columns have much greater resolution capacities than either TLC plates or SPE columns but for many applications the separation achieved by using the latter two methods is considered adequate.

The resolution ability of a single SPE column is insufficient to completely separate a complex lipid mixture. Nevertheless, by using a single octadecyl bonded cartridge [6], isolation of total phosphatidylcholines (PCs) from serum has been achieved. A single aminopropyl bonded column has been employed for the separation of a broad variety of lipid mixtures of different origins [7–12]. Fungal lipid mixtures [7] and lipid extracts from mixed microbial

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00888-3

cultures [8] have been separated into three fractions comprising: neutral lipids, free fatty acids and phospholipids. Four fractions were isolated from serum lipids [9] and total lipid extracts from *Escherichia coli* and human spermatozoa [10]. The serum lipids were separated into cholesteryl esters, triglicerides, free fatty acids and phospholipids fractions. The phosphatidylethanolamine (PEA) and phosphatidylglycerol (PG) from *E. coli* were completely separated as third (neutral polar lipids) and fourth (polar acidic lipids) fractions, respectively. PC and PEA from human spermatozoa were eluted together in the fraction of neutral polar lipids and phosphatidylinositol (PI) – in the fraction of polar acidic lipids. In both of these lipid fractions, PG was present. In two other reports [11,12], acid phospholipids could not be completely eluted from an aminopropyl stationary phase with methanol, in contrast to what was reported in Ref. [13]. Acid phospholipids were recovered, from a 500 mg aminopropyl column, with 4 ml of hexane–2-propanol–ethanol–0.1 *M* ammonium acetate in water–formic acid (420:350:100:50:0.5, v/v) containing 5% phosphoric acid [12]. Polar lipids were eluted from a 600 mg silica gel stationary phase with a combination of methyl *tert*.-butyl ether–methanol–ammonium acetate (pH 8.6) [14]. In this case, PEA and PI eluted together in one fraction, whereas the most polar lipids, such as PC, sphingomyelin and lyso-PC, were eluted as a second fraction. Again, by adaptation of the solvent mixture alone, lipid extracts from small amounts of neural tissue were fractionated into neutral lipids, phospholipids, nonsialylated sphingolipids and gangliosides by using a silica gel column [15].

SPE, as a technique, readily permits the combination of multiple stationary phases. The serial combination of identical SPE columns (aminopropyl bonded phase columns [13]) increases the separation capacity of the SPE manifold. When different SPE columns are combined (silica gel/aminopropyl cartridges [11,16]), both capacity and selectivity of separation can be increased. Kaluzny et al. [13] first employed this idea by constructing a flexible manifold of three aminopropyl bonded columns which was used to isolate different lipid classes from a bovine adipose tissue extract. A serial manifold of silica gel and aminopropyl bonded cartridges was employed to separate neutral lipids, glycolipids and phospholipids from wheat flour into individual classes [11]. The silica gel stationary phase was used to separate neutral lipids into eight classes, as well as monogalactosyldiglycerides (MGDGs) and digalactosyldiglycerides (DGDGs). Phospholipids were separated by a series of silica gel and aminopropyl bonded stationary phases into PC and lyso-PC, *N*-acyl-PEA and *N*-acyl-lyso-PEA. In another case, a similar manifold of silica gel and aminopropyl bonded cartridges was used to separate five glycerophospholipids from animal tissue [16]. First, the fractions of neutral (PC and PEA) and acidic (phosphatidylserine, PG and cardiolipin) glycerophospholipids were eluted from an aminopropyl bonded cartridge. The individual glycerophospholipid classes were subsequently isolated from a serial manifold of silica gel and aminopropyl cartridges. Neutral glycerophospholipids were separated under a polarity gradient of the elution system and acidic glycerophospholipids – under a pH gradient of eluents.

All reviewed approaches involved collection of an intermediate fraction of a mixture of lipid classes, which require subsequent loading onto a new SPE manifold for future separation. We have not found in the literature a previous description of the continuous separation of lipid extracts from which individual lipid classes were collected directly as separate fractions.

