



# Metodologies analítiques per a l'estudi de compostos al·leloquímics en conreus de blat

Marta Villagrasa Giménez



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## **METODOLOGIES ANALÍTIQUES PER A L'ESTUDI DE COMPOSTOS AL·LELOQUÍMICS EN CONREUS DE BLAT**

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Novembre 2013

# CAPÍTOL IV.- ANÀLISI DE COMPOSTOS AL·LELOPÀTICS EN MOSTRES DE BLAT I SÒL AGRÍCOLA

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## CAPITOL IV.- ANÀLISI DE COMPOSTOS AL·LELOPÀTICS EN MOSTRES DE BLAT I SÒL AGRICOLA

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### IV.1. INTRODUCCIÓ I OBJECTIUS

En els darrers anys, hi ha hagut un interès creixent per explotar l'al·lelopatia com a alternativa al control de les males herbes, però també a insectes i altres malalties. Per dur a terme tal objectiu i fer ús de cultius amb majors propietats al·lelopàtiques o bé sintetitzar nous compostos i usar-los com a herbicides o insecticides naturals, és necessari dur a terme estudis com els realitzats dins del projecte FATEALLCHEM. El possible ús de l'al·lelopatia no tan sols hauria d'incrementar el potencial al·lelopàtic en cultius convencionals, on s'usen productes químics, sinó també en cultius orgànics on l'ús de plaguicides no és permès i l'ús de cultius amb propietats al·lelopàtiques compensaria la pèrdua de la producció causada per la presència de males herbes i/o plagues. Per tant, en qualsevol sistema de producció, els indicadors sobre nous o majors nivells de compostos al·lelopàtics en diferents varietats de blat són necessaris per tal d'avaluar el progrés en la millora de la sostenibilitat dels sistemes agrícoles.

Els processos de degradació que afecten als àcids hidroxàmics, les aglucones i les benzoxazolinones, podrien incrementar o disminuir els efectes fitotòxics d'una planta envers un agent extern. Qualsevol procés de degradació que tingui lloc en el sòl es veurà afectat per l'entorn biològic de les plantes presents. Aquests processos defineixen les propietats al·lelopàtiques i el destí final del compost alliberat [1]. El transport dels compostos al·leloquímics cap al sòl pot donar-se principalment per la lixiviació des de les parts aèries [2, 3], exsudació de les arrels [4], descomposició dels residus de la planta per l'acció microbiana [5] o directament per la transformació associada als microorganismes presents en les arrels [6].

Els mètodes desenvolupats en els capítols II i III d'aquesta tesi van ser aplicats en l'anàlisi de mostres de blat i sòl agrícola amb la finalitat d'identificar i quantificar el contingut de compostos al·leloquímics. Els principals objectius plantejats són:

- Identificar i quantificar la quantitat de compostos al·lelopàtics en:
  - o Diferents varietats de blat conreades en diferents sistemes de cultiu (Convencional i orgànic).
  - o Diferents estadis de creixement del cultiu de blat descrits segons l'escala BBCH. L'escala BBCH és un sistema de codificació uniforme d'identificació fenològica dels estadis de creixement per a totes les espècies de plantes mono i dicotiledònies. Utilitza un sistema de codi decimal que es divideix en etapes de creixement principal i secundàries i es basa en el sistema de codi de cereals de Zadoks.
  - o Diferents parts de la planta com la fulla, arrel i llavors germinades.
  - o En l'ambient del sòl circumdant a l'arrel sota les condicions esmentades anteriorment.

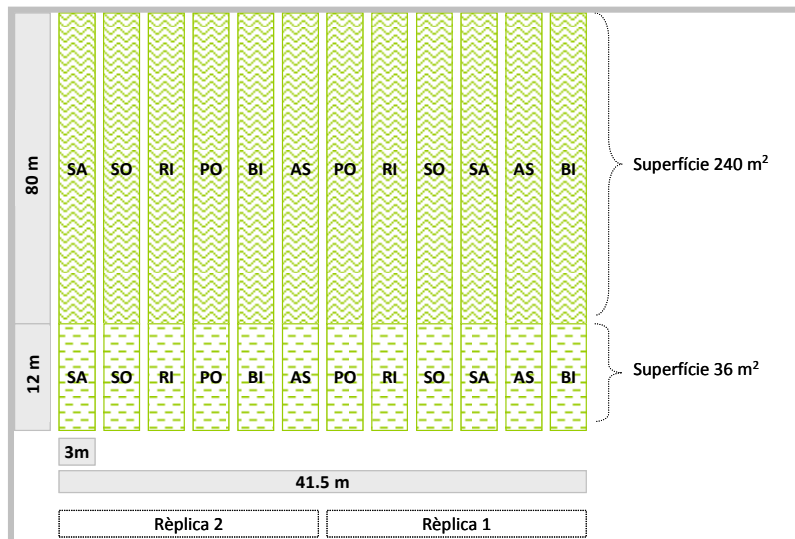
## IV.2. DESCRIPCIÓ DELS CULTIUS EXPERIMENTALS

Les mostres de blat i sòl agrícola que s'han analitzat en aquest treball han estat cultivades a Lleida pel grup del Dr. Andreu Taberner del Departament d'Hortofruticultura, Botànica i Jardineria de l'Escola Superior d'Enginyeria Agrària de la Universitat de Lleida. Tanmateix la presa de mostra la va realitzar el personal del mateix grup. Es van realitzar dues campanyes en les quals diferents varietats de blat varen ser cultivades sota l'agricultura convencional (ús de plaguicides) i orgànica. En cadascuna d'aquestes campanyes es van dur a terme 5 collites corresponents a diferents estadis de creixement de la planta, descrits per l'estat fenològic del blat, segons l'escala BBCH (veure apartat I.1 El cultiu del blat del Capítol I.- Introducció i objectius).

Algunes accions realitzades durant la presa de mostra en la primera i segona campanya varen ser diferents, les característiques de cadascuna d’elles s’exposen a continuació.

**IV.2.1. Primer disseny experimental**

Les mostres de la primera campanya es van cultivar en dos espais iguals però diferenciats un per a l’agricultura orgànica i l’altre per a la convencional. El disseny del camp es mostra a la Figura IV.1, on sis varietats de blat van ser cultivades per duplicat, Stakkado (SA), Solist (SO), Ritmo (RI), Portal (PO), Bill (BI) i Astron (AS) en una superfície total de 240 m<sup>2</sup>. Paral·lelament es van cultivar les mateixes varietats ocupant una parcel·la de 36 m<sup>2</sup> destinada als estudis de degradació.



**Figura IV.1.-** Disseny del camp dels cultius de les diferents varietats de blat en la primera campanya

Les dates de la sembra i de la presa de mostra per a les mostres cultivades en l’agricultura orgànica i convencional es mostren a la Taula IV.1.

**Taula IV.1.-** Dates de sembra i collita de les mostres de la primera campanya

|   | Cultiu Orgànic | Cultiu Convencional |
|---|----------------|---------------------|
| Data Sembra                                     | 09/11/2001     | 14/12/2001          |
| 1 <sup>a</sup> collita (1 <sup>er</sup> estadi) | 11/12/2002     | 31/01/2002          |
| 2 <sup>a</sup> collita (2 <sup>on</sup> estadi) | 08-16/01/2002  | 12-13/02/2002       |
| 3 <sup>a</sup> collita (3 <sup>er</sup> estadi) | 05-11/02/2002  | 18-19/03/2002       |
| 4 <sup>a</sup> collita (4 <sup>t</sup> estadi)  | 16/04/2002     | 24/04/2002          |
| 5 <sup>a</sup> collita (5 <sup>è</sup> estadi)  | 16/05/2002     | 28/05/2002          |

La presa de mostra es va dur a terme agafant 10 plantes de cada varietat, separant la fulla de l'arrel i un cop arribades al nostre laboratori conservant-les a -20°C fins al moment del seu anàlisi. El fet de no haver congelat les mostres en el moment de la collita, tal i com s'exposa en els articles presentats en aquest capítol, va tenir com a conseqüència una degradació dels compostos glucosats, fet que va suposar no poder detectar-los en les mostres de fulla i arrel de la primera campanya. Per a cada cultiu en cada estadi hi ha un total de 24 mostres, de manera que el resultat total de mostres recollides va ser de 240. Durant la conservació de les mostres hi va haver un problema amb el congelador, i part de les mostres es van descongelar de manera que no van poder ser analitzades, i finalment el total de mostres analitzades de la primera campanya varen ser 120, corresponents a les dues rèpliques del primer, segon i tercer estadi del cultiu convencional i al quart i cinquè de l'orgànic. A part de la fulla i arrel també es van analitzar les llavors germinades del primer i segon estadi del cultiu convencional.

#### IV.2.2. Segon disseny experimental

Una vegada avaluats els resultats de l'anàlisi de les mostres de la primera campanya es va decidir reduir el nombre de varietats a analitzar, escollint les varietats AS, RI, SA que són les que més nivells de compostos al·lelopàtics presentaven i augmentant el nombre de rèpliques a analitzar. El disseny del camp es mostra a la figura IV.2.

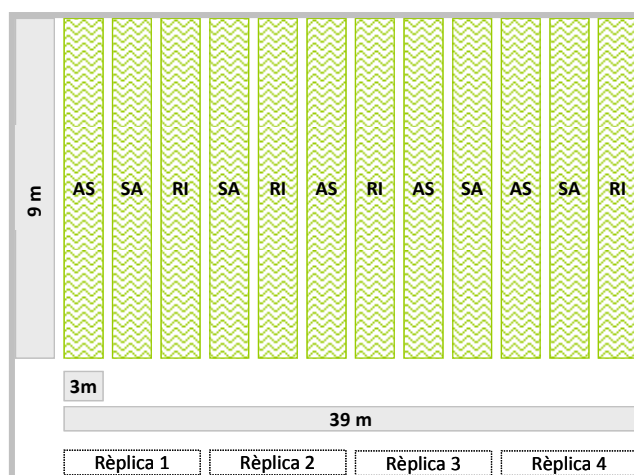


Figura IV.2.- Disseny del camp mostres segona campanya



De la mateixa manera 10 mostres de cada varietat es van recol·lectar als diferents estadis de creixement, en el mateix camp es va separar la fulla de l'arrel i es van conservar a -20°C immediatament en el mateix moment de la presa de la mostra. A més a més 100g de sòl agrícola propers a la zona de l'arrel va ser collit i conservat a -20°C. Tot i que inicialment es van cultivar 4 rèpliques només tres d'elles van ser analitzades. Les dates de la sembra i de la presa de mostra de la segona campanya per a les mostres cultivades en l'agricultura orgànica i la convencional es mostren a la Taula IV.2.

**Taula IV.2.-** Dates de sembra i collita de les mostres de la segona campanya

|   | Cultiu Orgànic | Cultiu Convencional |
|---|----------------|---------------------|
| Data Sembra                                     | 06/11/2002     | 06/11/2002          |
| 1 <sup>a</sup> collita (1 <sup>er</sup> estadi) | 28/11/2002     | 28/01/2002          |
| 2 <sup>a</sup> collita (2 <sup>on</sup> estadi) | 12/12/2002     | 13/12/2002          |
| 3 <sup>a</sup> collita (3 <sup>er</sup> estadi) | 15/01/2003     | 16/01/2003          |
| 4 <sup>a</sup> collita (4 <sup>t</sup> estadi)  | 24/04/2003     | 25/04/2003          |
| 5 <sup>a</sup> collita (5 <sup>è</sup> estadi)  | 22/05/2003     | 22/05/2003          |

D'aquesta manera, per a cada cultiu en cada estadi s'analitzen un total de 12 mostres. El total de mostres de planta analitzades va ser de 180, mentre que de mostres de sòl van ser analitzades, finalment, una rèplica corresponent al primer i segon estadi tant del cultiu orgànic com convencional resultant en un total de 12 mostres. A part de la fulla, l'arrel i el sòl agrícola també es van analitzar les llavors germinades del primer i segon estadi del cultiu convencional i orgànic.

### IV.3. PUBLICACIONS CIENTÍFIQUES

El treball referent a l'aplicació dels mètodes desenvolupats en els anteriors capítols en l'anàlisi de les plantes de blat i el sòl agrícola ha donat lloc a dues publicacions científiques. El treball #6, que es troba descrit en la secció IV.3.1 del present capítol i que porta per títol : *"Benzoxazinoid Allelochemicals in Wheat: Distribution among Foliage, Roots, and Seeds"*, es presenten els resultats de la distribució dels diferents analits en les matrius de la fulla, arrel i les llavors corresponents a les mostres dels dos primers estadis d'ambdues campanyes. Mentre que en el treball #7 descrit en la secció IV.3.2 del present capítol amb el títol: *"Concentration of benzoxazinoid allelochemicals*

*in wheat samples (Triticum aestivum)*” es mostren els resultats de tots els analits en la fulla i l’arrel de totes les mostres de blat analitzades del primer i segon disseny experimental.

#### IV.3.1. Distribució dels compostos al·lelopàtics en diferents parts del blat

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Publicació Científica #6

“Benzoxazinoid Allelochemicals in Wheat: Distribution among Foliage, Roots, and  
Seeds”

Per:

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Damià Barceló

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## Benzoxazinoid Allelochemicals in Wheat: Distribution among Foliage, Roots, and Seeds

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In this study, the distribution of eight allelochemicals among the foliage, roots, and seeds of different wheat varieties is reported for two different sampling campaigns, corresponding to two consecutive years. The determination of benzoxazinoid derivatives was performed by combining pressurized liquid extraction–solid-phase extraction followed by liquid chromatography–electrospray mass spectrometry. To the authors' knowledge, there are no previous works about the content of allelochemicals in seed tissue of germinated wheat seedlings. Allelochemicals found in seeds were detected at levels similar to those found in foliage and roots. The results showed that the type of metabolites detected depends strongly on the working up procedure of the plant material, as well as of plant growth stage. A general decrease of the total amount of allelochemical content in the plants was observed with plant age. There was a significant difference in the total amount of benzoxazinoid derivatives in the different wheat varieties analyzed.

**KEYWORDS:** Allelochemicals; hydroxamic acids; wheat varieties; foliage; roots; seeds

### INTRODUCTION

Plants produce a wide variety of secondary metabolites that play important roles in complex interactions among living organisms in the natural environment. Allelopathy has been defined as "any process involving secondary metabolites produced by plants, microorganisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects" (1). The term allelopathy has mostly been used by plant ecologists who have focused on allelopathic effects between agricultural crops and weeds. There is an enormous diversity of allelochemicals in nature. About 10 000 compounds are known, whereas another 400 000 are estimated to exist (2). Poaceae such as wheat, rye, and maize contain secondary metabolites at different concentrations, depending on varieties and environmental conditions. One group of allelochemicals that has been heavily in focus during the past decade is the benzoxazinoid derivatives. The group of chemical compounds named benzoxazinoid derivatives is subdivided into hydroxamic acids (Hx), lactams, benzoxazolinones, and methyl derivatives of the hydroxamic acids. Benzoxazinoids and particularly their aglycone forms have been reported to be involved in the defense of the plant against a wide variety of organisms, including fungi, bacteria, and a range of insects. The concentration of these

compounds in plants is influenced by a range of factors, such as light intensity (3), temperature (4), and water availability (5). The latter authors suggested that these compounds could be stress metabolites.

The cyclic Hx and lactams are naturally present in the seedling of several Poaceae as 2-*O*- $\beta$ -D-glucosides. The glucosides are enzymatically converted into aglycone forms by the action of  $\beta$ -glucosidases after the plant tissue is crushed (6). Aglycones are unstable and are easily converted to benzoxazolinones. The main structures are shown in **Figure 1**. Previous studies showed that maximal concentrations of hydroxamic acids in roots and aerial parts of cereal seedlings occurred between 4 and 6 days after germination (7, 8). Despite the subsequent decline in concentrations of Hx, the total amount within the whole seedling continued to increase, albeit at a slower rate. This increase continued up to 8 days after germination (9). Hx has not been detected on nongerminated seeds (9–12). Copaja et al. (13) analyzed germinated seeds of wheat and detected no benzoxazinoids. Several authors present the levels of some allelochemicals in wheat seedlings. The concentration of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) found in the shoots of wheat seedlings ranged from 1.4 to 10.9 mmol/kg of fresh weight in a collection of Chilean cultivars (8), from 0.99 to 8.07 mmol/kg of fresh weight in a worldwide collection (7), and from 0.21 to 16.0 mmol/kg of fresh weight in a collection of wheat progenitors (14).

The aim of our study was to determine the distribution of the eight allelochemicals [2- $\beta$ -D-glucopyranosyloxy-4-hydroxy-

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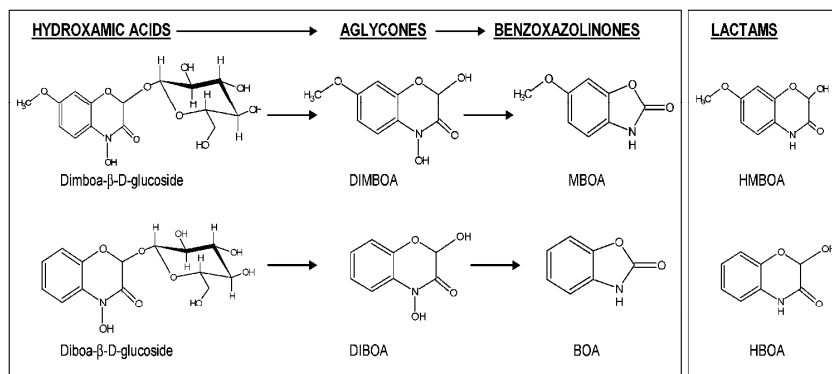


Figure 1. Structures of main benzoxazinoid derivatives.

1,4-benzoxazin-3-one (DIBOA-β-D-glucoside), 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-β-D-glucoside), 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), DIMBOA, 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), benzoxazin-2-one (BOA), and 6-methoxybenzoxazin-2-one (MBOA) among the foliage, root, and seed of different wheat seedlings cultivated under different cultivation conditions. To our knowledge, there are no previous works about the content of allelochemicals in seed tissue of germinated wheat seedlings.

## MATERIALS AND METHODS

**Chemicals and Materials.** The benzoxazinoid standards were obtained from commercial and private sources as available: DIBOA-β-D-glucoside and DIMBOA-β-D-glucoside, from Prof. Dr. Hajime Iwamura (Kyoto University), Prof. Dr. Lisbeth Jonsson (Södertörn University College), and Dr. F. Macías (University of Cadiz); DIMBOA from Dr. Scott Chilton, University of North Carolina; HBOA, HMBOA, DIMBOA, MBOA, and BOA, from Dr. F. Macías (University of Cadiz); DIBOA and the non-naturally occurring synthetic derivative 2-methoxy-2H-1,4-benzoxazin-3(4H)-one (2-MeO-HBOA) from Dr. Sicker (University of Leipzig).

HPLC-grade solvents water (H<sub>2</sub>O), methanol (MeOH), and 98% pure acetic acid (HOAc) were purchased from Merck (Darmstadt, Germany). Diatomaceous earth was obtained from Varian Inc. LiChrolut RP C<sub>18</sub> (500 mg) solid-phase extraction (SPE) cartridges were purchased from Merck.

