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FOOD PRODUCTION FOR SPACE MISSIONS.

SOYBEAN (*GLYCINE MAX* L.) AND SOYMEAL CHARACTERIZATION.

Candidato:

Dott.sa Mariantonella Palermo

Docente tutor:

Ch.mo Prof. Giancarlo Barbieri

Docente co-tutor:

Ch.mo Prof Vincenzo Fogliano

Coordinatore: Ch.mo Prof. Giancarlo Barbieri

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ABSTRACT

In the context of space mission designing, physical and chemical systems which regenerate the resources with production "in situ" of air, food and water are increasing their importance. The MELiSSA (Micro-Ecological Life Support System Alternative) project aims at developing of an artificial ecosystem of microorganisms and higher plants able to support the life of a crew during long-term space missions. Particular attention has been given to the choice of plant species and technologies for their processing. Among several candidate species, potato, soybean and two wheat species were selected.

Soybean (*Glycine max (L.) Merr*.) is an important crop wordwide because of its high nutrient content and because of the main ways it can be processed. Within a closed system for a space mission, yield, energy consumption, sensorial acceptability and numerous other factors must be taken into account. For these reasons, the simultaneous production of soymilk and okara was considered optimal: it allows high nutrient recovery without excessive energy consumption.

In order to identify optimal parameters for soybean processing into milk and okara, water for soaking at room temperature was chosen: it provides long times but low nutrient leaking in the water and no energy expenditure. Optimal processing parameters were seeds milling in water at room temperature and extraction by 30 min boiling; next steps were filtration to separate aqueous extract from the pulp and milk sanification by boiling for15 min.

Regarding sensorial aspects, consumers do not always appreciate soymilk, but it is possible to improve taste by adding sugar and vanilla. A study with non-expert panelists showed that salt and vanilla sugar addition fails to mask the typical bean aroma but it can improve taste up to global levels of pleasantness comparable to market soymilk.

The possibility of soybean production cycle shortening was evaluated: for this purpose effects on seeds and transformed products were studied.

Harvest of immature seeds results great management and economic benefits and, from a nutritional point of view, it is possible to obtained processed products with lower phytic acid content. On the other hand, it is well known that the accumulation of dry matter and nutrients in soybeans seeds increases with advancing of maturation steps. For dry matter, this trend was mitigated with transformation but for fat and protein the highest concentration was observed in soymilk and okara from seeds at complete maturation so, optimal harvest moment within a Life Support System is commercial ripeness.

Based on this preliminary information, nutritional and compositional comparison, among four different cultivars of soybean seeds and their derived products were performed. Influence of hydroponic cultivation in controlled environment (simulation of a Life Support System in the space) was evaluated, too. Hydroponic cultivation in controlled environment is not only a method to produce food in space, but also a system to increase lipid, fiber and mineral accumulation in soy seeds. Hydroponic cultivation in controlled environment determined different performances compared to open field cultivation and, for this reason, selecting optimal cultivar, only data from experimental cultivation must be considered.

In particular during soymilk and okara production, seeds cultivation system did not significantly modify aqueous extract composition but only residue pulp characteristics. The choice of the best cultivar will be contextualized within the diet of the astronauts: good protein and fiber values were observed in transformed products from Atlantic seeds but, if you need a higher fat content, you will choose other cultivars such as Cresir or Regir.

After this stage of the study about food production and preparation on Earth, a second phase with the study of technology in the absence of gravity and the evaluation of space environment effect on biological processes will perform.

ABSTRACT

Nell'ambito degli studi di progettazione delle missione spaziale, sempre più interesse si sta dedicando ai sistemi di rigenerazione fisico-chimica delle risorse con produzione "in situ" di aria, acqua e cibo. In particolare, il progetto MELiSSA (Micro-Ecological Life Support System Alternative) mira allo sviluppo di un ecosistema artificiale di microrganismi e piante superiori in grado di supportare la vita di un equipaggio durante missioni spaziali a lungo termine. Particolare attenzione merita la scelta delle specie vegetali destinate a tale scopo e delle tecnologie per la loro trasformazione in alimenti. Tra numerose specie candidate, la selezione è ricaduta su soia, patata, grano duro e grano tenero.
La soia (Glycine max (L.) Merr.) rappresenta, una coltivazione sempre più diffusa in tutto il mondo grazie al suo elevato contenuto in nutrienti ma anche grazie alle numerose possibilità di trasformazione. Nel contesto di un sistema chiuso per missioni spaziali, però, la processazione delle materie prime deve anche tener conto anche di fattori come resa, dispendio energetico, accettabilità sensoriale etc. Per queste ragioni si è reputata come ottimale la simultanea produzione di latte ed okara che consente, un quasi totale recupero dei nutrienti della soia senza eccessivo consumo di energia.
In particolare nell'individuazione dei parametri ottimali di processo per la trasformazione dei semi in latte e okara si è scelto di procedere con l'idratazione a temperatura ambiente che prevede, rispetto all'acqua a temperature maggiori, tempi più lunghi ma anche minore perdita di nutrienti nel mezzo e nessun dispendio energetico. Le condizioni reputate ottimali, sono state poi la macinazione dei semi con acqua a temperatura ambiente e l'estrazione mediante 30 minuti di ebollizione: seguono la filtrazione per separare l'estratto acquoso dalla polpa residua ed il risanamento del latte mediante ebollizione per 15 minuti.
Per quanto riguarda gli aspetti organolettici, il sapore del latte di soia non è sempre ben accetto dai consumatori europei, ma può essere facilmente migliorato mediante additivi: uno studio con pannellisti non esperti ha dimostrato come l'aggiunta di sale e zucchero vanigliato, pur non riuscendo a mascherare il tipico aroma di fagiolo, riesce a migliorarne il sapore fino a livelli di gradimento globale paragonabili a quello del latte di soia della principale marca commerciale.
Si è voluta valutare la possibilità di un accorciamento del ciclo di coltivazione della soia studiandone gli effetti sui semi ma anche sui trasformati. La raccolta dei semi ad uno stadio di maturazione ceroso porterebbe indiscutibili vantaggi economici e gestionali ma è, ben noto che l'accumulo di sostanza secca e di nutrienti nei semi è funzione del progredire della maturazione. Se per la sostanza secca, questo andamento si attenua con la trasformazione, per le proteine ed i grassi si sono osservate concentrazioni nettamente più alte sia nel latte che nell'okara derivanti dai semi a maturazione commerciale. Si è dovuto, quindi, concludere che, anche nel contesto di un Life Support System, il momento ottimale per la raccolta della soia è la cosiddetta "maturazione commerciale".
Sulla base di queste informazioni preliminari si è proceduto al confronto in termini nutrizionali e composizionali, tra quattro differenti cultivar di soia e tra i loro prodotti di derivazione. In parallelo si verificata l'influenza della coltivazione in idroponica in ambiente strettamente controllato tale da simulare le condizioni di Life Support System. E' stato possibile, in conclusione, affermare che la coltivazione controllata, non solo rappresenta un valido sistema di produzione di alimenti nello spazio, ma consente anche di migliorare, il valore nutrizionale dei prodotti di soia favorendo l'accumulo di lipidi, fibra alimentare e sali minerali quali fosforo e magnesio.. La coltivazione controllata, inoltre, ha determinato prestazioni differenti rispetto all'allevamento in pieno campo e, pertanto, nell'ottica di scelta delle cultivar ottimali, sono stati considerati i soli dati della coltivazione sperimentale e non del controllo. Nel caso specifico della produzione simultanea di latte e okara, il sistema di produzione non ha influenzato fortemente la composizione dell'estratto acquoso, mentre le variazioni composizionali e nutrizionali osservate nei semi si sono andate a ripercuotere maggiormente sulla polpa residua. La scelta della cultivar ottimale, in ogni caso, andrà contestualizzata all'interno della dieta degli astronauti: buoni valori di proteine e fibra alimentare si sono osservati nei trasformati di soia Atlantic ma, se dovesse risultare necessario un maggiore apporto lipidico, bisognerà indirizzarsi verso altre cultivar quali Cresir o Regir.
A questa prima fase relativa alle problematiche di produzione e trasformazione degli alimenti sulla Terra seguirà, comunque una seconda fase con lo studio delle tecnologie in assenza di gravità e la valutazione dell'effetto dell'ambiente spaziale sui processi biologici.

0 PREFACE

0.1 Food for space

In the contest of space missions designing, a great problem is related with correct feeding of the crew. First of all, the development of a proper diet, able to combat any physiological damage related to the absence of gravity, is crucial to the health of astronauts. The intake of energy and calcium, for example, is often not sufficient while the introduction of sodium is excessive: this can greatly contribute to the development of osteoporosis, especially in conditions of microgravity because the absence of mechanical forces has a significant impact on the metabolism of bone calcium (Heer, 2002).
It is necessary to solve a series of logistical problems related to sanitation, conservation, preparation and consumption of food in the absence of gravity (Popov & Bychkov, 1994): for example, the crumbs of cookies or crackers in the absence of gravity can create problems for equipment or crew, and foods with high water activity, if not completely consumed, can deteriorate causing a health disorder.
Finally, we have not neglect all hedonistic and social aspects related to food: the consumption of palatable meals helps the crew in tolerating psychological and emotional stresses they are subjected during missions (Kerwin & Seddon, 2002).
Food for the first space missions, in the 60s, was packaged in tubes (similar to toothpaste): they can be squeezed directly into the mouth. Soon, this system was replaced by packaging "spoon-bowl" that allowed for a convenient use of the spoon to eat the meal: individual portions of food were freeze-dried and packaged in flexible films with a valve for rehydration. This new system allowed for consumption of foods, such as meat or vegetable, with more pleasing texture but a further improvement occurred with the introduction of special cutlery and trays that allow making food intake more enjoyable, similar to a normal meal. Improving the packaging system, the variety of foods increased and today astronauts can choose, at the time of departure, from a wide range of dishes. In the absence of refrigerators or freezers, however, they must always choose stable products: thermostabilized products such as pouched soups, sides, desserts, puddings and entrees, irradiated products (irradiation is not typically used for commercial foods but NASA received special dispensation from the Food and Drug Administration to prepare nine irradiated meat items), freeze-dried and rehydratable products such as spicy green beans, cornbread, dressing and cereals, extended shelf life bread products (waffles, tortillas etc.), natural form foods with low moisture such as almonds and peanuts. Although processing systems provide safe food, this level of processing can reduce the quality of the food, including nutritional value and acceptability. Major limitations, are also due to the lack of fresh products such as fruits and vegetables: they have short shelf life and, therefore, are provided in very small quantities.
This food supply system creates major problems in the management of empty containers, too. They currently represent the largest contribution to the waste during space missions, both in terms of volume in terms of mass (Cooper et al., 2011).
A viable alternative to stocks of food from Earth is the development of "Life Support Systems" (LSS); with a continual recycling of resources, they allow for production "in situ" of air, water and food. The hypothesis of a physical-chemical regeneration (air and water but not food) or biological regeneration were evaluated (Dry dale et al., 2003): these systems are called “Bioregenerative Life Support System” or “Controlled Ecological Life Support System” (CELSS) o “Environmental Closed Life Support System” (ECLSS).

0.2 MELiSSA project

In the context of CELSS developing, European Space Agency (ESA) is working on the MELiSSA (Micro-Ecological Life Support System Alternative) project for studying and developing an artificial ecosystem of microorganisms and higher plants able to support the life of a crew during long-term space missions.
Main targets of the project are food and oxygen production and waste recycling through closing of a cycle with 5 separate compartments (**Figure 1**).

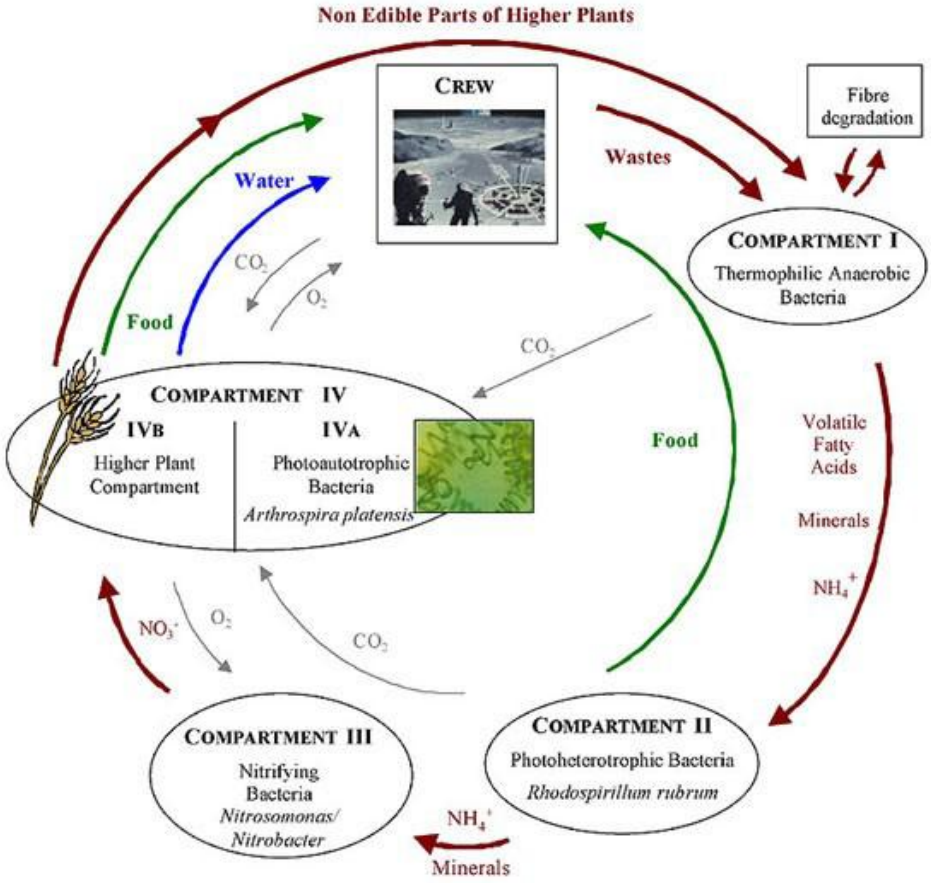

Fig. 1: The MELiSSA Ecosystem

Waste from crew (feces, urine, paper) and waste from non-edible plant cultivation enter in the compartment I of the MELiSSA loop. Thermophilic anaerobic bacteria convert the waste into ammonium, H_2, CO_2, volatile fatty acids and minerals. Limiting factor in the management of this compartment is lignin, which, among all the components of the waste, is the most refractory to anaerobic decomposition.

Compartment II is colonized by Rhodospirillaceae: they are able to metabolize low molecular weight carbon compounds, volatile fatty acids in particular, from compartment I. The biomass produced by *Rhodospirillum rubrum* can be also considered as an edible biomass and it could eventually be used as a complementary food source.

In the compartment III, nitrifying bacteria perform ammonium oxidation: nitrates are the most available form of nitrogen by higher plants and by photo-autotrophic bacteria.

In the compartment IV there are the production of food and water and the regeneration of CO_2 into oxygen. Compartment IV is divided into two sub-compartments: in the compartment IVa there are higher plants and in the compartment IVb there are cyanobacteria such as *Arthrospira platensis*. It produces spirulina with very high nutritional value (protein content above 70%, well balanced aminogram, good content in vitamins and unsaturated fatty acids).

The crew is end-users of food, water and CO_2 and O_2 and producer of the waste for compartment I. It closes the cycle of the ecosystem (Hendrickx et al., 2006).

The MELiSSA project is organized into five phases: during Phase 1, each compartment of the loop is analyzed separately and the overall loop is then simulated on the Earth, during Phase 2 influences of space environment on biological processes are evaluated, within Phase 3, the technologies developed in Phases 1 and 2 will be tested and adapted taking into account life support hardware constraints, with Phases 4 scientific knowledge will be transferred to industrial technologies, in Phases 5 communication will performed.

0.3 References

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1. STATE OF THE ART

1.1 Genetic factors and environmental conditions influence on nutritional soybean characteristics

Soybean (*Glycine max* (L.) Merr.) is an important crop worldwide because of its nutritional properties. Growing evidence showed that consumption of soybean may prevent certain cancers (Alekel et al., 1998; Anthony et al., 1996), reduce the risk of osteoporosis (Arjmandi et al., 1998; Barnes et al., 1994), lower plasma cholesterol (Franke et al, 1995; Ho et al., 2000) and decrease the risk of coronary heart (Lucas et al., 2001). Epidemiological studies suggest that populations consuming high amounts of soybean products have both lower incidences of cancer and lower mortality rates for the major cancer types commonly found in the Western hemisphere (Spector et al., 2003).
As a first approximation, soybean seed contains 20% lipid, 40% protein, 35% carbohydrate, and 5% ash on a dry-weight basis.

1.1.1.Proteins

Soybean is an important source of protein and the most abundant seed storage proteins are the salt-soluble proteins termed globulins. Glycinin and β-conglycinin account for about 70–80% of the total seed globulin fraction: because of their abundance, these proteins are mainly responsible for nutritional quality of soybean foods. Like in other legumes, storage soybean proteins are deficient in sulfur-containing aminoacids (cysteine and methionine) (Nielsen, 1996).
Several authors studied protein variation as cultivar or environmental effect.
Kumar et al. (2006) studied seven Indian cultivars from different locations and reported protein percentage ranged from 32.2% to 42.1% dry basis.
Zarkadas et al. (2007) studied protein content in soybean from different cultivars, too. Respect to Kumar, they reported lower values and smaller but significant differences (from 29.8% to 36.1% dry basis). In this study, it was found that genotypes with higher protein content had lower sulfur amino acid content.
Jurgonski et al. (1997) cultivated soybean within CELSS with two different CO_2 concentrations. In both cultivations, seeds from CELSS showed higher protein content than field control (48.0 and 49.4% dry basis, respectively vs 42.5 % in field soybean). Cysteine and methionine content increased in soybean from CELSS respect to field control, too.
Wheeler et al. (1995), instead, did not report significant differences between protein content in soybean seeds from CELSS and soybean seeds from field.

1.1.2 Fat

Soybean is one of the best sources of high quality plant oil. Soy oil is a good source of essential fatty acids such as linoleic and linolenic acid but a very high presence of polyunsaturated fatty acids makes it easily susceptible to oxidation (Frankel, 1980).
Cultivar and environmental conditions can influence soybean fat content: Kumar et al. (2006) reported, in seven Indian cultivars values from 15.4 to 22.0% dry basis. A number of reports (Wolf et al., 1982; Rennie & Tanner, 1989) suggest that soybeans developing under warmer temperatures possess elevated oleic acid and low polyunsaturated fatty acids.
Jurgonski et al. (1997) cultivated soybean cv. Hoyt within a CELSS and reported higher fat content than field control (19.0% and 20.2% dry basis at two different CO_2 concentrations vs 18.1% in seeds from field).
Wheeler et al (1995), instead, did not report significant differences in fat content between soybean seeds from CELSS and soybean seeds from field.
In the soybean lipid fraction, tocopherols are also present. α-, β-, γ-, and δ-tocopherols constitute a family of lipophilic antioxidants that protect biological membranes against peroxyl radicals, but only the 2R chiral form of α-tocopherols is retained by humans (Traber & Atkinson, 2007). Soybean seeds, contain high concentrations of total tocopherols, but α- tocopherols, usually, represents only 5–10% of total tocopherols.
Significant differences among soybean cultivars were detected in tocopherols forms distribution, but relatively small increases in temperature or severe drought during seed development cause large increases in fraction α-tocopherols/tot tocopherols (Britz & Kremer, 2002; Ujiie et al., 2005).

1.1.3 Carbohydrates

Cultivar can influence not only fat and protein content but also carbohydrates content in soybean seeds. Hymowitz &Collins (1974) evaluated 195 soybean cultivars grown in the United States and Canada: carbohydrates content ranged from 6.2 to 16.6% dry basis.
In a CELSS, Jurgonski et al. (1997) noticed carbohydrates contents lower than field control (19.8 and 21.6 % dry basis in two different CO_2 concentrations, respectively vs 29.7% in control seeds). In a similar study Wheeler et al. (1995) agreed on this trend.

1.1.4 Dietary fiber

Nutritional interest in soybeans is also done to the dietary fiber presence.
Although the effects of dietary fiber (reduction of cholesterol level, positive effect on diarrhea and constipation, anti-inflammatory and anti-carcinogenic effects on the digestive system) are well known, few studies was found in the literature on characterization of dietary fiber in soybean seeds (Van Laar et al., 2000; Van Laar et al., 1999).
Grieshop & Fahey (2001) analyzed fiber content in 48 soybean samples collected from Brazil, China and United States of America. Significant differences were found among cultivars (from 11.5% to 18.52% dry basis) but not among countries.
Wheeler et al. (1995) reported in soybean seeds from CELSS higher fiber content than field control. Constituents of soybean seeds fiber include cellulose, pectin substances, galactomannans, lignin and hemicelluloses (Selvendran et al., 1987).

1.1.5 Ash

Both studies on CELLS soybean cultivation report increasing in ash content respect to filed control. Main minerals in soybean seeds are potassium (1740 mg/100 g fresh weight), phosphorus (591 mg/100 g fresh weight) and calcium (257 mg/100 g fresh weight).

1.1.6 Phenolic compounds and isoflavones

Soybean seeds contain many phenolic compounds such as chlorogenic acid, caffeic acid, ferulic acid and p-coumaric acid. Important differences in total phenolic compounds were detected: from 2.13 to 3.45 mg gallic acid equivalent/g in Tepavcevic et al. (2010), from 6.4 to 81.7 mg gallic acid equivalent/g in Prakash et al. (2006).
Important compounds of soybeans are isoflavones, phenolic compounds comprised of aglucones, β-glucosides and β-glucosides conjugated with malonyl and acetyl groups (**Figure 2**).
Twelve different isoflavones were detected in soybeans: daidzein, genistein and glycitein are the aglycons which can shape three glucoside forms (Murphy et al., 2002).

