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## Nutritional and Proteomic Profiles in Developing Olive Inflorescence

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### 1. Introduction

Flowering is a crucial phase of the plant life cycle hardly programmed through floral time genes, cadastral genes and floral organ identity genes (Jack, 2004). Four pathways control flowering time, floral meristem identity genes and floral organs genes: light and photoperiod, temperature, energy supplies (mainly through carbohydrates) and hormones (Taiz and Zeiger, 2010).

Plant nutrient status and especially nitrogen is determinant for flowering and completion of the plant life cycle. Tree nutrient status, as well as its seasonal nutrient fluctuations have been based almost exclusively on leaf analysis. Flower analysis has been used for the study of the distribution of different nutrients through the various parts of the fruit tree (Drossopoulos et al., 1996, Sanchez et al., 1992), as well as for the detection and handling of deficiencies during this early stage of growth (Sanz and Montanes, 1995).

Olive tree (*Olea europaea* L.) is a very important indeterminate long lived, evergreen fruit tree, cultivated mainly in the Mediterranean basin, since experimental evidences have demonstrated both fruits and leaves to be important organs for synthesis of several interesting biological compounds. The tree has been cultivated for approximately 6000 years. Some specimens have been reported to live for thousand, and it may be in the spirit of peace that olive branches appear on the flag of the United Nation Organization (Rugini and Fedeli, 1990; Martin, 2009).

Olive tree has a plentiful bloom but a low percentage of normal fruit set. (Reale et al., 2006). In plants nutrient status affects flowering, and play a crucial role for the completion of the life cycle. Except from nutrients, proteins play a special role in inflorescence development, as they play crucial role in flower organ development (Taiz and Zeiger, 2010). In olive tree, nitrogen status affects flower quality and ovule longevity (Fernandez-Escobar et al., 2008).

Proteomic analyses in trees have also gained little interest, as they are difficult as plant materials. Especialy, olive tree has major difficulty, because of the presence of many phenolic compounds. In spite of the importance of olive tree for human diet, no interest has been focused on olive inflorescence development. In this work we studied the profile of nutrients, as well as the proteomic profile in olive inflorescence cv "Konservolia" in order to add data on this interest subject.

## 2. Materials and methods

### 2.1 Plant material

Three about 30-year-old fruit bearing olive trees (*Olea europaea* L. cv "Konservolia"), were selected. The experiment started about one week after floral bud burst, nine weeks before full bloom, in early March, and lasted up to one week after full bloom, in late May. Hanging or horizontal reproductive shoots 15-25 cm long with ten or more inflorescence bearing nodes were cut from positions symmetrically distributed around the crown of each tree at weekly intervals. The inflorescences from the central region of each reproductive shoot (3<sup>rd</sup> to 6<sup>th</sup> node measuring from the top of the shoot) were used for further analysis. Three groups of samples were collected each sampling date at 09:00 h i.e. (i) for measurements of developmental parameters of inflorescences (length, FW, DW, and morphological observations), (ii) for nutritional profile analyses and (iii) for proteomic analyses.

### 2.2 Developmental parameters of inflorescence development

Twenty inflorescences per tree were used for measurements of the inflorescence length and fresh and dry weight of the organ. For detailed morphological and anatomical observations two representative inflorescences with four lateral branches were selected from four reproductive shoots of each of the three trees and fixed in FAA.

Anatomy (median longitudinal section of perfect flowers) were observed on flowers from the first branch (counting from the apex of the inflorescence). Photographs were taken with an Olympus SZX12 stereoscope, equipped with an Olympus DP71 P-30N camera.

### 2.3 Nutritional analyses

Three samples per tree were separately analysed. Fresh weight per sample was recorded, samples were oven-dried at 80 °C, dry weight was recorded and the samples were ground to pass a 40-mesh screen using an analytical mill (IKA, model A10) prior to chemical analysis (Mills and Jones 1996). N was analyzed by micro-Kjeldahl digestion followed by distillation (Jones, 1991). P, K, Mg, Ca, Cu, Fe, Zn and Mn were determined following a wet acid digestion procedure based on the combination of HNO<sub>3</sub> and 30% H<sub>2</sub>O<sub>2</sub> (Mills and Jones 1996). Phosphorus quantitative analysis in the diluted digests was carried out colorimetrically by determining the absorption of the blue phosphomolybdate complex at 660 nm, using the ammonium molybdate and stannous chloride procedure (Peach and Tracey 1956). The concentrations of all other nutrients were determined in the diluted digests by atomic absorption spectrophotometry using a GBC Avanta spectrophotometer. For the determination of Ca and Mg, 1% (w/v) lanthanum was added in the digests.

### 2.4 Proteomic analyses

Three separate replicates were used for each of the proteomic analyses we followed. A modified protocol based on those suggested by Garcia et al. (2000) Süle et al. (2004), and Wang et al. (2003, 2004) for olive was followed for protein extraction and purification. Proteins were extracted using a 10fold volume/weight buffer (50 mM sodium borate, 50 mM ascorbic acid pH 9.0, 1% β-mercaptoethanol, 1% soluble PVP, 1% insoluble PVP, 10 mM PMSF) under continuous vortexing for 60 min at 4 °C. The homogenate was centrifuged at 4 °C for 30 min at 35,000 g and the pellet was discarded. Proteins in the supernatant were precipitated by adding equal volume of cold 10% TCA, 0.07% β-mercaptoethanol in acetone. The precipitation was

carried out overnight at -20 °C. A 10 min centrifugation in cold at 10,000 g resulted in a three phase system, an upper aqueous, an intermediate protein phase, and a lower organic phase. The upper aqueous phase was discarded and the remained proteins were homogenized with the lower organic phase by vortexing. Proteins were then rinsed three times by adding a twofold volume of 10% aqueous TCA, 0.07%  $\beta$ -mercaptoethanol, vortexing and centrifugation for 5 min in cold at 10,000 g. Purification of proteins completed by washing once with 5fold volume ddH<sub>2</sub>O. Finally, the protein pellet was washed twice with ice cold acetone, 0.07%  $\beta$ -mercaptoethanol and centrifuged for 5 min in cold at 10,000 g. The pellet was dried under a gentle stream of air. Total water extractable proteins (WSP) were quantified using bovine serum albumin as standard (Bradford, 1976; Bearden, 1978).

The pellets for SDS-PAGE analysis (about 50  $\mu$ g) were solubilised in sample buffer 62,5 mM Tris-HCl, pH 6.8, 2% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol and traces of bromophenol blue (Garcia et al. 2000) enhanced with 8.0 M urea, and denatured by heating in boiling water for 5 min. Then cooled to room temperature and centrifuged for 5 min at 14,000g. Electrophoresis was carried out on a Perfect Blue M 14x16 cm (Peqlab Biotechnologie GmbH) vertical electrophoresis system using 12,5% continuous pH 8,3 SDS running gel (1,5 mm thick), with a 4% stacking gel parallel with Sigma markers (14 to 205 kDa), according to Hofer (1994) at 30 mA for about 1.5 hours. The gels were stained with Coomassie Brilliant Blue R-250, according to Sigma (1994) protocol.