Plant membrane lipids are mainly composed of glycerolipids [17]. The complete resolution of all glycerolipids is a demanding and complicated process because glyceroglycolipids tend to co-chromatography with glycerophospholipids [18,19]. Additional difficulties arise also from the presence, in plant tissues, of chlorophyll and other pigments [20]. Because of these difficulties, most probably we have not found in the literature information concerning the separation of plant membrane lipids by SPE.

The aim of the present work was to investigate the efficiency of separation of glycerolipids [glyceroglycolipids, such as MGDG, DGDG and sulfoquinovosyldiacylglicerol (SQDG), and glycerophospholipids – PG, PC, PEA, PI] from photosynthetic and non-photosynthetic plant tissues by means of SPE.

2. Experimental

2.1. Reagents

Lipid standards for glyceroglycolipds: MGDG, DGDG and SQDG and glycerophospholipids: PG (soya), PI (wheat germ, sodium salt), PC (soya) and PEA (corn germ) were purchased from Larodan (Malmö, Sweden). A standard mixture of fatty acid methyl esters (Supelco 37 component FAME mix) was obtained from Supelco (Bellefonte, PA, USA) and heptadecanoic acid from Sigma (USA). All other reagents were of analytical or HPLC grade. Solvents, such as chloroform, acetone, diethyl ether, tetrahydrofuran (THF), acetonitrile, propan-2-ol, methanol, *n*-hexane and heptane and acids, such as sulfuric, formic and acetic, as well as ammonium acetate, were obtained from Merck (Darmstadt, Germany) or Sigma.

2.2. Procedures

2.2.1. Extraction of total lipids from plant tissue

Rice seedlings (*Oriza sativa* L., cv. *Indica*) were cultivated in a growth chamber (E. Chrisagis, Athens, Greece). The growth conditions were 25°C/22°C day/night and 16-h photoperiod (700 μmol m^{-2} s^{-1}) [21]. At the tillering stage, leaves, leafy stems and roots were collected from individual plants. The maize leaves (*Zea mays* L.) were collected from mature plants growth in a greenhouse (John Innes Center, Norwich, UK) Immediately after cutting, the plant tissues were immersed in liquid nitrogen and then stored at −80°C until used.

For successful semi-preparative separation of all glycerolipids of interest, 0.3 g of plant tissue was ground to fine powder in the presence of liquid nitrogen. While the tissue was still frozen, methanol–chloroform–water (2:1:0.8, v/v) [22] was added in a ratio of one part tissue to three parts solvent. The extraction was continued until the sample completely lost its color. After filtration, the cleared extract was transferred into a separation funnel. A 5-ml volume of chloroform and water was added for complete separation of the two phases. The chloroformic fraction was collected and evaporated to dryness using a vacuum evaporator (Heidolph WB2001, Germany), at 40°C. The dry residue was then dissolved in 0.2–0.5 ml chloroform.

2.2.2. SPE optimization

The selection of stationary phases and elution solvents for SPE was directed by previously reported data for column [6–16] or HPLC [5,23] lipid separations together with the results of pilot runs. The quantities and composition of all elution systems were determined by extensive experimentation. The efficiency of separation was verified by visual examination following TLC. The SPE optimization was performed with 0.45 ml standard glycerolipid mixture (0.11 mg ml^{-1} of MGDG, DGDG, SQDG, PEA, PI, 0.22 mg ml^{-1} of PG and 0.19 mg ml^{-1} of PC).

All of our efforts for glycerolipid separation with a single or paired identical SPE column (silica gel or aminopropyl) led to a mixed elution mode.

The pigments from leaves and leafy stems (pigment containing tissues) were preliminarily separated from glycerolipds at the unpaired aminopropyl column. The aminopropyl stationary phase was selected in order to prevent the breakdown of chlorophylls, which is catalyzed by silica gel [24]. The composition and temperature of elution system used reflected the conclusions in Refs. [25] and [26], respectively.