**Sample Collection.** The distribution of the allelochemicals among foliage, roots, and seeds was analyzed in two different sampling campaigns, corresponding to two consecutive years. The wheat plants were grown in Lleida (Spain). In the first sampling campaign (autumn 2001), four different wheat varieties, Astron (As), Ritmo (Ri), Bill (Bi), and Solist (So), were grown in conventional cultivation conditions; the samples were collected and were then frozen in the laboratory and stored at -20 °C until further manipulation. In the second sampling campaign (autumn 2002), three wheat varieties, As, Ri, and Stakado (Sa), were grown in conventional and organic cultivation. Immediately after the harvest, the samples were frozen and stored at -20 °C. In each sampling campaign, 10 plants of each wheat seedling were collected at the Zadocks stages 10 and 12 days, corresponding to two different stages. The stages were defined by BBCH scale (a system for uniform coding of phenologically similar growth stages of all mono- and dicotyledonous plant species).

**Sample Preparation Procedures.** The water in wheat samples was removed by lyophilization until weight loss was no longer observed. The samples were divided into foliage, roots, and seeds; roots and seeds were cut finely, whereas foliage was ground with a pestle in a mortar. Pooled samples were obtained by combining the 10 plants collected at each Zadocks stages. A 0.1 g subsample was extracted by pressurized liquid extraction (PLE) using an ASE 200 (Dionex, Idstein, Germany) apparatus, equipped with 11 mL stainless steel extraction cells.

Diatomaceous earth was used to fill the extraction cells, with the matrix and sample thoroughly mixed to ensure good dispersion of the sample. The diatomaceous earth was cleaned by ultrasonication with the same solvent of the extraction and dried at 70 °C prior to use. Conditions of pressure (1500 psi), static times (3), cell purge (60 s), flush volume (60%), solvent composition [100% acidified MeOH (1% HOAc)], and temperature (150 °C) were used. A purification of the samples prior to instrumental analysis is recommended because in the raw extracts of plants the broad variety of substances (salts, lipids, glycosides, phosphates, peptides, macromolecules, and chlorophyll) can influence the quantification. The organic extracts were concentrated to dryness by rotary evaporation and redissolved in 2 mL of acidified water (1% HOAc) prior to the cleanup step. The reconstituted extracts in acidified H<sub>2</sub>O resulted in a turbid solution; a filtration step prior to purification was therefore required to prevent clogging of the cartridge. Filtration was performed using 1 μm 25 mm syringe-driven filter units. Purification was performed via LiChrolut RP C<sub>18</sub> SPE cartridges activated and preconditioned with 5 mL of acidified MeOH (1% HOAc) followed by 5 mL of acidified H<sub>2</sub>O (1% HOAc). The concentrated and filtered extracts were then applied to the purification step. A two-step elution procedure was used: first, with 6 mL of acidified H<sub>2</sub>O (1% HOAc) and second with 5 mL of acidified MeOH/H<sub>2</sub>O (1% HOAc) (60:40). Benzoxazinoid derivatives were well recovered in the second fraction (52–99%), with the exception of the most polar compounds (DIBOA-β-D-glucoside, HBOA, and DIBOA), which eluted in the first fraction (56–69%) (15). Both fractions were then analyzed by LC-ESI-MS.

**Chromatographic Conditions.** Analyses were performed on a HP 1100 LC-MS. A Synergi Max-RP 80A (C-12 TMS) LC column (250 × 4.6 mm Phenomenex) with a solvent flow rate of 1 mL/min was used. The sample injection volume was set at 50 μL. Acidified H<sub>2</sub>O (0.05% HOAc) and MeOH were used as the elution solvents A and B, respectively. The solvent gradient adopted was as follows: 0–2 min, 100–70% A; 2–19 min, 70–40% A; 19–21 min, 40–5% A; 21–23 min, 5–5% A; 23–25 min, 5–70% A; 25–30 min, 70–100% A. Total run time was 35 min with the benzoxazinoid derivatives eluted over 8–20 min, and the final 15 min was used for column cleaning and regeneration. Detection was carried out by a UV-visible detector using a HP 1040 M diode array detector over the range of 190–500 nm (15).

**Mass Spectrometry Conditions.** MS analyses were carried out in selected ion monitoring (SIM) mode. The LC-MSD HP 1100 mass selective detector equipped with an atmospheric pressure ionization source was used with electrospray interface. The ESIMS was operated in negative ion mode with the following instrument settings: nebulizer pressure, 5 V; gas temperature, 350 °C; capillary voltage, 3500 V; fragmentor, 0–15 min, 250 V, and 15–35 min, 70 V (15).

**Quantification.** In this study, internal calibration was used for quantification. The use of internal standards has not been described for the quantification of benzoxazinoids. The only quantitative method describing the use of an internal standard for the analysis of these compounds used the naturally occurring degradation product BOA as internal standard (12). As this product can potentially occur in samples,

**Table 1.** Levels (Micrograms per Gram of Dry Weight) of Benzoxazinoid Derivatives in Wheat Foliage, Roots, and Seeds: Total Allelochemical Content in Each Tissue of Wheat Seedlings of the First Sampling Campaign<sup>a</sup>

|  |         | DIBOA-Glc | DIMBOA-Glc | HBOA | DIBOA | HMBOA | DIMBOA | BOA  | MBOA | Σ tissue |
|--|---------|-----------|------------|------|-------|-------|--------|------|------|----------|
| First Stage, Conventional Cultivation  |         |           |            |      |       |       |        |      |      |          |
| Astron                                 | foliage | <LOD      | <LOD       | <LOD | <LOD  | 4     | <LOD   | <LOD | 28   | 32       |
|  | root    | <LOD      | <LOD       | <LOD | <LOD  | 3     | 4      | <LOD | 90   | 97       |
|  | seed    | <LOD      | <LOD       | <LOD | <LOD  | 7     | <LOD   | <LOD | 62   | 69       |
| Ritmo                                  | foliage | <LOD      | <LOD       | <LOD | <LOD  | 13    | 2      | <LOD | 38   | 53       |
|  | root    | <LOD      | <LOD       | <LOD | <LOD  | 12    | 0.5    | 0.7  | 117  | 130      |
|  | seed    | <LOD      | <LOD       | <LOD | <LOD  | 3     | <LOD   | 0.5  | 45   | 49       |
| Bill                                   | foliage | <LOD      | <LOD       | <LOD | <LOD  | 3     | <LOD   | <LOD | 29   | 32       |
|  | root    | <LOD      | <LOD       | <LOD | <LOD  | 4     | 0.6    | <LOD | 80   | 85       |
|  | seed    | <LOD      | <LOD       | <LOD | <LOD  | 4     | <LOD   | 0.2  | 46   | 50       |
| Solist                                 | foliage | <LOD      | <LOD       | <LOD | <LOD  | 19    | 19     | <LOD | 86   | 124      |
|  | root    | <LOD      | <LOD       | <LOD | <LOD  | 17    | 4      | <LOD | 128  | 149      |
|  | seed    | <LOD      | <LOD       | 6    | <LOD  | 15    | 38     | <LOD | 66   | 125      |
| Second Stage, Conventional Cultivation |         |           |            |      |       |       |        |      |      |          |
| Astron                                 | foliage | <LOD      | <LOD       | 0.5  | <LOD  | 0.8   | <LOD   | <LOD | 26   | 27       |
|  | root    | <LOD      | <LOD       | <LOD | <LOD  | 0.5   | 0.7    | 0.6  | 57   | 59       |
|  | seed    | <LOD      | <LOD       | <LOD | <LOD  | 2     | <LOD   | 0.4  | 69   | 72       |

<sup>a</sup> <LOD, below limit of detection; <LQD, below limit of quantification.

it cannot be considered as an appropriate internal standard. In a previous study (15), different internal standards were tested: a non-naturally occurring structural analogue of HBOA (2-MeO-HBOA), a natural flavonoid (quercetin-3-O-rutinoside), and indoxyl-β-D-glucoside. A non-naturally occurring structural analogue of HBOA (2-MeO-HBOA) showed the best results and was used as internal standard for benzoxazinoid derivatives in the current study. Two ions for each analyte were selected according to specificity and sensitivity, with the primary ions used for quantification and the secondary ion providing confirmation. The ions selected for each analyte were the following: DIBOA-β-D-glucoside (134, 342); DIMBOA-β-D-glucoside (164, 372); HBOA (164, 108); DIBOA (134, 78); HMBOA (194, -); DIMBOA (164, 149); BOA (134, -); and MBOA (164, 149). The instrumental detection limits (LOD<sub>inst</sub>) ranged between 0.010 and 0.002 ng/μL (15).

The applied methodology to plant material gave recoveries of the analytes between 66 and 110% with coefficients of variation ranging from 1 to 14%. The method detection limits (LOD) ranged between 1 and 27 μg/g of dry weight and gave limits of quantification (LQD) between 3 and 89 μg/g of dry weight (15).

## RESULTS AND DISCUSSION

The benzoxazinoid derivatives were detected in foliage, roots, and also in seeds. None of the previous results presented in the bibliography (10–13) detected allelochemicals in germinated seeds.

**First Sampling Campaign.** Levels of benzoxazinoid derivatives are presented in Table 1. Five allelochemicals were detected, but only HMBOA and MBOA were present in all wheat extracts, whereas the glucoside derivatives and DIBOA were not detected in extracts. MBOA was the major metabolite detected in foliage, root, and seed extracts and present at the highest levels in root tissue with 128 μg/g of dry weight in So, at 117 μg/g of dry weight in Ri, at 90 μg/g of dry weight in As, and at 80 μg/g of dry weight in Bi. The total amount of allelochemicals in seed extracts was detected at a concentration similar to those found in foliage and root extracts. The variety with highest levels was So followed by Ri, As, and Bi. The total amount of allelochemicals found in the As seedling in the second stage of cultivation was lower than in the first one. Figure 2a shows the distribution of the total amount of allelochemicals (expressed as a percentage) in wheat seedlings. As can be observed, the total amount of the allelochemicals was distributed evenly among foliage, roots, and seeds in all cases. However, the percentage was slightly higher in root

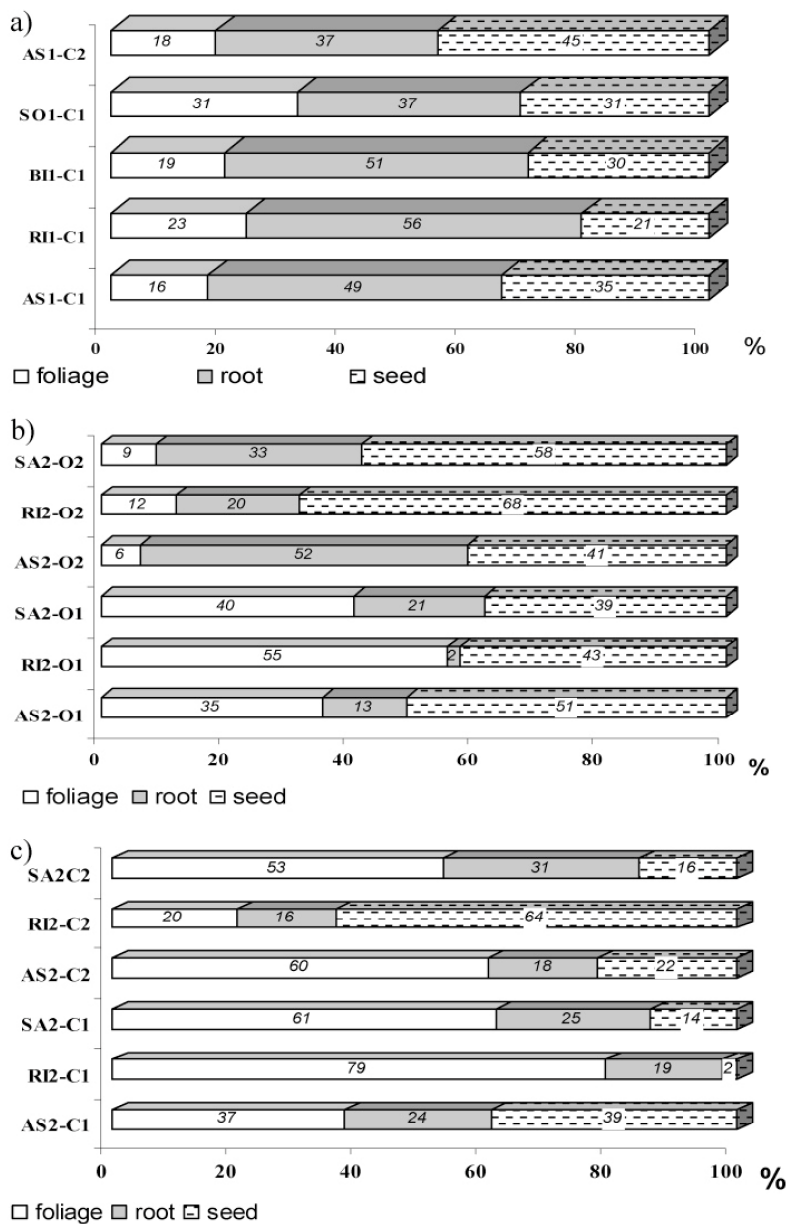
extracts. The percentages obtained for foliage, roots, and seeds ranged from 16 to 31%, from 49 to 56%, and from 21 to 35%, respectively, in wheat seedlings at the first stage of conventional cultivation.

**Second Sampling.** Table 2 presents the levels of individual allelochemicals as well the total amount of allelochemical content in each tissue. Whereas five allelochemicals were detected in the first sampling, all of the target compounds (DIBOA-β-D-glucoside, DIMBOA-β-D-glucoside, DIBOA, DIMBOA, HBOA, HMBOA, BOA, and MBOA) were detected in wheat seedlings of the second sampling. Figure 3 shows the chromatogram of methoxy derivatives (DIMBOA-β-D-glucoside, DIMBOA, HMBOA, and MBOA) present in foliage, root, and seed extracts of the Ri variety.

The differences observed in the content of the allelochemicals between the first and second sampling campaigns were ascribed to the stability of the compounds. In a previous study (16), the stability of selected compounds was checked using different acidified standard solutions stored at different temperatures (-20, 4, and 20 °C). The trials showed that in order to stabilize the analytes, the solution must be stored at -20 °C, including the glucoside derivatives. For this reason, freezing the samples immediately after harvesting prevented the degradation of glucoside derivatives, and it was possible to detect the glucoside derivatives in sample extracts. A similar behavior was observed by Baumeler et al. (17).

Differences between benzoxazinoid concentrations in conventionally and organically grown wheat in this study are influenced by the soil types and cultivation history as well. Some correlations between allelochemicals content and cultivation characteristics were found as discussed below.

**Conventional Cultivation.** The major metabolites detected were DIMBOA, MBOA, HMBOA, and DIMBOA-β-D-glucoside. Nevertheless, MBOA was the only metabolite detected in all extracts. The detected compounds in foliage samples showed a different distribution between the first and second stages. In the first stage, DIMBOA presented the highest levels followed by MBOA and HMBOA, whereas DIMBOA-β-D-glucoside was not detected. In the second stage, HMBOA was the major metabolite present in the Ri and Sa varieties, and HBOA presented 54% of the contribution of allelochemicals in the As variety. The results obtained from root samples showed a different distribution of compounds among varieties and between



**Figure 2.** Distribution of total allelochemical content (expressed as percentage) among foliage, roots, and seeds: (a) first sampling; (b) second sampling, organic cultivation; (c) second sampling, conventional cultivation.

the first and second stages. Whereas DIMBOA- $\beta$ -D-glucoside was not detected in foliage, this metabolite was detected in root extracts. In the first stage, MBOA, DIMBOA- $\beta$ -D-glucoside, and DIMBOA were the major metabolites detected in the As, Ri, and Sa varieties, respectively. In the second stage, DIMBOA was the major metabolite detected in As, DIMBOA- $\beta$ -D-glucoside in Ri, and MBOA in Sa of root extracts. Several benzoxazinoid derivatives were also detected in seed extracts. DIMBOA was the major metabolite detected in the first stage of the As and Sa varieties, whereas MBOA was the major one in Ri. Concerning the second stage, the major metabolite detected in the As and Sa varieties was MBOA, whereas DIMBOA presented the highest levels in seed extract of the Ri variety (Table 2A).

*Organic Cultivation.* The most relevant metabolites detected were DIMBOA, MBOA, HMBOA, and DIMBOA- $\beta$ -D-glucoside. Nevertheless, the results obtained for foliage samples showed also different distributions of compounds between the first and second stages of cultivation. In the first stage of cultivation, DIMBOA was the major metabolite detected, whereas MBOA presented the highest levels in foliage extracts of the second stage for the As and Ri varieties; HMBOA was the major metabolite detected in the foliage extract of the second stage of the Sa variety. The main metabolites in root extracts were MBOA and DIMBOA for the As and Sa varieties, respectively, in each stage of cultivation, whereas for the Ri variety a difference existed between the first and second stages. Whereas MBOA was the main metabolite in the first stage,