For 2-DE about 300  $\mu$ g of purified proteins were diluted with rehydration buffer (9.5 M urea, 2% CHAPS, 2% IPG buffer pH 4-7 and 20 mM DTT (Amersham Biosciences). The samples were then put on Immobiline Drystrip 13 cm with a linear pH range 4.0-7.0, the strips were covered with cover fluid and left to rehydrate for at least 10 h. Isoelectric focusing was carried out on a horizontal Multiphor II Amersham Biosciences system under a four phases program (300 V/1 Vh, 300 V/1800 Vh, 3500 V/9500 Vh and 3500 V/19250 Vh). Prior to running the second dimension the proteins were reduced with 65 mM DTT followed by alkylation with 135 mM iodoacetamide and traces of bromophenol blue, both diluted in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol and 2% SDS). The second dimension was carried out as in SDS-PAGE analysis but with 0.8 mm thick gels, without stacking gel. Sigma markers (14-205 KDa) were run parallel to samples. Proteins were stained with silver nitrate and data were assessed by mean of Photoshop CS3 program.

### 3. Results and discussion

Flower and inflorescence development is one of the most complicated and well regulated plant process (Rolland-Lagan et al., 2003). Flowering time genes, floral meristem identity genes, and floral organ identity genes have well studied in *Arabidopsis*, *Pisum* and *Antirrhinum*, as well as in other annual plants. Tetramers of MADS proteins are thought to determine floral organ identity (Jack, 2004; Taiz and Zeiger, 2010). The size and the shape of petals as well as of the rest whorls is genetically determined (Laitinen et al., 2007). However little data are available until now concerning flower proteomic (Dafny-Yelin et al., 2005; Moccia et al., 2009; Sun et al., 2009; Logacheva et al. 2011; Zhu et al., 2011).

#### 3.1 Olive inflorescence development

Olive inflorescence is a panicle which arises with a central axis terminated by a flower, with lateral axes branching from the peduncle. The lateral axes may in turn branch, resulting in a tertiary branched panicle (Weis et al., 1988; Seifi et al., 2008; Ganino et al., 2011).

Under normal conditions, olive inflorescence development last a prolonged period starting in March and full bloom occurs in May or June (Cirik, 1989; Weis et al., 1991).

The inflorescence development in “Konservolia” (Fig. 1, photos above plate a) seemed to constitute of three stages, as was also reported for “Kalamon” inflorescence (Bouranis et al., 2010) and lasted about 10 weeks until full bloom. Nine to seven weeks before full bloom inflorescence looked as a small berry covered with bracts and the elongation of the main axis prevailed, obviously due to the elongation of its cells. Six to four weeks before full bloom lateral axes having compact floral meristems on them were developed on the inflorescence, which suggest mitotic activity, as well as cell enlargement in the organs. At this stage the development of floral meristems and their whorls on the lateral axes indicates the implication of proteins derived from floral organ identity genes transcription. The next three weeks up to full bloom the development of flower whorls tended to complete. The week after full bloom only fertilized ovaries were remained on inflorescence, while lateral axes, unfertilized flowers and bracts were massively abscised during this week.

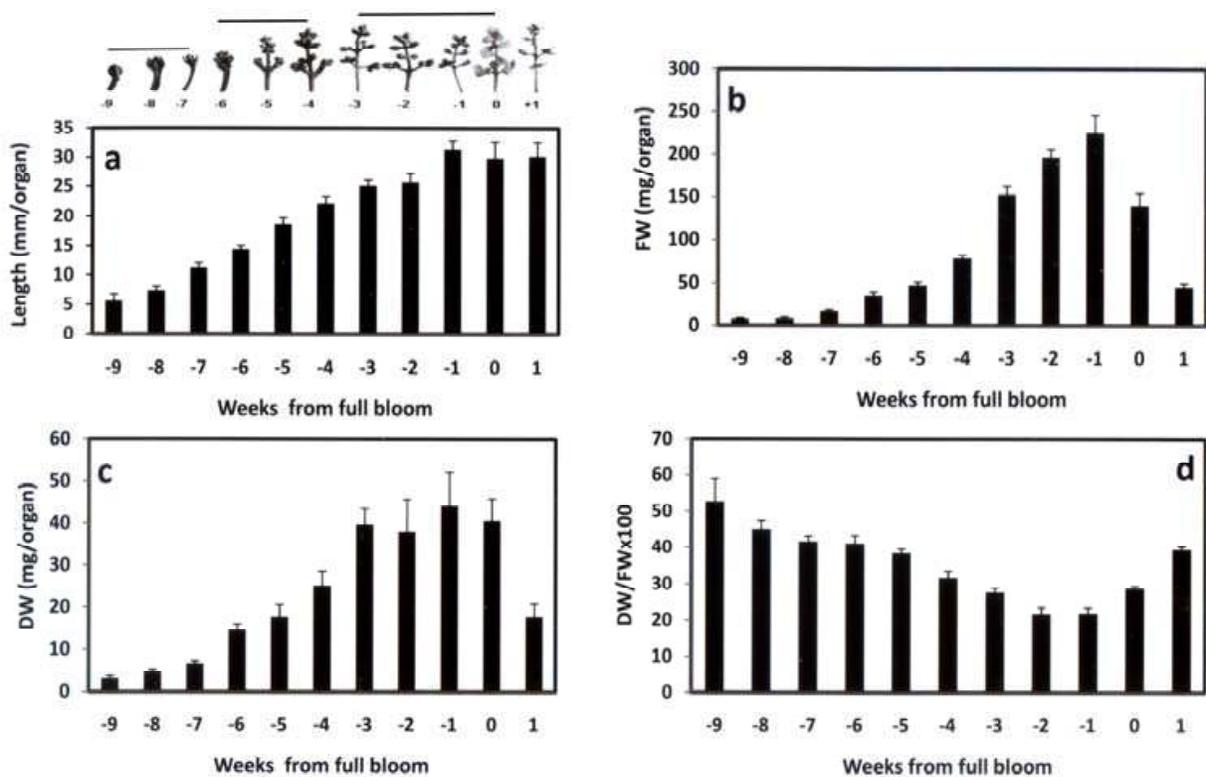


Fig. 1. The developmental stages of olive cv “Konservolia” inflorescence from floral bud burst 9 week before full bloom (plate a) up to one week after full bloom as well as the profiles of the changes of the length (plate b), of the fresh weights (plate c), dry weights (plate d) and the DW/FW ratio (plate d) (full bloom week 0). Vertical bars represent SE.

The increase of inflorescence length (Fig. 1, plate a) followed a sigmoid type of development and reached a maximum one week before full bloom. The fresh weight of inflorescence (Fig. 1, plate b) was progressively increased for a period of five weeks, with an intensive increase up to one week before full bloom, decreasing rapidly afterwards. Dry weight presented a similar but milder progress in comparison to that of FW during the same period (Fig. 1,

plate c). The DW/FW ratio (plate d) showed a decreasing trend for a period of seven weeks before full bloom, stabilized on the lower level the two weeks before full bloom, increased at full bloom when floral whorls were separated by each other, and increased sharply the week after full bloom. This decreasing trend is obviously due to the enlargement of the floral organ cells and the contemporary hydration of them. The opening of petals and thereafter the higher transpiration rate, as well as the abscission of floral organs even before full bloom (Weis et al., 1988) and mainly after full bloom leads to a recovery of the DW/FW ratio at full bloom and the next week.