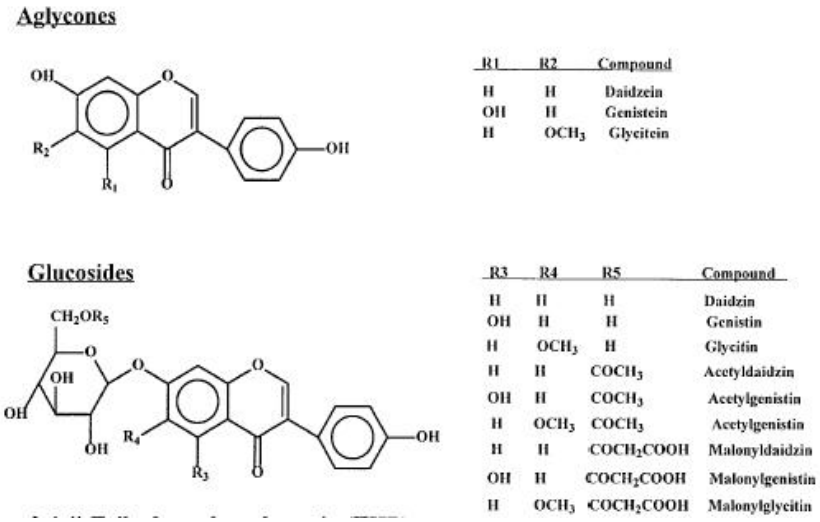

Fig. 2: Soy isoflavones chemical structures

These compounds are very important because of their pharmacological and antioxidant properties: they reduce risk of heart diseases (Ho et al., 2000; Wong et al., 1998) and risk of several types of cancer including the breast, prostate and colon (Messina & Barnes, 1991); they reduce menopausal symptoms (Chiechi, 1999) and promote bone health (Anderson & Garner, 1997). On the other hand, recent research on isoflavones revealed not only beneficial but also adverse effects, for instance, on the reproductive system (Andres et al., 2011). The literature suggests that biological activity of soy isoflavones does not depend upon glycoside form, because for their absorption in organism the hydrolysis of the glycosides is necessary (Xu et al., 2000). On the contrary, activity depends on the type of aglycon: for example daidzein and glycitein has less estrogenic activity than genistein (Potter et al., 1998). Ribeiro et al. (2007) reported in 18 soybean cultivars total isoflavones content from 61.83 to 174.30 mg/100 g dry matter. Isoflavones profile was, more or less, constant: amount of total daidzein was the highest (47.2% on average), followed by total genistein (40.2% on average) and total glycitein was the lowest (12.6% on average) (Tepavcevic et al., 2010). Tepavcevic et al. (2010) found a correlation between polyphenol content, total isoflavones content and antioxidant activity: radical scavenging antioxidant activity was mainly done to polyphenol compounds different from isoflavones.

1.1.7 Phytic acid

In addition to numerous pro-nutritional characteristics of the soymilk, the presence of anti-nutritional compounds such as trypsin and trypsin-chyme inhibitors, hemagglutinins and phytic acid (Birk, 1996; Grant, 1989) should be mentioned. Structure of phytic acid in a solution is the chair conformation of hexaphosphorylated myoinositol (**Figure 3**) and it usually occurs as a mixed calcium–magnesium–potassium salt (Oberleas, 1983) but it can also form complexes with proteins by electrostatic interactions: for this reason, phytic acid contributes to mineral deficiencies and reduces the bioavailability of protein (Liu et al, 1998). On the other hand, many recent evidence suggested that phytate also have antioxidant positive effect which can be considered more prominent than the reduction of mineral bioavailability for the majority of consumers (Greiner et al., 2006).

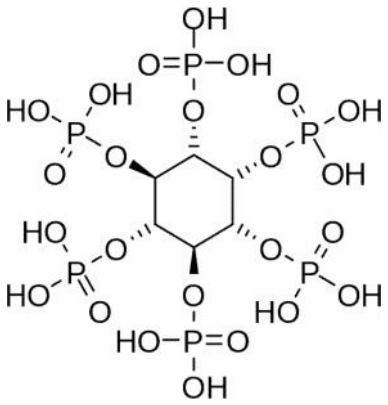

Fig. 3: Phytic acid chemical structure.

Kumar et al. (2006) in their study on Indian soybeans in different location worked on phytic acid, too: concentration varies from 27.8 to 45.0 mg/g dry weight basis in function of location and genotypic effects.

1.2 Changes in soybeans composition during maturation steps

Lifecycle of soybean plants is divided into two phases: development and flowering. 10-15 days after flowering, pods develop from the flowers: this process takes 3 weeks on average, and most of dry matter accumulation is realized within 30-40 days.
At physiological maturity, accumulation of dry mass ends and pods lose their green color. In the following 10-15 days there is a rapid loss of water, pods become dark, leaves turn yellow and fall: this is the time for seeds harvest.
At present, only few studies evaluated accumulation of nutrients in soybean seeds during pods formation and filling phases: protein content varied from 28% (very small seeds) to about 38%

(mature seeds) (Krober & Gibbson, 1962), triglycerides content increased from 6.5% on 30 days after flowering to 85% at maturity (Roehm & Privett, 1970). Moreover, correlations between the maturity stage and isoflavones content in soybeans were found. Kim & Chung (2007) reported rapid increase in isoflavones content from the complete pods filling (when they are still green) (296 μg /g dry matter) to the beginning of pods maturity (when they are getting brown) (611 μg /g dry matter); conversely, only a further small increase was observed at full ripeness (669 μg /g dry matter). During maturation steps, glucosides and malonyl-glucosides concentrations increased while aglicones and acetyl-glucosides decreased.
Plant phytic acid biosynthesis comes through the progressive phosphorylation of inositol monophosphate (Cosgrove, 1980). In many legumes an increase in phytic acid content was found starting from the flowering (Honke et al., 1998).

1.3 Compositional and nutritional characteristics of soymilk and okara

Soymilk is a water extract of soybeans, closely resembling dairy milk in physical appearance and composition; because of its optimal nutritional profile, it can be used as an animal milk complement or substitute.
After separation from the fiber residues, soymilk is composed of about 2% lipid, 3–4% protein, 1-4% carbohydrate, 0.6-0.9% ash and 8–12% total solids (Cruz et al., 2007; Iwuoha & Umunnakwe, 1997). INRAN (Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione) reports values for minerals and vitamins in soymilk: 32 mg Na, 120 mg K, 0.4 mg Fe, 13 mg Ca, 47 mg P, 0.06 mg tiamin, 0.10 mg niacin.
Soymilk does not taste like dairy milk, but it presents a characteristic flavor of beans. Pentanol, hexanol, heptanol, hexanal and ethyl vinyl-ketone are the most important volatile flavor compounds present in soy foods. Lipoxygenase, an enzyme naturally present in soybeans, mediates conversion of polyunsaturated fatty acids to hydroperoxides; subsequent degradation products are responsible for the off-flavors generated. The primary off-flavor precursors in soybean are C18:2 linoleate which produces hexanal, and C18:3 linolenate which produces 2-hexenal and 3-hexenol (Kobayashi et al., 1995).

1.3.1 Okara

The residue pulp after extraction of the water extractable fraction is named okara (Noguchi, 1987); about 1.1 kg of fresh okara is produced from 1 kg of soybeans processed for soymilk (Khare et al., 1995).
Okara composition depends on pressure during filtration and therefore, data quite variable can be found in the literature (Bourne et al. 1976; Guermani et al.1992; Ma et al., 1996). Van de Reit et al. (1989) reported composition of okara from three different soybean cultivars (**Table 1**).

	Average value of 3 cultivars
Protein	25.4 -28.4 g/100 g dm
Fat	9.3 – 10.9 g/100 g dm
Phytic acid	0.5 – 1.2 g/100 g dm
Fiber	52.8 – 58.1 g/100 g dm
Ash	3000- 3700 mg/100 g dm
Calcium	260 – 428 mg/100 g dm
Magnesium	158 – 165 mg/100 g dm
Iron	6.2 – 8.2 mg/100 g dm
Sodium	16.2 – 18.4 mg/100 g dm
Potassium	1046 – 1233 mg/100 g dm
Zinc	3.5 – 6.4 mg/100 g dm
Phosphorus	396 – 444 mg/100 g dm
Thiamine	0.48 – 0.59 mg/100 g dm
Riboflavin	0.03 – 0.04 mg/100 g dm

Tab 1: Okara composition

1.3.2 Production techniques

Traditional Chinese method, involves a relatively low level of technology: soybeans are soaked overnight in excess water (ratio water/soybeans should be variable from 8:1 to 10:1), drained, grinded with additional fresh water. The paste obtained is boiled in order to inactivate tripsin inhibitors, improve the flavor, sanitize the product. In the end, raw soymilk is separated from the pulp by filtration and then subjected to further boiling. According to the Japanese method, hydrated seeds are boiled and then ground: the last stages of production are filtration and sanitation of the milk (Liu, 1997).
Many process modifications took place over the years, with the development of new processing technologies and considering consumer requirements.
For example, it is possible to add a preliminary dehulling operation: it causes slightly better flavor, improved digestibility, less of the oligosaccharides, fewer bacteria, better protein yield, lower viscosity. On the other hand, dehulling requires more energy and additional machineries.
In general, in large-scale production, high-temperature and short-time processes often substitute normal low-temperature and long-time thermal processes.
For examples, soaked soybeans may be ground not with cold water but with hot water.
Prabhakaran & Perera (2006) confronted two preparation methods: dehulled soybeans were either hot ground (95°C) or cold ground (45°C). Soymilk by hot-grind process contained significantly higher isoflavones concentration: this improvement could be due to the high solubility of isoflavones in hot water than in cold water (Gugger & Grabiel, 2000).
Jung et al. (2008) evaluated thermal effect on soymilk isoflavones: there was no statistical difference in the final isoflavones concentration, between raw and sanitized soymilks, but there are changes in isoflavones distribution for effect of thermal treatment. The raw soymilk had the same isoflavones distribution as the soybeans; in soymilk treated at 95°C for 15 min, as compared to soybeans and raw soymilk, the distribution of the individual isoflavones was shifted towards the β-glucoside and aglucon forms at the expense of a decrease in the malonyl-β -glucoside content.
Wang & Murphy (1996) studied the impact of soymilk production process on isoflavones content and profile. The beans lost about 10% of isoflavones during soaking due to leaching into soaking water, but raw soybeans possessed comparable profiles of isoflavones. According to Jung et al. (2008), cooking of soy slurry decreased malonylic forms and increased aglycons daidzein, genistein and glycitein and glucosides daidzin and genistin. Filtration did not show considerable alteration of isoflavones and after this step, isoflavones concentration in milk is higher than in residue pulp.

1.3.1 Cultivar effect

Cai et al. (1997) studied soymilk from 13 different cultivars: the higher protein in seeds, the higher protein content in soymilk and the higher protein recovery. Same trend was observed for fat and ash contents.
In a similar study Mullin et al. (2001) reported this trend for fat content but not for protein content: significant differences were not found among soymilks from four different soybean seeds cultivars.

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2. AIMS OF THE STUDY

Among targets of the MELiSSA Phase 1, there is the study of food production and preparation (Food Production and Preparation System - FPPS) for a typical Mars mission. A single crew needs, on average, 3kg of combined food and water a day, but the closing of the MELiSSA ecosystem loop can be achieved by a food production equivalent to 40% dry mass of the crew dietary needs.
Several factors must be considered in the selection of plants (and then specific cultivars) to use: ability to provide oxygen and consume carbon dioxide and water, compact size, high productivity, tolerance to osmotic stress (Wheeler, 2011), high harvest index, ease of processing (Hoff et al.,1982; Tibbitts & Alford,1982). In a former study, among 29 candidate species, potato, soybean and two wheat species were selected: they are easily processable products and their combination can meet crew's nutritional needs (Schaafsma, 2000).
Within this context, our study is aimed to the selection of soybean cultivars most suitable for space purpose and to the identification of the optimal transformation methods.

2.1 References

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3. RESULTS AND DISCUSSIONS

3.1 Topic 1: processed soybean products preliminary assessment

The use of soybeans as a food is increasing worldwide not only because of their nutritional properties but also because of the many way it can be processed: generally, higher protein content, low oil content, lighter seed coat and a clear hilum are desirable characteristics for food uses (Liu et al., 1995).
The aim of this study was the selection of most suitable processes for space purpose.

3.2 Materials and methods 1

Evaluation of possible food transformation processes for space missions was based on 7 standardized criteria known ALiSSE (Advanced Life Support System Evaluator):

1. Mass criteria: the mass of equipments and spare parts.
2. Volume mass: the volume of equipments.
3. Crew time: the time required for system management and the time needed to manage errors. We must remember that, if the food is difficult to prepare, then the overall acceptability of the food is reduced (Vickers, 1999).
4. Energy criteria.
5. Risk to human (chemical, physical or biological risks).
6. Efficiency (yield, production of wastes etc.).
7. Reliability.

Nutritional assessment of production processes, in terms of macronutrients and micronutrients percentage recovered from raw materials in final products is very important, too.
The food, however, must be attractive and it must meet criteria of appearance, color, smell, taste and texture. In order to make meals appreciable during the long period in a space mission to the Moon or Mars, lassitude effect must be avoid by a suitable food variability: as it is in general accepted, breakfast can be quite standardized, but lunch and dinner need a menu rotation.
In this first phase of the MELiSSA project, fermented products were not considered because they involve bacterial, fungal and yeast strains with possible stability problems.
Processes evaluated were:

- Soymilk production from dehulled seeds and from whole seeds (layout in **Figure 4**).
- Okara texturization (layout in **Figure 4**) (Nolan, 1983).
- Oil extraction: in CELSS, chemical solvents must be avoided and, for this reason, only physical extraction of soybean oil was taken into account (layout in **Figure 5**).
- Isolated protein production from defatted flour (layout in **Figure 5**).
- Germination (layout in **Figure 6**).

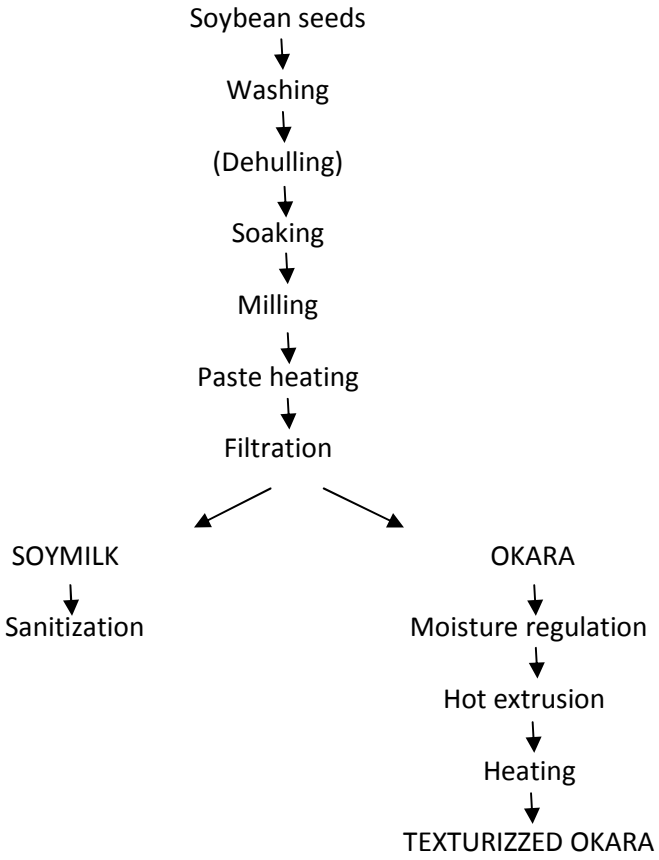

Fig 4: Soymilk and texturized okara layout

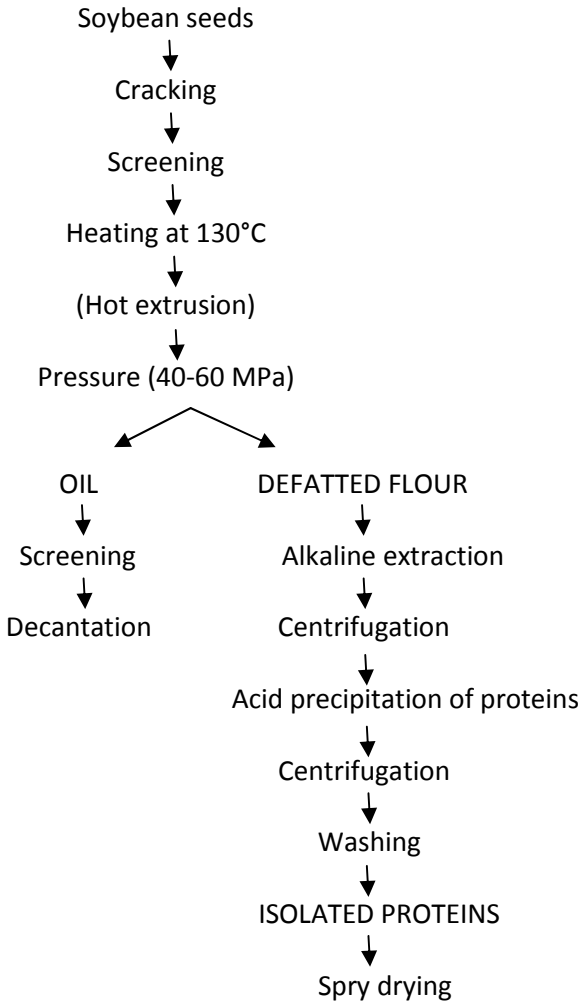

Fig. 5: Oil and isolated protein layout

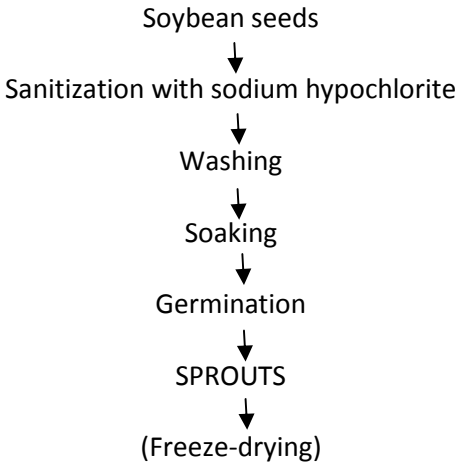

Fig. 6: Soy sprouts layout

3.2 Results and discussions 1

Oil and isolated protein production systems were deemed unfit for CELSS and, therefore, they were excluded from subsequent evaluations. They provide, in fact, a considerable energy request and are also associated with important risks to crew (for oil production, there are risks associated with high pressures and release of potentially explosive dust during breaking and screening of the seeds, for isolates protein production there are risks associated with high concentrations of acids and alkalis).
Preceded by hot extrusion, oil extraction allows for 90% recovery of fat phase (Bargale et al., 1999) but proteins recovery from defatted flour is less than 70% (Berk, 1992).
Soymilk extraction and okara reutilization were chosen.
Simultaneous production of milk and okara, in fact, allows for a high nutrients recovery from seeds without excessive energy consumption. For soymilk production, we decided to by-pass dehulling step: it causes additional energy expenditure and could generate potentially explosive dust.
Soymilk taste is not always well accepted by European consumers, but it can be easily improved by adding sugar and /or vanilla.
Similarly, in order to reduce risks and energy consumption, we decided to avoid okara texturization and use residue pulp for "home recipes".
A possible research side line in this phase of the MELiSSA project is the study of soybean sprouts production. Germination, in fact, is an economical and effective technology which does not involve loss of nutrients or energy request (only in the optional step of freeze-drying there is energy consumption); there is a single chemical risk because of sanitation by hypochlorite, but it is used at very low concentrations. Soy sprouts are not a typical European food but their consumption is increasing: they can be used in many recipes.

3.4 Conclusions 1

Within a closed system for space missions, simultaneous production of soymilk and okara was considered optimal: it allows for high nutrient recovery without excessive energy consumption. A possible research side line in this phase of the MELiSSA project is the study of soybean sprouts production with the identification of optimal parameters, the assessment of potential effects due to the seed cultivation systems and the selection of most suitable cultivars. Germination, as well as soymilk and okara production, in fact, is a well suited technology for space missions: it is very simple, does not involve loss of nutrients or energy expenditure and sprouts are very versatile for several recipes.

3.5 References

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3.6 Topic 2: identification of optimal parameters for soymilk preparation

The aim of the experiment was the comparison among different parameters for soymilk production.
Compositional and nutritional characteristics of milk and okara obtained with different production techniques were evaluated; ALiSSE criteria (energy requirement and sensorial acceptability, in particular) were taken into account, too. After selecting optimal process parameters, the effect of each technological step on macronutrients was evaluated.

3.7 Materials and methods 2

3.7.1 Plant materials

Seeds available on the market were used (Soia Gialla, Fertitecnica Colfiorito).

3.7.2 Soymilk extraction

First of all, soaking tests were carried out.
40g of seeds were kept deionized water (ratio 1:10) at three different temperatures: room temperature (~25°C), 30°C and 40°C. At different times the weight of the hydrated seeds and the electrical conductivity of soaking water (index of mineral releasing from seeds in the water) were determined.
Optimal soaking condition were identified and applied to subsequent experiments. After soaking, seeds were rinsed and grinded in the same amount of water. The effect of temperature of water for milling and the effect of time/temperature parameters for extraction were evaluated: **Table 2** shows different tested production techniques.

Grinding \ Extraction	boiling for 30 min	Heating 90 °C – 90 min
Room temperature water	**Sample 1**	**Sample 3**
80 °C water	**Sample 2**	**Sample 4**

Tab. 2: Soymilk production parameters

For **Sample 1** and for **Sample 3**, water at room temperature was used for milling, for **Sample 2** and for **Sample 4** water at 80 °C was used for milling.
The obtained paste was heated, in order to extract soymilk: for **Sample 1** and for **Sample 2**, paste was boiled for 30 min, for **Sample 3** and for **Sample 4** paste was heated at 90°C for 90 min.
After extraction the paste was filtered in order to separate soymilk from okara and the last step was soymilk sanitization (boiling for 15 min).
Milk extraction was performed twice for each technique.

3.7.3 Proximate composition

Moisture was determined by oven-drying at 105±1°C (AOAC, 1995). Fat content was measured by extraction with diethyl ether in a Soxhlet system (James, 1995). Proteins were analyzed as total nitrogen content by the Kjeldahl procedure (AOAC, 1995) and the conversion factor was 5.71.
All compositional determinations were performed three times for each sample.

3.7.4 Antioxidant activity determination

The antioxidant activity was measured on the water-soluble fraction using the N,N-dimethyl-phenylenediamine (DMPD) method (Fogliano et al., 1999); the antioxidant activity of the water soluble fraction was expressed as equivalent millimolar of ascorbic acid. The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method performed as described by Pellegrini et al. (1999) was used to assess the antioxidant activity of water-insoluble fractions; the antioxidative activity of the lipophilic fraction was expressed in millimoles of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).
Determinations were performed three times for each sample.

3.7.5 Isoflavones analysis

1 g of dried materials was extracted by 30mL of methanol/water (70:30, v/v) and sonicated at room temperature for 30 minutes (Otieno et al., 2007). The mixtures were centrifuged at 4000 rpm, filtered through a Whatman filter paper and then used for LC-MS/MS analysis using a method previously described (Ferracane et al., 2010). Chromatographic separation was performed using an HPLC apparatus equipped with two Micropumps Series 200 (PerkinElmer, Shellton, CT, USA), a UV/VIS series 200 detector (PerkinElmer, Shellton, CT, USA) settled at 280 nm and a Prodigy ODS3 100Å column (250 mm ×4.6 mm, particle size 5 µm) (Phenomenex, CA, USA). The eluents were: A water 0.2% formic acid; B acetonitrile/methanol (60:40, v/v). The gradient program was as follows: 20-30% B (6 min), 30-40% B (10 min), 40-50% B (8 min), 50–90% B (8 min), 90-90% B (3 min), 90-20% B (3 min) at a constant flow of 0.8 mL/min. The LC flow was split and 0.2 mL/min was sent to the mass spectrometry. Injection volume was 20µL. Two injections were performed for each sample. MS and MS/MS analyses of soybean extracts were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source working in the negative ion mode. Six glucosides (daidzin, genistin, glycitin, acetyl-genistin, malonyl-daidzin, malonyl-genistin) and only one aglycone (genistein) were identified: LC/MS/MS conditions of identified isoflavones are shown in **Table 3.** After peak identification the isoflavones quantification was performed by HPLC as follows: filtered extract (20 µL) was injected into an HPLC (Shimadzu LC 10, Shimadzu, Kyoto, Japan) with photodiode array detector. Separations were achieved on the same column with the same gradient program. The flow rate was 0.8 mL/min, and chromatograms were recorded at 280 nm. Isoflavones were quantified using, as external standard, genistein and genistin for all glucosides. Genistein stock solution was prepared by dissolving standard (Sigma Aldrich) in methanol while genistin stock solution was prepared by dissolving standard (Sigma Aldrich) in a mixture methanol/water (70:30, v/v).