**Table 2.** Levels (Micrograms per Gram) of Benzoxazinoid Derivatives in Wheat Foliage, Roots, and Seeds: Total Allelochemical Content in Each Tissue of Wheat Seedling of the Second Sampling Campaign<sup>a</sup>

|         |         | A. Conventional Cultivation |            |      |       |       |        |      |      |          |
|---------|---------|-----------------------------|------------|------|-------|-------|--------|------|------|----------|
|         |         | DIBOA-Glc                   | DIMBOA-Glc | HBOA | DIBOA | HMBOA | DIMBOA | BOA  | MBOA | Σ tissue |
|         |         | First Stage                 |            |      |       |       |        |      |      |          |
| Astron  | foliage | <LOD                        | <LOD       | <LOD | <LOD  | 270   | 833    | <LOD | 552  | 1655     |
|         | root    | 46                          | 155        | <LOD | <LOD  | 59    | 303    | 11   | 476  | 1050     |
|         | seed    | 89                          | 59         | <LOD | 60    | 48    | 1380   | <LOD | 119  | 1755     |
| Ritmo   | foliage | <LOD                        | <LOD       | <LOD | <LOD  | 489   | 1775   | <LOD | 942  | 3206     |
|         | root    | <LOD                        | 318        | <LOD | <LOD  | 34    | 124    | <LOD | 290  | 766      |
|         | seed    | <LOD                        | <LOD       | <LOD | <LOD  | <LQD  | <LOD   | 17   | 72   | 89       |
| Stakado | foliage | <LOD                        | <LOD       | <LOD | <LOD  | 326   | 3261   | <LOD | 695  | 4282     |
|         | root    | 27                          | 329        | <LOD | <LOD  | 83    | 860    | <LOD | 420  | 1719     |
|         | seed    | <LOD                        | <LOD       | <LOD | <LOD  | <LQD  | 896    | <LOD | 70   | 966      |
|         |         | Second Stage                |            |      |       |       |        |      |      |          |
| Astron  | foliage | <LOD                        | <LOD       | 177  | 66    | 56    | <LOD   | <LOD | 30   | 329      |
|         | root    | <LOD                        | <LOD       | <LOD | <LOD  | <LQD  | 62     | <LOD | 34   | 96       |
|         | seed    | <LOD                        | <LOD       | <LOD | <LOD  | 42    | <LOD   | <LOD | 80   | 122      |
| Ritmo   | foliage | <LOD                        | <LOD       | <LOD | <LOD  | 161   | 58     | <LOD | 94   | 313      |
|         | root    | <LQD                        | 134        | <LOD | <LOD  | <LQD  | 57     | <LOD | 57   | 248      |
|         | seed    | <LOD                        | 97         | <LOD | <LQD  | 59    | 721    | <LOD | 130  | 1007     |
| Stakado | foliage | <LOD                        | <LOD       | <LOD | <LQD  | 126   | 51     | <LOD | 91   | 268      |
|         | root    | <LOD                        | <LQD       | <LOD | <LOD  | <LQD  | 55     | <LOD | 103  | 158      |
|         | seed    | <LOD                        | <LOD       | <LOD | <LQD  | <LQD  | <LOD   | 10   | 69   | 79       |
|         |         | B. Organic Cultivation      |            |      |       |       |        |      |      |          |
|         |         | DIBOA-Glc                   | DIMBOA-Glc | HBOA | DIBOA | HMBOA | DIMBOA | BOA  | MBOA | Σ tissue |
|         |         | First Stage                 |            |      |       |       |        |      |      |          |
| Astron  | foliage | <LQD                        | 122        | <LOD | <LQD  | 179   | 413    | <LOD | 197  | 911      |
|         | root    | <LOD                        | <LOD       | <LOD | <LOD  | <LQD  | <LOD   | 23   | 324  | 347      |
|         | seed    | 164                         | <LOD       | <LOD | 50    | 50    | 925    | <LOD | 130  | 1319     |
| Ritmo   | foliage | <LOD                        | <LOD       | 395  | 65    | 355   | 1570   | <LOD | 142  | 2527     |
|         | root    | <LOD                        | <LQD       | <LOD | <LOD  | <LOD  | <LOD   | <LQD | 97   | 97       |
|         | seed    | 75                          | 77         | <LOD | 43    | 67    | 1544   | <LOD | 134  | 1940     |
| Stakado | foliage | <LOD                        | <LOD       | <LOD | <LQD  | 237   | 1270   | <LOD | 433  | 1940     |
|         | root    | <LOD                        | 144        | <LOD | <LOD  | 82    | 463    | <LOD | 319  | 1008     |
|         | seed    | <LOD                        | <LOD       | <LOD | 105   | 91    | 1536   | <LOD | 128  | 1860     |
|         |         | Second Stage                |            |      |       |       |        |      |      |          |
| Astron  | foliage | <LOD                        | <LOD       | <LOD | <LOD  | <LOD  | <LOD   | <LOD | 24   | 24       |
|         | root    | <LQD                        | <LOD       | <LOD | <LOD  | 48    | <LOD   | <LOD | 152  | 200      |
|         | seed    | <LOD                        | <LOD       | <LOD | <LQD  | 68    | <LOD   | <LOD | 89   | 157      |
| Ritmo   | foliage | <LOD                        | <LOD       | <LOD | <LOD  | <LOD  | <LOD   | <LOD | 279  | 279      |
|         | root    | <LOD                        | 385        | <LOD | <LOD  | <LOD  | <LOD   | 10   | 82   | 457      |
|         | seed    | <LOD                        | 165        | <LOD | <LQD  | 69    | 1166   | 20   | 170  | 1590     |
| Stakado | foliage | <LOD                        | <LOD       | <LOD | <LQD  | 89    | <LOD   | <LOD | 56   | 145      |
|         | root    | <LOD                        | <LOD       | <LOD | <LOD  | 116   | 311    | <LOD | 125  | 552      |
|         | seed    | 371                         | <LOD       | <LOD | <LQD  | <LQD  | 462    | <LQD | 147  | 980      |

<sup>a</sup> <LOD, below limit of detection; <LQD, below limit of quantification.

DIMBOA- $\beta$ -D-glucoside presents the highest level in the second stage. In seed extracts DIMBOA was the main compound found for all varieties in the first stage, and a different result was obtained in the second stage between varieties. In this case, MBOA presents the highest levels in the As and Sa varieties and DIMBOA in the Ri variety (**Table 2B**).

As can be observed, our results showed that the type of benzoxazinoid derivatives found depends strongly on plant growth stage and the working procedure of the plant material. Several authors showed different methodologies for the quantification of benzoxazinoid derivatives (17–23). In our case, the glucoside derivatives were detected in the wheat seedlings of the second sampling campaign; the samples were frozen immediately after harvesting. This fact also explains why the major metabolite detected in samples of the first sampling was MBOA, originated from the degradation of DIMBOA and HMBOA. Nevertheless, DIMBOA and HMBOA were the major metabolites detected in wheat extracts of the second sampling.

**Conventional versus Organic Cultivation.** **Figure 4** presents the total amount of allelochemical content of wheat plants in

conventional and organic cultivation. As can be observed, similar levels were found for conventional and organic cultivation. The As2-C1 (As variety, second sampling, first stage of conventional cultivation), Sa2-C1, and As2-C2 showed slightly higher levels in conventional cultivation than in organic cultivation. However, the Ri2-O1 (Ri variety, second sampling, first stage of organic cultivation), Ri2-O2, and Sa2-O2 presented higher levels in organic cultivation.

**Stage of Cultivation.** A general decrease of the total amount of allelochemical content in plants was observed from the first to the second stage. The decrease was also observed in each tissue. Like the total amount of allelochemical content in plants and tissues, the general behavior of individual compounds was a decrease of concentration from the first to the second stage except for some metabolites. The first exception was DIMBOA- $\beta$ -D-glucoside, HMBOA, DIMBOA, and MBOA, which presented an increase of concentration from the first to the second stage in seed extracts of the Ri variety in conventional cultivation. This increase ranged from <LOD to 97  $\mu$ g/g of dry weight for DIMBOA- $\beta$ -D-glucoside, from <LQD to 59  $\mu$ g/g



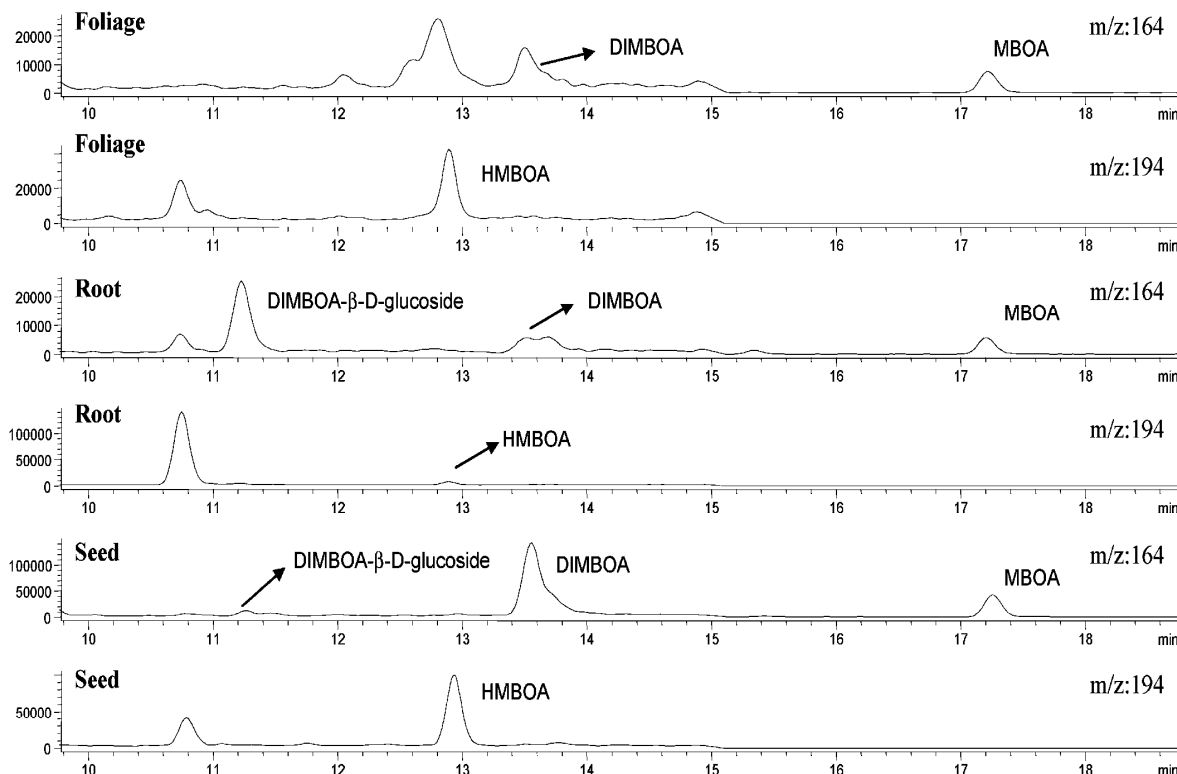


Figure 3. Chromatogram of methoxy derivatives (DIMBOA- $\beta$ -D-glucoside, DIMBOA, HMBOA, and MBOA) present in foliage, root, and seed extracts of Ritmo variety.

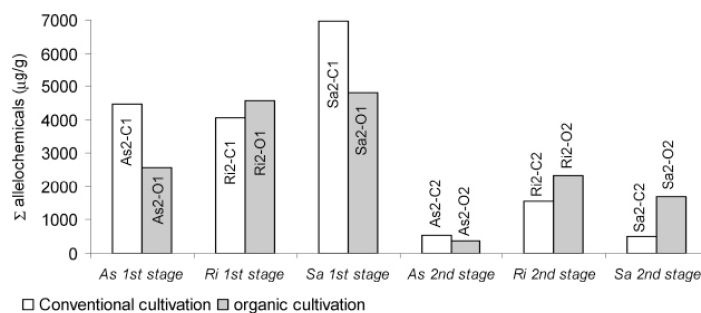


Figure 4. Correlations of total allelochemical content between conventional and organic cultivation in wheat seedlings of the second sampling.

of dry weight for HMBOA, from <LOD to 721  $\mu\text{g/g}$  of dry weight for DIMBOA, and from 72 to 130  $\mu\text{g/g}$  of dry weight for MBOA. Whereas in conventional cultivation the increase was present only in one variety, in organic cultivation the increase was present in all varieties. Then, DIMBOA- $\beta$ -D-glucoside showed an increase from <LOD to 371  $\mu\text{g/g}$  of dry weight in seed extract of Sa variety, DIMBOA- $\beta$ -D-glucoside increased in concentration from 144 to 365  $\mu\text{g/g}$  of dry weight in root extract of Ri and from <LOD to 165  $\mu\text{g/g}$  of dry weight in seed extract of the Ri variety, HMBOA showed an increase from <LOD to 48  $\mu\text{g/g}$  of dry weight in root extract of the As variety and from 82 to 116  $\mu\text{g/g}$  of dry weight in root extract of the Sa variety and, finally, MBOA showed an increase from 134 to 170  $\mu\text{g/g}$  dry weight in seed extract of Ri and from 128 to 147  $\mu\text{g/g}$  of dry weight in seed extract of the Sa variety.

A general decrease of the total allelochemical content was observed in wheat seedlings. These results were consistent with

those presented in the literature. According to Argandona et al. (9), concentration in the plant increases abruptly a few days after germination and then decreases progressively with plant age. Nevertheless, an increase of concentration of some metabolites from the first to the second stage indicated that the allelochemicals were synthesized during the growth of plant.

**Wheat Varieties.** The highest levels of total amount of allelochemicals were found in Sa in the first stage of conventional cultivation and for Ri in the second one (Table 2A). For organic cultivation, the highest levels were also found for Sa and Ri in the first and second stages, respectively (Table 2B). The content of benzoxazinoid derivatives in wheat plants showed variations between different species (9, 17).

**Distribution in Foliage/Roots/Seeds.** The distribution of the total amount of allelochemicals (expressed as a percentage) among different tissues in the three varieties is shown in Figure 2. In organic conditions (Figure 2b), the large amount of allelo-

chemicals was distributed between foliage and seeds during the first stage, and the highest percentage of compounds was distributed in the seeds for the second stage of cultivation. The distribution of the total amount of allelochemicals was not regular in wheat seedlings cultivated in conventional conditions (Figure 2c).

Copaja et al. (13) suggested that the allelochemical production must be initiated during germination because they observed that the total amount of benzoxazinoids increased during early growth and that no allelochemicals were detected in germinated seeds. In the current study, allelochemicals found in germinated seeds were detected at levels similar to those found in foliage and root. These findings suggest that this matrix must be taken into account for evaluation of total content of allelochemicals in wheat seedlings. It is important to take into account that the occurrence of benzoxazinoids in seeds 10 days after sowing does not prove that the benzoxazinoids were present in the dry seeds. Our results suggest that the dry seeds could contain precursors from which the benzoxazinoids are formed as soon as they are put into the soil. Future work will be focused in this direction, to determine the occurrence of benzoxazinoids in dry seeds. This information could be important from the health perspective.

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IV.3.2. Anàlisi de les mostres de blat

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Publicació Científica #7

“Concentration of benzoxazinoid allelochemicals in wheat samples (*Triticum aestivum*)”

Per:

Marta Villagrasa, , Andreu Taberner, Romà Tauler, Ethel Eljarrat i Damià Barceló

En preparació per enviar a Revista:  
Journal of Agricultural and Food Chemistry

# Concentration of benzoxazinoid allelochemicals in wheat samples (*Triticum aestivum*)

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**ABSTRACT**

The content of different benzoxazinoid derivatives were analyzed in several wheat varieties under different conditions. Two consecutive sampling campaigns in which different varieties of wheat were grown under conventional and organic systems were performed. The samples were collected at five different growth stages (BBCH 9-10, 12, 21, 31 and 53) and the foliage and root were analyzed. Significant differences were found on the total content of benzoxazinoid derivatives between growth stages, foliage and root and between agricultural systems, but no significant difference were found between wheat varieties. The methoxy derivatives were the compounds mostly detected, being MBOA and DIMBOA the major benzoxazinone derivatives found in the first and second sampling, respectively. At the early growth stages, the total concentrations in foliage were higher than in root, but the root levels were more constant over the time. Differences on the concentration of allelochemicals due to different experimental factors (growth stage, type of cultivation, tissue sample and wheat variety) were assessed by means of N-way ANOVA. Our results were compared with other studies found in the literature, in which the DIMBOA derivative was the compound mostly reported. Higher concentrations of DIMBOA were obtained in our study. It should be pointed that previous results were obtained by studies under controlled conditions; however, our results corresponded to analyses in field conditions.

**KEYWORDS: Benzoxazinone derivatives; wheat; allelochemicals, DIMBOA.**

## INTRODUCTION

Since it has been found that allelochemical compounds and their decomposition products play an important role in the resistance of plant to insect pests and plant pathogenic fungi, scientific interest for allelopathy has increased, meaning a potential for selective biological weed management [7-9]. One of several alternatives to the use of synthetic pesticides is the possible exploitation of allelopathy for protecting agricultural plants against weeds, insects, pathogens, and other pests. To be able to evaluate the consequences of the widespread use of allelopathic crop and to facilitate the understanding of the mechanism of crop allelopathy, it is important to identify the allelochemicals responsible for the weed suppression.

Several types of allelochemicals in wheat have been identified, namely, phenolic acids, hydroxamic acids, and short chain fatty-acids [10]. The chemical family of benzoxazinones is the main active allelopathic group in different crops such as wheat, rye or maize. These compounds are naturally present in the plants as  $\beta$ -glucosides [11, 12]. When the plant is injured, glucosides are hydrolyzed by  $\beta$ -glucosidases yielding the respective aglycones [11]. As the same time, the aglycones are unstable and rapidly converted into the benzoxazolin-2-ones [13]. When these compounds are leached to the soil, a chemical and microbial transformation of the compounds will take place. According to literature, the major products of the benzoxazolinones are aminophenoxazinones and corresponding malonamic acids [6, 14, 15]. Poaceae such as wheat, rye, and maize contain secondary metabolites at different concentrations, depending on plant varieties and environmental conditions such as light intensity, temperature, and water availability [16-18]. Several authors reported concentrations of benzoxazinone derivatives in Poaceae, but most of their research was performed at

seedling stage [19-22] and few studies have been performed in field conditions [23, 24]. The seeds of wheat did not contain hydroxamic acids but their synthesis begins at very early stages of germination. In a previous work, the allelochemicals in the germinated seeds at a very early stage of germination were found [25]. Most of the studies about the analysis of allelochemicals in wheat showed that the highest concentration of allelochemicals are present in the early stages of growth, normally between 4 and 6 days after germination and then decreases. The 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one (DIMBOA) has been the most analyzed compound. The range of concentration of DIMBOA in wheat seedling was quite different depending on the studies reviewed. The concentration of DIMBOA in shoots of wheat seedling ranged from 1.4 to 10.9 mmol/kg of fresh weight (fw) in a collection of Chilean cultivars, from 0.99 to 8.07 mmol/kg of fw in a worldwide collection and from 0.21 to 16.0 mmol/kg of fw in a collection of wheat progenitors. Other studies performed in field conditions also reported the content of DIMBOA jointly with other derivatives such as glucoside compounds [2- $\beta$ -D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA- $\beta$ -D-Glucoside) and 2- $\beta$ -D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA- $\beta$ -D-glucoside)], the aglucones [2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and DIMBOA], the lactams [2-hydroxy-1,4-benzoxazin-3-one (HBOA) and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA)], and benzoxazolinones [benzoxazolin-2-one (BOA) and 6-methoxy-benzoxazolin-2-one (MBOA)], being DIMBOA the compound found in highest amount. The concentration of DIMBOA in root ranged from 40 to 1996 mg/kg dry weight (dw) in Polish wheat varieties [24] and from 2 to 163 in modern wheat varieties [23].

The aim of our study focuses on the determination of the concentration of different allelopathic compounds present in modern wheat varieties. These wheat varieties were grown under two contrasting farming systems. Samples were collected at five different growth stages and foliage and roots of each plant were analyzed. A test of analysis of variance to try to determine which factor or factors significantly influence the concentration of allelopathic compounds in wheat samples were applied.

## **MATERIALS AND METHODS**

**Chemicals and Materials.** The benzoxazinoid standards were obtained from commercial and private sources as available: DIBOA- $\beta$ -D-Glucoside and DIMBOA- $\beta$ -D-Glucoside from Prof. Dr. Hajime Iwamura (Kyoto University), Prof. Dr. Lisbeth Jonsson (Södertörn University College).; DIMBOA from Dr. Scott Chilton (University of North Carolina); HBOA, HMBOA, DIMBOA, MBOA and BOA from Dr. F.Macías (University of Cadiz).; DIBOA and the non naturally occurring synthetic derivative 2-methoxy-2H-1,4-benzoxazin-3(4H)-one (2`MeO-HBOA) from Dr. Sicker (University of Leipzig). HPLC-grade solvents water (H<sub>2</sub>O), methanol (MeOH) and 98% pure acetic acid (HOAc) was purchased from Merck (Darmstadt, Germany). Diatomaceous earth was obtained from Varian Inc. LiChrolut® RP C18 (500 mg) SPE cartridges were purchased from Merck (Darmstadt, Germany).