In Fig.2 are shown representative developmental stages of perfect flowers of the first lateral branch of four-branching inflorescence on median longitudinal sections five weeks before full bloom up to full bloom. Bracts which covered individual flowers were already shed five weeks before full bloom (plate -5). The stamen sac development found to be completed about two weeks before full bloom (plate -2). Pistil development followed that of the stamen and continued until full bloom in perfect flowers. Nucellus was already visible in the ovary five weeks before full bloom, while two well developed embryosacs were found on sections two and one week before full bloom (plates -2, -1). At full bloom, the opened petals revealed the flattened anthers and a global-like ovary (plate 0).

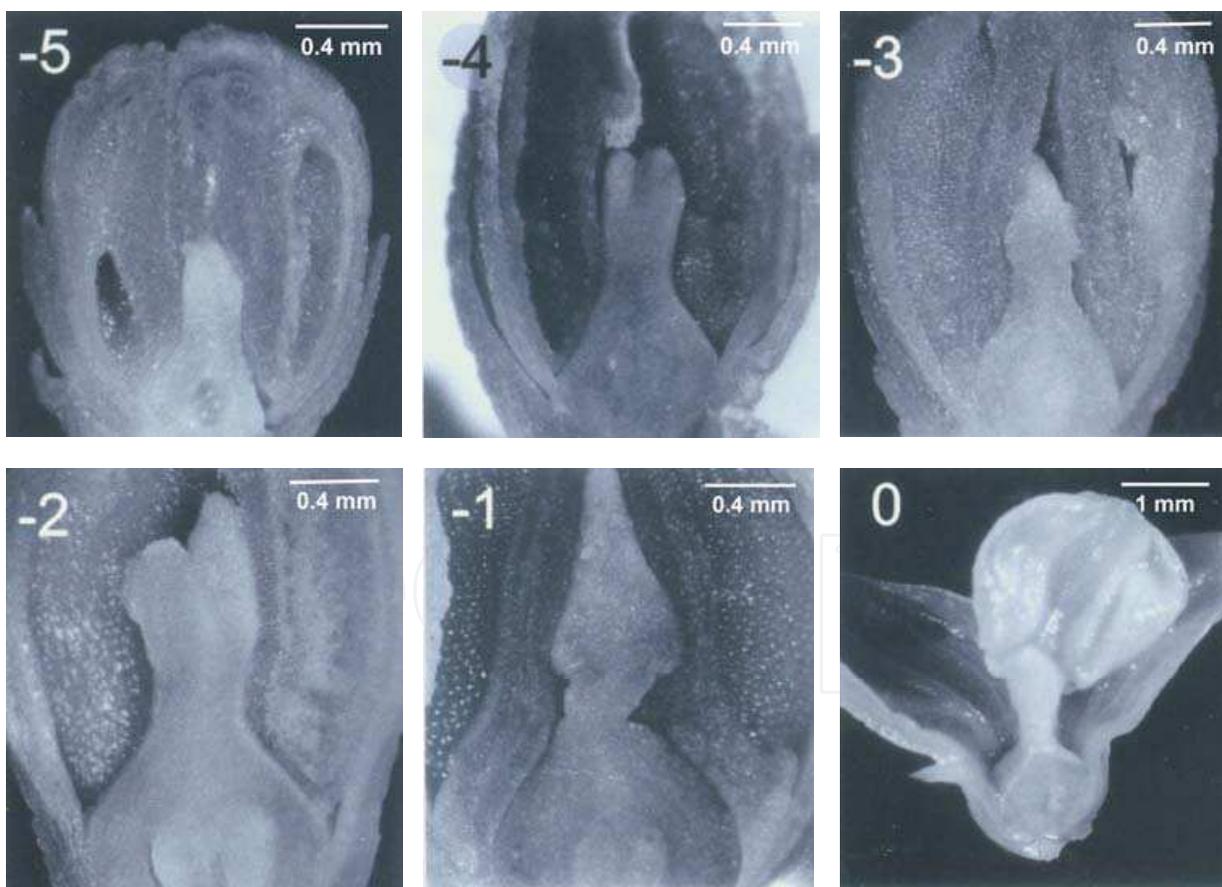


Fig. 2. Median longitudinal sections of olive perfect flowers (*Olea europaea* L. cv "Konservolia") showing the developmental stages of the organ at weekly intervals from 5 weeks before full bloom (plate -5) to full bloom (plate 0).