Compound	**Precursor ion [M-H$^+$] (*m/z*)**	**Product ion**
Genistein	271	243 215 187
Daidzin	417	199 255
Genistin	433	215 271
Glycitin	447	285
Acetyl-genistin	475	431 417
Malonyl-daidzin	503	417
Malonyl-genistin	519	433

Tab 3: LC/MS/MS conditions for the detection of soybean isoflavones.

3.7.6 Sensorial evaluation

Optimal production technique was used in soymilk extraction. Three soymilk formulations with different amounts of salt, sugar and vanilla sugar were analyzed (**Table 4**).

Formulation 1	1.5 g salt (~½teaspoon) + 5 g sugar (~2 teaspoons) /L
Formulation 2	1.5 g salt (~½teaspoon) + 2.5 g sugar (~1 teaspoon) / L
Formulation 3	1.5 g salt (~½teaspoon) + 2 g vanilla sugar (~1 teaspoon) / L

Tab. 4: Different soymilk formulations

Thirteen panelists (staff and students of the University) considered three soymilk brands (Valsoia, Sojasun and Alprosoya): they evaluated pleasantness of appearance, pleasantness of flavour, pleasantness of taste and global pleasantness. A numeric scale from 1 to 10 was used.
Then panelists analyzed three soymilk experimental formulations: they evaluated the same four aspects of pleasantness and the main descriptors reported in the literature (Torres & Reitmeir, 2001). A numeric scale from 1 to 10 was used.
Samples were presented, one at a time, placed in small plastic cups, in random order. The evaluation form that was used is shown on the next page. Results were plotted on a rays graph.

CAMPIONE N. ……

1. GUARDA IL CAMPIONE

Colore giallo: 1 2 3 4 5 6 7 8 9

Quanto ti piace? 1 2 3 4 5 6 7 8 9

2. ODORA IL CAMPIONE

Aroma di fagiolo crudo: 1 2 3 4 5 6 7 8 9

Aroma di fagiolo cotto: 1 2 3 4 5 6 7 8 9

Quanto ti piace? 1 2 3 4 5 6 7 8 9

3. ASSAGGIA IL CAMPIONE

Sapore di fagiolo: 1 2 3 4 5 6 7 8 9

Sapore dolce: 1 2 3 4 5 6 7 8 9

Sapore di vaniglia: 1 2 3 4 5 6 7 8 9

"Sabbiosità": 1 2 3 4 5 6 7 8 9

Astringenza: 1 2 3 4 5 6 7 8 9

Persistenza sapore: 1 2 3 4 5 6 7 8 9

Quanto ti piace? 1 2 3 4 5 6 7 8 9

3. DAI UN GIUDIZIO GLOBALE

Quanto ti piace? 1 2 3 4 5 6 7 8 9

NOTE

3.7.7 Statistical analysis

Data were analyzed by ANOVA and means were compared by Duncan's Multiple Range Test.

3.8 Results and discussions 2

3.8.1 Seeds characterization

Table 5 and **6** show macronutrient and micronutrient composition and isoflavones content and profile in Fertitecnica Colfiorito seeds, respectively.

Dry matter (%±SE)	87.62±0.21
Protein content (%/dm±SE)	37.43±0.08
Fat content (%/dm±SE)	17.53±0.11
Hydrophilic antioxidant activity (mg ascorbic acid equivalent/ 100 g dm±SE)	6.06±0.14
Lipophilic antioxidant activity (mg Trolox equivalent/ 100 g dm±SE)	0.07±0.03

Tab. 5: Soybean seeds (Fertitecnica Colfiorito) macronutrient and micronutrient composition

	mg/100 g dm±SE	% of total±SE
Daidzin	21.81±1.52	13.91±1.03
Glycitin	9.30±2.51	5.92±1.58
Genistin	27.50±0.30	17.54±0.13
Malonyl-daidzin	37.58±0.28	23.97±0.09
Malonyl-genistin	47.17±1.23	30.09±0.90
Acetyl-genistin	8.73±0.20	5.57±0.11
Genistein	4.69±0.04	2.99±0.01
Total content	156.76±0.58	

Tab.6: Soybean seeds (Fertitecnica Colfiorito) isoflavones content and profile

3.8.2 Seeds soaking

Figure 7 shows soybean seeds soaking trends at three different temperatures.
Soaking temperature did not influence amount of absorbed water (plateau is 120 g water/100 g dry seeds, approximately) but it influenced soaking time: full rehydration was observed at 700 min, 360 min and 240 min at room temperature, 30°C and 40°C, respectively.

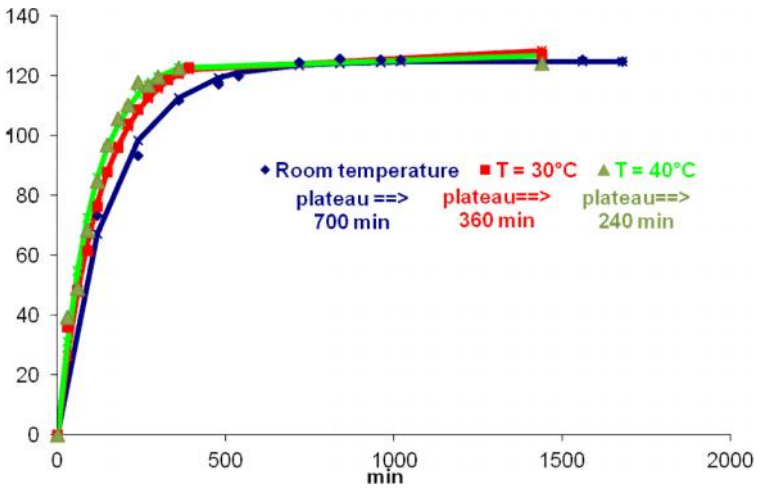


Fig. 7: Soaking trends (g H_2O absorbed/ 100 g dry seed) at three different temperatures

At the same times, electrical conductivity in soaking water was observed (**Figure 8**): the higher temperature, the higher releasing of substances from seeds in water.

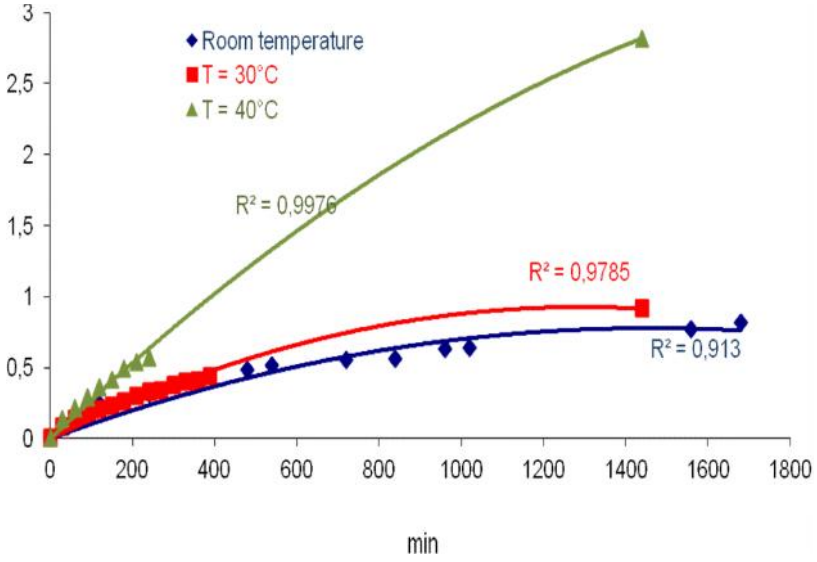
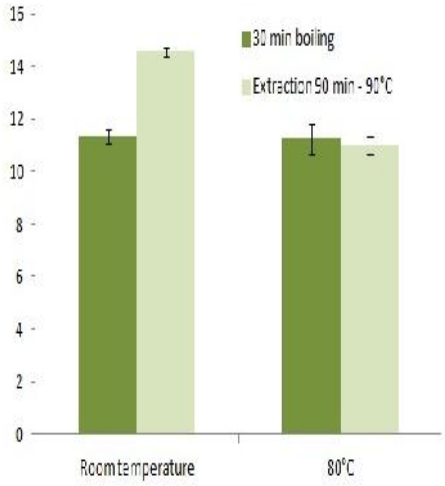


Fig. 8: Electrical conductivity in soaking water at three different temperatures

3.8.3 Comparison among production techniques

Great differences among samples involved okara but not soymilk.
Okara **Sample 1** (milling in water at room temperature and extraction by boiling for 30 min) showed the highest protein content (11.5% dry matter). Longer process favors fat extraction and, for this reason okara **Sample 3** (milling in water at room temperature and 90 min extraction at 90°C) showed the highest lipid content (14.6% dry matter).



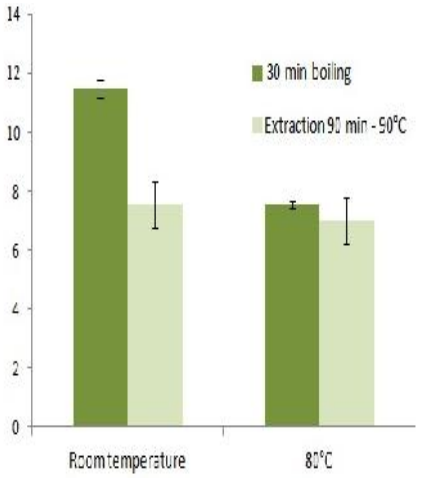
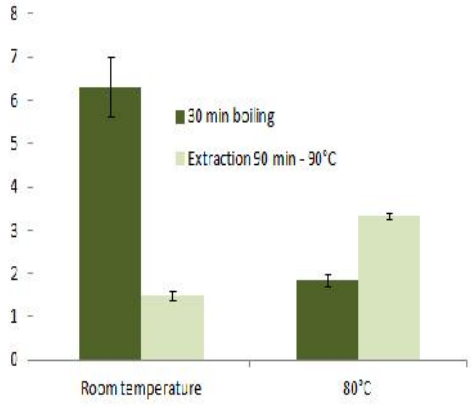


Fig. 9 and **10**: Fat (%/dm) and protein (%/dm) content in okara obtained with different process parameters (average values±SE)

Okara **Sample 1** showed the highest hydrophilic and lipophilic antioxidant activity (9.87 mmol ascorbic acid equivalent/100 g dry matter and 2.14 mmol Trolox equivalent/100 g dry matter, respectively).



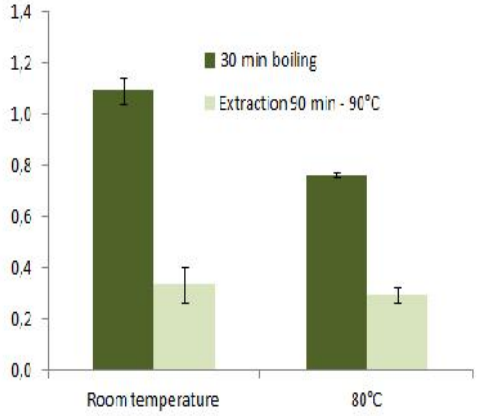


Fig. 11 and **12**: Hydrophilic antioxidant activity (mmol ascorbic acid equivalent/ 100 g dm) and lipophilic antioxidant activity (mmol Trolox equivalent / 100 g dm) in okara obtained with different process parameters (average values±SE)

In soymilk samples significant differences in macronutrient content were not noticed but variations in antioxidant activity were observed. **Sample 1** showed the highest lipophilic antioxidant activity not only in okara but also in soymilk (2.14 mmol Trolox equivalent/100 g dry matter). Soymilk **Sample 2** showed the highest hydropilic antioxidant activity (9.87 mmol ascorbic acid equivalent/100 g dry matter).
Figure 15 shows total isoflavones content in soymilk samples. According to Prabhakaran and Perera (2006), hot water grinding improved isoflavones extractability. The effect of extraction process parameters was significant, too. Longer extraction with slightly lower temperature allowed for higher total isoflavones content in soymilk.

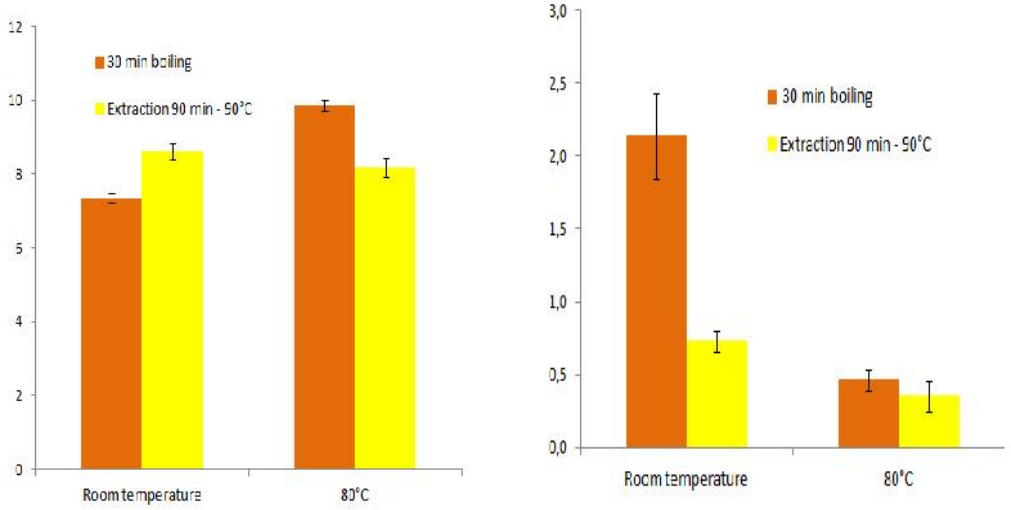


Fig. 13 and **14** Hydrophilic antioxidant activity (mmol ascorbic acid equivalent/ 100 g dm) and lipophilic antioxidant activity (mmol Trolox equivalent / 100 g dm) in soymilk obtained with different process parameters (average values±SE)

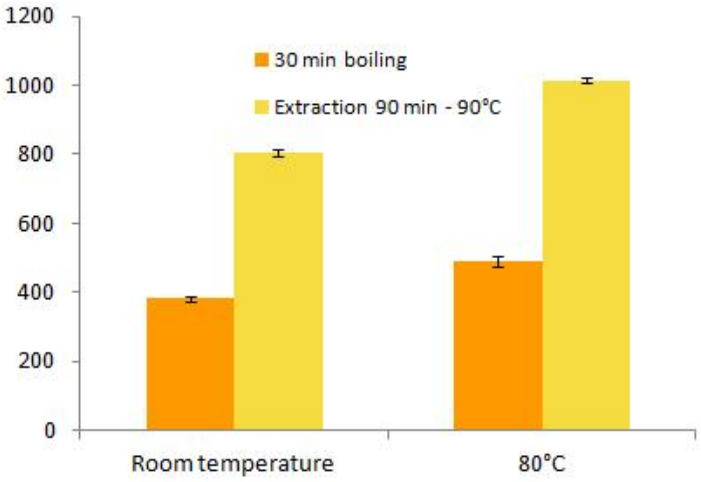


Fig. 15: Total isoflavones content (mg /100 g dm) in soymilk obtained with different process parameters (average values±SE)

Figure 16 shows a representative HPLC chromatograph of soymilk extracted. Glucosides daidzin and genistin, followed by malonyl-genistin and malonyl-daidzin were the most abundant isoflavones in soymilk; malonyl-genistin and malonyl-daidzin were the most abundant forms in raw seeds. In general, transformation of soybean seeds in soymilk, regardless of process parameters, caused percent decrease in malonyl-genistin and malonyl-daidzin and percent increase in glucosides daidzin and genistin. In contrast with Jung et al. (2008) "hot effect" did not caused increase in aglucones but the highest incidence of genistein was observed in soymilk **Sample 1** and the lowest incidence of genistein was observed in soymilk **Sample 4**. The same trend was observed in acetyl-genistin incidence (the highest percentage in **Sample 1**, values under detection limit in **Sample 4**). **Sample 4** presented, instead, the highest content in glucosides, daidzin and genistin in particular.

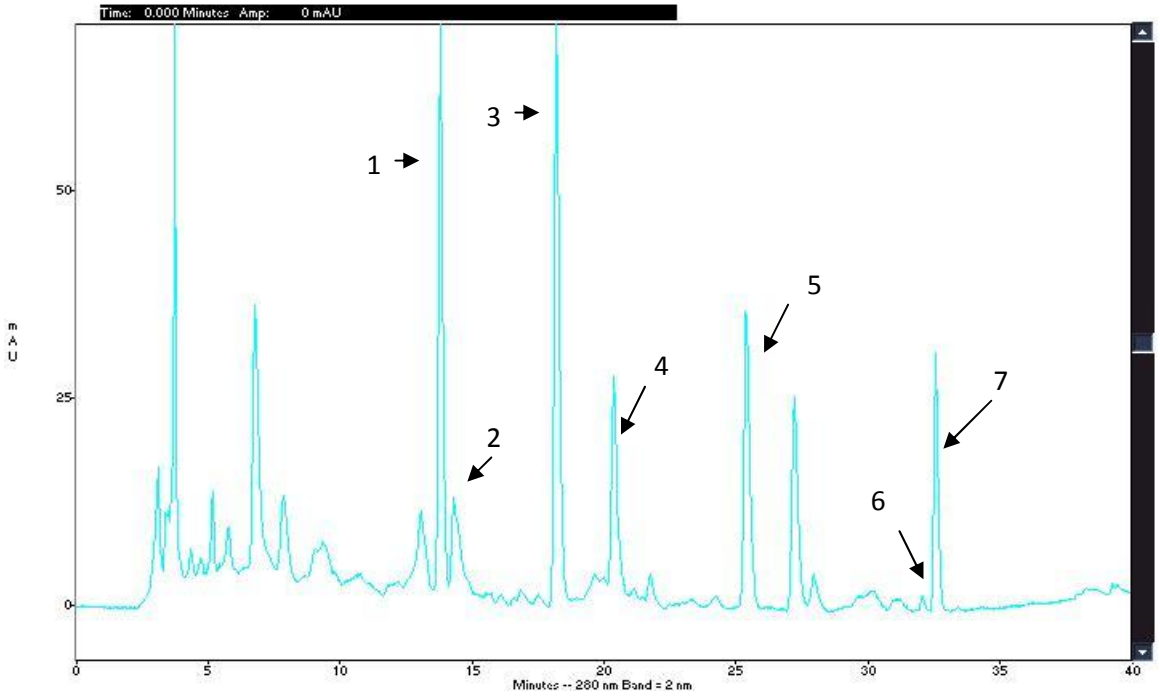


Fig. 16: HPLC chromatograph of extracts from soybean. 1, daidzin; 2, glycitin; 3, genistin; 4, malonyldaidzin; 5, malonylgenistin; 6, acetylgenistin; 7, genistein.

		Grinding Room T / boiling for 30 min	Grinding 80°C/ boiling for 30 min	Grinding Room T Extraction 90 min – 90°C	Grinding 80°C/ Extraction 90 min – 90 °C
Daidzin	mg/100g dm	83.89^{b}	114.75^{b}	248.05^{a}	298.95^{a}
	(%tot)	(22.7^{b})	(23.4^{b})	(30.8^{a})	(29.4^{a})
Glycitin	mg/100g dm	33,24^{a}	33.01^{a}	28.69 ^{a}	33.03^{a}
	(%tot)	(8.7^{a})	(6.7^{a})	(3.6^{ab})	(3.3^{b})
Genistin	mg/100g dm	107.99^{d}	136.17^{c}	237.39^{b}	280.00^{a}
	(%tot)	(29.2^{a})	(27.7^{a})	(29.5^{a})	(27.6^{a})
Malonyl-daizin	mg/100g dm	59.18^{c}	72.87^{c}	129.58^{b}	201.47^{a}
	(%tot)	(16.0^{b})	(14.8^{b})	(16.1^{b})	(19.9^{a})
Malonyl-genistin	mg/100g dm	47.85^{c}	105.89^{bc}	129.53^{b}	192.26^{a}
	(%tot)	(12.9^{b})	(21.4^{a})	(16.1^{ab})	(18.9^{ab})
Acetyl-genistin	mg/100g dm	11.34^{a}	11.06^{a}	11.22^{a}	
	(%tot)	(3.1^{a})	(2.3^{b})	(1.4^{c})	
Genistein	mg/100g dm	27.74^{a}	18.39^{b}	20.53 ^{b}	9.28^{c}
	(%tot)	(7.5^{a})	(3.7^{b})	(2.5^{c})	(0.9^{d})

Tab. 7: Isoflavones profile in soymilk obtained with different process parameter. Different letters within the same line indicate significant differences at P<0.05

3.8.4 Sensorial evaluation

Figure 16 reports results of preliminary analysis on three soymilk brands.