**Sample Collection.** The wheat plants were grown in Lleida (Spain) in two different sampling campaigns, corresponding to two consecutive years. In the first sampling campaign (autumn 2001), six different wheat varieties, *Astron (As)*, *Ritmo (Ri)*, *Bill (Bi)*, *Solist (So)*, *Porta (Po)* and *Stakado (SA)* were cultivated under conventional (C) as



well as organic (O) farming conditions, each variety being sown in two random plots in each of the two fields. Wheat was sown on November 12, 2001. In the second sampling campaign (autumn 2002), three wheat varieties, *As*, *Ri* and *Stakkado* (*Sa*) were cultivated under conventional as well as organic farming conditions, each variety being sown in four random plots in each of two fields. Samples of ten wheat plants were collected at five growth stages defined by BBCH scale (system for a uniform coding of phenologically similar growth stages of all mono-dicotyledonous plant species: BBCH9-10 (1), BBCH12 (2), BBCH21 (3), BBCH31(4) and BBCH51 (5). Immediately after sampling, roots (R) were separated from foliage (F), and were frozen at -20°C in separate bags. It is important to note that the samples of the first sampling campaign were frozen in the laboratory whereas the samples of the second sample campaign were frozen immediately after harvest in the same field.

**Sample preparation.** Wheat plant samples were frozen and stored at -20°C until further analysis. Excess water was removed from the frozen plants by lyophilization for 24 hours. The benzoxazinoid derivatives were isolated from the foliage and root samples using pressurized liquid extraction (PLE) followed by solid phase extraction (SPE) cleanup. A more detailed description of the method is given elsewhere [26].

**Wheat plant analysis.** LC-MS system consisted of a HP 1100 LC with a binary high pressure pump, a solvent degassing unit and an automatic sample injector from Hewlett-Packard (Palo Alto, CA, USA). An 1100 series diode array detector (DAD) was connected in line with a bench top mass selective detector for the HP 1100 series equipped with ESI source. The instrument control and data processing utilities included the use of LC-MSD ChemStation software. A Synergi Max-RP 80A (C-12 TMS) LC

column (250x4.6mm Phenomenex) with a solvent flow rate of 1 mL/min was used. The sample injection volume was set 50  $\mu$ L. Mobile phase consisted of acidified H<sub>2</sub>O (0.05% HOAc) as solvent A and acidified MeOH (0.05% HOAc) as solvent B. [26]. MS analyses were carried out in selected ion monitoring (SIM) mode. The ESI-MS was operated in negative ion mode. Two ions for each analyte were selected, according to specificity and sensitivity, with the primary ions used for quantification and the secondary ion providing confirmation [26].

**Quantification.** A non-naturally occurring structural analogue of HBOA (2-MeO-HBOA) was used as internal standards for benzoxazinoid derivatives in wheat plants samples.

### Statistical Analysis

A multivariate data analysis is performed to statistically assess the possible effects of different factors (growth stage, type of cultivation, tissue sample and wheat variety) on the concentration of allelochemicals. In order to find the influence of these four factors (on the allelochemical content, one-way and multiway analysis of variance (N-way ANOVA) were applied to the experimental data. N-way ANOVA allows estimating if means of data grouped according to different factors differ significantly in statistical terms. If they do differ, this test allows to identify which factors or combination of factors are associated to the observed differences. This statistical test allows the separation of the different variance sources and check for their significance. There are different models for N-way ANOVA, like the additive model and the interaction model. The additive model assumes that the effect on the outcome of a particular level change

for one explanatory variable does not depend on the level of the other explanatory variable. If an interaction model is needed, then the effects of a particular level change for one explanatory variable depend on the level of the other explanatory variable. Treatment means were considered to be statistically different at the significance level of 5% . Results are shown using the standard ANOVA-table, which by default divides the variability of allelochemical concentrations into the variability due to differences between the levels of each factor accounted for in the model (one row for each factor) and the remaining (residual) variability not explained by any of the tested systematic sources. The N-way ANOVA test evaluates the hypothesis that the different levels of a factor have the same effect, against the alternative that they do not all have the same effect. As there are many group of means, there are also many pairs to compare. The alpha value would apply to each comparison, so the chance of incorrectly finding a significant difference would increase with the number of comparisons. Instead of ordinary t-tests a multiple comparison test procedure was applied (multcompare). This test is designed to provide an upper bound on the probability that any comparison will be incorrectly found significant. Population marginal means were also computed. In the multcompare function we used critical values from Scheffes's procedure, derived from the F distribution. This procedure provides a simultaneous confidence level comparisons of all linear combinations of means.

One-way ANOVA, N-way ANOVA and multiple comparison test (Mulcompare) of the experimental data were performed using Statistic Toolbox under MATLAB (version 7, The Mathworks Inc, Manson, WA USA) computer and visualization environment.

## RESULTS AND DISCUSSION

Part of the results of the content of allelochemicals in wheat samples was already presented in a previous work [25], but only the samples of the early growing stages were showed. Here we present the results of the quantification of allelochemicals in all samples related in the first and second sampling campaigns.

### **First sampling campaign.**

*Conventional farming.* The samples analyzed correspond to the growth stages BBCH10 (1S), BBCH12 (2S), and BBCH21 (3S). All benzoxazinone derivatives were detected in foliage (F) and roots (R) at all growth stages. The only exception is for DIBOA that is not present at the first growth stage and appears in the second and third one in all varieties. The methoxylated compounds were present in a highest concentration than non-methoxylated analytes. MBOA showed the highest concentration of any derivatives (from 23 to 309 mg/kg dw). Figure 1 shown the concentration in foliage (Figure 1a) and roots (Figure 1b) of the different benzoxazinone derivatives analyzed. The bars represent mean concentration of samples collected in two different plots. The total content of allelochemicals decreases from the first (up to 233 mg/kg dw in foliage and 309 mg/kg dw in root to the third growth stage ( up to 175 mg/kg dw in foliage and 151 mg/kg dw in root)in both wheat tissue samples. Stakkado was the variety showing the higher content of allelochemicals in foliage (from 478 to 157 mg/kg dw) and root sample (from 410 to 121 mg/kg dw) .

*Organic farming.* The analyzed samples in the first campaign in the organic cultivation are related only to the wheat growth stages BBCH 31 (3O) and 52 (4O). Figure 1c and

It is shown the concentration in foliage and roots of the different benzoxazinone derivatives analyzed. The bars represent mean concentration of samples collected in two different plots. The total content of allelochemicals decreases from the third (up to 74 mg/kg dw in foliage and up to 243 mg/kg dw ) to the fourth growth stage up to 24 mg/kg dw in foliage and up to 132 mg/kg dw ) in both wheat tissue samples. The highest content was observed in root samples, with values up to 243 mg/kg dw. As it was observed in conventional samples, the methoxylated compounds are the metabolites mostly detected. MBOA was also detected in all samples and it shows also the highest concentrations (from 65 to 1.4 mg/kg dw in foliage and from 230 to 6 mg/kg dw).

### **Second sampling campaign.**

*General trends in wheat samples.* Figure 2 shows the concentrations in F and R of the different benzoxazinoid derivatives analyzed. The largest concentration of allelochemicals occurred in the first stage of growth and then decreased from a growth stage to another one. This decrease is more pronounced in F (Figure 2a and 2c) than in R (Figure 2b and 2d). In general, the foliage showed higher total levels (from 7988 to 267 mg/kg dw in conventional cultivation and from 2526 to 24 mg/kg dw in organic cultivation) of allelochemicals than the root (from 1719 to 55 mg/kg dw in conventional cultivation and from 1120 to 63 mg/kg dw in organic cultivation) in the early stages. A greater range of allelochemicals was detected in the second sampling campaign compared to the first one. As it was observed in the first sampling campaign, the methoxylated derivatives presented higher concentrations than the non-methoxylated compounds (Table 1). Nevertheless, the compound that was found at highest concentration was the aglucone DIMBOA. The concentration varied from 0.68 to 3026

mg/kg dw and 0.31 to 786 mg/kg dw in the first growth stage of conventional farming in foliage and roots, respectively. For the organic agriculture, DIMBOA ranged from 129 to 1.468 mg/kg dw in the foliage and from 21.7 to 323 mg/kg dw in the roots of the first stage of growth. As it can be observed, levels in conventional agriculture were larger than those of organic agriculture. A possible explanation could be that the plant responds to application of pesticides producing largest amounts of allelopathic compounds. The second analyte detected in higher concentration was MBOA. It varied in the first stage of growth of conventional agriculture from 2.64 to 646 mg/kg dw and 9.24 to 427 mg/kg dw in the foliage and roots, respectively. At the first growth stage of organic agriculture MBOA ranged from 27.5 to 414 mg/kg dw in the foliage and from 56.4 to 298 mg/kg dw in the root. HMBOA was also detected in the samples but at lower concentrations than DIMBOA and MBOA. Unlike the first sampling campaign, the glycoside compounds were present in the second sampling campaign samples. This may be because the samples of the first campaign were not conserved at -20°C immediately after their collection. The concentration ranges of the methoxylated compounds in foliage and root during the different growth stages grown under conventional and organic manner are shown in Table 1. DIMBOA-Glc was not detected in any sample of foliage with an exception for the Astron variety in the first stage of growth of the organic farming. However, it was detected in all the samples of root in the first stage of growth in the conventional farming and in the As and Sa varieties of the first stage of growth in organic farming. Regarding to the different varieties of wheat, a similar pattern in the distribution of the compounds in both organic and conventional farming was observed (Figure 2). However, Sa variety showed the highest amount of allelochemicals in most of the samples analyzed.

*Statistical analysis of the data of the second sampling campaign.* In order to accomplish the assumptions of normality and homoscedasticity in ANOVA, data were transformed to logarithmic values. In Figure 3, data boxplots grouped for each factor are given. As it can be seen, the growth stage factor is the one which presents higher variation in the total concentration of allelochemicals between the different levels. One-way ANOVA was first applied to find out if there are significant differences between different levels within each factor. The p-value obtained for the growth stage was a very low value ( $p < 2.2 \times 10^{-10}$ ). This is a strong indication that the total content of allelochemicals between the different growth stages was not equal. The p-value for the types of cultivation was 0.02472, which is also significant, but less than the observed for the growth stage. Non significant differences were found for the variable type of tissue sample matrices ( $p = 0.1994$ ) nor for wheat varieties ( $p = 0.4064$ ). To check whether the large variations due to the different growth stages could hide the possible variability present in the other factors, separation of the different sources of variation was performed using N-way ANOVA. The n-way ANOVA model that best fit our data has been the interaction model for the factors of tissue sample, growth stage and type of cultivation with the interaction between growth stage and tissue sample matrix (Table 2). This model indicates that the total concentration of allelochemicals seems to be related to the factors: tissue sample matrix ( $p = 0.001$ ), growth stage ( $p = 0$ ) and type of cultivation ( $p = 0$ ). But as the interaction of E\*M is significant ( $p = 0$ ), this indicates that the difference between tissue sample matrices is not the same in the five levels of growth stage. The multiple comparison test was then applied. This test determined which pairs of levels were significantly different and which ones were not. The plot of the results obtained by the application of this multiple comparison test for the interaction E\*M is

shown in Figure 4. As it can be observed, significant differences between foliage and root on the first growth stage and fourth growth stage are detected.

### **Range of DIMBOA concentration found in several studies.**

Most of the published papers on the content of allelochemicals in wheat, reported the concentration of DIMBOA. That because, our DIMBOA concentration data (second sampling campaign) will be compared with the published data. However, make the comparison of the data available is not easy since most studies does not specify the stage of growth of the plant analyzed. Also, it should be noted that the majority of studies have been conducted under controlled conditions and not in field studies. The concentration range of the analytes from the reviewed studies is presented in Figure 5. In early reports, the DIMBOA content data was expressed as mmol/kg fw (Figure 5a). In order to express the results as mg/kg of fw, we transform our data considering that the dry matter content of the plants was approximately of 10%. Niemeyer [19] found that DIMBOA content in four wheat cultivars ranged from 0,45 to 3,88 mmol/kg fw. Copaja et al [21] showed that several wheat varieties differed significantly in the production of DIMBOA, ranging from 1,4 to 10,9 mmol/kg fw. A range of 0,99 to 8,07 mmol/kg fw was found in a worldwide screening of 47 of Triticum [27]. In a study carried out by Niemeyer et al [20] a collection of wheat progenitors, the DIMBOA ranged from 0,21 to 16,0 mmol/kg of fw. Copaja et al [28] found DIMBOA in three wheat varieties in a range from 4,03 to 8,12 and 2,20 to 4,95 mmol/kg fw in foliage and root, respectively. In our study, the DIMBOA ranged from 0,39 to 1,03 in foliage and from 0,01 to 0,33 mmol/kg fw in root of the first growth stage, whereas in the second growth stage the DIMBOA ranged from nd to 0,37 and from 0,01 to 0,08 mmol/kg fw in foliage and root, respectively. The range of concentrations presented in all these



studies showed values of DIMBOA higher than those found in our study. However it should be taken into account that these studies were conducted under controlled conditions, while our study was carried out in field conditions. Nevertheless, our data was in good agreement with the data presented by Mogensen et al [23] which corresponded also to a study in field conditions. Figure 5b presented the concentration ranges found in the literature expressed as mg/kg of dw. DIMBOA was found in 17 days old wheat seedling ranging from 20 to 728 and from 75 to 727 mg/kg of dw in foliage and root, respectively [29] In a study of 17 days old seedling of 58 different wheat accessions DIMBOA ranged from nd to 730 mg/kg of dw in shoots and from nd to 734 mg/kg of dw in roots [30]. The growth stage at which the plants have been harvested was not showed in these studies, but the age of 17 days old corresponds to samples harvested at the first stage of our study (about 22 days). However the range of concentrations is more similar to that obtained in samples harvested in the second stage of growth in our study.

### **Acknowledgment**

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Comesaña and R. Chaler (IDAEA-CSIC) is most appreciated. Finally, we thank Merck for supplying the SPE cartridges.

**FIGURE CAPTIONS**

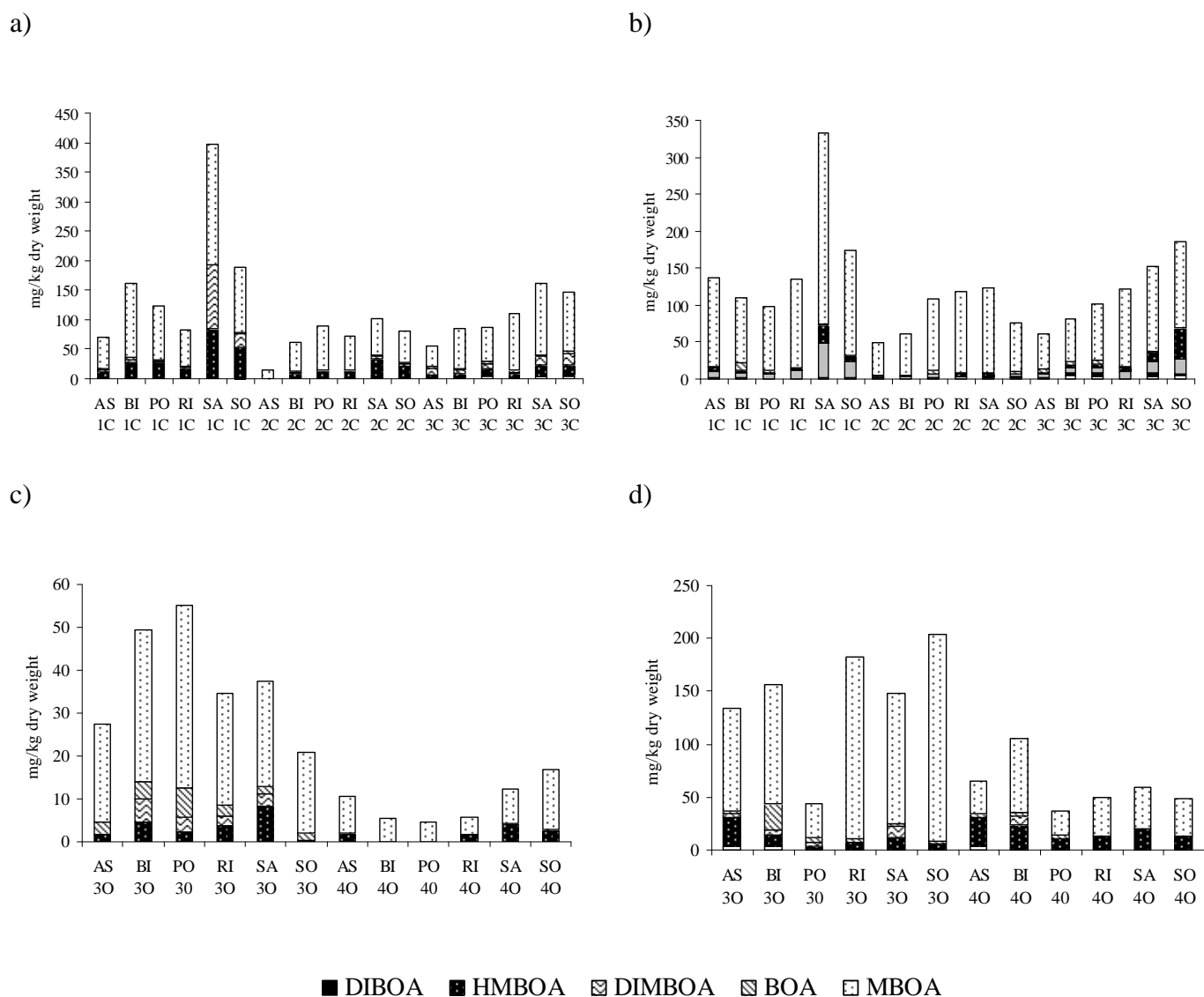
**Figure 1.-** Concentration of different benzoxazinone derivatives in six winter wheat varieties at different growth stages of the samples of first sampling campaign: a) foliage and b) root in conventional grown; and, c) foliage and d) root in organic grown.

**Figure 2.-** Concentration of different benzoxazinone derivatives in six winter wheat varieties at different growth stages of the samples of second sampling campaign: a) foliage in conventionally grown, b) root in conventional grown, c) foliage in organic grown and d) root in organic grown.