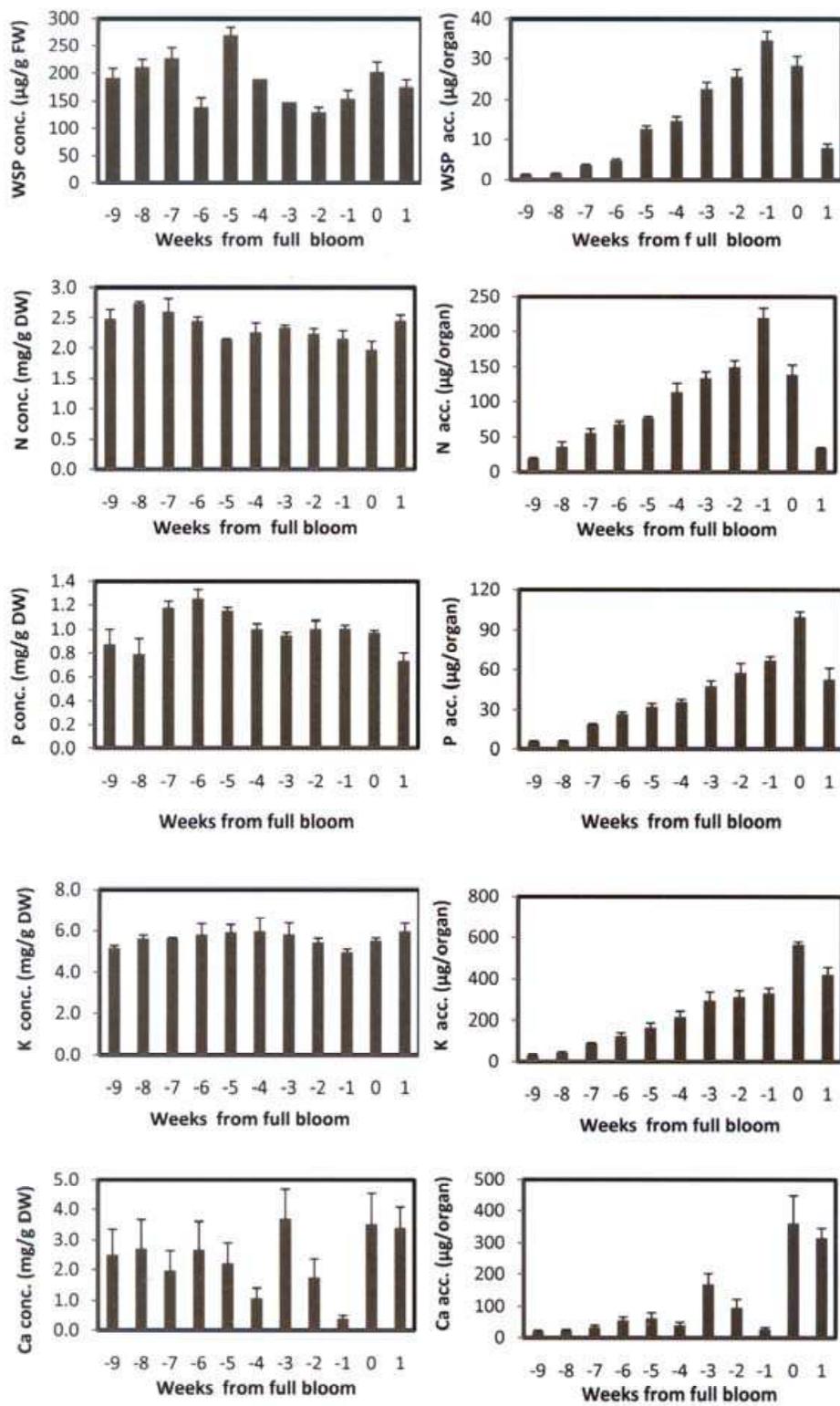


Fig. 3a. The profiles of concentration and accumulation of water soluble proteins, total nitrogen, phosphorus, potassium and calcium in olive inflorescence (*Olea europaea* L. cv "Konservolia") from floral bud burst (nine weeks before full bloom) to one week after full bloom.

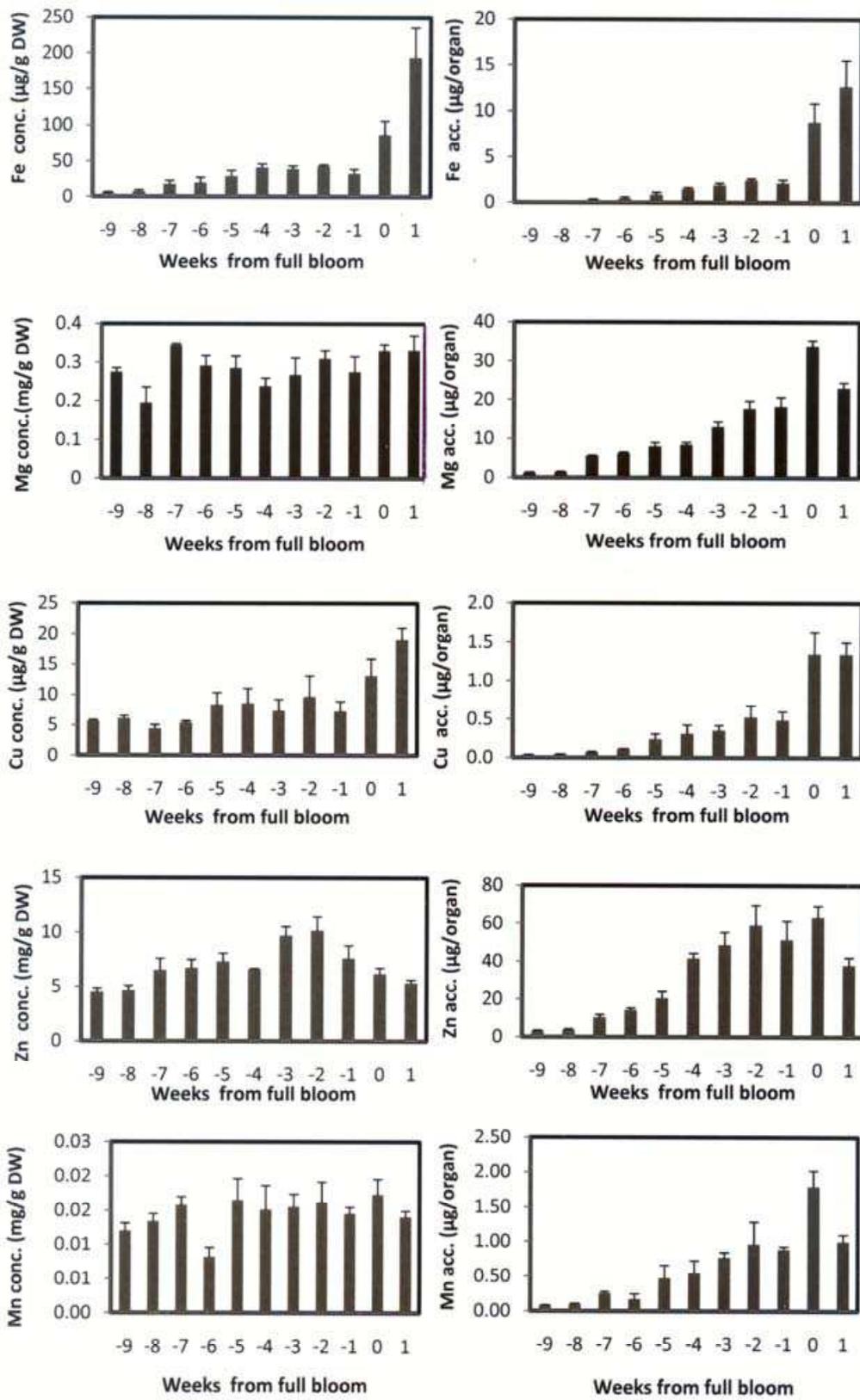


Fig. 3b. The profiles of concentration and accumulation of iron, manganese, copper, zink, and manganese in olive inflorescence (*Olea europaea* L. cv “Konservolia”) from floral bud burst (nine weeks before full bloom) to one week after full bloom.

### 3.2 Profiles in total water extractable proteins and macro- and micro-nutrients

A pulse like profile found in total water extractable proteins in olive inflorescence which seemed to be consisted of three phases. The two lower levels at water soluble proteins found six and 2 weeks before full bloom. This profile showed to be similar to that of the progress of inflorescence development as discussed above. Water soluble protein accumulation showed a sigmoid like profile in which the phase of the higher rate of accumulation localized in the period of five to one week before full bloom, which corresponds to the period of flower and floral whorls development on inflorescence. On the other hand, the profile of nitrogen concentration showed to be more uniform, having a decreasing trend. Nitrogen accumulation showed an increasing trend up to one week before full bloom. In spite the similar trend, the rate of increase of nitrogen accumulation was lower to that of DW accumulation, resulting in a progressive dilution of nitrogen level in the developing inflorescence. A pulse like profile on water soluble proteins was found in differentiating olive floral buds during late winter and thought to be due to a need of a relaxing phase of the dividing cells (Kitsaki et al., 2010). Proteins may be enzymes for the different metabolic processes among which mitosis, structural proteins, membrane proteins, or storage proteins. In flowers tetramers of proteins which determine floral organ identity and development, as well as those implicated to meiosis have a special role (Jack, 2004; Luu and Maurel 2005; Ahsan and Komatsu, 2009; Malumbres et al., 2009; Echalié et al., 2010; Taiz and Zeiger, 2010). As different physiological processes take place during floral organ development, among them meiosis, a fluctuated profile of water soluble proteins during this period could be expected. The observed fluctuations in the water soluble proteins could also be attributed to interchanges between soluble and insoluble protein fractions in the organ.