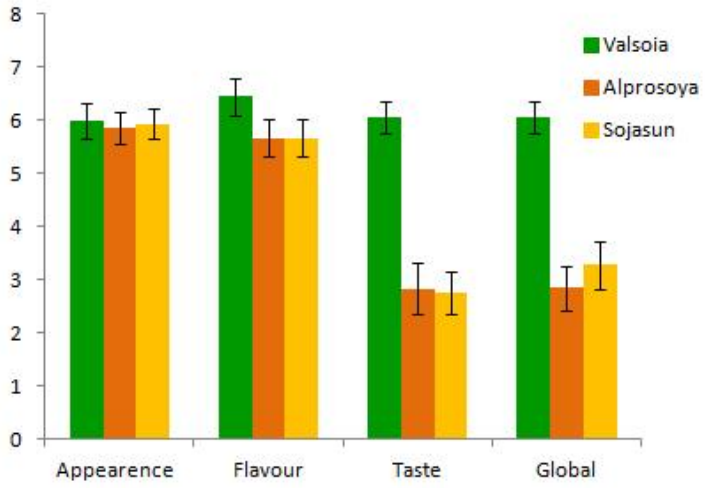


Fig. 17: Pleasantness values in three soymilk brands (Average values±SE)

Differences in appearance and in flavor were not detected but, as concern taste pleasantness and global pleasantness, panelists preferred leader market brand. European consumers are not used to consume soymilk and panelists gave, on average, very low scores (Valsoia soymilk just reached sufficient values).
For this reason, different formulation scores were not evaluated in absolute terms but they were related to Valsoia soymilk scores.
Figure 18 reports pleasantness of three soymilk formulations. Salt, sugar and vanilla adding did not influence appearance: panelists gave similar scores to three experimental formulations and to Valsoia soymilk. In term of flavor, **Formulation 3** was the best with values above the reference. In term of taste **Formulation 3** was the best, but the difference between **Formulation 3** and **Formulation 1** was not large. **Formulation 2** was less appreciated: because of lower sugar concentration, panelist noticed excessive salty taste. Just adding salt, sugar and vanilla it was not possible to reach taste pleasantness values observed for Valsoia (in commercial soymilk there are several and different additives): average taste pleasantness values, compared with Valsoia, were 78% and 70% in **Formulation 3** and in **Formulation 1**, respectively. In term of global pleasantness, **Formulation 1** was the best (99% on average); followed **Formulation 2** and **Formulation 1.**

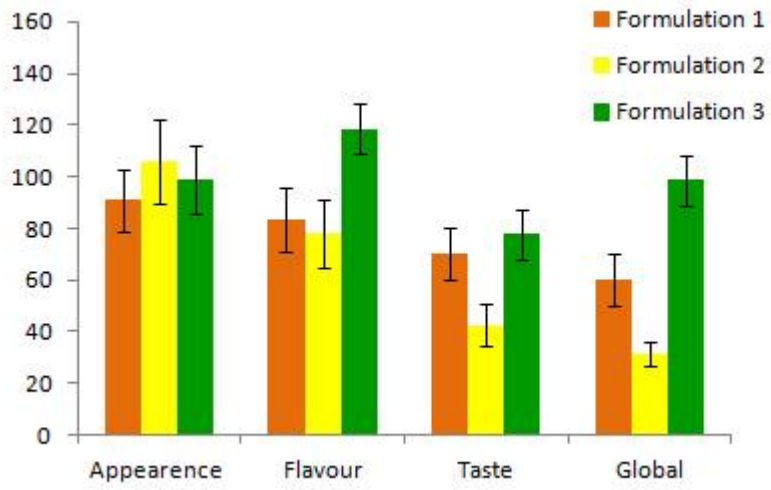


Fig. 18: Pleasantness (%) in three soymilk experimental formulation (% respect to market control) (Average values±SE)

Figure 19 reports sensorial profile in three analyzed soymilk formulations. The only visual parameter (yellow intensity) did not show differences among samples and the values were around the center of the scale: soymilk has a yellow color and this can negatively affect the overall rating, especially if soymilk is associated with cow's milk. The most important aroma "flaws" in soymilk is the bean flavor. The perception of the beans flavor in the three samples was around 3 - 4, without important differences between raw beans and cooked beans flavor. Panelists clearly recognized vanilla flavor in **Formulation 3** but it did not mask bean flavor. Panelist strongly recognized bean taste (values around 5-6) but vanilla masked it in **Formulation 3**. **Formulation 1** was the sweetest but it showed the greatest taste persistence, too. **Formulation 3** presented lower sugar concentration than **Formulation 2** but the presence of vanilla caused higher perception of the sweet taste. Astringency is a typical sensorial characteristic in soymilk: it is due to the presence of isoflavones (Okubo et al., 1992), but it is not always appreciated by consumers. Panelists found astringency below the value 4 without important difference among samples.

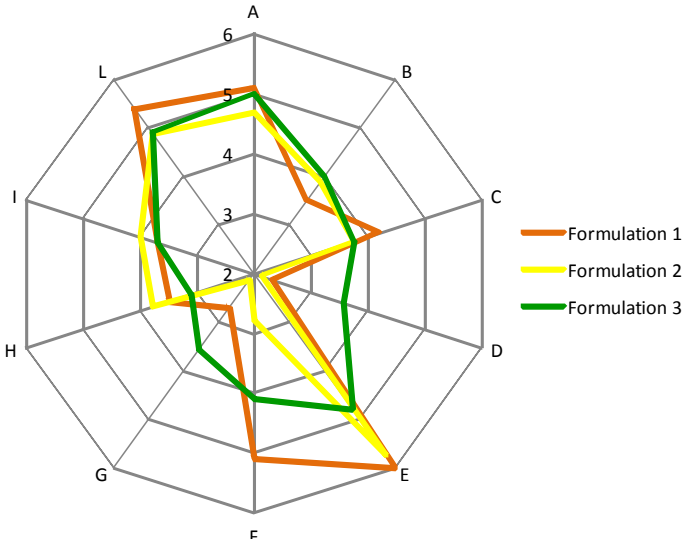


Fig. 19: Sensory profile in three soymilk experimental formulations (Average values) A, Yellow color intensity B, Raw bean flavor C, Cooked bean flavor D, Vanilla flavor E, Bean taste F, Sweetness G, Vanilla taste H, Chalkiness I, Astringency L, Taste persistence

3.8.5 Effect of each technological stages

Figure 20 shows technological layout of 100 g seeds.
Soaking caused an increase of 240% in weight and sanitization caused, because of evaporation, a reduction of 34% in weight.
The process did not cause significant losses in dry matter amount: dry matter was split, almost equally, between liquid and solid phase (53% in okara and 46% in okara). **Figure 21** and **22** show dry matter content in different process steps, in percentage and absolute values, respectively.
Protein and lipid contents did not show variation in soaking and sanitization steps, too (**Figure 23** and **Figure 24**, respectively). As shown in Figure **25** and **26** process allowed for a total macronutrient recovery but concentration was higher in soymilk than in okara: from 100 g of seeds (30.16 g of protein and 16.33 g of fat) we obtained 16.68 g of protein in soymilk and 13.55 g of protein in okara (55% and 45% dry matter, respectively) and 9.61 g of fat in soymilk and 5.80 g in okara (62.3% and 37.6% dry matter, respectively).
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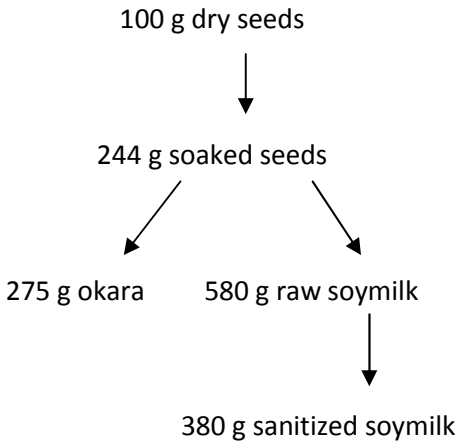

Fig. 20: Soymilk and okara production layout

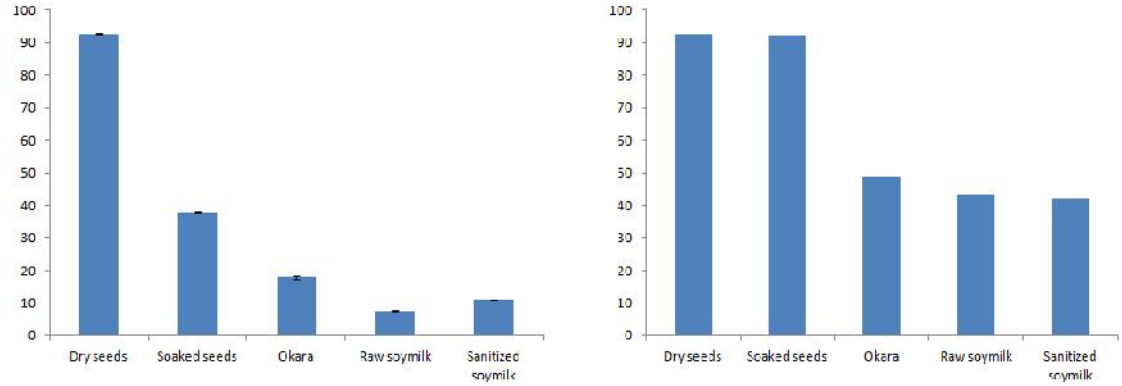

Fig. 21 and **22**: Dry matter content (% and g, respectively) in different process steps (average values±SE)

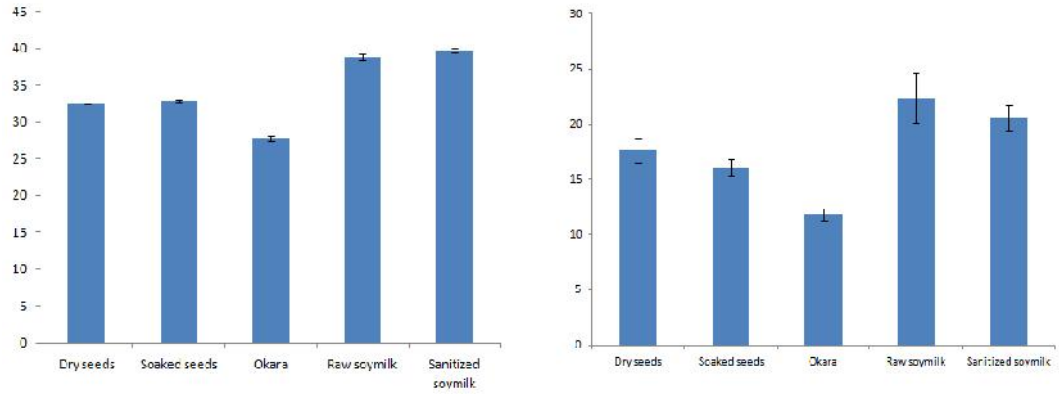

Fig. 23 and **24**: Protein and fat contents (%/dm) in different process steps (average values±SE)

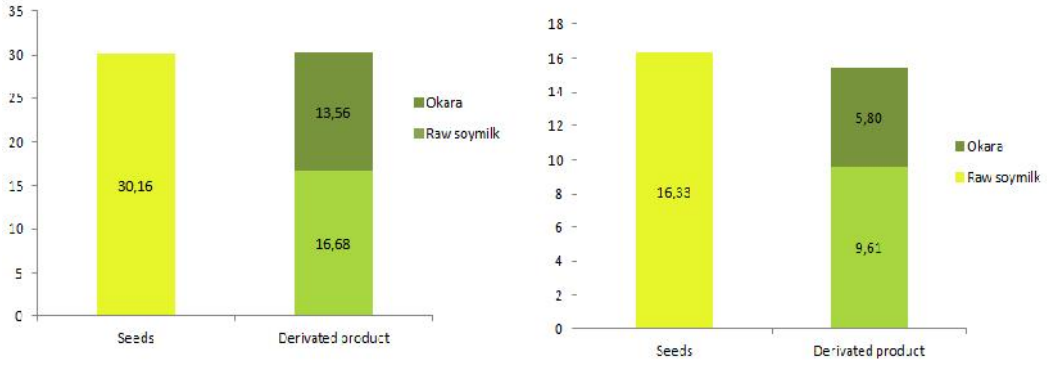

Fig. 25 and **26**: Protein and fat contents in different process steps

As shown in **Figure 27**, hydrophilic antioxidant activity did not vary during seeds soaking but it greatly increased during extraction up to 4.50 mmol ascorbic acid equivalent/100 g dry matter in okara and 13.03 mmol ascorbic acid equivalent/100 g dry matter in raw soymilk (maybe extractability of antioxidants was improved). Soymilk boiling step reduced hydrophilic antioxidant activity up to 8.61 mmol ascorbic acid equivalent/100 g dry matter. Significant variations in lipophilic antioxidant activity was not observed

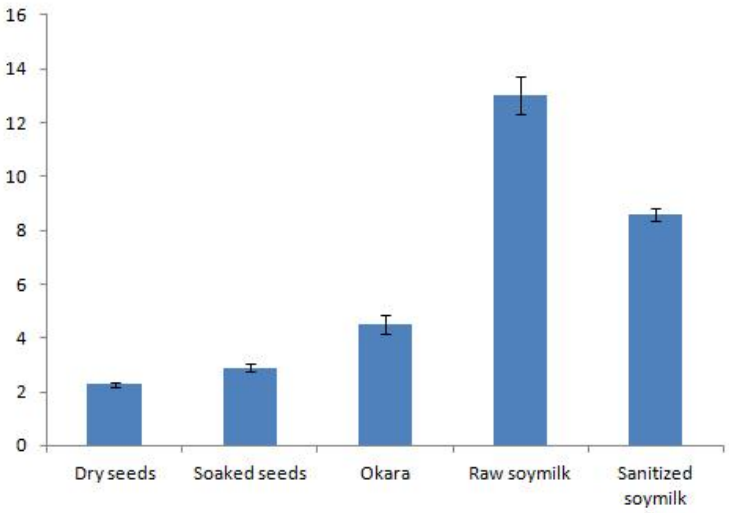

Fig. 27: Hydrophilic antioxidant activity (mmoli ascorbic acid equivalent/ 100 g dry matter) in different process steps (average values±SE)

3.9 Conclusions 2

Choosing optimal process parameters, nutritional and ALISSA criteria were taken into account. Soaking in water at room temperature slows down seed hydration but it minimizes loss of hydrophilic substances; this condition is optimal because of lack of energy expenditure, too. Energy rating had great importance in choosing grinding and extraction condition, too. Milling seeds in water at room temperature and extracting paste by 30 min boiling, okara with the highest protein content and the highest antioxidant activity and soymilk with the highest lipophilic antioxidant activity were obtained. Less importance was attributed to isoflavones content: because of their hot extractability, selected conditions allowed to obtain the lowest concentration. Adding salt and vanilla sugar, the best soymilk formulation was obtained: additives did not mask bean flavor but they improved soymilk taste. Global pleasantness was similar to market control but further improvements are possible: during space missions, in fact, taste perception can change.

3.10 References

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3.11 Topic 3: characterization of soybean seeds and production of soymilk at different ripening stages

In a CELSS, the shortening of plant biologic cycle provides many economic and management benefits.
The aim of this experiment was the characterization of soybean seeds at different maturation stages, in order to identify the optimal harvest time in a CELSS.
Seed composition and nutritional characteristics were investigated, then the seeds were transformed into milk and okara and these products were analyzed: this is the first work about soy meal from seeds at different maturation steps.

3.12 Materials and methods 3

3.12.1 Soybean cultivation

Soybean (cv. Condor), was sown on 29 May 2009 in soil (50 m^2) with a density of 40 pts m^{-2}. During sowing, fertilization was performed with 1.75kg of nutrient (N:P:K 1:1:1). During the cultivation, 5 kg of organic fertilizer were used twice and a treatment against aphids, whiteflies and leafhoppers was performed.
Green seeds were harvested 88 days after soaking and a second harvest was carried 100 days after soaking selecting immature and commercial maturated seeds.

3.12.2 Soymilk extraction

Based on previous experiment results, soybean seeds were processed as follows:

- Soaking in water at room temperature (seed:water =1:10)
- Rinsing
- Grinding in water at room temperature (seed:water = 1:10)
- Extraction by boiling for 30 min
- Filtration
- Sanitization by boiling for15 min

3.12.3 Nutritional and compositional analysis

According to already described protocols samples were analyzed in:

- Protein content
- Fat content
- Hydrophilic and lipophilic antioxidant activity
- Isoflavones content and profile

All determinations were performed three times for each sample.

Phytic acid content concentration was determinate, too. It was measured by Ishiguro et al (2003) method; in order to extract phytic acid from seeds and from okara, 0.5 g of milled product were mixed in 10 mL of 0.5 M HCl and shaken for 1 hour at room temperature, then the mixture was centrifuged

for 6 min at 18000xg and supernatant was analyzed. For soymilk samples, extraction step was not necessary. Phytic acid determination was performed three times for each sample.

3.12.4 Statistical analysis

Data were analyzed by ANOVA and means were compared by Duncan's Multiple Range Test.

3.12 Results and discussions 3

As expected, with advancing of maturation stages, a progressive dry matter accumulation in seeds was observed (**Figure 28**): 34% in green seeds, 52% in immature seeds, 73% at commercial ripeness. Same trend was observed in processed products, but in soymilk were not significant differences between green and immature seeds and in okara were not significant differences between immature and commercial steps.

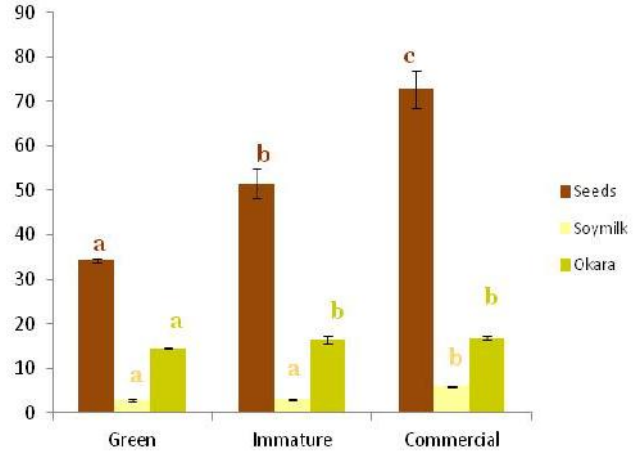

Fig. 28: Dry matter (%) in soybean and soy meat at different maturation stages (average values±SE)

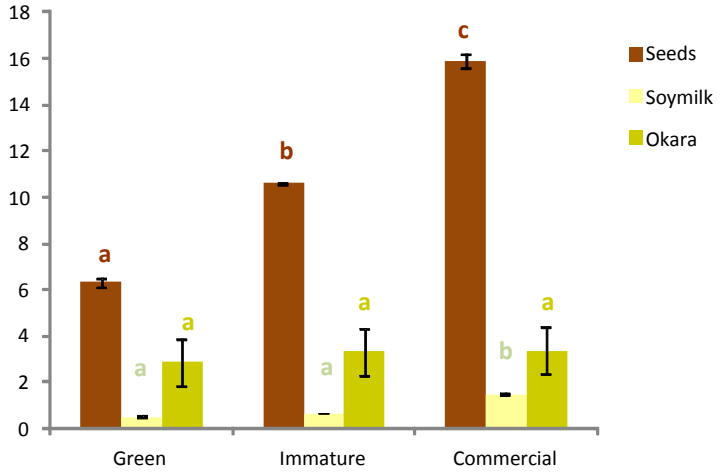

Fig. 29: Protein content (%/fresh weight) in soybean and soy meat at different maturation stages (average values±SE)

As expected, with advancing of maturation stages, a progressive protein accumulation in seeds was observed (6.32% fresh basis in green seeds, 10.60% fresh basis in immature seeds, 15.91% fresh basis at commercial ripeness) In soymilk a difference was observed in product from seeds at commercial ripeness, only (1.50% fresh basis) but differences among okara samples were not detected (see **Figure 27**). Seeds showed gradual and steady increase in fat content but in okara and soymilk differences between green and immature steps were not found (**Figure 30**).

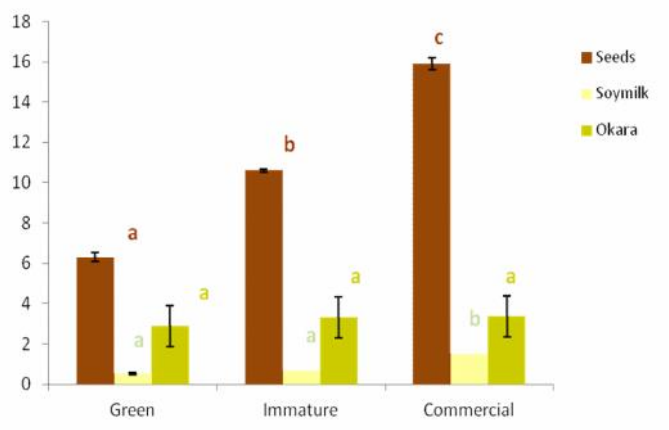

Fig. 30: Fat content (%/fresh weight) in soybean and soy meat at different maturation stages (average values±SE)

Antioxidant activity increased with advancing of maturation steps, too. Hydrophilic component of antioxidant activity (**Figure 31**) showed in seeds a steady and progressive increase but, in okara and soymilk, differences between green and immature steps were not found. Lipophilic antioxidant activity (**Figure 32**) steadily increased in seeds and in soymilk during maturation steps but in okara differences between green and immature steps were not found.

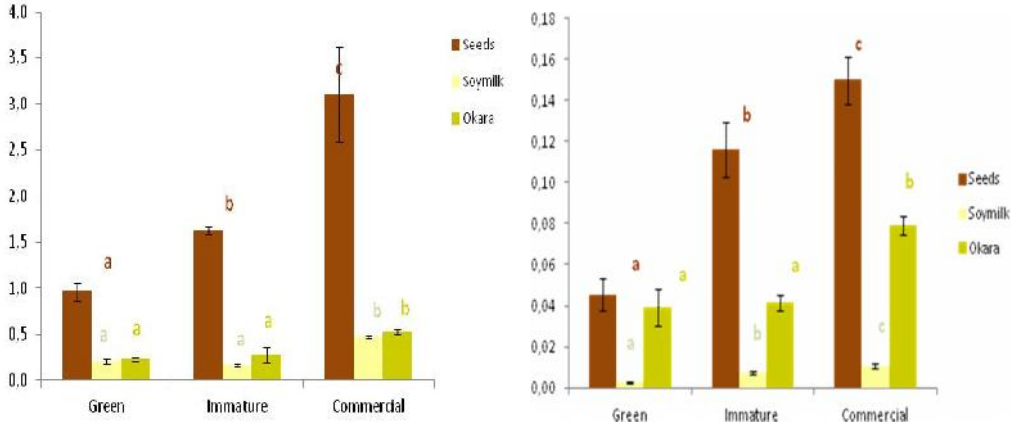

Fig. 31 and **32**: Hydrophilic antioxidant activity (mmol ascorbic acid equivalent/100 g fresh weight) and lipophilic antioxidant activity (mmol Trolox equivalen/100 g fresh weight) in soybean and soy meat at different maturation stages (average values±SE)

According Kim & Chung (2007), total isoflavones content in soybean seeds greatly increased from green seeds (27.46 mg/100g fresh basis) to immature seeds (84.84 mg/100g fresh basis) and lightly increased to commercial ripeness (120.90 mg/100g fresh basis). Same trend was found in okara and in soymilk.

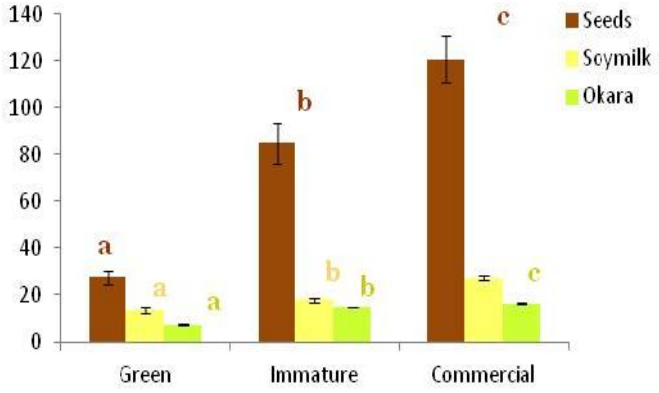

Fig. 33: Total isoflavones content (mg/100 g fresh weight) in soybean and soy meat at different maturation stages (average values±SE)

Tables 8, 9and **10** report isoflavones profile in soymilk and processed products: important differences were found at immature step. According Kim & Chung (2007), soybean seeds showed, with advancing of maturation steps, a percent reduction in genistein and acetyl-genistin. Kim & Chung (2007) reported, with advancing of maturation steps, an increasing in glucosides and malonyl-glucosides, too. On the contrary, our data showed an increasing in genistin but not in daidzin, an increasing in malonyl-daidzin but not in malonyl-genistin. Similarly in soymilk, reduction in acetyl-genistin and increasing in genistin were observed with advancing of maturation stages. Genistein showed increasing from green to immature stages. No differences were observed in malonylic forms. Similarities between seeds and okara were observed in decreasing trend for malonyl-genistin, daidzin and glycitin and in increasing trend for genistein. Acetyl-genistin was not detected in pulp from soymilk extraction.