MGDG, PEA, PG and DGDG demonstrated promising elution behavior in serial SPE manifold of aminopropyl and silica gel columns utilizing solvent mixtures as simple as hexane–propan-2-ol varying from 1:1 to 1:8 (v/v). The major challenge was separation of MGDG from PEA as individual fraction. The addition of small amount of acetic acid (0.2 ml glacial) to the solvent mixture however allowed full (approx. 90%) elution of MGDG with a low level of contamination with PEA (approx. 20%). Likewise addition of acetic acid in the second elution system resulted in an almost exclusive isolation of PEA (approx. 80%) as a second fraction. The decreased temperature of the solvent mixture for MGDG elution (4°C) prevented tailing of the MGDG spot with PEA during TLC examination. The subsequent addition of chloroform, THF and acetonitrile to the solvent mixture produced reproducible and more complete separations of MGDG and PEA,

PG and DGDG without addition of acetic acid to the third and fourth solvent systems.

No residual lipids remained in the uncoupled aminopropyl column when the above elution process was completed. This was verified by passing of 30 ml of chloroform–methanol (1:5, v/v) through the aminopropyl column followed by TLC of the collected fraction.

To continue the separation process, the silica gel column (containing the remaining glycerolipids) was connected to a new aminopropyl column and a series of elution systems were tested for stepwise removal of the remaining components. Chloroform–methanol–0.1 *M* ammonium acetate [27] did not give satisfactory separation. SQDG, PI and PC co-eluted in all fractions. The preliminary activation of the aminopropyl stationary phase as weak anion exchanger resulted in the predominant elution of SQDG, PI and PC as the fifth, sixth and seventh fractions, respectively. According to the approach adopted by the end, the aminopropyl column was preconditioned (for weak anion-exchange) with 2 ml each of methanol and deionized water, and conditioned by passing first 2 ml of 0.1 *M* HCl, then 2 ml each of deionized water and methanol, and last 2 ml heptane ($-CH_2CH_2CH_2NH_2 \rightarrow -CH_2CH_2CH_2-N^+H_3Cl^-$). However, TLC analysis indicated cross-contamination among separated lipids at approximately 25–30%. The addition of propan-2-ol and acetonitrile to the elution system resulted in the complete resolution of the last three separated glycerolipids. The supplementation of propan-2-ol in different quantities to the elution mixture allows precise polarity adjustment. The polarities of the fifth, sixth and seventh elution systems (Table 1), measured as conductivity, were 184, 415 and 719 μS, respectively. The acetonitrile [5] addition resulted in more rapid and effective elution of the separated lipids.

2.2.3. Separation of plant lipid extract

In the proposed approach for SPE continuous elution took place, and only individual fractions of glycerolipids were collected. For all elution processes, the flow velocity was maintained at approximately 1 ml min^{-1} and the pressure was applied with a 20-ml glass syringe. The glycerolipid SPE separation process can be divided into three steps:

First step: after loading of the whole lipid extract as a continuous plug into an unconditioned aminopropyl column (500 mg, from Macherey-Nagel, Düren, Germany), pigments were removed with elution system 0 (for the nature, volume and temperature of all elution systems, see Table 1).

Second step: the aminopropyl column with the sample loaded was connected to a silica gel column (1000 mg, from Macherey-Nagel), which was preliminary conditioned using 4 ml heptane. The individual fractions of MGDG, PEA, PG and DGDG were recovered in sequence with elution systems 1, 2, 3 and 4 (Table 1).