In plant cells except from proteins nitrogen is a constituent of free amino acids, amides, nucleic acids nucleotides, coenzymes, amines and others substances Therefore, nitrogen deficiency rapidly inhibits plant growth and if persists transition of plant from vegetative to reproductive phase may not be completed (Marschner, 1995; Taiz and Zeiger, 2010). Differences in the level of nitrogenous compounds between vegetative and reproductive shoots on the same plant have been reported for the majority of plant, among them for olive (Bouranis et al., 2004). In olive tree, nitrogen status affects flower quality and ovule longevity (Fernandez-Escobar et al., 2008).

The higher levels of phosphorus concentration observed during the early stages of flower development (-7 to -5 weeks), while the nutrient was accumulated progressively up to full bloom in the inflorescence. Potassium also accumulated in the inflorescence up to full bloom but changes in the concentration showed a rather mild profile. The accumulation of calcium remained at low levels up to full bloom when a huge increase was observed. On the other hand the fluctuations in calcium concentration were the most dramatic (Fig. 3a). Except from the special role of P in energy storage, of K in osmoregulation and of Ca in the structure of cell wall and as a second messenger, as well as their participation in many enzymes and coenzymes, they occupy a special role in the pollen grain germination as components of phytate salts (Fu et al., 2008; Taiz and Zeiger, 2010). The decreasing trend in Ca up to full bloom is possibly the result of the immobile character of this nutrient (Marschner, 1995), while the rapid increase in its accumulation after full bloom may be due to the dehydration of inflorescence at this stage and the consequent increase of percentage of cell walls in the organ.

Profiles of iron and copper concentration showed an increasing trend during inflorescence development, zink concentration also tended to increase up to two weeks before full bloom,

while magnesium and manganese showed not considerable fluctuations during inflorescence development. On the other hand accumulation of the above nutrients showed a slow or medium increasing rate up to full bloom, when a rapid increase was observed in the accumulations (except from that of Cu). All the above nutrients are constituents of enzymes which implicate in the basic metabolic activity and especially Mg and Fe. Iron and copper seemed to have special interest during the massive flower organ abscission one week after full bloom, as their concentrations was increased considerably. On the other hand three to two weeks before full bloom (late stages of ovary and stamens development) zink concentration showed to be of increased interest. The strong positive linear regression among DW and water soluble proteins, total N, as well as macro- and micro-nutrients accumulation during inflorescence development (Table 1) reinforces the concept that olive inflorescence is a strong sink organ.

	WSP	N	P	K	Mg	Ca	Cu	Fe	Zn	Mn
<b>R<sup>2</sup></b>	0.78**	0.98**	0.97**	0.98**	0.97**	0.64**	0.81**	0.67**	0.76**	0.90**
<b>SE</b>	14.2	7.0	6.3	18.6	1.8	70.6	0.25	3.1	0.5	0.4
<b>n</b>	11	11	11	11	11	11	11	11	11	11

Table 1. The R<sup>2</sup> values among dry weight accumulation and of total water soluble proteins (WSP), as well as mineral nutrients (N, P, K, Mg, Ca, Cu, Fe, Zn and Mn) in developing olive inflorescence (*Olea europaea* L. cv "Konservolia").

### 3.3 Proteomic changes in developing olive inflorescence

In Fig. 4 is shown the profile of proteins revealed by SDS-PAGE analysis in developing olive inflorescence nine weeks before full bloom up to one week after full bloom. In table 2 are summarized the number and the kDa of the detected protein bands as well as the most abundant and the common protein bands in developing olive inflorescence by SDS-PAGE analysis.

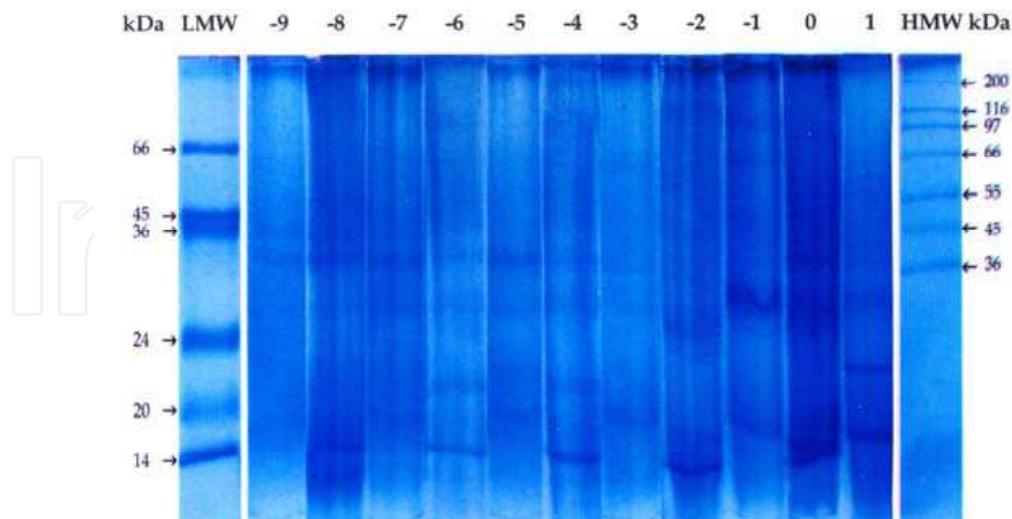


Fig. 4. The protein profile in developing olive inflorescence (*Olea europaea* L. cv "Konservolia") nine weeks (-9) before full bloom up to one week (1) after full bloom. Proteins (50 µg) were resolved in 12,5% SDS/acrylamide gels (1,5 mm thick) parallel with Sigma markers (14 to 66 and 36 to 205 kDa) and gels were stained with Coomassie Brilliant Blue R-250, according to Sigma protocol.

W	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1
1								121.5			
2									<b>101.6</b>	101.1	
3							80.26		<b>79.74</b>		
4								<b>77.36</b>		<b>77.36</b>	
5		65.56								<b>67.25</b>	
5					53.81			54.85		<b>54.51</b>	
6	51.06	<b>52.80</b>	50.73								
7									49.04		
8				45.90							
9	<b>42.78</b>	<b>43.62</b>			<b>43.34</b>	<b>43.89</b>				<b>43.89</b>	<b>44.20</b>
10			<b>41.39</b>		<b>39.02</b>		<b>39.54</b>	<b>40.15</b>	<b>40.59</b>		<b>40.41</b>
11											
12				<b>33.18</b>						<b>33.60</b>	32.27
13		<b>31.93</b>	<b>31.22</b>		<b>31.22</b>	<b>32.33</b>	<b>31.43</b>		<b>31.85</b>		
14								29.58			
15				<b>24.44</b>				<b>24.28</b>			
16					<b>20.13</b>	<b>20.45</b>	<b>20.66</b>				<b>20.78</b>
17	<b>19.48</b>	<b>19.07</b>	<b>19.86</b>	<b>19.43</b>		<b>19.06</b>					
18			<b>15.28</b>		<b>15.90</b>		<b>15.08</b>			<b>15.00</b>	
19	<b>14.31</b>			<b>13.40</b>					<b>13.94</b>	<b>13.87</b>	<b>13.58</b>
20		<b>12.53</b>				<b>12.37</b>				<b>12.45</b>	
21								<b>11.83</b>			
22		<b>10.61</b>									<b>10.52</b>

Table 2. The kDa of the detected protein bands as well as the most abundant (bold numbers) and the common (shadowed cells) protein groups in developing olive inflorescence by SDS PAGE analysis nine weeks before (-9) full bloom to one week after full bloom (1).