		Green	Immature	Commercial maturation
Daidzin	mg/100g dm	8.87[a]	14.01[a]	15.97[a]
	(%tot)	(11.7[a])	(8.3[b])	(9.3[ab])
Glycitin	mg/100g dm	10.93[a]	11.26[a]	12.18[a]
	(%tot)	(14.4[a])	(6.7[b])	(7.2[b])
Genistin	mg/100g dm	11.89[b]	44.56[a]	48.66[a]
	(%tot)	(17.8[b])	(27.3[a])	(29.6[a])
Malonyl-daizin	mg/100g dm	74.31[a]	71.69[a]	23.67[b]
	(%tot)	(31.2[b])	(45.2[a])	(43.3[a])
Malonyl-genistin	mg/100g dm	7.47[a]	9.17[a]	8.27[a]
	(%tot)	(10.0[a])	(5.7[b])	(4.9[b])
Acetyl-genistin	mg/100g dm	6.89[b]	9.57[a]	7.76[b]
	(%tot)	(9.0[a])	(5.7[b])	(4.8[b])
Genistein	mg/100g dm	4.30[a]	1.59[a]	1.66[a]
	(%tot)	(5.76[a])	(0.83[b])	(0.9[b])

Tab 8. Isoflavones profile in soybean at different maturation stages. Different letters within the same line indicate significant differences at $P<0.05$

		Green	Immature	Commercial maturation
Daidzin	mg/100g dm	139.51 ^{ab}	152.13^{a}	107.39^{a}
	(%tot)	(29.54^{a})	(25.86^{b})	(23.27^{b})
Glycitin	mg/100g dm	88.51^{a}	39.43^{b}	37.70^{b}
	(%tot)	(19.08^{a})	(6.820^{c})	(8.18^{b})
Genistin	mg/100g dm	69.64^{b}	186.39^{a}	149.14^{a}
	(%tot)	(14.89^{b})	(32.23^{a})	(32.26^{a})
Malonyl-daizin	mg/100g dm	110.60^{a}	138.41 ^{a}	109.61^{a}
	(%tot)	(23.88^{a})	(23.44^{a})	(23.86^{a})
Malonyl-genistin	mg/100g dm	35.46^{a}	40.49^{a}	30.76^{a}
	(%tot)	(7.42^{a})	(6.87^{a})	(6.69^{a})
Acetyl-genistin	mg/100g dm	17.29^{a}	10.48^{a}	11.41^{a}
	(%tot)	(3.54^{a})	(1.80^{b})	(2.48^{b})
Genistein	mg/100g dm	8.74^{a}	17.62^{a}	14.97^{ab}
	(%tot)	(1.65^{b})	(2.98^{a})	(3.26^{a})

Tab 9. Isoflavones profile in soymilk from seeds at different maturation stages. Different letters within the same line indicate significant differences at $P<0.05$

		Green	Immature	Commercial maturation
Daidzin	mg/100g dm	10.88^{b}	16.13^{a}	16.03^{a}
	(%tot)	(13.19^{a})	(11.38^{b})	(11.11^{b})
Glycitin	mg/100g dm	8.44^{a}	8.20^{a}	8.76^{a}
	(%tot)	(16.72^{a})	(9.03^{b})	(9.05^{b})
Genistin	mg/100g dm	12.24^{b}	28.14^{a}	28.59^{a}
	(%tot)	(24.22^{c})	(31.02^{a})	(29.50^{b})
Malonyl-daizin	mg/100g dm	10.28^{c}	23.30^{b}	27.44^{a}
	(%tot)	(21.56^{c})	(25.67^{b})	(28.32^{a})
Malonyl-genistin	mg/100g dm	6.65^{b}	10.33^{a}	10.76^{a}
	(%tot)	(13.19^{a})	(11.38^{b})	(11.11^{b})
Acetyl-genistin	mg/100g dm	1.40^{c}	4.63^{b}	5.30^{a}
	(%tot)	(2.78^{b})	(5.10^{a})	(5.47^{a})

Tab. 10: Isoflavones profile in okara from seeds at different maturation stages. Different letters within the same line indicate significant differences at $P<0.05$

As in other legumes (Honke et al., 1998), soybean seeds showed, with advancing of maturation steps, a gradual and steady phytic acid accumulation: 0.44 % fresh basis in green seeds, 1.13% fresh basis in immature seeds, 1.37% fresh basis in commercial matured seeds. Less clear is trend in transformation products. Soymilk from different matured seeds did not show significant differences and okara showed differences between green step (0.062% fresh basis) and commercial maturation step (0.094 % fresh basis) (see **Figure 34**) .

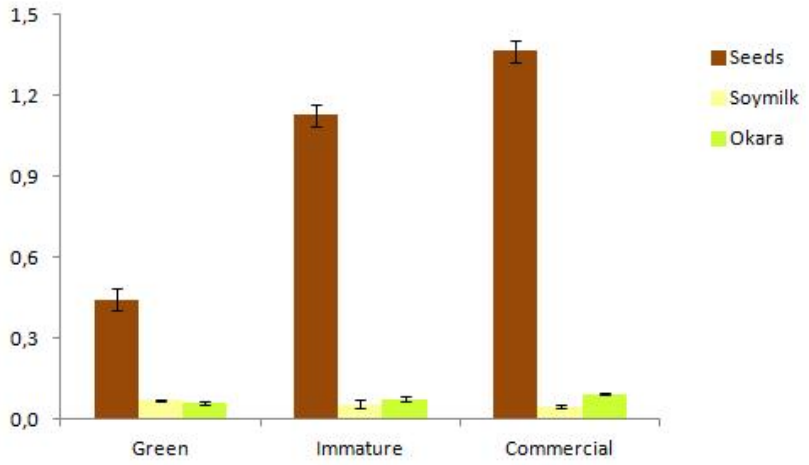

Fig. 34 Phytic acid content in soybean and soy meat at different maturation stages (average values±SE)

3.13 Conclusions 3

Harvest of immature seeds and consequent shortening in production cycle would result great management and economic benefits within a CELLS system. From a nutritional point of view, seeds and transformed products with lower phytic acid content could be obtained. On the other hand, it is well known that accumulation of dry matter and nutrients in soybeans seeds increases with advancing of maturation steps. For dry matter, this trend was mitigated with transformation but for fat and protein, the highest concentration was observed in soymilk and okara from commercial matured seeds. Antioxidant activity, also, greatly increased with advancing of maturation steps. Advantages of production cycle shortening can not justify the large losses nutritional and, for these reasons, optimal harvest moment within a CELSS is commercial maturation (when pods become dark, leaves turn yellow and fall).

3.14 References

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3.15 Topic 4: comparison among soybean cultivars: suitability to CELSS cultivation and to milk transformation

The aim of this study was to compare nutritional composition of seeds and their derived products soymilk and okara of four soybean cultivars obtained by traditional cultivation system in soil (open field) and by cultivation in a CELSS.

3.16 Materials and methods 4

3.16.1 Plant material

Commercial seeds, field grown in the year 2009, were obtained from Italian sellers (see **Table 11**).

Cultivar	**Seller**
Atlantic	Venturoli Sementi Srl
Cresir	Pioneer Hi-Bred Italia Sr
PR91M10	Pioneer Hi-Bred Italia Sr
Regir	Pioneer Hi-Bred Italia Sr

Tab 11: Commercial seeds origin

Cultivars were selected among not genetically modified varieties admitted in European Union, on the basis of agronomical and technological properties (yield, tolerance to biological and abiotic stresses, suitability to industrial uses). Seeds were analyzed, used for soymilk extraction and okara production and for hydroponic cultivation.

3.16.2 CELSS cultivation

The experiment was carried out in growth chamber with controlled environment (**Figure 35**). Light was provided by High Intensity Discharge lamps (400 W) with a light/dark regime of 12/12 hours. Temperature regime was established at 26/20 °C (light/dark) and relative humidity was kept within the optimum range of 65-75% using a fog system; air change and dehumidification was guaranteed by

two air extractors. Plants were grown in plastic double gullies using a re-circulating Nutrient Film Technique (NFT). The nutrient solution was based on the standard Hoagland recipe 1/2 strength modified by Wheeler et al. (2008), according to specific requirement of soybean. Electrical conductivity and pH were kept at 2.0 dS m^{-1} and 5.8, respectively, and adjusted every two days. Soybean pods were harvested twice a week, starting from 114 day after soaking at the moment of commercial ripeness.

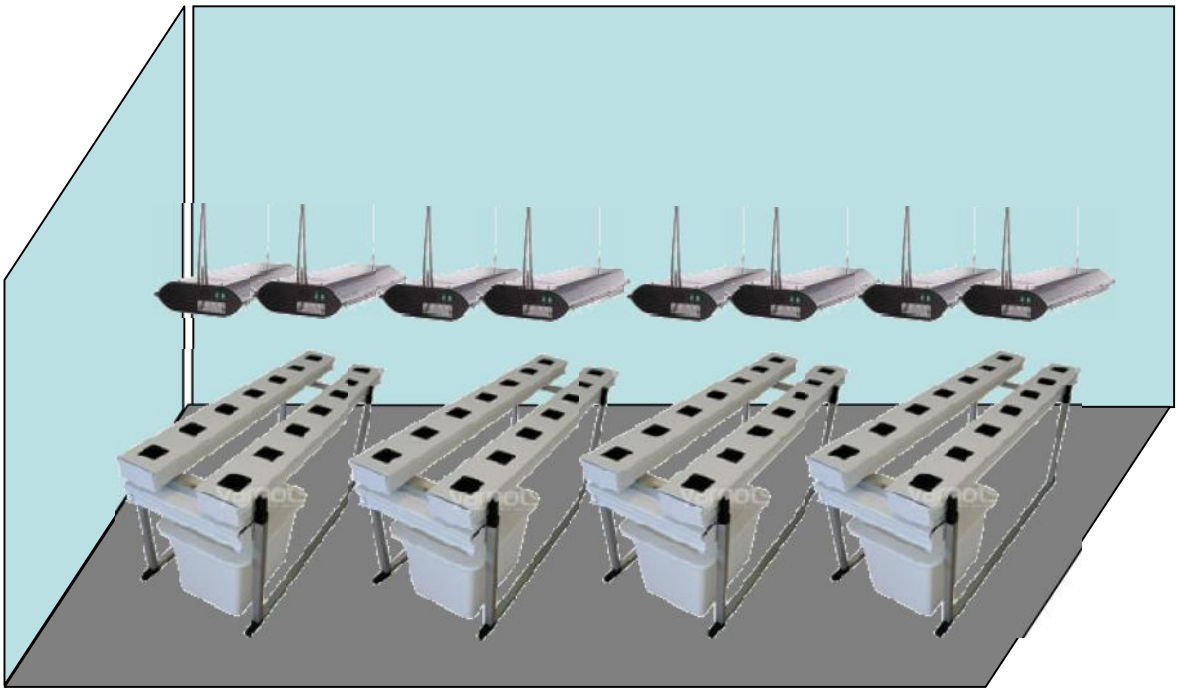

Fig. 35: CELSS cultivation simulation

3.16.3 Seeds analysis

According to already described protocols seeds from field and from CELSS were analyzed in:

- Protein content
- Fat content
- Hydrophilic and lipophilic antioxidant activity
- Isoflavones content and profile
- Phytic acid content

Total fiber content was determined, too. AOAC 985.29 gravimetric method (Prosky et al., 1998) was performed.
Ash content was determined by treatment at 550±1°C in a muffle furnace (AOAC, 1995).
In order to quantify mineral contents, a single extraction was performer: 0.5 g dry and milled seeds were dissolved in 100 mL water and clarified by Activated Charcoal Powder. Specific reagents then were used. PhosVer3 Phosphate Reagent (sodium molybdate and ascorbic acid) was used for phosphorus determination by spectrophotometric reading at 890 nm. Sodium tetraphenylborate was used for potassium determination by spectrophotometric reading at 650 nm. Calmagite was used for calcium and magnesium determination by spectrophotometric reading at 522 nm.
All determinations were performed three times for each sample.

3.16.4 Soymilk extraction

Based on previous experiment results, soybean seeds were processed as follows:

- Soaking in water at room temperature (seed:water =1:10)
- Rinsing
- Grinding in water at room temperature (seed:water = 1:10)
- Extraction by boiling for30 min
- Filtration
- Sanitization by boiling for 15 min

3.16.5 Statistical analysis

Differences among cultivars were determined by analysis of variance and Duncan's multiple range test ($P \leq 0.05$). Differences between field and hydroponically grown samples were determinate by Student's t-test ($P \leq 0.05$).

3.17 Results and discussions 4

Figure 36 summarized the variations in proximate composition of soybean seeds determined by the cultivation method. The data were the average of the four different cultivars and, despite the variability among cultivars, results are significant: hydroponic cultivation in controlled environment increased fats, total dietary fiber and phytic acid and reduced the amount of isoflavones, compared to soil cultivation in open field.

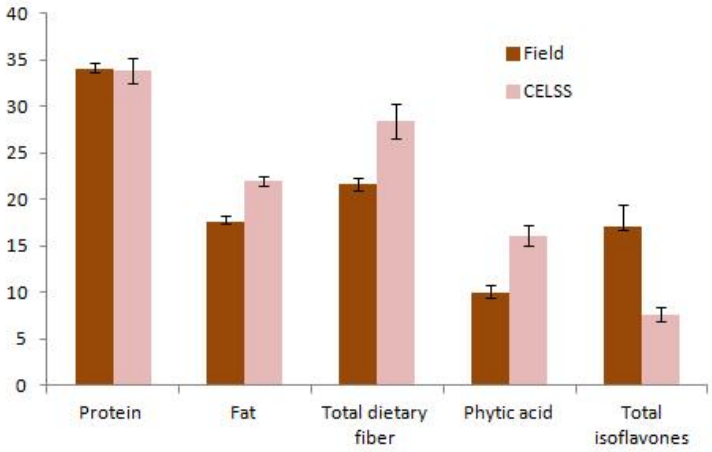

Fig. 36: Protein content (%/dry mass), fat content (%/dry mass), total dietary fiber content (%/dry mass), phytic acid content (g/Kg dry matter) and total isoflavones content (mg/Kg dry matter) in seeds from field and from CELSS. Data are the mean values of the four analyzed cultivars ± SE.

Figure 37 shows the effect of cultivation system on ash and mineral content. According to previous works (Jurgonski et al., 1997, Wheeler et al., 1995), soybean seeds from CELSS showed higher ash content values than field control without significant differences among cultivars.
In each analyzed mineral, differences among cultivars were not detected but, respect to field control, seeds from CELSS showed higher magnesium content (2.2 vs 1.5 g/Kg dry matter as average values of four cultivars) and phosphorus content (2.5 vs 1.9 g/kg dry matter as average values of four cultivars). Differences in potassium and calcium content were not found.

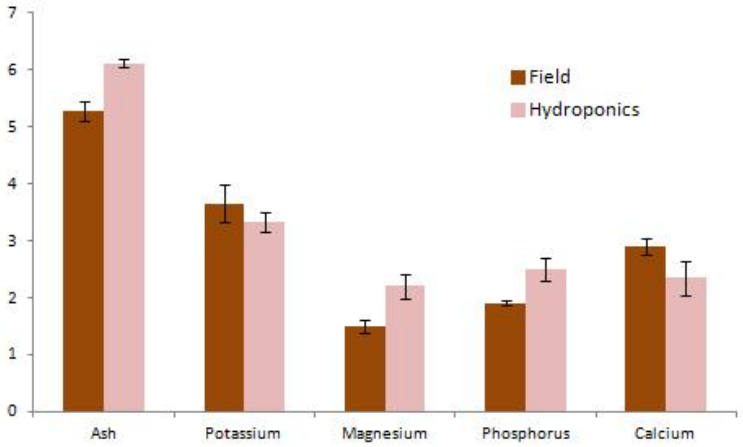

Fig 37: Ash (% dry basis), potassium (% dry basis), magnesium (g/Kg dry basis), phosphorus (g/Kg dry basis) and calcium (g/Kg dry basis), in seeds from field and from CELSS. Data are the mean values of the four analyzed cultivars ± SE.

Protein content presented a significant interaction cultivation system X cultivar. Atlantic cultivar is very suitable to CELSS cultivation because it increased its protein content from 32.52% dry basis (field control) to 33.80% dry basis. On the other hand, Cresir seeds showed a reduction from 35.95% dry basis in field control to 34.11 % dry basis in CELSS. Seeds from Pr91m10 and Regir cultivars did not show significant difference between two analyzed cultivation methods (**Table 12**).
Previous studies are not in agreement: according to Dornbos & Mullen (1992), the higher water availability reduces protein accumulation in soybean seeds, Jurgonski et al. (1997) reported an increases in soybean seeds protein content as CELSS effect, Wheeler et al. (1995) did not reported significant differences between soil and CELSS cultivations, studies on different pulses, such as peanuts, reported that protein concentrations in the seeds harvested from hydroponic system and field cultivation are not different (Liu et al., 2003).
Table 12 compares composition of soybean seeds and their transformed products from two cultivation systems.

	Seeds			Soymilk			Okara		
	Field	*Hydroponics*		*Field*	*Hydroponics*		*Field*	*Hydroponics*	
	Protein content (g/100 g dry matter)								
Atlantic	32.48 ^{b}	33.92 ^{b}	*	35.63 ^{b}	37.79 ^{a}	*	34.65 ^{b}	35.83 ^{a}	*
Cresir	35.95 ^{a}	34.11 ^{b}	*	39.60 ^{a}	36.81 ^{ab}	*	43.25 ^{a}	32.04 ^{bc}	*
Pr91M10	35.27 ^{a}	35.55 ^{a}	*n.s.*	37.98 ^{a}	36.31 ^{ab}	*n.s.*	33.60 ^{b}	33.12 ^{b}	n.s.
Regir	32.52 ^{b}	31.96 ^{c}	*n.s.*	36.25 ^{b}	35.39 ^{b}	*n.s.*	32.86 ^{b}	31.60 ^{c}	n.s.
	Fat content (g/100 g dry matter)								
Atlantic	17.60 ^{a}	21.96 ^{ab}	*	18.13 ^{a}	18.24 ^{a}	*n.s.*	11.86 ^{a}	19.97 ^{ab}	*
Cresir	19.28 ^{a}	22.09 ^{a}	*	19,09 ^{a}	17.06 ^{a}	*n.s.*	9.45 ^{b}	20.98 ^{a}	*
Pr91M10	16.70 ^{a}	21.19 ^{a}	*	15.22 ^{a}	16.96 ^{a}	*n.s.*	9.40 ^{a}	19.11 ^{b}	*
Regir	16.99 ^{a}	22.50 ^{b}	*	18.90 ^{a}	18.12 ^{a}	*n.s.*	12.59 ^{b}	20.80 ^{a}	*
	Total dietary fiber content (g/100 g dry matter)								
Atlantic	21.65 ^{a}	27.51 ^{b}	*	n.d.	n.d.		50.11 ^{a}	64.84 ^{a}	*
Cresir	19.26 ^{b}	27.32 ^{b}	*	n.d.	n.d.		42.91 ^{b}	55.10 ^{c}	*
Pr91M10	22.10 ^{a}	27.57 ^{b}	*	n.d.	n.d.		48.78 ^{a}	57.61 ^{b}	*
Regir	23.68 ^{a}	31.44 ^{a}	*	n.d.	n.d.		50.92 ^{a}	65.35 ^{a}	*
	Phytic acid content (g/kg dry matter)								
Atlantic	14.04 ^{a}	15.71 ^{a}	*	14.80 ^{a}	16.63 ^{a}	*n.s.*	12.66 ^{a}	15.39 ^{a}	*
Cresir	11,47 ^{ab}	16,25 ^{a}	*	12,33 ^{ab}	9,49 ^{b}	*n.s.*	13,86 ^{a}	15,33 ^{a}	*
Pr91M10	12.12 ^{a}	16,84 ^{a}	*	12.69 ^{ab}	7.93 ^{b}	*n.s.*	10.48 ^{b}	15.25 ^{a}	*
Regir	8.94 ^{b}	15.69 ^{a}	*	8.95 ^{b}	9.88 ^{b}	*n.s.*	9.26 ^{b}	14.49 ^{a}	*
	Total isoflavones content (mg/kg g dry matter)								
Atlantic	12.09 ^{c}	7.67 ^{a}	*	44.80 ^{b}	48.58 ^{b}	*n.s*	13.64 ^{b}	18.27 ^{a}	*
Cresir	27.08 ^{a}	7.27 ^{a}	*	65.28 ^{a}	44.96 ^{b}	*	28.25 ^{a}	16.74 ^{b}	*
Pr91M10	10.39 ^{d}	7.27 ^{a}	*	37.19 ^{b}	45.40 ^{b}	*n.s*	11.66 ^{c}	15.42 ^{c}	*
Regir	18.61 ^{b}	8.41 ^{a}	*	44.60 ^{b}	54.60 ^{a}	*	13.44 ^{b}	14.91 ^{d}	*

Tab 12: Proximate composition, phytic acid and total isoflavones content of field and hydroponic grown soybean cultivars and derivates products. Different letters within the same column indicate significant differences at P<0.05; * and ns indicate significant and not significant differences between cultivation systems at P<0.05.

The protein concentrations in the seed parallel those observed in soymilk and okara: products from field and hydroponically grown plants did not show differences in Pr91m10 and Regir, while they gave higher values in CELSS for Atlantic and in soil for Cresir. Protein content ranges from 35.39 to 39.60 g/100 g dry mass in soymilk and from 31.60 to 43.25 g/100 g dry mass in okara, confirming previous results reported by other authors for the same products (Cruz et al., 2007; Peñalvo et al., 2004; Prèstamo et al., 2007; Ma et al., 1997).

Values of fat content in soybean seeds fall within the range reported in the literature, with no relevant differences among cultivars (see **Table 12**). However, it is worth to notice that, in agreement with Jurgonski et al. (1997). CELSS cultivation caused an increase in fat content from 17.37 to 21.94 g/100 dry matter, on the average of the tested cultivars; according to Dornbos & Mullen (1992) this increase can be due to the constant water availability favoring oil accumulation in soybean.