Third step: the already used aminopropyl column

Table 1
Composition, volume and temperature of used elution systems

Solvent	Composition of elution systems (ml)							
	0	1	2	3	4	5	6	7
n-Hexane		45	33	10				
Chloroform	80	5	5	5	15	10	10	10
Acetone	20							
Tetrahydrofuran		10	10	10				
Acetonitrile		10	10	10		10	10	10
Propan-2-ol		40	52	75	30	30	20	10
Methanol				20	50	50	50	50
Acetic acid, glacial		0.2	0.1					
Methanolic 0.1 *M* ammonium acetate (pH 8.4)						5	10	15
Volume of elution (ml)	4	10	10	15	15	5	5	5
Temperature of elution (°C)	4	4	25	25	25	25	25	25

was disconnected from silica gel column and the former column (containing the remaining part of the lipid extract) was connected to a new aminopropyl (weak anion exchanger) column (500 mg, from Macherey-Nagel). The elution process continued with fifth, sixth and seventh elution systems (Table 1). In this manner individual fractions of SQDG, PI and PC were eluted.

The quantification of separated plant lipid classes was carried out using a Hewlett-Packard 8452A diode array spectrophotometer. Calibration curves were produced for every lipid standard by absorbance determination at 205 nm [4]. Similarly, the efficiency of pigment removal was also assessed spectrophotometrically at 430 nm [28].

2.2.4. Thin-layer chromatography

Routine visual checks of the chromatographic purity of each eluted lipid class were made using a silica gel HR 60 TLC plate (Merck). Samples were spotted manually using a 100-μl Hamilton syringe. The plate had previously been thermally activated at 130°C for 1–2 h. Each plate was developed vertically using a solvent system of chloroform–methanol–acetic acid–water (170:30:20:7, v/v) [3]. Visualization was achieved by spraying with 50% sulfuric acid and heating for 10 min at 100°C. The glycerolipid identification was carried out by comparison with commercial standards.

2.2.5. Fatty acid analysis

The whole glycerolipid extract or 20 μl of the monogalactosyldiacylglycerol or phosphatidylinositol standards before and after SPE were subjected to acid transmethylation [29] at 100°C for 2 h with 0.5 ml methanol containing 2% H_2SO_4, 0.05 ml benzene and heptadecanoic acid as internal standard. Extracts were then left to cool and extracted against 1 ml light petroleum and 0.5 ml water. The light petroleum phase was evaporated under a nitrogen stream and 50 μl heptane was added. Fatty acid methyl esters were injected into a Hewlett-Packard 5890, series II gas chromatography (GC) system equipped with a flame ionization detection (FID) system. A HP-FFAP (crosslinked) capillary column (50 m×0.20 mm I.D.) with a 0.25 μm film thickness was employed. Oven temperature was isothermal at 220°C, while injector and detector temperatures were 240°C. Fatty acid methyl esters were identified by their retention times on comparison with those of the commercial standards.

3. Results and discussion

The interaction of the non-polar functional groups and the acyl moieties of MGDG, DGDG, PEA and PC with the aminopropyl stationary phase is weaker than their interaction with silica gel because of the reduced number of hydrogen groups at the surface of the former [30]. The acidic polar membrane lipids (SQDG, PI and PG) are attracted to the aminopropyl stationary phase through dipole–dipole interactions. Due to this series of different interactions, the aminopropyl bonded column initially separates non-polar and polar lipids.

The elution order of non-polar glycerolipids from the serial SPE manifold of one aminopropyl and one silica gel column under polarity, pH and temperature gradients was: MGDG, PEA, PG and DGDG. Elution of PG as the third fraction of the lipid mixture, before DGDG, provided evidence that the separation was predominantly determined by the interaction of lipids with the silica gel stationary phase. The pH difference between the first and the second elution systems (Table 1) was approx. 0.14 (calculated by assuming that the dissociation constant of acetic acid was the same in both solvent mixtures). For the non-aqueous systems used, this pH gradient was sufficient to resolved MGDG and PEA. On the other hand, the acidity of first and second elution systems was high enough to interrupt the interaction between the aminopropyl stationary phase (free-base form) and the acidic functional group of PG. Because of this, hydrogen bonds between the acyl moiety and the silanol group of silica gel determined the elution order. Decreasing the temperature of the first elution system (Table 1) proportionally decreased the power of the hydrogen bond interaction between lipids and the two stationary phases (aminopropyl and silica gel). However, lowering the temperature to 4°C affected the phase behavior of MGDG during SPE. The inclusion of 36% propan-2-ol in the first elution system (Table 1), which was estimated to be equivalent to approximately 17% water in terms of polarity [31], resulted in a reduction of the area of the polar