Protein bands molecular weights were fluctuated between 10 and about 100 kDa. Protein bands of 44, 40, 32, 19, 15 and 14 kDa were the most frequently appeared, while the groups of 14 and 12 kDa were the most abundant. The number of protein bands was fluctuated between 5 and 9 during the three developmental stages of inflorescence. Most of protein groups were visualized at full bloom (9 groups), at the middle of the phase of inflorescence elongation (-8 week), as well as at the late stage of floral organ development (-2 week), 7 protein groups. As full bloom was coming, new groups of high molecular weights were appeared (3 weeks before full bloom to full bloom). These groups were disappeared the week after full bloom. In this week the range of molecular weights of protein groups was reduced to 10 to 44 kDa (Table 2). The fluctuation of the number of protein bands may be related with the intensity of meristematic activity, as well as cell enlargement processes, which are correlated with enzyme systems relative to mitosis, as cyclin-dependent-kinases, structural proteins for daughter cells and others, as mentioned above (Dembinsky et al., 2007; Malumbres et al., 2009; Echaliier et al., 2010; Nafati et al., 2011). This concept is reinforced by the pulse like data we found in the profile of water extractable proteins. At full bloom new physiological processes take place e.g pollen tube growth, pollination and fertilization which need specific enzymatic activity (Chen et al., 2009). Proteome analysis of soybean

flowers and leaves at various developmental stages revealed organ specific functional differentiation of proteins (Ahsan and Komatsu, 2009). Moreover, Dafny-Yelin et al. (2005) through expression analysis of resolved proteins from rose petals at advanced stage development found about 30% of them to be stage specific. Recently Zhu et al. (2011) identified differentially expressed proteins in mature and germinated maize pollen and found 26 proteins (among about 470) to be changed between the two stages, 13 of them (up-regulated) were mainly involved in tube wall modification, actin cytoskeleton organization and energy metabolism. However, although genetic control of flowering and floral organ development has been extensively studied (Jack, 2004; Leitanen et al., 2007) much remains to be done concerning proteomic approach. Three groups of proteins (about 20 kDa, 31 kDa and 40 kDa) which were present in a wide range of developmental stages of inflorescence may be related to the basic cell metabolic activity or may be storage proteins. Ahsan and Komatsu (2009) referred an abundance of a glycoprotein of 31 kDa in soybean petals, which may be similar to that we also realized. Except from any relation to pollen germination, pollination and fertilization, the presence of the high molecular weight proteins exclusively at the late stage of inflorescence development, may also be related to genes that regulate synthesis of secondary metabolites, as flavonoids, carotenoids and other aromatic substances, which have been reported that appear at the late stage of floral organ development (Ben-Meir et al., 2002).

Further proteome analyses with 2-D electrophoresis carried out in three sampling dates distributed in the three phases of inflorescence development: (a) eight weeks before full bloom (middle of the phase of inflorescence axis elongation), (b) four weeks before full bloom (middle of the phase of floral organ development and (c) at full bloom. In Fig. 5a, 5b, 5c are shown the correspond to the above sampling dates profiles of proteins revealed by 2-DE analysis. In table 3 are summarized the kDa and the pIs of the detected proteins by 2-DE analyses. Proteins were distributed all over the range of 4.0 to 7.0 pH, while molecular weights fluctuated between about 10 to 55 kDa the -8 and the -3 weeks and between 10 to 105 kDa at full bloom (Table 3). Most of the abundant proteins were found at full bloom. Two protein groups (42.5 kDa/4.12-4.60 pIs and 28.2 kDa/4.64-5.83 pIs) were visualized in the three sampling dates. Some of proteins were common in the three stages of development, while other were present in one or two of the three phases. The most abundant protein was that of 44.3 kDa/4.67-5.19 pIs, especially at -8 week and at -4 week. Proteins with high molecular weights (over 60 kDa) were monitored exclusively at full bloom.

Early tree proteome studies were focused on the process of xylogenesis mainly in *Populus* and *Pinus* (Costa et al., 1999; Mijnsbruge et al., 2000), but some fruit trees have recently gained interest for proteome analysis (Cartu et al., 2008; Paiva et al., 2008). However, little effort have been done on olive proteomic, probably because of the difficulty on sample handling, due to the presence of many phenolic compounds (Garcia et al. 2000, Wang et al., 2003, Wang et al., 2004, Wang et al., 2010).

The pIs of most of the proteins we visualized by 2-DE in developing olive inflorescence were localized in the low pH region. Similar data have been reported for olive leaf ( Wang et al., 2003), as well as for other plants (Dafny-Yelin et al., 2005; Ahsan and Komatsu, 2009; Chen et al., 2009). The fluctuation in abundance in the three stages of inflorescence development may be related to developmental stages specific functional proteins (Ahsan

and Komatsu, 2009). The majority of the abundance found at full bloom is in accordance with the recent data of Ahsan and Komatsu (2009) and Zhu et al. (2011), concerning stage specific functional proteins.