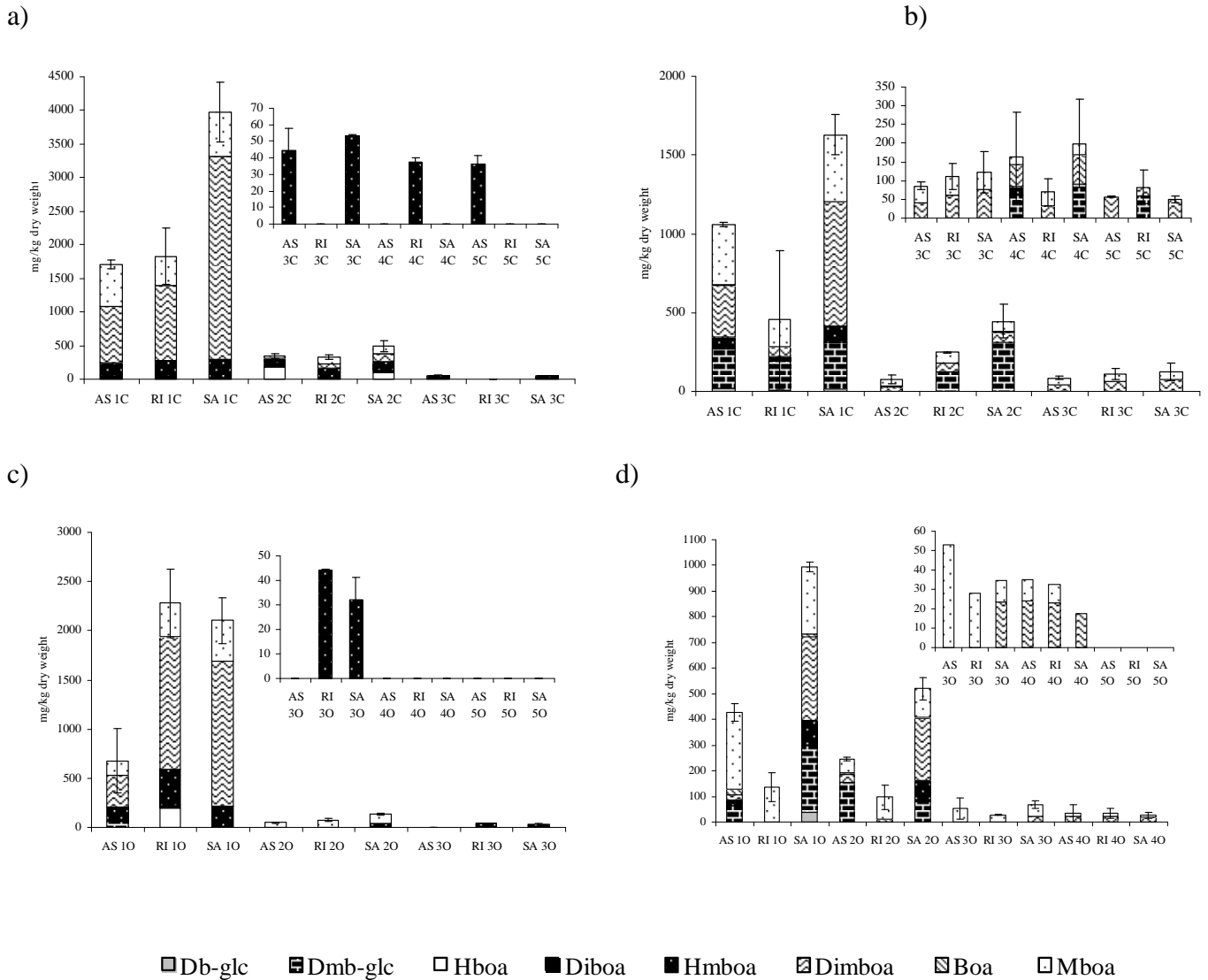
**Figure 3.-** Boxplots of the concentrations found in wheat a) type of cultivation b) growth stage c) varieties and d) type of matrix.

**Figure 4.-** Population marginal means with standard error of the total content of benzoxazinod derivatives related to foliage and root in the different growth stages. Two means are significantly different if their intervals are disjoint, and are not significantly different if their intervals overlap.

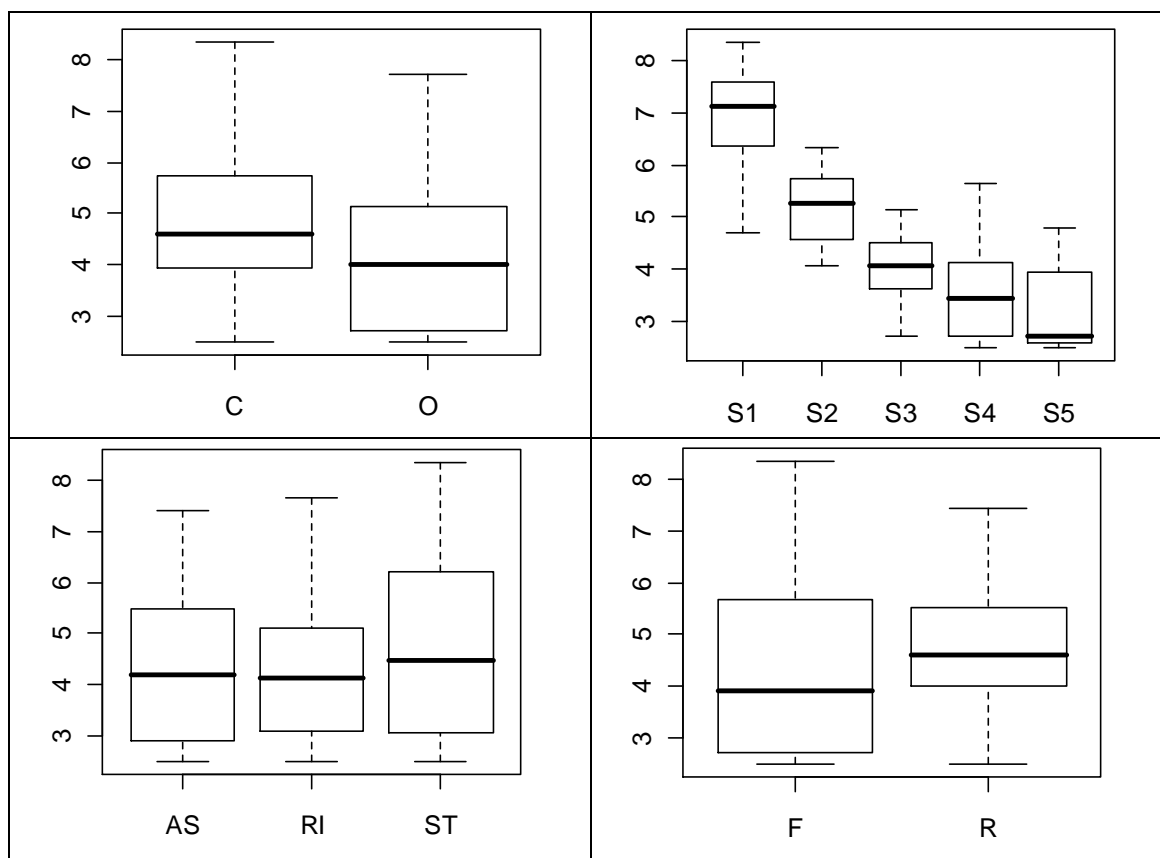
**Figura 5.-** Range concentration of DIMBOA found in literature for foliage (F) and root (R) of wheat expressed as a) mmol/kg of dw and b) mg/kg dw.



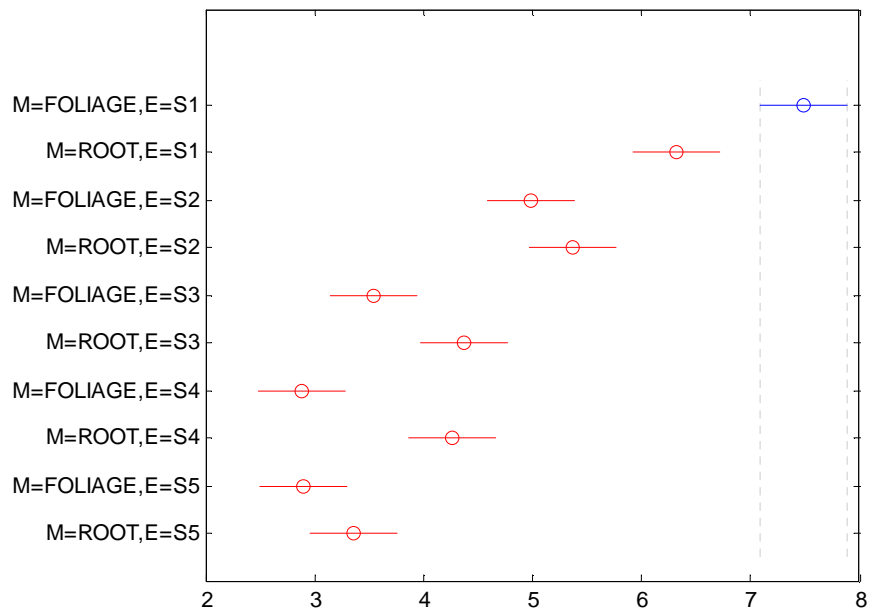
**Figure 1.-** Concentration of different benzoxazinone derivatives in six winter wheat varieties (AS, BI, PO, RI, SA and SO) at different growth stages (1, 2, 3 and 4) in a) foliage in conventionally grown (C), b) root in conventional grown, c) foliage in organic grown (O) and d) root in organic grown.



**Figure 2.-** Concentration of different benzoxazinone derivatives in three winter wheat varieties (AS, RI, SA) at different growth stages (1, 2, 3, 4 and 5) of the samples of second sampling campaign: a) foliage in conventionally grown (C), b) root in conventional grown, c) foliage in organic grown (O) and d) root in organic grown.

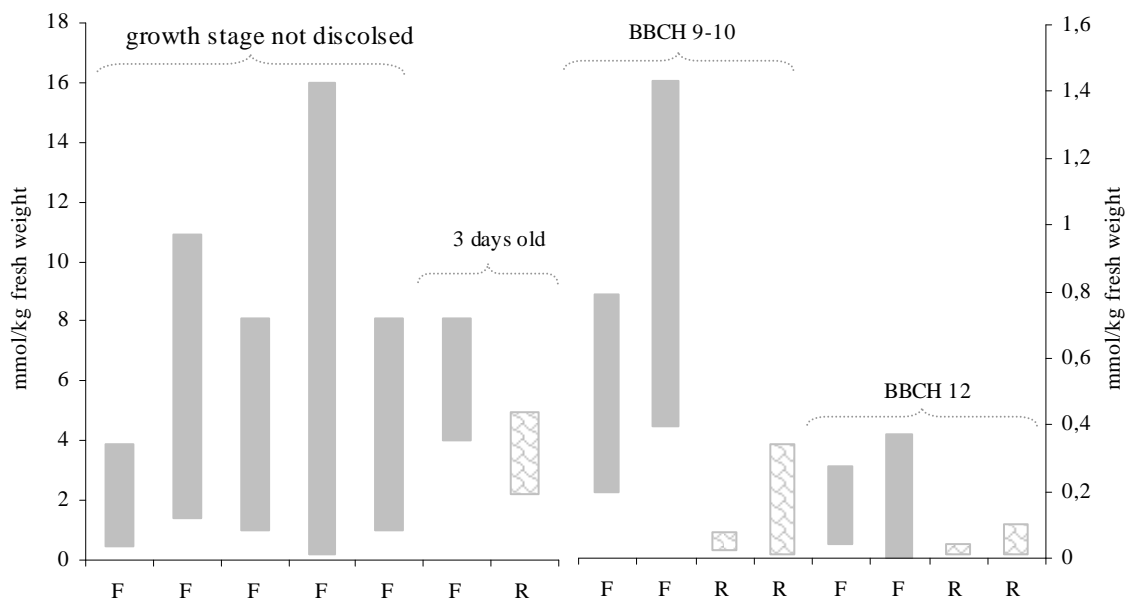


**Figure 3.-** Boxplots of concentrations found in wheat grouped according to a) type of cultivation (conventional grown (C), organic grown (O) b) growth stages (first stage (S1), second stage (S2), third stage (S3), fourth stage (S4) and fifth stage (S5)) c) wheat varieties (Astron (AS), Ritmo (RI) and Stakkado (ST)) and d) tissue sample matrix (Foliage (F) and root (R)).

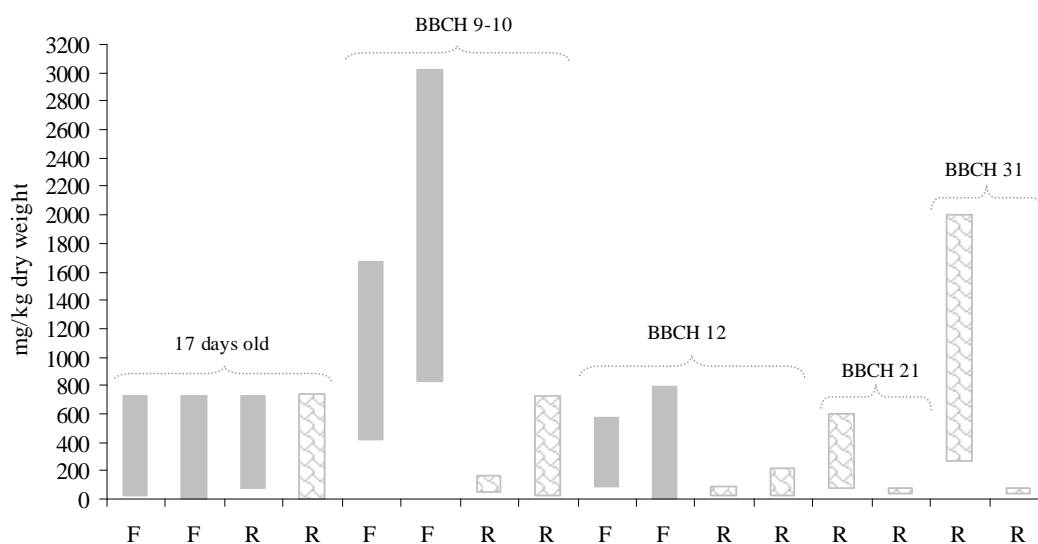


**Figure 4.-** Population marginal means with standard error of the total content of benzoxazinod derivatives related to foliage and root in the different growth stages. Two means are significantly different if their intervals are disjoint, and they are not significantly different if their intervals overlap.

a)



b)



**Figura 5.-** Range concentration of DIMBOA found in literature for foliage (F) and root (R) of wheat expressed as a) mmol/kg of dw and b) mg/kg dw.



**Table 1.-** Concentration range (mg/kg dw) of the methoxylated derivatives in foliage and roots in each growth stage under conventional and organic farming

| <b>Conventional farming</b> | <b>Growth stage</b> | <b>Dimboa-Glc</b> | <b>HMBOA</b> | <b>DIMBOA</b> | <b>MBOA</b> |
|-----------------------------|---------------------|-------------------|--------------|---------------|-------------|
| Foliage                     | 1C                  | <LOD              | 124-489      | 833-3026      | 433-646     |
|                             | 2C                  | <LOD              | 65-157       | <LOD-108      | 47-115      |
|                             | 3C                  | <LOD              | <LOD-54      | <LOD          | <LOD        |
|                             | 4C                  | <LOD              | <LOD-38      | <LOD          | <LOD        |
|                             | 5C                  | <LOD              | <LOD-36      | <LOD          | <LOD        |
| Root                        | 1C                  | 183-301           | 27-101       | 62-786        | 174-427     |
|                             | 2C                  | <LOQ-309          | <LOQ         | 31-67         | 42-66       |
|                             | 3C                  | <LOQ              | <LOQ         | 41-75         | 44-48       |
|                             | 4C                  | <LOQ-91           | <LOQ-24      | 31-78         | 22-37       |
|                             | 5C                  | <LOQ-57           | <LOQ         | 26-57         | <LOQ        |
| <b>Organic farming</b>      | <b>Growth stage</b> | <b>Dimboa-Glc</b> | <b>HMBOA</b> | <b>DIMBOA</b> | <b>MBOA</b> |
| Foliage                     | 10                  | <LOD-61           | 143-360      | 321-1468      | 154-414     |
|                             | 20                  | <LOD              | <LOD-44      | <LOD          | 49-93       |
|                             | 30                  | <LOD              | <LOD-44      | <LOD          | <LOD        |
|                             | 40                  |                   | <LOD         |               |             |
|                             | 50                  |                   | <LOD         |               |             |
| Root                        | 10                  |                   |              |               |             |
|                             | 20                  | <LOQ-155          | <LOQ-82      | <LOQ-242      | 52-114      |
|                             | 30                  | <LOQ              | <LOQ         | <LOQ-23       | 28-53       |
|                             | 40                  | <LOQ              | <LOQ         | 17-24         | 9-11        |
|                             | 50                  |                   | <LOQ         |               |             |

**Table 2 .-** Mutiway analysis of variance of total content of analytes

|   | <b>Df</b> | <b>Sum Sq</b> | <b>Mean Sq</b> | <b>F value</b> | <b>Pr(&gt;F)</b> |
|---|-----------|---------------|----------------|----------------|------------------|
| <b>M</b>  | 1         | 4.19          | 4.19           | 11.29          | 0.00107**        |
| <b>E</b>  | 4         | 222           | 55.5           | 149            | <2.20e-16***     |
| <b>C</b>  | 1         | 12.6          | 12.6           | 34.1           | 5.52e-08***      |
| <b>M:E</b>  | 4         | 21.5          | 5.38           | 14.5           | 1.56e-09***      |
| <b>Residuals</b>  | 109       | 40.4          | 0.37           |                |                  |
| Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 |           |               |                |                |                  |

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**IV.4. DISCUSSIÓ DELS RESULTATS**
**IV.4.1. Anàlisi de les mostres de blat**

A les Taules IV.3 i IV.4 es mostren els descriptors estadístics (mitjana, mediana, concentracions mínimes, màximes, i freqüència de detecció) per a cadascun dels analits determinats en les mostres del cultiu orgànic i convencional del primer i segon disseny experimental, respectivament. Els resultats del segon disseny experimental es presenten separant els estadis de creixement, donat que a mida que la planta creix la concentració dels diferents analits disminueix, essent en la majoria d'ells negligibles a partir del tercer estadi de creixement.