Fat content in soymilk and okara (**Table 12**) are consistent with previous works (Cruz et al., 2007; Peñalvo et al., 2004; Prèstamo et al., 2007; Ma et al., 1997). In agreement with Cai et al. (1997) and with Mullin et al. (2001), cultivar selection did not affect lipid content in soymilk as it did in okara. This distribution of fats in soybean products is not surprising as fat extractability in water is limited independently from the amount of fats in the starting material. At the same time data from hydroponics showed that the higher the fat content in seeds, the higher the fat content in okara.
CELSS cultivation increased dietary fiber content in soybean seeds from 21.67 to 28.46 g/100 g dry matter (mean values among the four analyzed cultivars); Grieshop & Fahey (2001) did not report significant difference in dietary fiber as environmental effect but our data agree with Wheeler et al. (1995). Significant differences among cultivars were detected and Regir seeds showed the highest value (27.56 g/100 g dry matter on the average between of the two cultivation systems). Dietary fiber from seeds is recovered in residue pulp and our data are in agreement with previous works. The same cultivation effect observed in seeds was found in okara .
Phytic acid content was significantly higher in seeds from CELSS than from field (1.61% vs 0.99% dry basis). This could be related to higher phosphorus content: in open field cultivation, phytic acid concentration has been demonstrated to be positively correlated to the available levels of phosphorus in the soil (Miller et al., 1980). CELSS cultivation eliminated differences among cultivars observed in seeds form field. The same trend was observed for phytic acid content in okara and not in soymilk, with higher concentration in okara from hydroponics soybean (1.51 g/100 g dry mass vs 1.16 g/100 g dry mass in open field, on the average of the four cultivars). Significant differences among cultivars were found in both processing products. Omosaiye & Cheryan (1979) and Beleia et al. (1993) reported slightly different distribution of phytic acid between milk and pulp (1.68 g/100 g dry matter in soymilk and 1.83 g/100 g dry matter in okara, respectively) however they analyzed market products and not simultaneous experimental production of milk and okara.
Figure 36 shows significant lower content of total isoflavones, in the hydroponically grown seeds compared to those from field (17.04 vs 7.66 mg/kg dry matter). Data for each cultivar confirmed that this trend was observed in all the analyzed cultivar; however it is worth to notice that, besides inhibiting isoflavones biosynthesis, hydroponic cultivation eliminated the differences among cultivars observed in field grown samples.
As observed in previous works (Eldridge & Kwolek, 1983; Wang & Murphy, 1994; Tepavcevic et al., 2010), significant differences among cultivars were detected. Among field grown cultivars, Cresir showed the best performance in total isoflavones content accumulation in seeds (171.80 mg/100 g dry matter) and in both derived products (551.21 and 224.97 mg/100 g dry matter in soymilk and okara, respectively). Among hydroponically grown cultivars significant differences were not detected in seeds and different isoflavones distributions between soymilk and okara were found: the highest value in soymilk was in Regir product, the highest value in okara was in Atlantic product, the lowest value in okara was in Regir product.
In **Table 13** the isoflavones pattern found in soybean seeds and soybean products was reported. In field-grown seeds, the most abundant component was malonyl-daidzin (36.6% as average of the four cultivars), followed by β-glucosides genistin and daidzin (26.5% and 18.0% on the average of the four cultivars, respectively); acetyl-genistin was not detected. This data are in agreement with those of previous reports (Eldridge & Kwolek, 1983*;* Hoeck et al., 2000; Lee et al., 2004; Romani et al., 2003). In seeds from CELSS, the percentage of malonyl-daidzin was drastically reduced (9.0% of the four cultivars) and the most abundant forms were β-glucosides daidzin and genistin (34.1% and 28.25% as average of the four cultivars, respectively). In Pr91m10 and Regir hydroponically grown seeds, acetyl-genistin was detected at very low level.
Compared to the seeds, transformation products showed wider distribution of isoflavones forms: according to Jung et al. (2008), heat treatment causes in soymilk a shift towards the β-glucoside and aglycone forms with a corresponding decrease in the malonyl-daidzin content. In soymilk obtained from field grown seeds, the most abundant component was genistin (30.2% as average of the four cultivars), followed by daidzin (20.8% as average of the four cultivars): these values did not significantly changes when soymilk was obtained from hydroponically grown seeds. Soymilk obtained from CELSS seeds showed an increase in malonyl-daidzin percent content up to 25.78% (average of the four cultivars) compared to soymilk from field grown seeds. In okara from field grown seeds, the most abundant components were malonyl-genistin and genistin (22.1% and 19.2%, respectively): comparing these data with okara from hydroponic seeds a decrease in first constituent and an increase in the second one was observed. An increase in daidzin and in malonyl-daidzin was observed too.

	Seeds			Soymilk			Okara		
	Field	*Hydroponics*		*Field*	*Hydroponics*		*Field*	*Hydroponics*	
Daidzin	18.05	34.08	**	20.79	22.68	*n.s.*	13.49	18.61	**
Glycitin	5.87	10.03	**	8.61	4.53	**	6.03	6.12	*
Genistin	26.50	28.25	**	30.19	31.12	*n.s.*	19.15	25.34	**
Malonyl-daidzin	36.55	8.97	**	18.17	25.78	**	17.65	23.87	**
Malonyl-genistin	10.96	9.00	**	12.88	9.77	**	22.13	14.10	**
Acetyl-genistin	*n.d.*	1.47		1.38	1.53	*n.s.*	4.74	4.18	**
Genistein	2.07	8.20	**	7.99	4.59	**	16.81	7.77	**

Tab 13 Isoflavones profile in field and hydroponic grown soybean seeds and derivates products (% of the total). Data were expresses as mean values of the four analyzed cultivars. ** and n.s. indicate significant and not significant differences between cultivation systems at P<0.01.

The biological activity of soy isoflavones is not related to the type of glycoside as deglycosilation is a prerequisite for their absorption (Xu et al., 2000). On the contrary, isoflavones activity depends on the aglycone moiety: daidzein and glycitein has less estrogenic activity than genistein (Potter et al., 1998). In field-grown seeds, amount of total daidzein was the highest (54.6%), followed by total genistein (39.5%) and total glycitein was the lowest (5.9%) considering the average concentrations of the four cultivars. In derivates products from field-grown seeds the amount of total genistein was the highest (52.4% in soymilk and 62.8% in okara), followed by total daidzein (39.0% and 31.1% in soymilk and okara, respectively) and total glycitein was the lowest (8.6% and 6.0% in the two analyzed products) considering the average concentrations of four analyzed soybeans. This evidence was in agreement with the previous observations that soybeans and soy foods usually contain similar amounts of genistein and daidzein and a much lower amount of glycitein (Hoeck et al., 2000). In seeds from CELSS, there was a shift towards the content of total genistein (45.4%) and total glycitein (10.0%) at the expense of total daidzein (43.0%) so, there was a reduction in total isoflavones content but, on the other hand increased the percentage of genistein, the form with greater biological activity.

3.18 Conclusions 4

Data showed that CELSS cultivation is not only a method to produce food in space, but also a system to increase macronutrient content and to improve nutritional value of soybean products. Hydroponic cultivation in a controlled environment promoted seeds accumulation of fat, total dietary fiber and minerals such as phosphorus and magnesium. On the other hand, the higher phosphorus concentration increased the phytic acid content. Accurately designed hydroponic systems do not induce stress in plants and, therefore, the concentration of isoflavones is dramatically reduced but higher percentage of genistein, the isoflavones with greater biological activity was recovered in seeds from CELSS cultivated plants.
CELSS determined different performances compared to open field cultivation and, for this reason, selecting optimal cultivar for food production in the space, only data from experimental cultivation must be considered. Anyway, identification of the best cultivar will always be strongly influenced by the final destination of the soybeans. If you assign more importance to the lipid fraction, the best cultivar is Regir. With a view to enhancing the protein component, the choice will fall on Pr91m10.
Looking at the soybean derived product seeds cultivation system did not significantly modify the soymilk composition; in okara products same compositional differences of the seeds were observed. Even in this case the choice of the best cultivar will be contextualized within the diet of astronauts:

good protein and fiber values were observed in transformed products from Atlantic seeds but, if you need a higher fat content, you will choose other cultivars such as Cresir or Regir.

3.19 References

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HYDROPONIC CULTIVATION IMPROVES THE NUTRITIONAL QUALITY OF SOYBEAN AND ITS PRODUCTS

Mariantonella Palermo[1], Roberta Paradiso[1], Stefania De Pascale[1], Vincenzo Fogliano[2]

[1]Dept. Agricultural Engineering and Agronomy, University of Naples Federico II, via Università 7 100, I-80055 Portici (Naples), Italy.

[2]Dept. of Food Science, University of Naples Federico II, Via Università 133, Parco Gussone 10 Edificio 84, I-80055 Portici (Naples), Italy.

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ABSTRACT

Hydroponic cultivation allows to control environmental conditions, save irrigation water, increase productivity and prevent plant infections. The use of this technique for large commodities such as soybean is not a relevant issue on fertile soils, but hydroponic soybean cultivation could provide proteins and oil in adverse environmental conditions. In this paper the composition of four cultivars of soybean seeds and their derivates, soymilk and okara, grown in hydroponics was compared to that of the same cultivar obtained from soil cultivation in open field. Beside proximal composition, the concentration of phytic acid and of isoflavones was monitored in the seeds, soymilk and okara.

Results demonstrated that, independently from the cultivar, hydroponic compared to the soil cultivations promoted the accumulation of fats (from 17.37 to 21.94 g/100 g dry matter) and total dietary fiber (from 21.67 to 28.46 g/100 g dry matter); reduced isoflavones concentration (from 17.04 to 7.66 mg/kg dry matter), while protein concentration was unaffected. The differences found in seed composition were confirmed in the respective okara products, while the effect of cultivation system was not significant looking at the soymilk composition.

Data showed that hydroponic cultivation improved the nutritional quality of soybean seeds as regard fats and dietary fibre. They also suggests that specific cultivar should be selected to obtain the desired nutritional features of the soybean raw material depending on its final destination.

INTRODUCTION

Hydroponic cultivation is an emerging technology as it allows a better control of water and nutrient supply, improves plant productivity, permits to avoid the crop rotation and reduces the use of pesticides *(1)*. Hydroponic methods are particularly useful in the region where soil or climate that are not suitable for crop cultivation. As population increases and arable land declines, hydroponics could replace traditional agriculture. There has already been a great deal of buzz in the scientific community for the potential use of hydroponics in third world areas and this technique will be important to provide fresh food according the Space programs for long-term colonization of Mars or the Moon *(2)*.

In these contexts also the cultivation of high nutritional density crops, such as soybean, will be considered. Soybean seeds [*Glycine max* (L.) Merr.] are an important source of protein and oil and contain high amounts of components with health benefits, such as dietary fiber and other biologically active substances such as isoflavones. Despite several health properties, the nutritional value of soybean and soy based meals is lower than expected: this is due to the presence of different compounds usually known as anti-nutritional factors reducing nutrient availability. The main one is phytic acid, the storage form of phosphorus in seeds, acting as antinutrient as it chelates various metals (Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}) and it binds to some proteins, resulting in an overall decrease of protein and minerals bioavailability *(3)*. On the other hand, many recent evidence suggested that phytate also have many positive effect which can be considered more prominent than the reduction of mineral bioavailability for the majority of consumers *(4)*.

Soybean seeds provide several transformed products with multiple gastronomic uses, particularly in vegetarian nutrition. Soymilk is one of the most popular: it is a water extract of soybean seeds, resembling animal milk in physical appearance. Because of its nutritional profile, it is used as an animal milk complement or substitute. Soymilk is not only a nutritious food but also a product made with a simple process involving a relatively low level of technology. The co-product of soymilk extraction, named okara or soypulpe, is rich in dietary fiber (50-60%), protein and fat and it contains a significant levels of B group vitamins *(5, 6)*. Okara is used in both animal and human nutrition; and

also to partially replace wheat flour for bread making and as a fermentation stock for the production of seasonings, spices, and tempeh *(7)*. Recently it has been also used as functional dietary additive in biscuits and snacks *(6)*.

Genetic factor and growth environmental conditions have a great impact on chemical composition and nutrient quality of soybean seeds. Protein content can range from 30 to 44% *(8,9,10)*; oil amount from 15 to 22% *(11,12,9)*, depending on soybean cultivar and soil or climate characteristics. Grieshop & Fahey *(13)* showed that dietary fiber content differed among cultivars from 11 to 19%, but not among countries of cultivation. The isoflavones content of different 65 soybean cultivars is extremely variable, ranging from 64 to 459 mg/100 g of dry matter of seed weight, depending on genetic factors, sowing conditions, geographic location and temperature during cultivation *(14,15,16,17)*. Also phytic acid content of soybeans can vary considerably; field type cultivars, which are the usual items of commerce, fall into the range of 1.0-4.5% *(18,19,9)*.

Many potential health benefits of isoflavones from soy products have been investigated, particularly on breast cancer, vascular disease, osteoporosis and menopausal symptoms, however they can also have negative effect for instance, on the reproductive system or during lactation (*20*)

The aim of this study was to compare the nutritional composition of soybean seeds and of the derived products soymilk and okara in commercial seeds of four soybean cultivars obtained by a traditional cultivation system in soil (open field), and by cultivation in the hydroponic system (Nutrient Film Technique - NFT).

MATERIALS AND METHODS

*Plant material*s. Commercial seeds of four soybean cultivars ('Atlantic', 'Cresir', 'Pr91m10' and 'Regir'), field grown in the year 2009, were obtained from Italian sellers ("Venturoli Sementi Srl" for 'Atlantic' seeds, "Pioneer Hi-Bred Italia Srl" for 'Cresir', 'Pr91m10' and 'Regir' seeds).

Cultivars were selected among not genetically modified varieties admitted for the cultivation in European Union. They were chosen on the basis of agronomical and technological properties (yield, tolerance to biological and abiotic stresses, suitability to industrial uses). Seeds were analyzed, used for soymilk extraction and okara production.

Hydroponic cultivation. The same varieties above described were cultivated under hydroponic conditions. The experiment was carried out in growth chamber with controlled environment. Light was provided by High Intensity Discharge lamps (400 W) with a light/dark regime of 12/12 hours.

Temperature regime was established at 26/20 °C (light/dark) and relative humidity was kept with in the optimum range of 65-75% using a fog system; air change and dehumidification was guaranteed by two air extractors. Plants were grown in plastic double gullies using a re-circulating Nutrient Film Technique (NFT). The nutrient solution was based on the standard Hoagland recipe ½ strength modified by Wheeler et al. *(21)*, according to specific requirement of soybean. Electrical Conductivity and pH were kept at 2.0 dS m-1 and 5.8, respectively, and adjusted every two days. Soybean pods were harvested twice a week, starting from 114 day after soaking, when pods had turned to brown color. The obtained seeds were analyzed and used for soymilk extraction.

Soymilk and okara preparation. **Figure 1** summarized the procedure to obtain soymilk and okara. Soybeans were soaked in water (ratio 1:10 weight /volume) at room temperature for 24 hours and soaked soybeans were milled with the same amount of water. The obtained 99 paste was boiled for 30 minutes and the material filtered to separate soymilk from okara. The last step was soymilk sanitization (15 minutes boiling). Milk extraction was performed twice for each cultivar.

Proximate composition. Each sample of seeds, soymilk and okara was freeze-dried, blended and homogenized by grinding to a fine powder so results for each analysis were expressed on dry basis.

Fat content was measured by extraction with diethyl ether in a Soxhlet system *(22)*. Proteins were analyzed as total nitrogen content by the Kjeldahl procedure (*23*), and the conversion factor used to transform nitrogen into protein was 5.71. Total fiber content was determined by AOAC 985.29 gravimetric method *(24)*. All compositional determinations were performed three times for each sample.

Phytic acid determination. The phytic acid content was measured by Ishiguro and others' method based on phytic acid precipitation as ferric phytate *(25);* in order to extract phytic acid from seeds and from okara, 0.5 g of milled product were mixed in 10 mL of 0.5 M HCl and shaken for 1 hour at room temperature, then the mixture was centrifuged for 6 min at 18000 x g and supernatant was analyzed. For soymilk samples, extraction step was not necessary. Phytic acid determination was performed three times for each sample.

Isoflavones analysis. 1 g of materials was extracted by 30mL of methanol/water (70:30, v/v) and sonicated at room temperature for 30 minutes. The extraction procedure was repeated twice for each

sample. The mixtures were centrifuged at 2500 g, filtered through a Whatman filter paper and then used for LC-MS/MS analysis using a method previously described *(26)*. Chromatographic separation was performed using an HPLC apparatus equipped with two Micropumps Series 200 (PerkinElmer, Shellton, CT, USA), a UV/VIS series 200 (PerkinElmer, Shellton, CT, USA) detector set at 280 nm and a Prodigy ODS3 100Å column (250 mm ×4.6 mm, particle size 5 μm) (Phenomenex, CA, USA). The eluents were: A water containing 0.2% formic acid; B acetonitrile/methanol (60:40, v/v). The gradient program was as follows: 20-30% B (6 min), 30- 40% B (10 min), 40-50% B (8 min), 50–90% B (8 min), 90-90% B (3 min), 90-20% B (3 min) at a constant flow of 0.8 mL/min. The LC flow was split and 0.2 mL/min was sent to the mass spectrometry. Injection volume was 20μL. Two injections were performed for each sample. MS and MS/MS analyses of soybean extracts were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source working in the negative ion mode. Six glucosides (daidzin, genistin, glycitin, acetyl-genistin, malonyl-daidzin, malonyl-genistin) and only one aglycone (genistein) were identified: LC/MS/MS conditions of identified isoflavones are shown in **Table 1.** After peak identification the isoflavones quantification was performed by HPLC as follows: filtered extract (20 μL) was injected into an HPLC (Shimadzu
LC 10, Shimadzu, Kyoto, Japan) with photodiode array detector. Separations 133 were achieved on the same column with the same gradient program. The flow rate was 0.8 mL/min, and chromatograms were recorded at 280 nm. Isoflavones were quantified using, as external standard genistein and genistin for all glucosides. Genistein stock solution was prepared by dissolving standard (Sigma Aldrich) in methanol while genistin stock solution was prepared by dissolving standard (Sigma Aldrich) in a mixture methanol/water (70:30, v/v).
Statistical analysis. Differences among cultivars were determined by analysis of variance and Duncan's multiple range test ($P \leq 0.05$). Differences between field and hydroponically grown
samples were determinate by Student's t-test ($P \leq 0.05$).

RESULTS AND DISCUSSION
Proximate composition. **Figure 2** summarized the variations in proximate composition of soybean seeds determined by the cultivation method (hydroponic vs soil). The data showed are the average of the four different cultivars and, despite the variability among cultivars, results are remarkable: hydroponic cultivation in controlled environment increased fats, total dietary fiber and phytic acid, and reduced the amount of isoflavones, compared to soil cultivation in open field.
The method of cultivation did not affect protein concentration when data of the four cultivars are averaged, however, when results for each cultivar are considered, this is the case only for 'Pr91m10' and 'Regir'. Conversely, protein concentration in hydroponic cultivation was higher ($p \leq 0.05$) in 'Atlantic' whereas it was lower ($p \leq 0.05$) in 'Cresir' compared to seed from field grown plant (see **Table 2**). According to Dornbos and Mullen *(27)*, the higher water availability reduces protein accumulation in soybean seeds, but studies on different pulses, such as peanuts, report that protein concentrations in the seeds harvested from hydroponic system and field cultivation are not different *(33)*. The protein concentrations in the seed parallel those observed in soymilk and okara: products from field and hydroponically grown plants did not show differences in 'Pr91m10' and 'Regir', while they gave higher values in NTF for 'Atlantic' and in soil for 'Cresir'. Protein content ranges from 35.39 to 39.60 g/100 g in soymilk and from 31.60 to 43.25 g/100 g in okara, confirming previous results reported by other authors for the same products *(28,4,29,30)*.
Values of fat content in soybean seeds fall within the range reported in the literature, with no relevant difference among the cultivars (see **Table 2**). However, it is worth to notice that hydroponic cultivation caused an increase in fat content from 17.37 to 21.94 g/100, on the average of the tested cultivars; according to Dornbos and Mullen *(27)* this increase 167 can be due to the constant water availability favoring oil accumulation in soybean. Fat content in soymilk and okara (**Table 2**) are consistent with previous works *(28,4,29,30)*. In agreement with Cai et al. *(31)* and Mullin et al. *(32)*, cultivar selection did not affect lipid content in soymilk as it did in okara. This distribution of fats in soybean products is not surprising as fat extractability in water is limited independently from the amount of fats in the starting material At the same time data from hydroponics showed that the higher the fat content in seeds, the higher the fat content in okara.
Hydroponic cultivation increased dietary fiber content in soybean seeds from 21.67 to 28.46 g/100 g (mean values among the four analyzed cultivars); according to previous works, significant differences among cultivars were detected and 'Regir' seeds showed the highest value (27.56 g/100 g on the average between of the two cultivation systems). Dietary fiber content values in okara samples fall

within the range reported in the literature and the same cultivation effect observed in seeds was found in okara *(31)*.

Phytic acid content. Phytic acid content was significantly higher in seeds from hydroponic system than from field (see **Figure 2**). This could be due to the better availability of phosphorus in the nutrient solution, in fact in open field cultivation, phytic acid concentration has been demonstrated to be positively correlated to the available levels of phosphorus in the soil *(34)*. The same trend was observed for phytic acid content in okara and not in soymilk, with higher concentration in okara from hydroponics soybean (1.51 g/100 g dry mass vs 1.16 g/100 g in open field, on the average of the four cultivars). Significant difference ($p \leq 0.05$) among cultivars were found in both processing products. Omosaiye & Cheryan *(35)* and Beleia et al. *(36)* reported slightly different distribution of phytic acid between milk and pulp (1.68 g/100 g dry matter in soymilk and 1.83 g/100 g dry matter in okara, respectively) however they analyzed market products and not simultaneous experimental production of milk and okara.

On the nutritional point of view the increase in dietary fibre, that is in accordance with all dietary guidelines, would almost inevitably be accompanied by a rise in phytate intake. However, many studies showed that dietary phytate may not be an undesirable component of plant foods, and, except for some specific categories prone to iron deficiency, the importance of increasing dietary fibre consumption should be considered a more important nutritional requirement (*37*).

Isoflavones content. **Figure 2** shows significant lower content of total isoflavones, in the hydroponically grown seeds compared to those from field (17.04 vs 7.66 mg/kg). Data for each cultivar confirmed that this trend was observed in all the analyzed cultivar (see **Table 2**); however it is worth to notice that, beside inhibiting isoflavones biosynthesis, hydroponic cultivation eliminated the differences among cultivars observed in field grown samples.

As observed in previous works *(14, 15, 17)*, significant differences among cultivars were detected. Among field grown cultivars, 'Cresir' showed the best performance in total isoflavones content accumulation in seeds (171.80 mg/100 g) and in both derived products (551.21 and 224.97 mg/100 g in soymilk and okara, respectively). Among hydroponically grown cultivars significant differences were not detected in seeds and isoflavones different distribution between soymilk and okara were found: the highest value in soymilk was in 'Regir' product, the highest value in okara was in 'Atlantic' product.