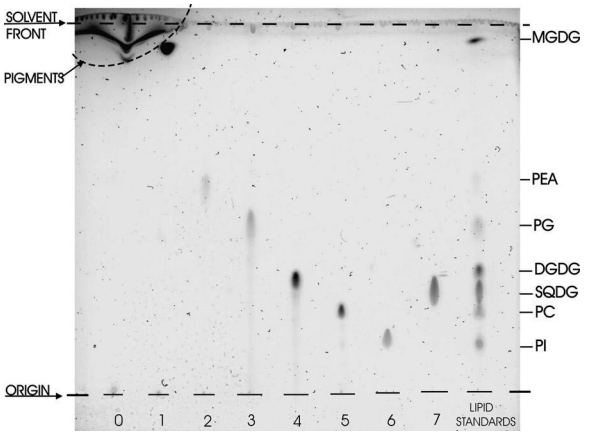

Fig. 1. Visual verification of the purity of glycerolipid classes separated by SPE from rice leaves. Lipid classes were fractionated as described in the Procedures section. Aliquots of each fraction were chromatographed and the plate was visualized as described in the Procedures section. The numbers under each band represent the chromatographed fraction (for details see Table 1).

head group, which in turn promoted the adoption of conical shape by the MGDG molecule [32]. Under these conditions, the MGDG will tend to form a reversed hexagonal phase [32], which can act as a stereospecific factor contributing to the separation of MGDG from the other plant membrane lipids. This turned out to be the case since lowering the elution temperature allowed isolation of the MGDG class without the co-elution of the other lipid classes (Fig. 1).

The separation of SQDG, PI and PC was continued on a serial SPE manifold combining the silica gel and an aminopropyl weak anion exchange (charged form) stationary phase. The separation process is supported by ammonium counter ions. The pH of the ammonium acetate (8.4) is slightly above the working pH diapason of the silica gel based stationary phase [33], which means that silica particles will partially solubilise. However, since SPE columns are cheap and can be easily replaced, column life reduction is not considered to be a problem.

As judged spectrophotometrically, 4 ml of chloroform–acetone (4:1, v/v) at 4°C effectively separated pigments from rice lipids on the aminopropyl SPE column. In a previous study [26], the same elution system had been used to separate pigments from lipids originating from spinach thylakoids by column chromatography with silicic acid (adjusted to pH 8.0 at 5°C). From a silica gel column (without adjustment of pH and at room temperature) elution with chloroform was reported to remove pigments together with neutral lipids [34]. In our case, SPE, the amount of pigments cross contaminating the MDGD fraction was as low as 1.3% of the totally applied chlorophylls and carotenoids. On the other hand, and as assessed by TLC (Fig. 1), none of the lipids co-eluted with the pigment fraction.

Following SPE fatty acids of the MGDG class tended to lose unsaturation (Table 2). The biggest changes (of 29.3% and 16.8%) were detected for linoleic and stearic acid, respectively. Since their absolute amounts were 0.40 μg and 0.53 μg it can be proposed that observed changes were, to a significant extent, attributable to instrumental error (GC–FID). The changes in stearic and linoleic acid content of the PI class after SPE ranged from 3.7% to −12.1%, respectively (absolute quantities of 0.15 μg and 0.95 μg). So, the overall fatty acid distribution in sepa-

Table 2
Fatty acid composition of monogalactosyldiacylglycerol and phosphatidylinositol standard before and after SPE

Lipid class	Fatty acid content (%)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
MGDG						
Before (n=2)	7.85	1.83	5.24	28.8	3.93	52.4
After (n=3)*	8.31±0.07	1.75±0.01	6.12±0.05	26.7±0.51	5.08±0.04	50.1±1.71
PI						
Before (n=2)	18.1	16.2	3.3	16.9	21.5	24.0
After (n=4)*	19.0±0.21	17.3±0.09	2.9±0.02	15.8±0.11	22.3±0.16	25.6±0.24

*Data are expressed as mean±SD.

rated glycorolipids was practically unaffected by the SPE procedure.