**Taula IV.3.-** Nivells màxims i mínims (mg/kg pes sec), mitjana, mediana i freqüència de detecció de les concentracions dels compostos al·leloquímics detectats en la fulla i l'arrel en el cultiu convencional (primer, segon i tercer estadi) i orgànic (tercer i quart estadi de creixement).

|                     | Compost | Nº mostres | FULLA   |         |           |           |                            | ARREL   |         |           |           |                            |
|---------------------|---------|------------|---------|---------|-----------|-----------|----------------------------|---------|---------|-----------|-----------|----------------------------|
|                     |         |            | Mitjana | Mediana | Valor MIN | Valor MAX | Freqüència de detecció (%) | Mitjana | Mediana | Valor MIN | Valor MAX | Freqüència de detecció (%) |
| Cultiu Convencional | Db-glc  | 36         | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |
|                     | Dmb-glc |            | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |
|                     | Hboa    |            | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | nd%                        |
|                     | Diboa   |            | nd      | nd      | nd        | nd        | 0%                         | 4,6     | 4,7     | 3,4       | 5,6       | 19%                        |
|                     | Hmboa   |            | 30,2    | 19,2    | 9,6       | 101,4     | 58%                        | 12,9    | 10,8    | 2,6       | 59,5      | 77%                        |
|                     | Dimboa  |            | 28,8    | 15,7    | 7,6       | 138,1     | 39%                        | 18,7    | 13,7    | 5,1       | 55,9      | 28%                        |
|                     | Boa     |            | 3,7     | 3,8     | 1,0       | 5,2       | 64%                        | 4,9     | 4,0     | 2,1       | 22,0      | 69%                        |
|                     | Mboa    |            | 83,5    | 79,1    | 24,8      | 233,4     | 94%                        | 101,0   | 107,8   | 22,6      | 309,8     | 97%                        |
| Cultiu Orgànic      | Db-glc  | 24         | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |
|                     | Dmb-glc |            | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |
|                     | Hboa    |            | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |
|                     | Diboa   |            | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |
|                     | Hmboa   |            | 13,0    | 13,0    | 9,3       | 16,7      | 8%                         | 20,6    | 17,6    | 10,9      | 42,8      | 63%                        |
|                     | Dimboa  |            | 10,6    | 10,6    | 10,6      | 10,6      | 4%                         | 13,1    | 12,5    | 5,7       | 21,7      | 17%                        |
|                     | Boa     |            | 3,9     | 3,4     | 1,2       | 8,5       | 42%                        | 6,8     | 3,8     | 1,9       | 46,2      | 63%                        |
|                     | Mboa    |            | 17,9    | 12,5    | 1,4       | 64,8      | 100%                       | 80,9    | 59,7    | 5,8       | 230,1     | 100%                       |

**Taula IV.4.-** Nivells màxims i mínims (mg/kg pes sec), mitjana, mediana i freqüència de detecció de les concentracions dels compostos al·leloquímics detectats en la fulla i l'arrel en el cultiu convencional i orgànic en a) el primer i segon estadi de creixement i b) el tercer, quart i cinquè estadi de creixement.

| a)                  |                |         | FULLA   |           |           |                            |         | ARREL   |           |           |                            |     |
|---------------------|----------------|---------|---------|-----------|-----------|----------------------------|---------|---------|-----------|-----------|----------------------------|-----|
| Compost             | Nº mostres     | Mitjana | Mediana | Valor MIN | Valor MAX | Freqüència de detecció (%) | Mitjana | Mediana | Valor MIN | Valor MAX | Freqüència de detecció (%) |     |
| Cultiu Convencional | Db-glc         | nd      | nd      | nd        | nd        | 0%                         | 18,1    | 16,3    | 8,6       | 32,5      | 50%                        |     |
|                     | Dmb-glc        | nd      | nd      | nd        | nd        | 0%                         | 162,4   | 158,3   | 16,7      | 309,4     | 83%                        |     |
|                     | Hboa           | 142,0   | 147,5   | 104,3     | 174,4     | 25%                        | nd      | nd      | nd        | nd        | 0%                         |     |
|                     | Diboa          | 50,7    | 51,7    | 22,8      | 73,2      | 58%                        | nd      | nd      | nd        | nd        | 0%                         |     |
|                     | Hmboa          | 126,7   | 103,1   | 12,3      | 296,4     | 92%                        | 50,3    | 27,0    | 22,5      | 101,2     | 42%                        |     |
|                     | Dimboa         | 669,1   | 332,2   | 21,8      | 3026,3    | 92%                        | 147,5   | 62,0    | 28,7      | 786,4     | 42%                        |     |
|                     | Boa            | nd      | nd      | nd        | nd        | 0%                         | 4,5     | 4,2     | 2,9       | 7,6       | 67%                        |     |
|                     | Mboa           | 188,8   | 87,3    | 2,6       | 646,3     | 100%                       | 134,9   | 66,5    | 9,2       | 426,6     | 92%                        |     |
|                     | Cultiu Orgànic | Db-glc  | nd      | nd        | nd        | nd                         | 0%      | 44,4    | 44,4      | 36,8      | 52,0                       | 17% |
| Dmb-glc             |                | 73,3    | 73,3    | 60,8      | 85,9      | 17%                        | 121,0   | 102,0   | 32,4      | 263,5     | 67%                        |     |
| Hboa                |                | 238,1   | 238,1   | 197,3     | 279,0     | 17%                        | nd      | nd      | nd        | nd        | 0%                         |     |
| Diboa               |                | 39,3    | 39,3    | 32,6      | 46,0      | 17%                        | nd      | nd      | nd        | nd        | 0%                         |     |
| Hmboa               |                | 129,1   | 62,8    | 22,6      | 360,2     | 58%                        | 50,1    | 39,6    | 20,8      | 96,8      | 50%                        |     |
| Dimboa              |                | 643,5   | 316,6   | 129,3     | 1468,5    | 50%                        | 122,6   | 68,7    | 21,6      | 323,2     | 67%                        |     |
| Boa                 |                | nd      | nd      | nd        | nd        | 0%                         | 9,2     | 7,8     | 2,0       | 20,4      | 83%                        |     |
| Mboa                |                | 130,9   | 67,8    | 6,8       | 414,0     | 100%                       | 106,5   | 83,0    | 12,2      | 297,8     | 100%                       |     |

| b)                  |                |         | FULLA   |           |           |                            |         | ARREL   |           |           |                            |    |
|---------------------|----------------|---------|---------|-----------|-----------|----------------------------|---------|---------|-----------|-----------|----------------------------|----|
| Compost             | Nº mostres     | Mitjana | Mediana | Valor MIN | Valor MAX | Freqüència de detecció (%) | Mitjana | Mediana | Valor MIN | Valor MAX | Freqüència de detecció (%) |    |
| Cultiu Convencional | Db-glc         | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
|                     | Dmb-glc        | nd      | nd      | nd        | nd        | 0%                         | 83,9    | 83,1    | 57,0      | 128,8     | 33%                        |    |
|                     | Hboa           | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
|                     | Diboa          | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
|                     | Hmboa          | 37,0    | 37,6    | 12,9      | 53,7      | 28%                        | 29,4    | 29,4    | 24,3      | 34,4      | 11%                        |    |
|                     | Dimboa         | nd      | nd      | nd        | nd        | 0%                         | 41,6    | 43,3    | 7,8       | 77,8      | 89%                        |    |
|                     | Boa            | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
|                     | Mboa           | nd      | nd      | nd        | nd        | 0%                         | 23,1    | 18,1    | 4,0       | 47,9      | 67%                        |    |
|                     | Cultiu Orgànic | Db-glc  | nd      | nd        | nd        | nd                         | 0%      | nd      | nd        | nd        | nd                         | 0% |
| Dmb-glc             |                | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
| Hboa                |                | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
| Diboa               |                | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
| Hmboa               |                | 28,5    | 32,0    | 9,2       | 44,3      | 17%                        | nd      | nd      | nd        | nd        | 0%                         |    |
| Dimboa              |                | nd      | nd      | nd        | nd        | 0%                         | 26,5    | 24,2    | 17,3      | 33,9      | 44%                        |    |
| Boa                 |                | nd      | nd      | nd        | nd        | 0%                         | nd      | nd      | nd        | nd        | 0%                         |    |
| Mboa                |                | nd      | nd      | nd        | nd        | 0%                         | 22,5    | 15,5    | 2,1       | 52,9      | 67%                        |    |

**Nivells entre fulla, arrel i llavors germinades.**

En general s'observen majors concentracions en la fulla que en l'arrel en els primers estadis de creixement, no obstant la disminució dels nivells d'al·leloquímics en la fulla a mida que la planta creix és molt més pronunciada que en l'arrel, on els nivells es

mantenen més constants i en alguns casos són superiors als trobats en la fulla en estadis de creixement més avançats (veure Figures 1 i 2 de l'article #7#).

Si bé, la majoria dels treballs publicats fins al moment, es centraven en l'anàlisi de les fulles i arrels (motiu pel qual varen ser les matrius seleccionades per l'estudi dins el projecte FATEALLCHEM), els nostres resultats mostren per primera vegada la presència dels compostos al·lelopàtics en les llavors germinades. Cal destacar que els nivells de compostos al·leloquímics trobats en les llavors germinades, van ser del mateix rang que els determinats en la fulla i l'arrel de la planta de blat. Tot i que s'hagin trobat els compostos al·leloquímics en les llavors germinades en els 10 dies posteriors a la sembra del blat, no prova que les llavors originàries continguin aquests compostos, però els resultats si que suggereixen que poden contenir els compostos precursors. En un estudi a nivell de laboratori dut a terme per Nakagawa et. al [31], van examinar la presència i els nivells dels àcids hidroxàmics en les llavors abans de la seva germinació. Els glucòsids (Diboa-Glc i Dimboa-Glc) es van trobar presents a partir de les 12-16 hores després de la sembra, mentre que les aglucones (DIBOA i DIMBOA) van ser perceptibles poc abans de la germinació, fins a 24 h després de la sembra. Per a un futur ús de les varietats de blat amb un elevat potencial al·lelopàtic, des d'un punt de vista de salut, seria interessant conèixer si les llavors de la planta de blat contenen compostos al·leloquímics i si aquests poden ser perjudicials o no per als humans a través de la seva ingesta. Com s'ha comentat anteriorment, fins fa relativament poc temps, els àcids hidroxàmics i els seus derivats no havien estat trobats en les llavors no germinades [7, 15, 32]. No obstant, després de la nostra publicació, han aparegut altres treballs en els que s'han analitzat diferents derivats de les benzoxazinones en grans de sègol, salvat de sègol i de blat a través de la LC acoblada a un detector de temps de vol (UPLC-qTOF-MS) [33]. El major compost identificat en aquest estudi va ser el derivat del DIBOA amb doble hexosa en la seva estructura, també es va identificar el DIBOA-Glc, el derivat del DIMBOA amb dues hexoses en la seva estructura però no el DIMBOA-Glc. Són necessaris més estudis d'aquest nivell per tal de provar quins compostos estan presents en les llavors no germinades i si aquests poden tenir repercussió en la salut alimentària.

### **Nivells dels diferents compostos al·lelopàtics.**

El primer que cal destacar, és la diferència entre el tipus de compostos trobats en les mostres del primer i segon disseny experimental. Els compostos glucosats només es troben presents en les mostres del segon disseny experimental. Aquests han estat trobats principalment en les mostres d'arrel, a excepció del Dimboa-Glc també present en el 17% de les mostres de fulla en els dos primers estadis de creixement de l'agricultura orgànica. Aquest mateix compost està present en un 83% de les mostres d'arrel del cultiu convencional en els dos primers estadis de creixement, i en un 67% en el cultiu orgànic. En la resta d'estadis la seva presència és inferior al 50% en el cultiu convencional i no es detecta en l'orgànic. El Diboia-Glc té una freqüència de detecció inferior que el seu homòleg metoxilat, essent del 50% en les mostres d'arrel dels dos primers estadis de creixement del cultiu convencional (Taula IV).

Com ja s'ha comentat durant els capítols anteriors, el pretractament de la mostra és important en l'anàlisi d'aquests compostos degut a la seva baixa estabilitat. Donat que les mostres en el primer disseny experimental no varen ser conservades immediatament a -20°C després de la seva collita, va donar lloc a una degradació dels compostos glucosats a les corresponents aglucones. Aquest tractament de la mostra no tant sols es veu reflectit en la no presència dels compostos glucosats sinó també en la major o menor presència de la benzoxazolinones envers les aglucones.

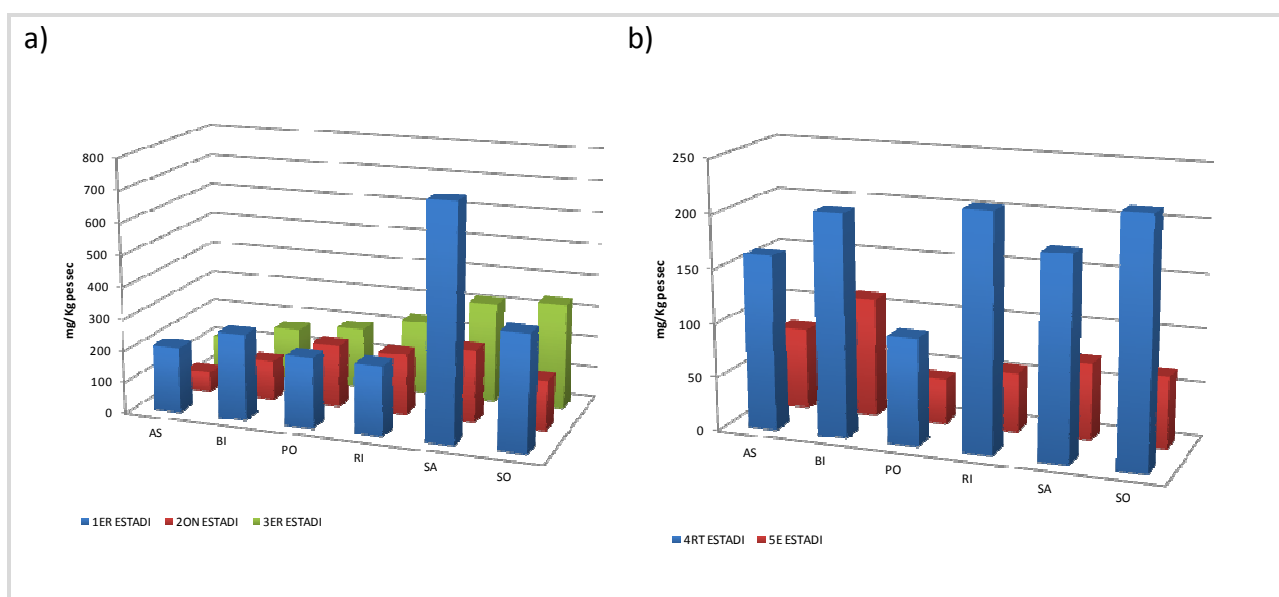
Mentre la benzoxazolinona MBOA es troba present en totes les mostres analitzades del primer disseny experimental, essent a la vegada el compost que presenta majors concentracions màximes. En les mostres del segon disseny experimental, tot i que el MBOA ha estat present en el 100% de totes les mostres del primer i segon estadi tant en la fulla com en l'arrel i en el 67% de les arrels de la resta dels estadis tant en el cultiu convencional com orgànic, el DIMBOA ha estat l'analit detectat a majors concentracions, essent a la vegada el compost que més àmpliament s'ha estudiat en el camp de l'al·lelopatia en cereals [12-16].



Els analits no metoxilats (HBOA, DIBOA i BOA) són els que es detecten en menor freqüència i els que a la vegada presenten menors concentracions. Aquest resultat és consistent amb el trobat en bibliografia on els derivats metoxilats (HMBOA, DIMBOA i MBOA) es troben en majors concentracions en el cultiu de blat [19].

### Nivells en funció l'estadi de creixement.

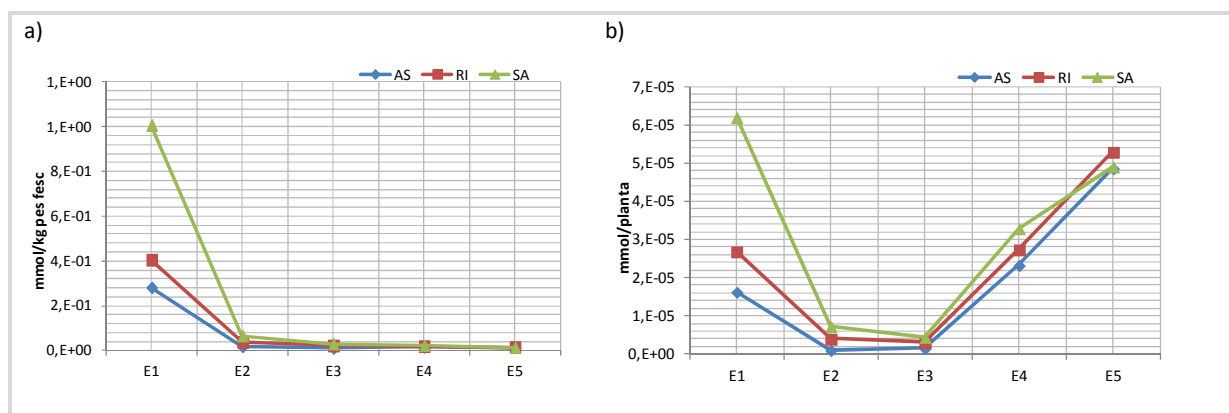
A la Figura IV.3 es mostra el contingut total d'al·leloquímics en la planta de blat (fulla+arrel) en els tres primers estadis del cultiu convencional, i el quart i cinquè estadi del cultiu orgànic per a les diferents varietats de blat del primer disseny experimental. En ambdós tipus de cultiu s'observa una disminució del contingut total de compostos d'un estadi de creixement cap al successiu superior.



**Figura IV.3.-**Contingut total de compostos al·leloquímics (fulla+arrel) de les diferents varietats de blat analitzades en a) Cultiu convencional i b) Cultiu orgànic

El mateix s'observa en les mostres del segon disseny experimental (Taula IV a i b). S'ha plantejat la hipòtesi que les plantes sintetitzen els diferents derivats de les benzoxazinones en els primers estadis de creixement, propers a la germinació de la planta, i que la reducció de la concentració observada al llarg del temps, és deguda a un procés de dilució dels compostos en la planta. Aquesta hipòtesi va ser descrita per Copaja et al. [28] en un estudi dut a terme en plantes de fins a 7 dies. No obstant, en

vista dels resultats obtinguts en el nostre estudi, és difícil pensar que sigui així en les plantes que creixen fins a la maduresa. Tenint en compte que el pes total promig de la planta és de 0.1, 0.2, 0.5, 5, i 12 grams en el primer, segon, tercer, quart i cinquè estadi, respectivament, i el promig de matèria seca de la planta és del 10%, podríem dir que la disminució observada no és deguda a un procés de dilució en la planta. La reducció en la concentració de les benzoxazinones del primer estadi de creixement al tercer és significativament major del que es pot explicar en termes de creixement de la planta (Figura IV.4a). Per exemple, la concentració total (mmol/kg de pes fresc) en la planta del derivat DIMBOA en el tercer estadi de creixement respecte el primer es veu reduït entre un 94-97% (depenent de la varietat) respecte el primer estadi, mentre que el pes fresc s'incrementa per un factor de 5. El mateix comportament s'observa en el total de compostos en la planta. En contraposició si es representa el contingut del compost en la planta (mmol/planta) no s'obté el mateix comportament, és a dir el contingut total en la planta disminueix del primer al tercer estadi però continua creixent del tercer al cinquè (Figura IV.4b). No està clar, en l'actualitat, com els canvis en el pes sec de la planta i les taxes de la biosíntesi i/o la degradació dels àcids interactuen per augmentar i disminuir posteriorment la concentració d'àcids hidroxàmics. Un comportament similar va ser observat en l'estudi d' Argadoña et al. On en l'anàlisi del contingut d'àcids hidroxàmics en mmol/kg pes fresc incrementa durant els 4 primers dies i posteriorment disminueix progressivament. De la mateixa manera el contingut total en la planta continua creixent després dels 4 dies.



**Figura IV.4.-** Concentració total de DIMBOA (fulla+arrel) de les diferents varietats de blat en els diferents estadis de creixement expressats com a) mmol/Kg pes fresc i b) mmol/planta

### Nivells entre agricultura convencional i orgànica.

Com a tendència general les concentracions trobades en el cultiu convencional són superiors a les trobades en el cultiu orgànic en les mostres d'ambdós dissenys experimentals. Aquest fet podria ser degut a que al sotmetre el cultiu al tractament amb plaguicides, la planta reaccioni a l'estrès provocat metabolitzant més compostos.