In both transformation products, the effect of cultivation system on isoflavones content was less clear: it depends on cultivar but, on the average, variations in seeds isoflavones content did not establish significant differences in soymilk (47.97 and 48.39 mg/kg in soymilk from field and from hydroponic, respectively) and in okara (16.75 and 16.34 mg/kg in soymilk from field and from hydroponic, respectively).

Figure 3 showed a representative HPLC chromatograph of soybean seed extracted as reported in method session, while in **Table 3** the isoflavones pattern found in soybean seeds and soybean products was reported. In field-grown seeds, the most abundant component was malonyl-daidzin (36.6% as average of the four cultivars), followed by β-glucosides genistin and daidzin (26.5% and 18.0% on the average of the four cultivars, respectively); acetyl-genistin was not detected. This data are in agreement with those of previous reports *(15,38,39,40)*. In hydroponically grown seeds, the percentage of malonyl-daidzin was drastically reduced ($p \leq 0.05$) (9.0% of the four cultivars) and the most abundant forms were β-glucosides daidzin and genistin (34.1% and 28.25% as average of the four cultivars, respectively). In 'Pr91m10' and 'Regir' hydroponically grown seeds, acetyl genistin was detected at very low level.

Compared to the seeds, transformation products showed wider distribution of isoflavones forms: according to Jung et al. *(41)*, heat treatment causes in soymilk a shift towards the β-glucoside and aglycone forms with a corresponding decrease in the malonyl-daidzin content. In soymilk obtained from field grown seeds, the most abundant component was genistin (30.2% as average of the four cultivars), followed by daidzin (20.8% as average of the four cultivars): these values did not significantly changes when soymilk was obtained from hydroponically grown seeds. Soymilk obtained from hydroponically grown seeds showed an increases in malonyl-daidzin content up to 25.78% (average of the four cultivars) compared to soymilk from field grown seeds. In okara from field grown seeds, the most abundant components were malonyl-genistin and genistin (22.1% and 19.2%, respectively): comparing these data with okara from hydroponic seeds a decrease in first constituent and an increases in the second one were observed. An increase in daidzin and in malonyl-daidzin was observed too.

The biological activity of soy isoflavones is not related to the type of glycoside as deglycosilation is a prerequisite for their absorption *(42)*. On the contrary, isoflavones activity depends on the aglycone

moiety: daidzein and glycitein has less estrogenic activity than genistein *(43)*. In field grown seeds, amount of total daidzein was the highest (54.6%), followed by total genistein (39.5%) and total glycitein was the lowest (5.9%) considering the average concentrations of the four cultivars. In derivates products from field-grown seeds the amount of total genistein was the highest (52.4% in soymilk and 62.8% in okara), followed by total daidzein (39.0% and 31.1% in soymilk and okara, respectively) and total glycitein was the lowest (8.6% and 6.0% in the two analyzed products) considering the average concentrations of four analyzed soybeans. This evidence was in agreement with the previous observations that soybeans and soy foods usually contain similar amounts of genistein and daidzein and a much lower amount of glycitein *(15,38)*. In hydroponically grown seeds, there was a shift towards the content of total genistein (45.4%) and total glycitein (10.0%) at the expense of total daidzein (43.0%) so, there was a reduction in total isoflavones content but, on the other hand increased the percentage of genistein, the form with greater biological activity.
Summarizing, data of this paper showed that hydroponic cultivation is not only a method to obtain valuable vegetable productions in adverse environmental conditions, but also a system to increase macronutrient content and to improve nutritional value of soybean products. Hydroponic cultivation promoted seeds accumulation of fat and total dietary fiber. On the other hand, the better availability of phosphorus in the nutrient solution increased, the phytic acid content. Accurately designed hydroponic systems such as the NFT system do not induce stress in plants and, therefore, the concentration of isoflavones is dramatically reduced. In our experimental conditions a higher percentage of genistein, the isoflavones with greater biological activity was recovered in seeds from hydroponically cultivated plants. Looking at the soybean derived product seeds cultivation system did not significantly modify the soymilk composition; while in okara products the same compositional differences of the seeds were observed.

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Table 1 LC/MS/MS conditions for the detection of soybean isoflavones.

COMPOUND	Precursor ion [M-H$^+$] (*m/z*)	Product ion
Genistein	271	243 215 187
Daidzin	417	199 255
Genistin	433	215 271
Glycitin	447	285
Acetyl-genistin	475	431 417
Malonyl-daidzin	503	417
Malonyl-genistin	519	433

Table 2 Proximate composition, phytic acid and total isoflavones content of field and hydroponic grown soybean cultivars and derivates products. Different letters within the same column indicate significant differences at P<0.05; * and ns indicate significant and not significant differences between cultivation systems at P<0.05.

	Seeds			**Soymilk**			**Okara**		
	Field	***Hydroponics***		***Field***	***Hydroponics***		***Field***	***Hydroponics***	
	Protein content (g/100 g dry matter)								
Atlantic	32.48 ^{b}	33.92 ^{b}	*	35.63 ^{b}	37.79 ^{a}	*	34.65 ^{b}	35.83 ^{a}	*
Cresir	35.95 ^{a}	34.11 ^{b}	*	39.60 ^{a}	36.81 ^{ab}	*	43.25 ^{a}	32.04 ^{bc}	*
Pr91M10	35.27 ^{a}	35.55 ^{a}	*n.s.*	37.98 ^{a}	36.31 ^{ab}	*n.s.*	33.60 ^{b}	33.12 ^{b}	n.s.
Regir	32.52 ^{b}	31.96 ^{c}	*n.s.*	36.25 ^{b}	35.39 ^{b}	*n.s.*	32.86 ^{b}	31.60 ^{c}	n.s.
	Fat content (g/100 g dry matter)								
Atlantic	17.60 ^{a}	21.96 ^{ab}	*	18.13 ^{a}	18.24 ^{a}	*n.s.*	11.86 ^{a}	19.97 ^{ab}	*
Cresir	19.28 ^{a}	22.09 ^{a}	*	19,09 ^{a}	17.06 ^{a}	*n.s.*	9.45 ^{b}	20.98 ^{a}	*
Pr91M10	16.70 ^{a}	21.19 ^{a}	*	15.22 ^{a}	16.96 ^{a}	*n.s.*	9.40 ^{a}	19.11 ^{b}	*
Regir	16.99 ^{a}	22.50 ^{b}	*	18.90 ^{a}	18.12 ^{a}	*n.s.*	12.59 ^{b}	20.80 ^{a}	*
	Total dietary fiber content (g/100 g dry matter)								
Atlantic	21.65 ^{a}	27.51 ^{b}	*	n.d.	n.d.		50.11 ^{a}	64.84 ^{a}	*
Cresir	19.26 ^{b}	27.32 ^{b}	*	n.d.	n.d.		42.91 ^{b}	55.10 ^{c}	*
Pr91M10	22.10 ^{a}	27.57 ^{b}	*	n.d.	n.d.		48.78 ^{a}	57.61 ^{b}	*
Regir	23.68 ^{a}	31.44 ^{a}	*	n.d.	n.d.		50.92 ^{a}	65.35 ^{a}	*
	Phytic acid content (g/kg dry matter)								
Atlantic	14.04 ^{a}	15.71 ^{a}	*	14.80 ^{a}	16.63 ^{a}	*n.s.*	12.66 ^{a}	15.39 ^{a}	*
Cresir	11,47 ^{ab}	16,25 ^{a}	*	12,33 ^{ab}	9,49 ^{b}	*n.s.*	13,86 ^{a}	15,33 ^{a}	*
Pr91M10	12.12 ^{a}	16,84 ^{a}	*	12.69 ^{ab}	7.93 ^{b}	*n.s.*	10.48 ^{b}	15.25 ^{a}	*
Regir	8.94 ^{b}	15.69 ^{a}	*	8.95 ^{b}	9.88 ^{b}	*n.s.*	9.26 ^{b}	14.49 ^{a}	*
	Total isoflavones content (mg/kg g dry matter)								
Atlantic	12.09 ^{c}	7.67 ^{a}	*	44.80 ^{b}	48.58 ^{b}	*n.s*	13.64 ^{b}	18.27 ^{a}	*
Cresir	27.08 ^{a}	7.27 ^{a}	*	65.28 ^{a}	44.96 ^{b}	*	28.25 ^{a}	16.74 ^{b}	*
Pr91M10	10.39 ^{d}	7.27 ^{a}	*	37.19 ^{b}	45.40 ^{b}	*n.s*	11.66 ^{c}	15.42 ^{c}	*
Regir	18.61 ^{b}	8.41 ^{a}	*	44.60 ^{b}	54.60 ^{a}	*	13.44 ^{b}	14.91 ^{d}	*

Table 3 Isoflavones profile in field and hydroponic grown soybean seeds and derivates products (% of the total). Data were expresses as mean values of the four analyzed cultivars. ** and n.s. indicate significant and not significant differences between cultivation systems at P<0.01.

	Seeds			**Soymilk**			**Okara**		
	Field	***Hydroponics***		***Field***	***Hydroponics***		***Field***	***Hydroponics***	
Daidzin	18.05	34.08	**	20.79	22.68	*n.s.*	13.49	18.61	**
Glycitin	5.87	10.03	**	8.61	4.53	**	6.03	6.12	*
Genistin	26.50	28.25	**	30.19	31.12	*n.s.*	19.15	25.34	**
Malonyl-daidzin	36.55	8.97	**	18.17	25.78	**	17.65	23.87	**
Malonyl-genistin	10.96	9.00	**	12.88	9.77	**	22.13	14.10	**
Acetyl-genistin	*n.d.*	1.47		1.38	1.53	*n.s.*	4.74	4.18	**
Genistein	2.07	8.20	**	7.99	4.59	**	16.81	7.77	**

Figure 1 Experimental procedure to obtain soymilk and okara

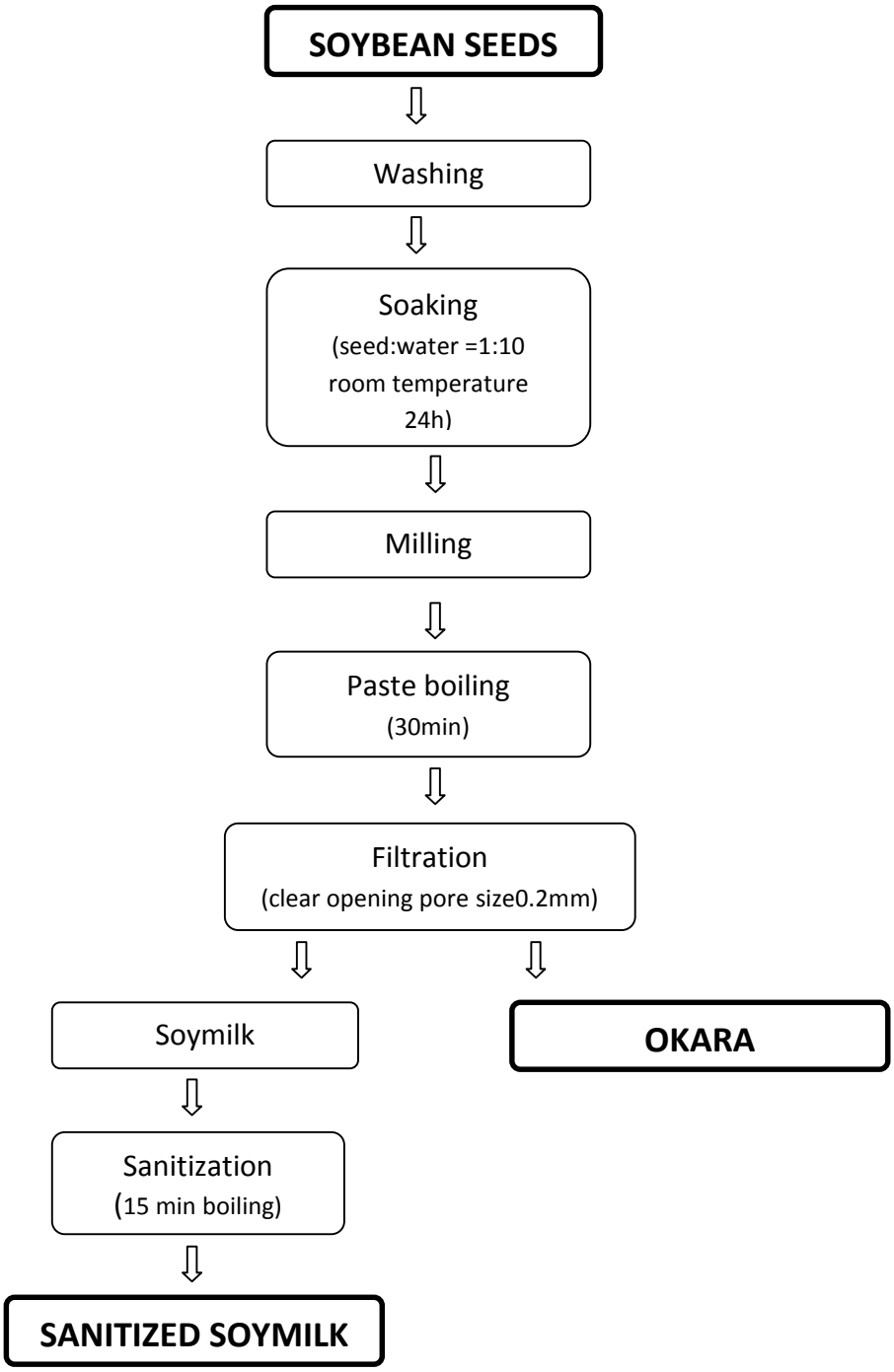


Figure 2 Protein content (%/dry mass), fat content (%/dry mass), total dietary fiber content (%/dry mass), phytic acid content (g/Kg dry matter) and total isoflavones content (mg/Kg dry matter) in seeds from field grown (black) and in hydroponic grow (gray) soybean plants. Data are the mean values of the four analyzed cultivars ± SD. * and n.s. indicate significant and not significant differences between cultivation systems at $P<0.05$.

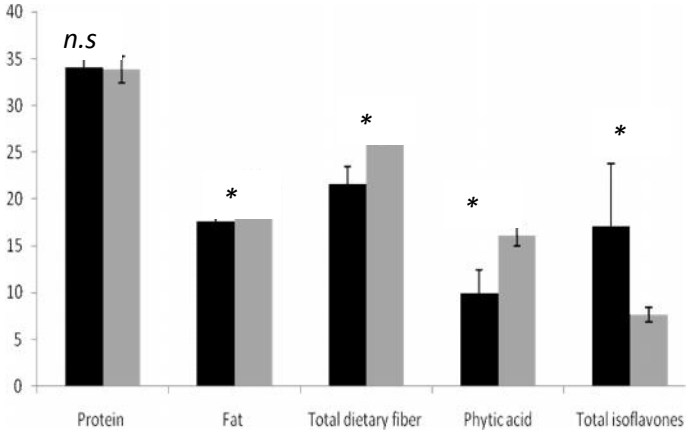
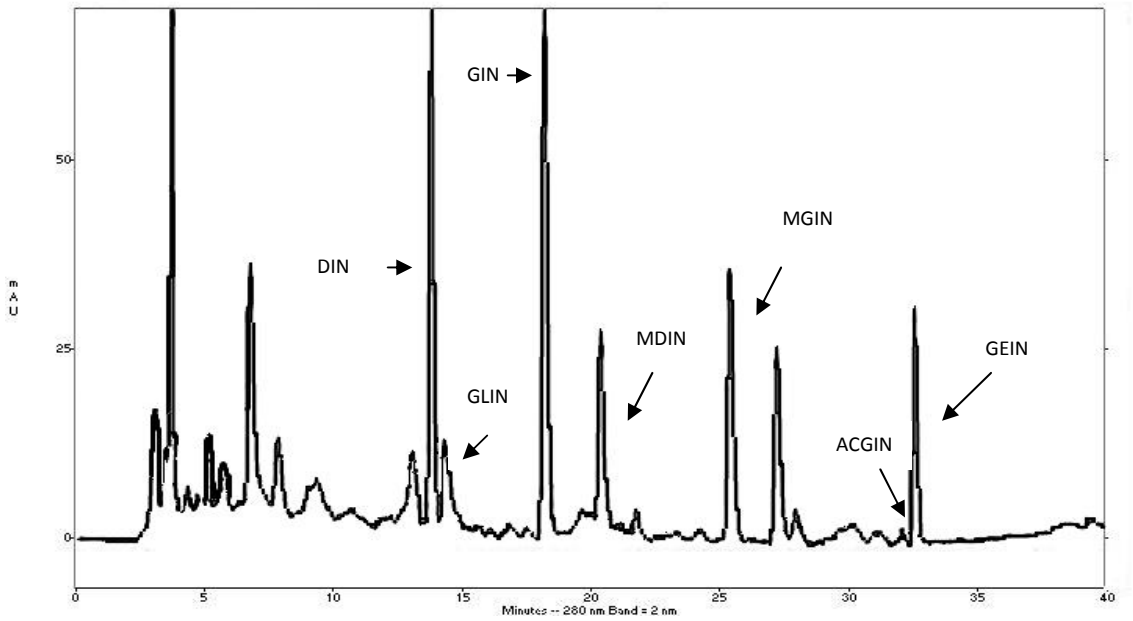


Figure 3 HPLC chromatograph of methanol water extracts from soybean seeds. UV absorbance at 280 nm was monitored. DIN, daidzin; GLIN, glycitin; GIN, genistin; MDIN, malonyldaidzin; MGIN, malonylgenistin; ACGIN, acetylgenistin; GEIN, genistein. The identification of the seven isoflavones was achieved by LC-MS-MS using the MRM as reported in **Table 1**.



SOYBEAN CULTIVAR SELECTION FOR BIOREGENERATIVE LIFE SUPPORT SYSTEMS (BLSSS) PART II: HYDROPONIC CULTIVATION

R. Paradiso [a], R. Buonomo [a], V. De Micco [b],G. Aronne [b], M. Palermo [a], G. Barbieri [a], S. De Pascale [a]

[a] *Dept. Agricultural Engineering and Agronomy, University of Naples Federico II, via Università 100, I-80055 Portici (Naples), Italy*

[b] *Dept. Arboriculture, Botany and Plant Pathology, University of Naples Federico II, via Università 100, I-80055 Portici (Naples), Italy*

Corresponding author:

E-mail address: roberta.paradiso@unina.it (Roberta Paradiso)

Telephone: 0039 081 2539135; fax 0039 081 2539156

Present address: Dep. Agricultural Engineering and Agronomy, Via Università 100, I-80055 Portici (Naples), Italy.

Abstract

Four soybean cultivars ('Atlantic', 'Cresir', 'Pr91m10' and 'Regir'), selected through a theoretical procedure as suitable for cultivation in BLSS, were evaluated in terms of growth and production. Germination percentage and Mean Germination Time (MGT) were measured. Plants were cultivated in a growth chamber equipped with a recirculating hydroponic system (Nutrient Film Technique). Cultivation was performed under controlled environmental conditions (12 h photoperiod, light intensity 350 µmol $cm^{-2}s^{-1}$, temperature regime 26/20 °C light/dark, relative humidity 65-75%). Fertigation was performed with a standard Hoagland solution, modified for soybean specific requirements, and EC and pH were kept at 2.0 dS m^{-1} and 5.5 respectively. The percentage of germination was high (from 86.9% in 'Cresir' to 96.8% in 'Regir')and the MGT was similar for all the cultivars (4.3 days). The growing cycle lasted from 114 in 'Cresir' to 133 days on average in the other cultivars. Differences in plant size were recorded, with 'Pr91m10' plants being the shortest (58 vs 106 cm). Cultivars did not differ significantly in seed yield (12 g $plant^{-1}$) and in non edible biomass (waste), water consumption and biomass conversion efficiency (water, radiation and acid use indexes). 'Pr91m10' showed the highest protein content in the seeds (35.6% vs 33.3% on average in the other cultivars). Results from the cultivation experiment showed good performances of the four selected cultivars in hydroponics. The overall analysis suggests that 'Pr91m10' could be the best candidate for the cultivation in a BLSS, coupling the small plant size and the good yield with high resource use efficiency and good seed quality.

Key words:
Glycine max (L.) Merr., soilless culture, resource use efficiency, seed yield, seed quality

Abbreviations

AR: Accumulation Rate

AUE: Acid Use Efficiency

BLSSs: Bioregenerative Life Support Systems

CELSS: Controlled Ecological Life Support System

Cv: Cultivar (cultivated variety)

DAS: Days after sowing

DM: Dry Matter

EC: Electrical Conductivity

FW: Fresh weight

HI: harvest index

LA: Leaf Area

MGT: Mean Germination Time

NFT: Nutrient Film Technique

PAR: Photosynthetically Active Radiation

RUE: Radiation Use efficiency

WUE: Water Use Efficiency

1. Introduction

The idea of using plants as key organisms in Bioregenerative Life Support Systems (BLSS) dates back the 50's and has been pursued through many ground-based projects worldwide (Myers, 1954; Gitelson et al., 1989; Wheeler et al., 1996; Edeen et al., 2000; Tako et al., 2005; Waters et al., 2002; Wheeler, 2010, 2011). Soybean [*Glycine max* (L.) Merr.] is one of the candidate species for cultivation in BLSS because its seeds have high nutritional value, being rich in proteins and lipids (Hoff et al., 1982). Moreover, the presence of soy-proteins in a diet can help hampering phenomena, such as osteoporosis and muscle atrophy, which are frequent in animal organisms exposed to weightlessness (Alekel et al., 2001; Tada et al., 2002). The objective of reaching a food production in BLSSs, stable in both quantity and quality, can be reached only starting by an appropriate choice of the cultivars which must be adaptable to specific growth conditions (technical requirements) and characterised by a suitable nutrient composition, in agreement with dietary needs. For instance, many experiments have been conducted by cultivating several crop species in the NASA's CELSS Biomass Production Chamber with the aim to regulate lighting, water, humidity, temperature and nutrient mineral supply to promote rapid growth and high yields, as well as to maximise the production of oxygen, fresh food and clean water (Myers, 1954; Gitelson et al., 1989; Edeen et al., 2000; Tako et al., 2005; Waters et al., 2002; Wheeler et al., 1996, 2010, 2011). Most of the studies aiming to characterise crop production under controlled conditions in the sight of their use in BLSS have been conducted by cultivating plants with soilless (or hydroponic) culture techniques, particularly with recirculating hydroponic systems (Wheeler et al., 2003). These methods guarantee a constant nutrient solution availability to the roots, avoiding water or nutritional stresses, and optimal aeration (Monje et al., 2003). In such systems, the development of plants, hence nutritional quality of harvested parts, is influenced by the maintenance of environmental factors at optimal levels and can be altered if compared with growth parameters available from cultivation trials carried out in open field (Jurgonski et al., 1997). Indeed for soybean, being a traditionally open field species, most available information about productivity derives from cultivation trials in the field. Knowing the response of this species after cultivation in controlled conditions can be useful for predicting food, potable water and oxygen production rates and dynamics in a bioregenerative system. The characterization of the plant growing environment is a crucial point for the control of the final crop yield and the waste composition. The continuous nutrient supply, typical of hydroponic systems, has been considered responsible for the higher content of proteins in wheat seeds, higher protein and ash levels in lettuce leaves and potato tubers, and higher ash and crude fibre levels in soybean seeds, if compared with data from field-grown plants (Wheeler et al., 1996). Our experiment was performed within the ESA project MELiSSA (European Space Agency - Micro Ecological Life Support System Alternative). The overall objective of MELiSSA is to conceive a bioregenerative system for resources regeneration in Space. Within this project, our aim was to select European cultivars of soybean suitable for cultivation in Space. This objective was pursued through: a) a theoretical procedure for a preliminary identification of four candidate cultivars for BLSS (De Micco et al., Part I); b) an experimental procedure for the evaluation of the behaviour of the selected cultivars grown in hydroponic system, under controlled environment. In this paper (Part II), we report the second phase of the selection procedure. Four soybean cultivars, 'Atlantic', 'Cresir', 'Pr91m10' and 'Regir', selected through the above mentioned procedure, were cultivated in a

recirculating hydroponic system and evaluated in terms of agronomic traits, including plant size and seed productivity, and technical parameters, such as the resource use efficiency and non edible biomass production.