The repeatability of the proposed method was examined by employing the glycerolipid standards and lipid extracts from rice leaves (Table 3), while the recovery was assessed using glycerolipid standards alone. Repeatability (presented as a relative standard deviation) varied from 2.3 to 6.1% for the standard glycerolipid mixture and from 3.1 to 7.3% for the rice leaf extracts. Recovery, of all seven of the studied standard lipid classes varied from 87 to 95% when 50–100 μg was loaded. The glycerolipid profile of the maize leaves determined using the proposed SPE method (Table 3) agreed very closely with the data (presented as percent of total) reported by Roughan and Batt [35] and Leech et al. [36]. The good agreement of the data produced using the SPE method with the independent data indicates that the proposed separation method provides reproducible results and ensures high recovery of the initial material.

The efficiency and repeatability of plant membrane lipid separation by the proposed SPE technique are strongly influenced by the sample loading conditions. The high polarity of some glycerolipids requires use of equally polar sample solvents. However, in SPE, the sample solvent also acts as an elution agent. The chloroform (eluotropic strength 0.31; [37]), in which glycerolipids are dissolved, partially competes with the analytes for polar interactions with the solid phase (aminopropyl and silica gel) and this in turn decreases the resolution of separation. For this reason, some researches have chosen to dissolve their samples, after extraction, in less polar solvents [9,11,14,16] or to work at lower levels of resolution [8,10,15] where repeatability is high. With our protocol, lipid samples were loaded onto an unconditioned SPE column as a continuous plug. This resulted in the activation of the stationary phase by the sample solvent and did not cause sample elution. In this way good repeatability and recovery were achieved, while high levels of resolution were maintained.

4. Conclusions

The proposed method for the separation of plant lipid extracts using multiple SPE is highly flexible in terms of resolution and selectivity of the stationary phase. During the continuous process of elution the entirety of each glycerolipid classes was collected as individual fraction. The modulation of the stationary phase properties, together with variation of the polarity, temperature, pH and ionic strength gradients of the elution systems, allows the separation of plant membrane lipids under mild conditions during a single SPE run and permits effective resolution of glyceroglycolipids and glycerophospholipids. Using this method, lipid extracts originating either from pigment-containing (rice and maize leaves and rice leafy stems) or pigment-free (rice roots) tissues were rapidly, effectively and reproducibly separated. The method is expected to be equally applicable and effective for the isolation of lipids from similar tissues of other plants.

Table 3
Glycerolipid composition of rice and maize plant tissues

Lipid class	Content of lipids (μg g^{-1} frozen tissue)			
	Leaves		Rice leafy stems (n=3)	Rice roots (n=3)
	Maize (n=2)	Rice (n=5)		
MGDG	781±6	637±20	527± 17	583±20
DGDG	520±30	500±10	401±12	300±14
SQDG	121±16	67±4	34±4	7±1
PEA	205±5	220±12	260±8	217±11
PC	26±7	323±24	367±12	383±13
PI	14±2	50±3	25±1	3±1
PG	84±3	243±11	141±7	47±6

Separation and quantification were performed as described in the Procedures section; data are presented as mean±SD.

Acknowledgements

I.R. was supported by a post-doctoral research grant from the State Scholarship Foundation of Greece (I.K.Y.) and by the Mediterranean Agronomic Institute of Chania (CIHEAM/MAICh).

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