<b>-8 week (middle of the phase of inflorescence axis elongation)</b>									
SN	kDa/pI	SN	kDa/pI	SN	kDa/pI	SN	kDa/pI	SN	kDa/pI
1	55.6/4.61	10	25.6/4.20	20	13.6/4.92	25	<b>11.8/6.28</b>	36	10.5/4.80
2	<b>46.8/4.96</b>	11	24.7/6.91	21	13.6/5.20	32	<b>11.5/5.34</b>	38	<b>10.0/4.40</b>
3	<b>44.3/4.95</b>	12	23.8/6.90	22	<b>12.9/6.03</b>	33	11.6/5.49	40	10.0/4.29
4	<b>42.7/4.96</b>	13	22.7/4.54	26	12.9/4.03	16	11.5/5.88	41	10.0/4.88
5	<b>40.9/4.96</b>	15	18.9/4.40	28	12.5/4.41	29	11.5/4.30	43	<b>10.0/5.81</b>
6	34.8/4.21	14	18.5/4.23	27	12.5/4.76	31	10.9/5.20	39	10.0/4.05
7	29.7/4.43	17	16.6/4.35	23	<b>12.3/5.95</b>	37	10.9/4.30	42	<b>10.0/5.29</b>
8	29.4/4.69	18	15.7/4.37	24	<b>12.3/6.27</b>	34	<b>10.5/5.77</b>	44	<b>10.0/6.03</b>
9	28.4/5.11	19	14.8/4.92	30	<b>12.3/4.84</b>	35	<b>10.5/5.93</b>	45	<b>10.0/6.10</b>
<b>-4 week (middle of the phase of floral organ development)</b>									
SN	kDa/pI	SN	kDa/pI	SN	kDa/pI	SN	kDa/pI	SN	kDa/pI
1	<b>52.8/5.2</b>	7	<b>42.3/4.21</b>	16	28.0/4.64	26	17.3/4.27	31	<b>14.4/4.84</b>
2	<b>50.5/5.19</b>	13	<b>35.4/5.51</b>	15	27.2/4.20	25	17.5/4.27	29	14.4/4.47
3	<b>48.7/5.19</b>	10	<b>33.5/4.20</b>	22	23.7/5.46	27	17.5/4.68	28	14.4/4.28
4	<b>47.5/5.19</b>	11	33.5/4.83	21	22.6/5.40	35	15.3/5.33	36	14.0/6.43
5	<b>44.8/5.19</b>	12	33.5/5.16	20	22.6/4.69	34	<b>15.3/5.27</b>	37	14.0/6.48
9	<b>44.7/4.67</b>	14	31.7/6.72	19	21.3/4.28	33	15.3/5.20	38	10.7/4.27
8	<b>43.5/4.38</b>	17	29.4/4.91	23	19.7/4.27	32	15.3/4.95	39	10.0/4.47
6	<b>43.5/5.19</b>	18	29.4/5.15	24	19.7/4.70	30	15.3/4.72	40	10.0/4.48
<b>0 week (full bloom)</b>									
SN	kDa/pI	SN	kDa/pI	SN	kDa/pI	SN	kDa/pI	SN	kDa/pI
8	106.5/6.29	6	93.5/5.34	17	<b>42.9/4.12</b>	25	32.3/4.84	32	16.5/4.39
10	105.2/6.47	4	92.0/5.19	19	<b>42.9/4.47</b>	26	<b>28.4/5.83</b>	34	<b>16.5/5.39</b>
9	104.3/6.45	12	68.7/4.57	20	<b>42.9/4.60</b>	27	<b>28.4/5.19</b>	35	<b>15.2/5.36</b>
2	<b>104.3/4.35</b>	11	67.3/4.38	21	<b>41.3/5.20</b>	28	26.3/4.50	36	<b>14.3/4.57</b>
3	<b>103.2/4.74</b>	13	<b>56.6/5.19</b>	18	<b>41.3/4.31</b>	29	<b>26.3/4.23</b>	37	14.3/4.36
7	103.2/5.86	14	<b>49.7/5.47</b>	22	<b>36.6/5.41</b>	30	20.7/4.70	38	13.2/4.16
1	<b>102.5/4.10</b>	15	<b>48.3/5.27</b>	23	<b>32.3/5.41</b>	31	20.7/4.34	39	<b>10.0/4.45</b>
5	93.5/5.34	16	<b>46.6/5.20</b>	24	<b>32.3/5.20</b>	33	17.2/4.59	40	<b>10.0/4.08</b>

Table 3. The kDa and the pIs of the detected proteins by 2-DE analyses in developing olive inflorescence (*Olea europaea* L. cv "Konservolia") eight weeks before full bloom (-8 week), three weeks before full bloom (-3 week) and at full bloom (0 week). The most abundant proteins are indicated with bold numbers (SN: spot number).

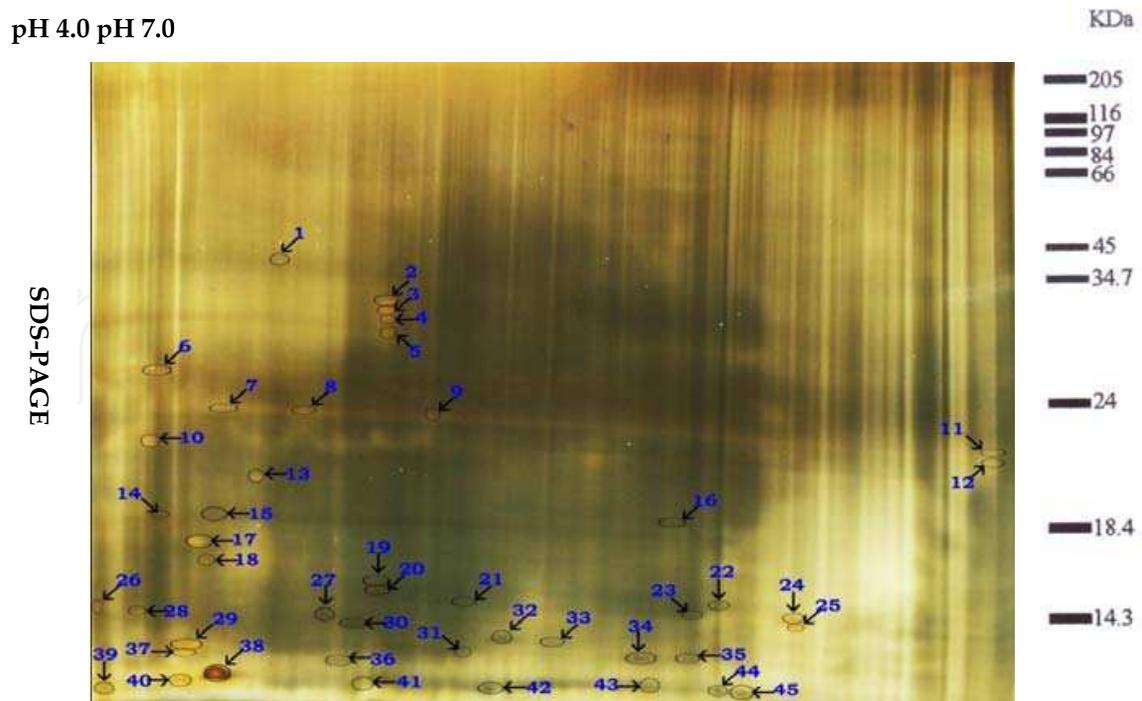


Fig. 5a. Protein profile in developing olive inflorescence eight weeks before full bloom. Proteins (300  $\mu$ g) were resolved in 13 cm linear immobilized dry strips pH 4.0-7.0 and in the second dimension in 12.5 % SDS/acrylamide 0.8 mm thick gels.