### Nivells entre varietats.

En general, les majors diferències en el contingut de compostos al·lelopàtics entre varietats s'observa en el primer estadi de creixement. En les mostres del primer disseny experimental (Figura IV.3), la varietat SA és la que presenta un contingut total de compostos major que en la resta de varietats en el cultiu convencional, mentre que en el cultiu orgànic en el tercer i quart estadi de creixement no s'observa una tendència clara. El mateix s'observa en el segon disseny experimental, on la varietat SA presenta les majors concentracions totals d'al·leloquímics tant en la fulla com l'arrel en el primer estadi de creixement del cultiu convencional, mentre que a partir del segon estadi les diferències no són tan evidents. No obstant, en el primer estadi de la fulla del cultiu orgànic la varietat que presenta majors nivells es la RI però en l'arrel torna a ser la SA tant en el primer com en el segon estadi.

#### IV.4.2. Anàlisi estadístic de les dades

Degut a que les mostres provinents del segon disseny experimental són més completes, es va dur a terme l'anàlisi estadístic dels resultats obtinguts per tal de determinar si alguns dels factors (variables independents) té algun pes significatiu sobre el contingut total d'al·leloquímics (variable dependent).

**Variable dependent:** concentració total de compostos en la mostra

**Variables independents:**

Estadi (5 nivells): S1, S2, S3, S4, S5

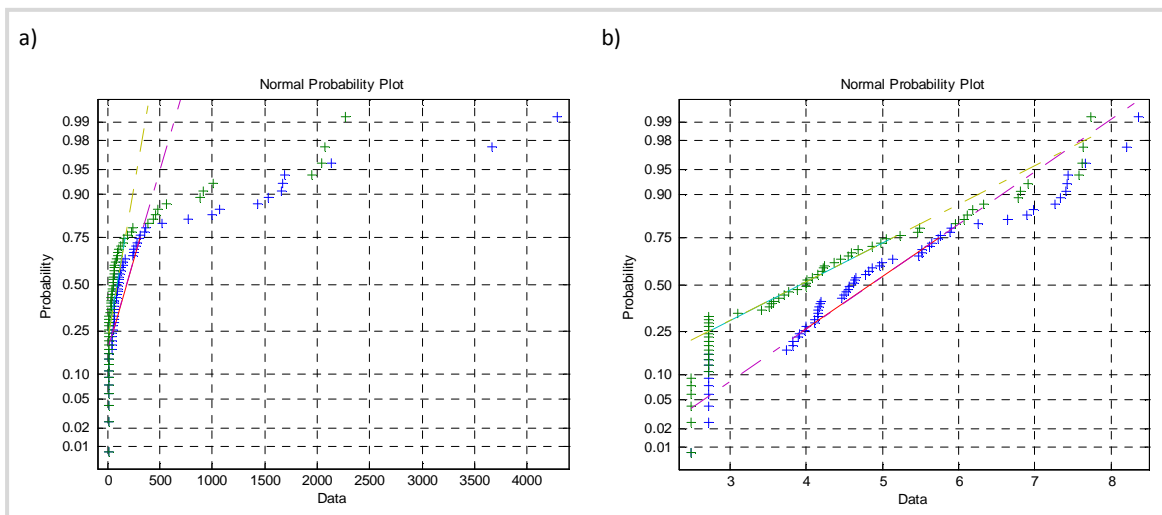
Cultiu (2 nivells): Convencional, Orgànic

Matriu (2 nivells): Fulla, Arrel

Varietat (3 nivells): Astron, Ritmo, Stakado

L'objectiu plantejat de l'anàlisi estadístic és conèixer si existeixen diferències significatives entre els 5 estadis, les 2 matris, les 3 varietats i els 2 tipus de cultiu.

Alhora de dur a terme un anàlisi estadístic i amb l'objectiu d'aplicar el test ANOVA, és important conèixer si el conjunt de dades que es volen analitzar compleixen amb els requeriments de l'anàlisi estadístic. Ens hem ajudat del programa MATLAB per dur a terme tots els càlculs estadístics. Donat que la variabilitat de les dades és elevada, es va decidir transformar-les en valors logarítmics per tal de complir amb els supòsits de normalitat i homogeneïtat requerits en l'anàlisi ANOVA. A la Figura IV.5 es mostra un exemple on la transformació de les dades agrupades per tipus de cultiu compleixen amb la normalitat.



**Figura IV.5.-** Gràfic de probabilitat normal de a) dades no transformades and b) dades transformades en valors logarítmics

Per tal de comprovar si les nostres dades compleixen amb el requeriment d'homogeneïtat es va aplicar la prova d'homoscedasticitat de Levene. Amb aquest test es comprova si les variàncies són iguals. Si la probabilitat associada a l'estadístic de Levene és  $\geq 0.05$ , es suposa que aquestes són iguals. Si pel contrari l'estadístic de Levene és  $< 0.05$ , llavors es suposa que les variàncies són diferents. A la Taula IV.5 es mostra el resultat d'aplicar el test de Levene per a les dades de les diferents etapes de

cultiu. Com es pot observar, transformar les dades a valors logarítmics assegura la homoscedasticitat de les mateixes.

**Taula IV.5-** Resultats del test de Levene per a les dades dels diferents estadis de creixement per a) matriu de dades original b) matriu de dades transformada

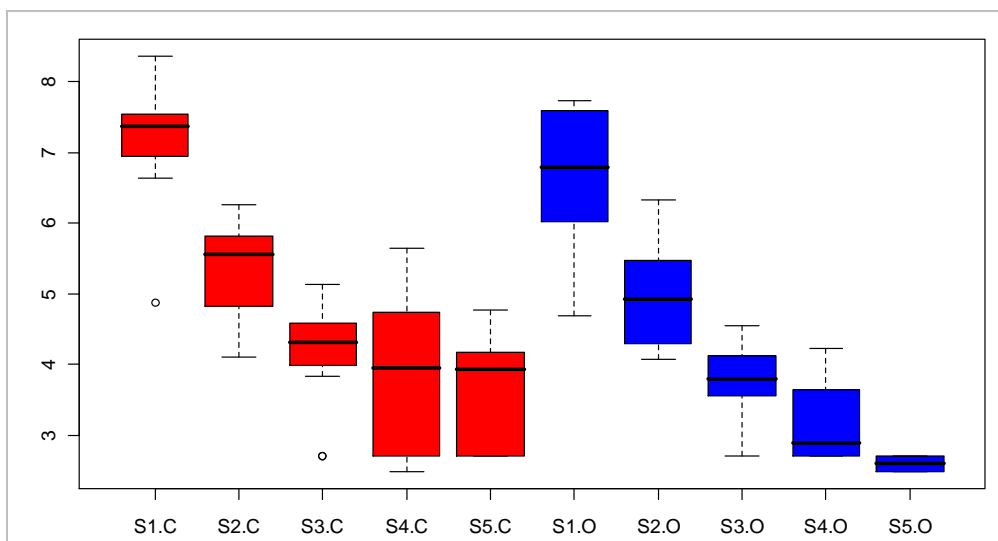
a)

|           | <i>Estadi 1</i> | <i>Estadi 2</i>  | <i>Estadi 3</i> | <i>Estadi 4</i> | <i>Estadi 5</i> |
|-----------|-----------------|--|-----------------|-----------------|-----------------|
| Mediana   | 1251            | 194  | 59              | 32              | 15              |
| Mitja     | 1406            | 223  | 64              | 58              | 31              |
| Variància | 1091172,22      | 22773,2846   | 1561,18752      | 5076,85658      | 775,909409      |
| n         | 24              | 24   | 24              | 24              | 24              |
| df        | 23              | 23   | 23              | 23              | 23              |
| Levene's  |                 |  |                 |                 |                 |
| Test      | 28,528          |  |                 |                 |                 |
| <b>p</b>  | <b>0,000</b>    | Rebutjar la hipòtesi nul · la perquè $p < 0,05$ (variàncies són diferents) |                 |                 |                 |
|           | $\alpha$        | 0,05   |                 |                 |                 |

b)

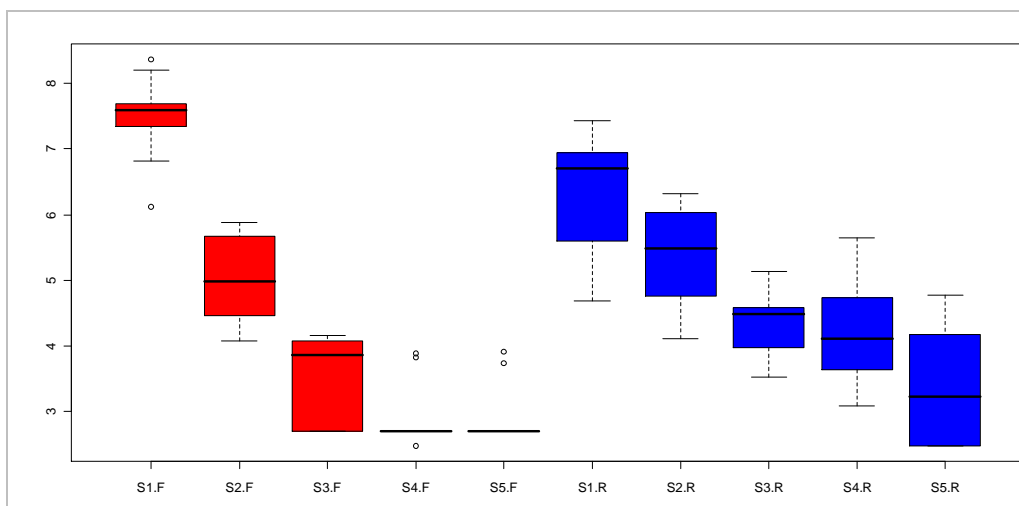
|           | <i>Estadi 1</i> | <i>Estadi 2</i>   | <i>Estadi 3</i> | <i>Estadi 4</i> | <i>Estadi 5</i> |
|-----------|-----------------|---|-----------------|-----------------|-----------------|
| Mediana   | 3,09            | 2,28  | 1,77            | 1,50            | 1,18            |
| Mitja     | 2,99            | 2,25  | 1,72            | 1,55            | 1,36            |
| Variància | 0,179           | 0,0977  | 0,0928          | 0,170           | 0,106           |
| n         | 24              | 24  | 24              | 24              | 24              |
| df        | 23              | 23  | 23              | 23              | 23              |
| Levene's  |                 |   |                 |                 |                 |
| Test      | 1,111           |   |                 |                 |                 |
| <b>p</b>  | <b>0,355</b>    | Acceptar la hipòtesi nul · la perquè $p > 0,05$ (variàncies són iguals) |                 |                 |                 |
|           | $\alpha$        | 0,05  |                 |                 |                 |

Després de comprovar que la transformació de les dades ens permet acomplir amb els requeriments d'homogeneïtat i homoscedasticitat requerit per l'ANOVA, el següent pas va ser dur a terme l'anàlisi descriptiu de les dades, on es varen representar els boxplot per a cadascun dels factors (Figura 3 de l'article #7#). D'aquest anàlisi descriptiu ja es pot intuir que el factor estadi és el que mostra més diferències entre els seus 5 nivells. A part de representar els boxplots per a cadascun dels factors individualment també es varen representar els provinents de la combinació de les diferents variables independents. A la Figura IV.6 es mostra el boxplot per a les mostres en els diferents estadis de creixement en el cultiu convencional (vermell) i en el cultiu orgànic (blau). S'observa una tendència similar entre ambdós cultius, amb disminució de la concentració total de compostos a mida que la planta creix.



**Figura IV.6.-** Representació boxplot de la concentració total de compostos al·leloquímics en els diferents estadis per al cultiu convencional (vermell) i orgànic (blau)

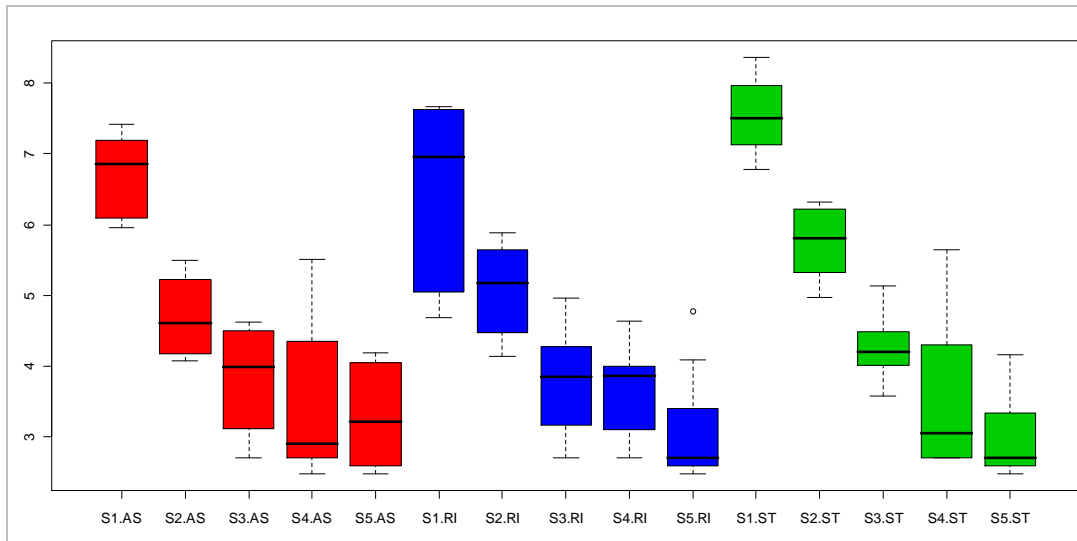
A la Figura IV.7 es mostra el boxplot de representar les dades en funció de les variables estadi de creixement per a la fulla i arrel. En aquest cas s'observen més diferències entre les matrius i es corrobora que en la fulla la disminució en la concentració total de compostos és molt més pronunciada que en l'arrel.



**Figura IV.7.-** Representació boxplot de la concentració total de compostos al·leloquímics en els diferents estadis per la fulla (vermell) i arrel (blau)

Representant el boxplot de la concentració total d'al·leloquímics en els diferents estadis de creixement per a les diferents varietats (Figura IV.8), també s'observa una

tendència general en la disminució del contingut total d'al·leloquímics durant el creixement de la planta en totes les varietats.



**Figura IV.8-** Representació boxplot de la concentració total de compostos al·leloquímics en els diferents estadis per la varietat Astron (AS; vermell), Ritmo (RI;blau) i Stakaddo (ST; verd).

Un cop dut a terme l’anàlisi descriptiu, el següent pas és veure si existeixen diferències significatives entre els nivells dels diferents factors i per aquesta raó es va aplicar el test estadístic ANOVA 1 factor.

A la taula IV.6 es mostra el resultat de l’ANOVA d’un factor per a cadascun de les variables independents (factors).

**Taula IV.6.-** Taula resultats ANOVA 1 factor (Codis: 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘.’ 0.1 ‘ ’ 1)

| Variables | Probabilitat | Resultat                        |
|-----------|--------------|---------------------------------|
| Estadis   | P< 2e-16 *** | ∃ diferències significatives    |
| Matriu    | P= 0.2       | No ∃ diferències significatives |
| Varietat  | P= 0.4       | No ∃ diferències significatives |
| Cultius   | P=0.02 *     | ∃ diferències significatives    |

Mentre els factors matriu i varietat no mostren diferències significatives entre els diferents nivells, els factors estadi i el tipus de cultiu si que en mostren. No obstant, aquestes són molt més marcades en el cas dels estadis. Per tal de comprovar si les grans variacions degudes als diferents estadis amaguen les de la resta de variables (varietats, cultiu, i matriu) la millor opció és separar les fonts de variació aplicant el test

estadístic ANOVA factorial (N-way ANOVA) que ens permet separar les diferents fonts de variació i distingir-les per observar si són significatives.

És important dur a terme un bon ajust del model d'acord amb la matriu de dades disponible. Cal comentar que construir el model amb tots els factors considerant totes les interaccions possibles fa que la interpretació dels resultats pugui arribar a ser realment complicada. Donat que no disposàvem d'un volum de dades el suficientment gran com per aplicar el model complet amb tots els factors i amb totes les interaccions, es va anar ajustant el model en funció dels factors que van anar mostrant les diferències més significatives. A la taula IV.7 es mostren els valors de p obtinguts en cadascun dels models testats. A nivell general, valors de  $p < 0.05$  pels factors individuals, indiquen que la concentració total d'al·leloquímics no segueix un comportament homogeni entre els diferents nivells de cada factor, mentre que valors de  $p < 0.05$  en les interaccions indica que hi ha una interacció significativa entre factors, és a dir que la concentració total d'al·leloquímics no segueix un comportament homogeni en tots els tractaments d'un factor respecte l'altre.

**Taula IV.7.-** Valors de p dels diferents factors en funció el model ANOVA factorial aplicat

| Nº | Descripció del model                  | E | C | M     | V    | Interacció         |
|----|---------------------------------------|---|---|-------|------|--------------------|
| 1  | Estadi-Cultiu sense interacció        | 0 | 0 | -     | -    | -                  |
| 2  | Estadi-Cultiu amb interacció          | 0 | 0 | -     | -    | 0.5                |
| 3  | Estadi-Matriu sense interacció        | 0 | - | 0.01  | -    | -                  |
| 4  | Estadi-Matriu amb interacció          | 0 | - | 0.004 | -    | 0                  |
| 5  | Estadi-Varietat sense interacció      | 0 | - | -     | 0.03 | -                  |
| 6  | Estadi-Varietat amb interacció        | 0 | - | -     | 0.03 | 0.2                |
| 7  | Estadi-Cultiu-Matriu sense interacció | 0 | 0 | 0.007 | -    | -                  |
| 8  | Estadi-Cultiu-Matriu amb interacció   | 0 | 0 | 0.001 | -    | 0 (E:M) ; 0.3(E:C) |

*Model nº 1:* de la mateixa manera que s'observa en l'anàlisi ANOVA per els factors individuals, tant el factor estadi com el de cultiu mostren diferències significatives entre els diferents nivells del factor amb valors de  $p=0$ .

*Model nº 2:* en el model amb interacció, mentre els factors principals segueixen mostrant diferències significatives, la interacció amb una  $p=0.5$  ens indica que les diferències en la concentració d'al·leloquímics entre els estadis és independent del nivell del factor cultiu.



*Model nº 3:* en el tercer model es consideren com a factors l'estadi i la matriu en un model sense interacció on s'obtenen diferències significatives entre els estadis ( $p=0$ ) i a diferència dels resultats de l'ANOVA d'un factor ara si que s'observen diferències significatives entre les matrius ( $p=0.01$ ).

*Model nº 4:* en el model amb interacció entre l'estadi i la matriu el valor de  $p$  per a la interacció és 0 el que ens indica que la concentració total d'al·leloquímics entre estadis no és igual entre els diferents nivells de la matriu.

*Model nº 5:* pels factors estadi i varietat s'observen diferències significatives entre els estadis ( $p=0$ ) i també a diferència del que es veia al ANOVA 1 factor també existeixen diferències significatives entre les diferents varietats ( $p=0.03$ ), tot i que cal comentar que entre les diferents varietats aquestes no són tant evidents donat que el valor de  $p$  no és molt inferior a 0.05.

*Model nº 6:* pels factors estadi-varietat, el valor de  $p$  per a la interacció és superior a 0.05 ( $p=0.2$ ) indicant que el contingut total d'al·leloquímics és independent de les varietats.