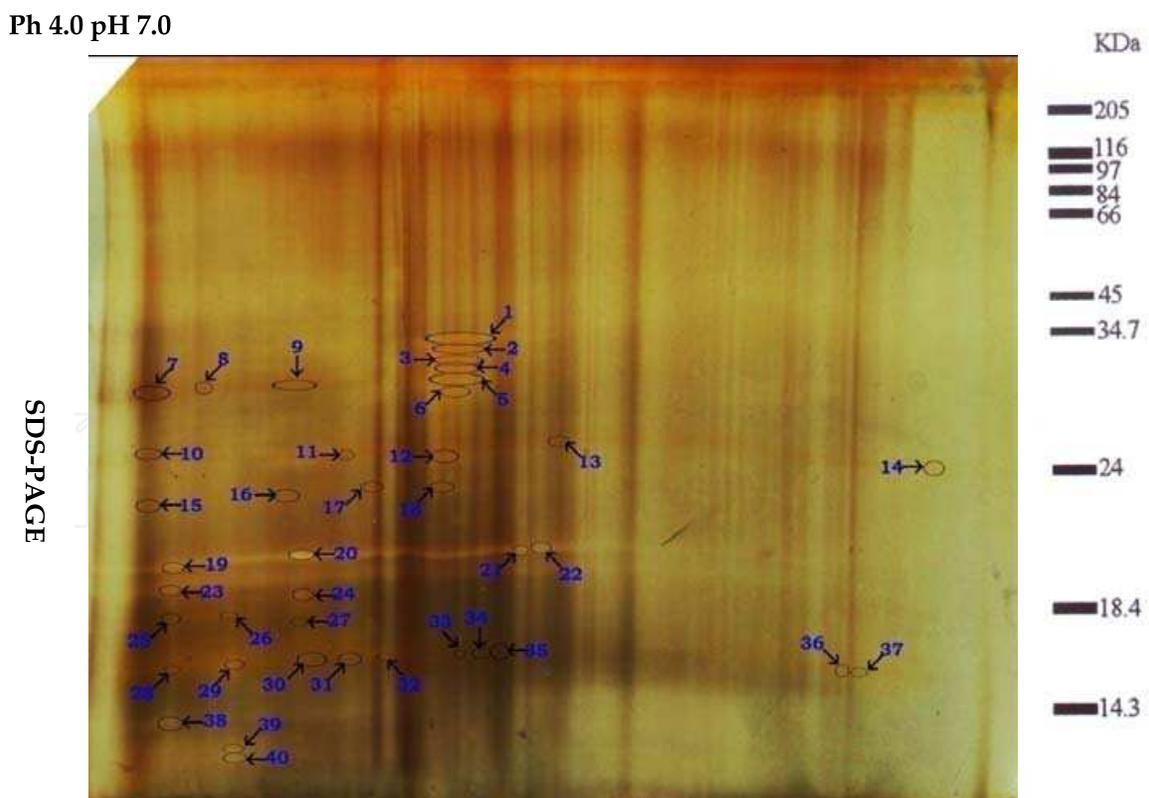


Fig. 5b. Protein profile in developing olive inflorescence four weeks before full bloom. Proteins (300  $\mu$ g) were resolved in 13 cm linear immobilized dry strips pH 4.0-7.0 and in the second dimension in 12.5 % SDS/acrylamide 0.8 mm thick gels.

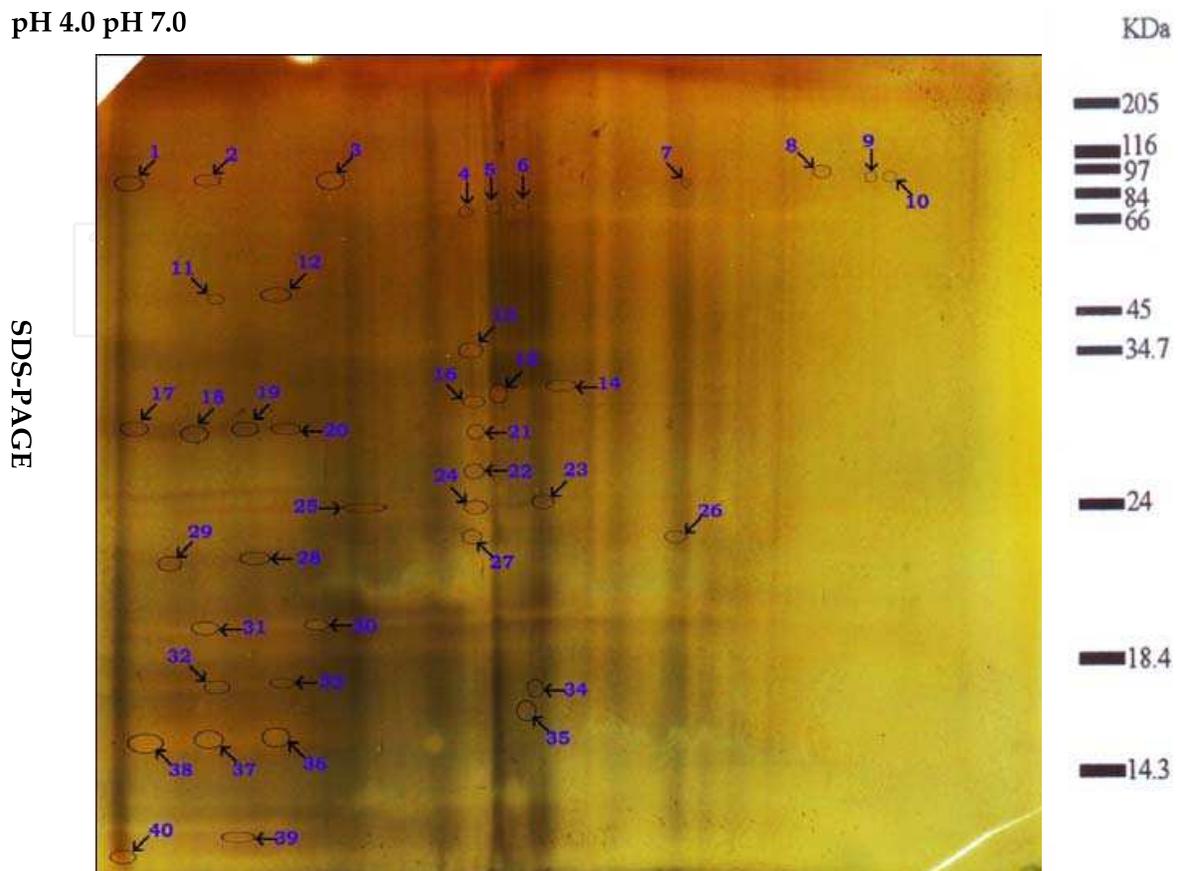


Fig. 5c. Protein profile in developing olive inflorescence at full bloom . Proteins (300  $\mu$ g) were resolved in 13 cm linear immobilized dry strips pH 4.0-7.0 and in the second dimension in 12.5 % SDS/acrylamide 0.8 mm thick gels.

#### 4. Conclusions

Olive inflorescence development seems to be separated in three stages: a first one corresponding to the inflorescence axis elongation, a second corresponding to the development of flowers and floral whorls development and the last one which corresponds to the full bloom and flower fertilization. Changes in DW/FW ratio revealed intense hydration of the organ two weeks before full bloom. Strong positive linear regression found among DW and water soluble proteins, as well as macro- and micronutrients. A pulse like profile found in total water extractable proteins in olive inflorescence which seemed to be consisted of three phases. This profile showed to be similar to that of the progress of inflorescence development. Most of protein groups were visualized at full bloom, at the middle of the phase of inflorescence elongation, as well as at the late stage of floral organ development. As full bloom was coming, new groups of high molecular weights were appeared. Proteins with high molecular weights (over 60 kDa) were monitored exclusively at full bloom. The pIs of most of the proteins we visualized by 2-DE in developing olive inflorescence were localized in the low pH region.

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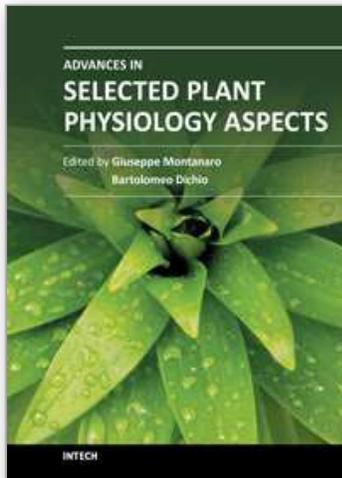
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