Tenint en compte aquests resultats, es pot extreure la informació que el contingut total d'al·leloquímics no és el mateix entre els diferents nivells dels factors estadi, cultiu i matriu. D'aquesta manera es van construir dos models més considerant aquests factors sense (nº7) i amb interacció (nº8). Mentre s'observen diferències significatives per als factors principals en els dos models, els valors de  $p$  per a la interacció entre els factors estadi-matriu i estadi-cultiu són  $p=0$  i  $p=0.3$ , respectivament. Donat que la interacció estadi-cultiu no és significativa, finalment es va fer l'ajust d'un model per als factors estadi, cultiu i matriu amb interacció entre els factors estadi i matriu. La taula ANOVA factorial obtinguda per al model definitiu es mostra a la taula IV.8.

**Taula IV.8.-** Taula ANOVA factorial

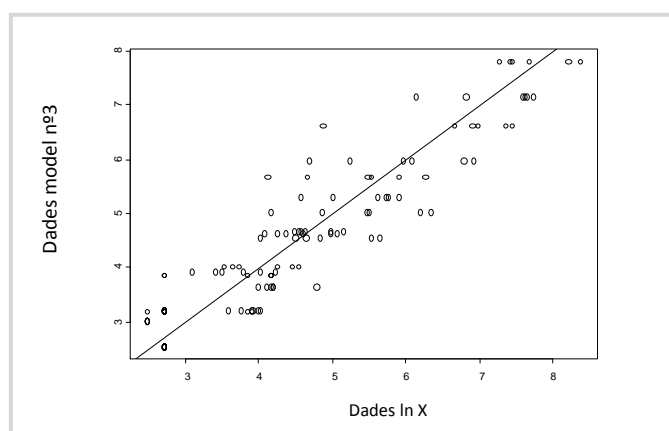
| Source | Sum sq. | d.f. | Mean Sq. | F    | Prob>F  |
|--------|---------|------|----------|------|---------|
| E      | 222     | 4    | 55.6     | 150  | 0       |
| M      | 4.19    | 1    | 4.19     | 11.3 | 0.00107 |
| C      | 12.6    | 1    | 12.6     | 34.1 | 0       |
| M:E    | 21.5    | 4    | 5.38     | 14.5 | 0       |
| Error  | 38.6    | 109  | 0.368    |      |         |
| Total  | 301     | 119  |          |      |         |

Finalment per comprovar que el model aplicat s’ajusta a les dades experimentals es va dur a terme el càlcul dels coeficients de correlació entre les dades experimentals i les obtingudes d’aplicar el model. En aquest cas es van comparar els valors de correlació entre tres dels models testats. Si comparem els coeficients de correlació ( $r^2$ ) obtinguts (Taula IV.9), podem concloure que el model finalment descrit és el que millor s’ajusta al conjunt de dades amb un  $r^2$  de (0.930).

**Taula IV.9.-** Coeficients de correlació obtinguts entre les dades experimentals i les predites per un model ANOVA factorial determinat

| Nº | Descripció del model                              | $r^2$ |
|----|---|-------|
| 1  | Estadi-cultiu                                     | 0.883 |
| 4  | Estadi-matriu amb interacció                      | 0.907 |
| 8  | Estadi-matriu-cultiu amb interacció estadi:matriu | 0.930 |

A la figura IV.9 es mostra el gràfic de correlació obtingut de representar les variables experimentals amb les predites pel model N°8.



**Figura IV.9.-** Gràfic de correlació obtingut entre les dades predites pel model i les dades experimentals

Un cop ajustat el model podem concloure que el contingut total de compostos al·leloquímics no és igual entre els diferents estadis ( $p=0$ ), entre la fulla i l'arrel ( $p=0.001073$ ) i entre l'agricultura orgànica i convencional ( $p=0$ ). De la mateixa manera es pot concloure que la concentració d'al·leloquímics en un estadi de creixement determinat depèn del tipus de matriu analitzada ( $p=0$ ).

#### IV.4.3. Anàlisi de compostos al·lelopàtics en el sòl agrícola

Una part dels resultats de l'anàlisi dels sòls es mostren en l'article #5# presentat en el capítol III de la present memòria, essent la primera vegada que s'analitzaven aquests compostos en mostres de camp, confirmant així les rutes de degradació prèviament determinades en experiments de laboratori. La totalitat dels resultats corresponents als diferents sòls es presenta a continuació. A les Figures IV.10 i IV.11 es mostren els nivells acumulats dels diferents analits determinats en les mostres de sòl sota l'agricultura convencional (Figura IV.10) i orgànica (Figura IV.11).

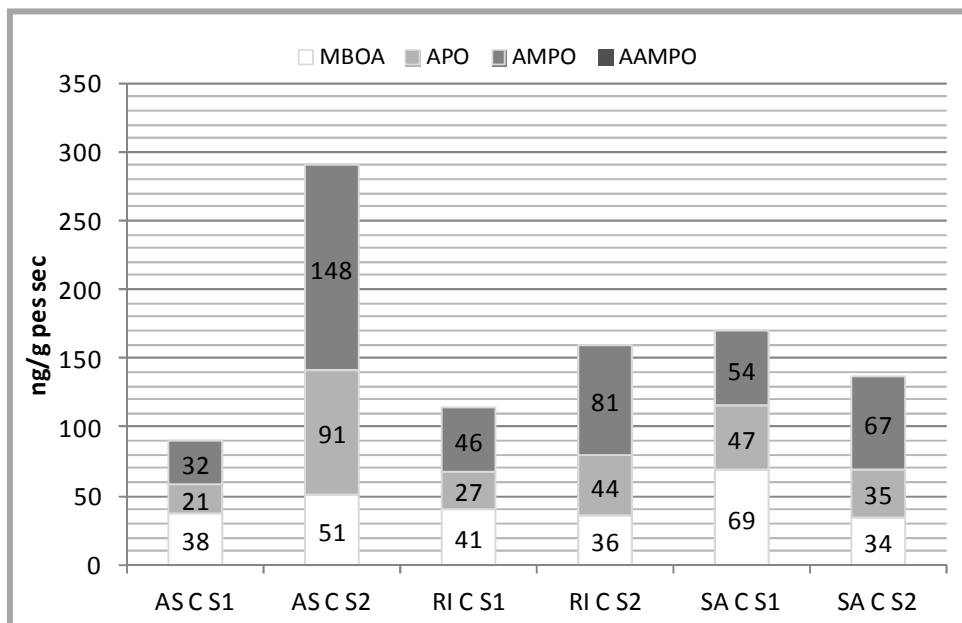


Figura IV.10.- Nivells de compostos al·lelopàtics en el primer i segon estadi del cultiu convencional

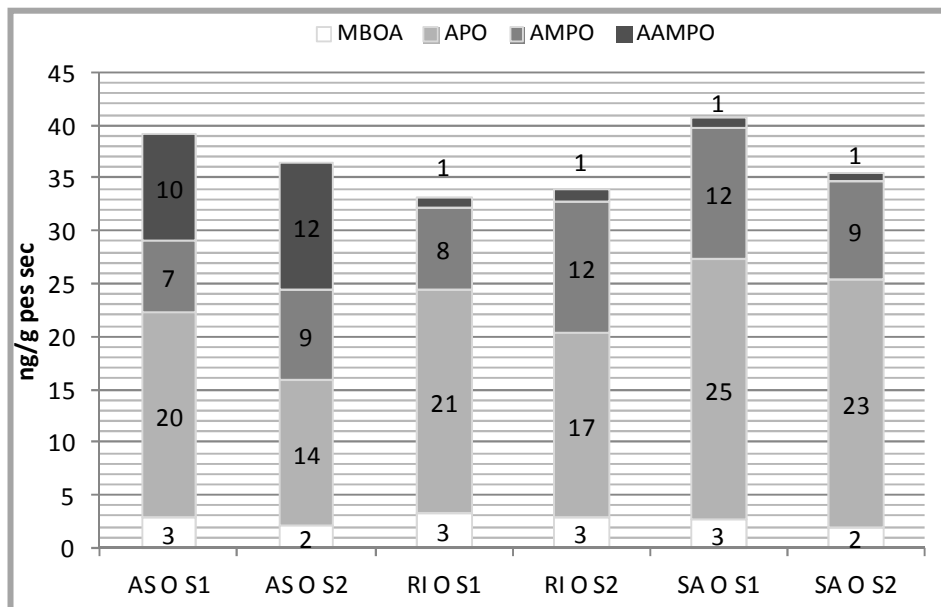


Figura IV.11.- Nivells de compostos al·lelopàtics en el primer i segon estadi del cultiu orgànic

Els compostos MBOA, APO i AMPO varen ser determinats en totes les mostres analitzades. No obstant, l'AAMPO també va ser determinat en les mostres del cultiu orgànic. En el cultiu convencional, el nivell total de compostos varia entre les diferents varietats de blat, mentre que aquest comportament no és tant pronunciat en el cultiu orgànic, on els nivells totals d'al·leloquímics són més similars entre les diferents varietats. De la mateixa manera com s'observava en les mostres de la planta, els nivells d'al·leloquímics trobats en els sòls del cultiu convencional (de 90 a 290 ng/g pes sec) són superiors als del orgànic (de 33 a 41 ng/g pes sec). És interessant destacar que els nivells trobats en les plantes són de l'ordre de mg/kg mentre que els nivells dels compostos de degradació en el sòl agrícola són del rang de ng/g el que ens confirma la necessitat d'haver desenvolupat una metodologia més sensible basada en la LC-MS/MS per a l'anàlisi d'aquests compostos.

En quant als compostos que han estat determinats en les diferents mostres de sòl, els resultats obtinguts són consistents amb els obtinguts en els diferents estudis duts a terme a nivell de laboratori enfocats a la determinació de les rutes de degradació de les benzoxazinones.

#### IV.4.4. Estimació del contingut de compostos de degradació en el sòl

A partir dels treballs de Macías et al. [1, 34], que van dur a terme estudis de la degradació de les benzoxazinones a nivell de laboratori en dos sòls agrícoles on havien estat cultivades les varietats AS i RI, analitzades en aquesta tesi, s'ha fet una estimació del contingut teòric dels compostos de degradació APO i AMPO. L'estimació d'aquests compostos en el sòl del segon estadi de creixement s'ha plantejat a partir del contingut total de compostos al·lelopàtics trobats en la planta en el primer estadi de creixement, considerant que:

- El contingut total de compostos presents en la planta en el primer estadi de creixement és alliberat totalment al sòl on tindrà lloc la degradació.
- La biomassa és de 820 g de planta/m<sup>2</sup> de sòl i en un cm<sup>2</sup> hi ha dos grams de sòl [35].

#### Estimació d'APO.

Per a l'estimació de l'APO, addicionalment a les consideracions esmentades anteriorment, s'han tingut en compte els resultats obtinguts del treball sobre l'estudi de degradació del Dibo-a-Glc [1] en el que:

- El Dibo-a-Glc un cop alliberat al sòl es transforma a DIBOA amb un temps de vida mitja de 23h per la varietat AS i de 25h per la RI.
- El DIBOA es transforma a BOA pràcticament al complet als 10 dies.
- Posteriorment el BOA és transformat ràpidament a APO en ambdós sòls agrícoles amb una conversió del 77 al 99% en 10 dies.
- L'APO no experimenta més degradació passats més de 90 dies des de l'inici de l'experiment.

Donada la ràpida conversió dels compostos Dibo-a-glc a DIBOA i del DIBOA a BOA es considera que si aquests compostos són alliberats al sòl seran ràpidament transformats a la benzoxazolinona BOA que posteriorment serà transformada a APO. A

la taula IV.10 es mostren els nivells totals de compostos no metoxilats (Diboa-glc, DIBOA i BOA) en la planta determinats en el primer estadi creixement, l'estimació del contingut de BOA en el sòl si tots els compostos no metoxilats en la planta són alliberats, l'estimació del contingut d'APO en funció del BOA estimat i el contingut d'APO trobat experimentalment en les mostres del segon estadi de creixement.

Taula IV.10.- Estimació del contingut d'APO en el sòl (\*Diboa-glc+DIBOA+BOA)

| Varietat | $\Sigma$ no metoxilats* | BOA estimat | APO estimat | APO 2n estadi | APO 1er estadi |
|----------|-------------------------|-------------|-------------|---------------|----------------|
|          | ng/planta               | ng/g sòl    | ng/g sòl    | ng/g sòl      | ng/g sòl       |
| AS       | 3,34E+02                | 137         | 103         | 91            | 21             |
| RI       | 2,15E+02                | 88          | 75          | 44            | 27             |

Si comparem les concentracions d'APO determinades experimentalment en el primer estadi de creixement amb les estimades, s'observa que són del mateix ordre, el que ens indicaria que la majoria dels compostos no metoxilats trobats en la planta han estat alliberats al sòl i transformats a APO pels microorganismes existents en el sòl. Tal i com s'ha comentat a l'inici d'aquest apartat, l'estimació d'APO en el sòl del segon estadi de creixement, s'ha fet tenint en compte els nivells dels compostos no metoxilats en la planta del primer estadi. D'aquesta manera i considerant que l'APO no es degrada passats 90 dies, segons l'experiment realitzat per Macías et al. [1], és important tenir en compte que en el sòl del segon estadi de creixement romandrà l'APO present en el primer, que és 21 i 27 ng/g per a les varietats AS i RI, respectivament. Això ens porta a considerar que el més correcte és fer la comparació dels valors d'APO estimats amb els valors experimentals d'APO del segon estadi de creixement menys els del primer estadi, essent doncs els nivells d'APO experimentals, corresponents a la degradació dels compostos presents en la planta del primer estadi de creixement, 70 ng/g per a la varietat AS i 17 ng/g per la varietat RI. Aquests nous valors s'allunyen més dels valors d'APO estimats, sobretot en el cas de la varietat RI, el que ens podria indicar que la quantitat de compostos alliberats al sòl és menor de l'esperada i varia en funció de la varietat estudiada, en aquest cas la varietat AS hauria alliberat al sòl el 68% dels compostos presents en el primer estadi de la planta de blat cap al sòl, mentre que la varietat RI ho hauria fet en un 23%.

## Estimació d'AMPO

De la mateixa manera que per a l'APO a part de les consideracions esmentades a l'inici del present apartat, per a l'estimació de l'AMPO s'han tingut en compte els resultats obtinguts de l'estudi de degradació del DIMBOA [34] en el que:

- El DIMBOA alliberat en el sòl es degrada en un 80% a MBOA en 50 min.
- El MBOA en el sòl es degrada a AMPO d'un 10% a un 40% depenent de la concentració (major degradació a menor concentració).
- L'AMPO tampoc presenta degradació passats 90 dies des de l'inici de l'experiment.

Segons els resultats de l'estudi de degradació del DIMBOA [34], en aquest cas cal considerar que l'AMPO en el sòl hauria de resultar de la degradació del MBOA. El MBOA present en el sòl pot provenir de la transformació del DIMBOA alliberat per la planta en el sòl, així com del propi MBOA present en la planta. Els resultats obtinguts en l'estimació de la concentració d'AMPO que caldria esperar en el sòl del segon estadi de creixement tenint en compte totes les consideracions establertes, es mostra a la Taula IV.11. Segons els nivells de DIMBOA i MBOA en la planta i suposant que tot s'allibera al sòl, caldria esperar una concentració de MBOA alliberat en el sòl de 3442 n/g per a la varietat AS i de 2273 n/g per a la RI. Aquest MBOA seria transformat a AMPO donant lloc a una concentració de 343 ng/g i 227 ng/g en el sòl per a les varietats AS i RI, respectivament. Si comparem el valor d'AMPO estimat amb el valor d'AMPO experimental s'observa que en aquest cas l'AMPO determinat a nivell experimental és molt inferior a l'estimat. A més a més, de la mateixa manera que el seu homòleg APO, l'AMPO no es degrada en el sòl passats 90 dies [34], de manera que s'ha de tenir en compte que els nivells d'AMPO en el sòl del primer estadi de creixement (36 ng/g per AS i 46 ng/g per RI) també es troben presents en el sòl del segon estadi de creixement, incrementant així la diferència entre el valor experimental i estimat. En aquest cas la planta hauria alliberat al sòl un 34% dels compostos presents en la planta, mentre que la varietat RI ho hauria fet un 15%.

Taula IV.11.- Estimació del contingut d'AMPO en el sòl

| Astron (AS) | DIMBOA    | MBOA estimat | AMPO estimat | AMPO estimat | AMPO 2n estadi | AMPO 1er estadi |
|-------------|-----------|--------------|--------------|--------------|----------------|-----------------|
|             | ng/planta | ng/g sòl     | ng/g sòl     | ng/g sòl     | ng/g sòl       | ng/g sòl        |
|             | 4.76E+03  | 1563         | 156          |              |                |                 |
| Ritmo (RI)  | MBOA      | MBOA estimat | AMPO estimat | 343          | 148            | 32              |
|             | ng/planta | ng/g sòl     | ng/g sòl     |              |                |                 |
|             | 4.58E+03  | 1879         | 187          |              |                |                 |
| Astron (AS) | DIMBOA    | MBOA estimat | AMPO estimat | AMPO estimat | AMPO 2n estadi | AMPO 1er estadi |
|             | ng/planta | ng/g sòl     | ng/g sòl     | ng/g sòl     | ng/g sòl       | ng/g sòl        |
|             | 3.78E+03  | 1240         | 124          |              |                |                 |
| Ritmo (RI)  | MBOA      | MBOA estimat | AMPO estimat | 227          | 81             | 46              |
|             | ng/planta | ng/g sòl     | ng/g sòl     |              |                |                 |
|             | 2.52E+03  | 1033         | 103          |              |                |                 |

Cal tenir en compte que tots aquests resultats són estimatius i han estat calculats tenint en compte diferents consideracions i a partir dels estudis duts a terme per Macias et al. [1, 34]. Aquests estudis s'han dut a terme a nivell de laboratori, on s'han addicionat diferents concentracions dels compostos a degradar i que aquestes concentracions en el sòl són majors que les trobades experimentalment en aquesta tesi. Dels estudis existents sobre la transformació de les benzoxazolinones en el sòl agrícola, s'ha vist que aquesta és dependent dels nivells de concentració en la que es troben el compostos en el sòl agrícola, del tipus de sòl i dels microorganismes associats. Les diferències en la transformació d'aquests compostos pot ser deguda a la densitat i diversitat de microorganismes associats a la planta i el sòl [36, 37]. De la mateixa manera, els compostos alliberats des de la planta pot variar en funció de la varietat o espècie i a la vegada pot influenciar en la microflora associada a les arrels. Els microorganismes a la vegada poden tenir diferents capacitats per metabolitzar aquests compostos.

No obstant, segons els resultats obtinguts de l'estimació sembla a ser que els compostos precursors de l'APO són alliberats de la planta en majors quantitats que els seus homòlegs metoxilats per donar lloc a l'AMPO. Una possible explicació a aquest fet podria venir donada pel fet que l'APO és el compost que mostra una major fitotoxicitat envers la resta de compostos, el qual té un rol important en el sistema de defensa de la planta. Segons els resultats obtinguts, la varietat AS seria la que mostraria una major fitotoxicitat envers la varietat RI tot i tenir quantitats de compostos precursors en la planta.



**IV.5. REFERÈNCIES**

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