2. Materials and Methods

2.1 Plant material and seed germination

Before starting the hydroponic cultivation, a germination test was performed on commercial seeds of the first 4 cultivars of soybean [*Glycine max* (L.) Merr.] selected after the application of the theoretical criterion developed by De Micco et al. (Part I): 'Pr91m10', 'Regir', 'Atlantic' and 'Cresir'. All the cultivars were characterised by indeterminate growth, early flowering and were photoperiod-independent. Seeds were screened for size, weight, uniformity and lack of defects and subjected to the germination method by Fernandez-Orozco *et al.* (2008). More specifically, seeds were soaked for 30 min with 0.07% sodium hypochlorite (50 ml) and rinsed in sterile distilled water until reaching neutral pH. Then, seeds were soaked with distilled water (50 ml) for 5 h and 30 min., being shaken every 30 min. Seeds were placed on wet filter paper and incubated in the dark (Temp. 20°C, R.H. 85%) for 8 days. Four replications (100 seeds per replicate) were tested per each cultivar, according to the *International Rules for Seed Testing* (1999). Germination percentage and Mean Germination Time (MGT) were evaluated after 8 days. MGT was calculated by counting the number of germinated seeds daily and according to the following formula:

$$MGT = \Sigma (n*d)/N$$

where n is the number of germinated seeds per day, d is the number of days from the beginning of the test, and N is the total number of germinated seeds.

2.2 Cultivation design, hydroponic system management and growth chamber environmental control

The experiment was carried out in a 16 m^2 walk-in growth chamber (atmospherically open), equipped with a recirculating NFT (Nutrient Film Technique) system, consisting in 12 polypropylene double gullies 60 cm high (Fig. 1). Each single gully was 12 cm wide, 93 cm long and 5 cm deep and was equipped with 3 sprinklers, to provide the nutrient solution. Three double gullies, arranged in randomised blocks, were used per each cultivar (14 plants per double gully; 42 plants per cultivar in total). Sowing was performed on March 2010 on moisturized perlite, in small plastic baskets placed in gully holes, at 20 °C, in the darkness. After sowing, baskets were covered with an acrylic cover for 4 days, to shade seedlings and to avoid water evaporation. Three seeds were used per each hole, then thinned to one after the emergence (8 DAS). Substrate was covered with a two-side polyethylene film, with the white side oriented to the plants, to reflect light, and the black side to the roots, to prevent the development of algae. Canopy support system was performed with vertical threads. Fertigation was performed with one separate 12-litre reservoir per each double gully: nutrient solution was pumped in the gullies by a submerged pump and returned to the reservoir by gravity dependent flow. Gully inclination was 1% and the flow rate was 2.4 l/min. The nutrient recipe was based on the standard Hoagland solution 1/2 strength (Hoagland and Arnon, 1950), modified by Wheeler *et al.* (2008), according to specific requirement of soybean. The ion concentration was (in mM): N 7.5, P 0.5, K 3.0, Ca 2.5, Mg 1.0, S 1.0; (in µM): Fe 60.0, Mn 7.4, Zn 0.96, Cu 1.04, B 7.13, Mo 0.01. EC and pH were kept at 2.0 dS m^{-1} and 5.5, respectively. Recirculating solution was monitored and adjusted every two days. Water depletion was measured and the volume of the nutrient solution in the reservoir was kept at a constant level, by adding deionised water and/or fresh nutrient solution and nitric acid (65% concentration), until the EC and pH target values. Nutrient solution was completely replaced once, at approximately the middle of the experiment (50 DAS). The experiment was carried out under controlled environmental conditions. Light was provided by High Intensity Discharge lamps (HID), working on hot vapour light emission. High Pressure Sodium lamps (HPS, 600 W) were used. A minimum PAR of 350 µmol $m^{-2}s^{-1}$ was obtained at the canopy level, according to a light/dark regime of 12/12 hours (light time from 8:00 a.m. to 8:00 p.m.). Temperature (T) regime was established at 26/20 °C (light/dark) and relative humidity (RH) in the light time was kept within the optimal range of 65-75%, using a fog system. Air change and dehumidification were guaranteed by two air extractors. Measurements were carried out to map the possible light, T and RH gradients, in order to minimize any border effect at the crop level. Particularly, the extinction profile of light distribution at different

distances from the lamps and the uniformity of temperature and RH were studied on vertical and horizontal planes.

2.3 Sampling and Measurements

Plant growth and yield
The plant growth was measured at 7-day intervals, on 6 plants per cv. Growth analysis was based on non-destructive measurements of plant height, number of nodes and leaf number and area. Plant leaf area (LA) was estimated by leaf length and width, using the formula of Wiersma and Bailey (1957) based on the specific soybean leaf types and shapes. Soybean pods were harvested twice a week, starting from 114 DAS, when pods had turned to brown colour (average water content 14%) (Gbikpi and Crookston, 1981). Harvest was staggered, to simulate a typical situation in CELSS, with limited space for seeds storing and the need of a sustainable work load for astronauts. Plant productivity was measured as grams of seeds per plant^{-1} and number of pods and seeds per plant^{-1}. At each harvest, yield data (number and fresh weight of pods and seeds) were determined per single plant (edible biomass). At the end of the experiment, plants were collected to determine fresh (FW) and dry weight (DW) and their partitioning in the different organs (non edible biomass). FW and DM of the different parts of plants (stems, leaves, seeds) were determined; measurements were carried out on 6 plants per cv (3 plants x double gully). DM was measured after the oven-drying at 60 °C until constant weight.

Plant water consumption and chemical analyses
Plant water consumption was calculated on the basis of the water balance of each double gully, by measuring the volume of the supplied and the drained nutrient solution, assuming the water uptake unaffected by evaporation thanks to the substrate covering.
The main ion concentration (N, P, K, Ca, Mg) in the different plant tissues was determined before the complete leaf falling (90 DAS), on water extract of DM (Walinga et al., 1995), using a spectrophotometer Hach DR 2000 (Hach Company, Loveland, CO). Organic nitrogen was determined by the Kjeldahl method (Kjeldahl, 1883). Proximate analyses on seeds (protein, fat, carbohydrates, fibre) were performed on 3 replicates per cv. Proteins were calculated on the basis of total nitrogen content determined by the Kjeldahl method (AOAC, 1995) and the conversion factor used to transform nitrogen into protein was 5.71. Fat content was measured by extraction with diethyl ether in a Soxhlet system (James, 1995). Total fiber content was determined by the gravimetric method AOAC 985.29 (Prosky et al., 1998).

Biomass accumulation rates and efficiency indexes
After the beginning of leaf abscission (70 DAS), fallen leaves were collected twice a week, separately for the different double gullies, and fresh and dry matter was determined and took into account to calculate the total biomass at the end of the growing cycle. At the end of the experiment, total and edible biomass accumulation rates, expressed as g of biomass per plant-1 d-1, and the following efficiency indexes were calculated: Harvest Index (HI), as dry weight of seeds in percentage of the total dry weight of plant (excluding roots); Water Use Efficiency (WUE), as g of edible DM per kg of nutrient solution; Radiation Use efficiency (RUE), as g of edible DM per mole of PAR; finally, Acid Use Efficiency (AUE, for the nitric acid used to control nutrient solution pH) was calculated, as g of edible DM per mmole of H+.
Data were analysed by ANOVA and means were compared by the Least Significant Differences (LSD) at P=0.05.

3. Results

Growth chamber environmental control
Temperature in the growth chamber was 24.2±0.03 °C (Mean ± Standard error) in the light time and 21.1±0.02 °C in the dark time, and Relative Humidity ranged from 73.5±0.25% to 80.9±0.21% respectively, with very small fluctuations in the 133 cultivation days (Fig. 2). The light distribution profile slightly changed with the distance from the single lamp and the position respect to more lamps, however, the light intensity in the vertical space occupied by the canopy (from 0 to 120 cm from the gully) was always sufficient for the crop requirement (from 350 to 510 μmol m-2s-1). Climatic parameters were not significantly affected by the distance from conditioning and lighting systems: as example, in the light time temperature within the vertical and the horizontal planes ranged between the interval 24.4-25.5 °C and RH from 67 to 73%, with no relevant gradient among the measurement positions.

Hydroponic system management

Temperature of recirculating nutrient solution varied with the air temperature in the growth chamber, from 22.0 °C in the light time to 17.9 °C in the dark time.

The evolution of pH and EC of the recirculating nutrient solution in the different cultivars, before and after the adjustment every two days, is shown in Figure 3. pH value always increased after two days of fertigation. The increase was smaller during the first week of the growing cycle, when the size of plant aerial part and roots was still small, while it was more severe and variable later, when plants were fully developed and during the leaf senescence. EC always tended to rise as consequence of plant water uptake and increases were very small in the first 20 days of measurements, became larger during the plant growth and decreased progressively after the beginning of leaf falling (around day 70).

On the average of the entire experiment, the value after two days of recycling was slightly higher in 'Atlantic' compared to the other cultivars for pH while it was not significantly different among the cultivars for EC (Table 1).

Plant growth

The percentage of seed germination after 8 days was high in all the tested cultivars, ranging from 86.9% in 'Cresir' to 96.8% in 'Regir'. MGT was not significantly different among cultivars (4.28±0.19 on average; Mean ± Standard Deviation). The time course of stem elongation followed a typical sigmoid pattern in all the soybean plants (Fig. 4), however cultivars showed relevant differences in the rate of stem elongation and in the maximum length, which were significantly lower in 'Pr91m10' and higher in 'Cresir' (Table 2).

Plant leaf area increased during the first 70 days of cultivation, because of the increasing of the number of trifoliate leaves, then drastically decreased because of leaf falling, which took place during the stage of pod filling (seed formation) (Fig. 4). The total number of leaves, before falling, ranged from 16.3 leaves per plant in 'Pr91m10' to 23.7 leaves per plant on the average of the other cultivars (Table 2), corresponding to the maximum plant leaf area of 854.2 and 1078 cm2, respectively.

Time for the beginning of the harvest was similar in the different cultivars, even though a slight advance was recorded in 'Cresir' (90 DAS vs 93 in 'Atlantic' and 'Regir' and 97 in 'Pr91m10'), which also had a significantly shorter duration of the harvest period (24 vs 39 days on average).

Seed production

Dry weight of total biomass at the end of the growing cycle had similar values in the four cultivars (19.2±2.6 g plant-1 on average; Mean ± Standard Deviation) (Table 3). Similarly, cultivars did not differ in seed production, neither in terms of total yield (12.0±2.4 g plant-1), nor in terms of number of pods and seeds (31.7±5.6 and 56.0±13 per plant, respectively).

Chemical composition

Chemical analyses of plant tissues revealed an average concentration of the main macronutrients (in g/100 g of DM) in the leaves of 2.50 for N, 0.15 for P and 2.13 for K, with no difference among the cultivars (Table 4). Stems had similar concentration for total nitrogen, potassium, magnesium and sulfur compared to the leaves, while higher content of P and lower content of Ca were found in stems (Table 4). NO3/total N ratio was always higher in the stems (Table 4).

The proximate composition of soybean seeds in the four cultivars is reported in Table 5. All the nutritional compounds varied among the cultivars: protein content was higher in seeds of 'Pr91m10' and lower in 'Regir', which also showed the highest content of fat and fiber. Regarding ash content, 'Pr91m10' and 'Cresir' showed intermediate values if compared with the other cvs.

Water use, biomass accumulation rates and efficiency indexes

Nutrient solution uptake followed the trend of plant leaf area, increasing rapidly during the early stage of growth, as the total evaporating surface of foliage increased, reaching the maximum about 65 days after sowing (440 ml plant-1 on average), then declining progressively with the leaf falling and the plant ageing (Fig. 4).

Daily and cumulative water consumption did not vary significantly among the cultivars (61.6±6.3 ml plant-1 d-1 and 7.9±0.9 litres plant-1, respectively; Mean ± Standard Deviation); this is also true for

the daily and the cumulative addition of nitric acid for the pH control in the nutrient solution (0.31±0.05 mmol plant-1 d-1 and 39.1±5.1 mmol plant-1) (Table 6).
The accumulation rate of total biomass and seed dry matter were similar for all the cultivars (Table 7), however DM partitioning in the different plant organs differed significantly at the harvest (Fig. 5). Particularly, DM accumulation in inedible parts (stem, leaves, pods) in 'Atlantic' plants was higher than in the other cultivars, determining a significantly lower HI (0.44 vs 0.56).
The efficiency of water, radiation and acid use did not differ significantly among the cultivars (1.34±0.23 g of seed per litre of nutrient solution, 0.24±0.05 g of seed per mole of PAR, 0.27±0.05 g of DM seeds per mol H+, respectively; Mean ± Standard Deviation) (Table 7).

4. Discussion

Hydroponic system management
In our hydroponic system, the passage through the root system and the plant uptake always resulted in increases of EC and pH of recirculating nutrient solution, as commonly observed in other studies on hydroponically-grown soybean (Grusak and Pezeshgi, 1994). However, the strategy of nutrient solution control adopted in the experiment, with adjustments at 2-day intervals, was efficient in containing the EC and pH fluctuations within acceptable values.
The EC increase constantly recorded during the growth cycle confirmed a preferential uptake of water compared to the mineral elements. The amplitude of EC increase changed in the time, being the proportion of water and nutrient uptake depending on the transpiration rate and the nutritional needs in the different phases of plant development (Jones, 2000).
Alkalinization of nutrient solution (and rhizosphere) in closed systems indicates a greater uptake of anions over cations and is typical in nitrate-based nutrient solutions (Willumsen, 1980). Particularly, it is demonstrated that pH goes acidic rather quickly when nitrate is depleted (Marschner, 1995). As a consequence, in the Hoagland solution (used in most the hydroponic cultivations in space-related experiments) the use of nitrate as the only N source determines a great input of acid for pH control. This approach results in the acid itself becoming a major source of N (up to 30% of the total supply) (Wheeler et al., 1999) and it can determine N over-supply. In our experiment, the total acid requirement reached 44 mmol plant-1 (corresponding to 4.3 ml plant-1 of HNO3 65%): in the view of long-duration missions and cultivation of several crops, this quantity can become substantial, suggesting the introduction of a NH4-N amount to maintain a better charge balance in the root zone and to reduce the acid use in BLSS (Marschner, 1995).
Temperature of the nutrient solution changed with the surrounding air temperature in light and dark periods, however it stayed within the range considered optimal to determine a good solubility of O2 in water, which strongly affects the root growth and function in hydroponic environment (Monje et al., 2000).

Cultivars performance
Results of the germination and the cultivation experiments demonstrated good performances of the four soybean cultivars, selected through the specifically designed procedure (De Micco et al., Part I).
Time for seed maturation was similar among the cultivars, according to their belonging to early flowering, however 'Cresir' had the earliest leaf senescence and falling and a slight advance of seed maturity compared to the others.
Cultivars did not show significant differences in the rate of biomass accumulation and in total biomass (as DM) at the end of the growing cycle, as well as in seed production. On these basis, the differences in plant size and in relative productivity (as ratio edible biomass/total biomass) represent important aspects in the judgement of plant performances for CELSS, due to the limited volume available for cultivation, also considering that soybean plants grown in controlled environment tend to be taller than in open field (Downs and Thomas, 1990). In our experiment, 'Cresir' was earlier in the seed maturation but it gave the greatest plant dimension while 'Pr91m10' had the shortest size, with no differences in edible yield. Our results are in contrast to previous researches on soybean grown in controlled environment under HPS lamps, which proved that plant height and seed production were positively correlated, thanks to the better light interception (and photosynthetic performances) in taller plants (Wells et al., 1993). Conversely, in our experiment the excessive stem elongation suggests a limited adaptation of 'Cresir' to the low light intensity in the growth chamber, probably related to the more dense canopy of this cultivar (higher LA) and the consequent shading of the inner and lower leaves.

Plant biomass and seed production obtained in the four tested European cultivars were lower than those reported by Wheeler et al. (1999; 2008) for two American cultivars grown in NFT with the same nutrient solution recipe. Similarly, water consumption was lower in our experiment. These results are probably due to the higher light intensities provided in the NASA's Biomass Production Chamber (500 to 800 μmol m-2 s-1), as postulated by Wheeler et al. (1999) who found a linear relationship between PAR and biomass production, nutrient and water uptakes and acid use.
HI ranged from 0.44 to 0.57, with 'Pr91m10' showing the highest score. These values are higher than those listed for hydroponically-grown soybean and revealed a favourable DM allocation in seeds compared to most tested cultivars for CELSS 'Hoyt' (Jurgonski et al., 1997; Dougher and Bugbee, 1997; Mackowiak et al., 1999; Wheeler et al., 2003) and 'McCall' (Wheeler et al., 1998; 2008).
DM accumulation rate and efficiency indexes are comparable to those reported by Wheeler et al. (2003; 2008) and Dougher and Bugbee (1997) in hydroponically-grown soybean, even though some slight differences were recorded among the experiments depending on the plant genotype or the experimental layout.
Plants did not show nutrient deficiency symptoms during the growth cycle and the values of the macro-nutrients concentration in stems and leaves were found to be in the optimal range for soybean (Duseja and Dennis, 2010). Nitrogen content in both the organs decreased from the vegetative to the reproductive phase, confirming that these tissues act as the source of nitrogen during the pod filling, to match the sustained demand for seed formation (Guiamét et al., 1986). NO3/total N ratio was always higher in stems than in leaves, since nitrate moves into the plant through the stem, while it is transformed in organic nitrogen in the leaves. A little amount of chloride was found; this can be ascribed to its presence as an impurity in the fertilizers (e.g. potassium nitrate).
The proximate composition of seeds showed some differences in the main nutritional compounds among the cultivars and in comparison to soybean seeds from field grown plants. Protein content was similar to those obtained by Wheeler et al. (1996) in hydroponics and was influenced by the cultivation method with different response in the cultivars (Jurgonski et al. (1997). Ash, fat and fiber values fall within the normal range for soybean (Bagger et., 1998; Redondo-Cuenca et., 2006; Kumar et al., 2006; Grieshop & Fahey, 2001) and showed in all the cultivars significant increase compared to open-field seeds (Jurgonski et al., 1997; Wheeler et al., 1995).

5. Conclusion

The overall analysis indicates good performances in hydroponic cultivation of the four cultivars chosen in the theoretical selection phase. The NFT system and the nutrient solution management adopted in the experiment proved to be efficient in growing healthy soybean plants, with no nutrient deficiency or other kind of stress (*e.g.* anoxic condition for the roots). Among the 4 tested soybean cultivars, 'Pr91m10' could be the most suitable for the cultivation in a CELSS, coupling the short size and the high resource use efficiency to a good production of seeds, having high nutritional quality.

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Table 1 - Average values of pH and EC of recirculating nutrient solution in the four selected cultivars of soybean, before and after the adjustment to the target values (pH 5.5; EC 2.0 dS/m) (Mean values ± Standard Deviation; n = 40).

	pH		EC	
	before	after	before	after
Atlantic	7.42 ± 0.35	5.37 ± 0.30	2.33 ± 0.17	2.01 ± 0.03
Cresir	7.09 ± 0.49	5.20 ± 0.43	2.39 ± 0.26	2.01 ± 0.03
Pr91m10	7.19 ± 0.45	5.24 ± 0.38	2.39 ± 0.18	2.01 ± 0.03
Regir	7.28 ± 0.34	5.23 ± 0.41	2.36 ± 0.22	2.01 ± 0.02

Table 2 - Main growth parameters of fully developed plants of the four selected cultivars of soybean (Mean values; n = 6; Different letters indicate significant differences at P=0.05).

	Plant height (cm)	Number of nodes (n./stem)	Number of leaflets (n./plant)	Maximum Leaf Area (cm^2/plant)
Atlantic	96.2	12.5	24.7	1005.4
Cresir	121.7	12.3	24.2	1237.3
Pr91m10	57.8	11.0	16.3	854.2
Regir	99.3	10.2	22.3	990.0
Lsd (P=0.05)	14.7	0.6	5.9	189.7

Table 3 - Total dry weight at the harvest and yield data of the four selected cultivars of soybean (Mean values; n = 6; ns = not significant at P=0.05).

	DM percentage (% of FW)	Total biomass DM (g plant^{-1})	Yield * (g seeds plant^{-1})	Number of pods (n. plant^{-1})	Number of seeds (n. plant^{-1})	Seeds per pod (n. per pod)	Weight 1000 seeds (g)
Atlantic	51.9	22.1	11.3	30.8	51.7	1.7	226
Cresir	59.3	19.3	12.4	29.0	51.7	1.8	243
Pr91m10	61.6	19.3	12.2	30.1	52.6	1.7	254
Regir	60.1	19.4	12.1	36.7	68.1	1.8	184
Significance	*	ns	ns	ns	ns	ns	*
[LSD P=0.05]	[8.5]						[46]

* at 14% water content

Table 4 - Chemical composition of stems and leaves of the four selected cultivars of soybean at the end of the growing cycle (90 DAS) (g/100 g DM; Mean value ± Standard Deviation; n=3).

	N_{tot}	P	K	Ca	Mg	NO_3/N_{tot}
Leaves						
Atlantic	2.50±0.02	0.18±0.04	2.60±0.17	2.07±0.75	0.52±0.02	4.0±0.8
Cresir	2.33±0.14	0.16±0.02	2.23±0.12	2.00±0.15	0.58±0.07	3.0±0.6
Pr91m10	2.42±0.15	0.17±0.02	2.27±0.12	1.81±0.20	0.38±0.03	4.5±1.0
Regir	2.76±0.28	0.12±0.01	1.97±0.06	1.36±1.15	0.45±0.01	5.1±1.8
Stem						
Atlantic	2.35±0.68	0.37±0.10	2.17±0.38	0.57±0.05	0.51±0.12	6.1±1.5
Cresir	2.10±0.50	0.26±0.03	2.50±0.35	0.52±0.13	0.36±0.06	9.3±3.6
Pr91m10	2.67±0.69	0.30±0.05	2.10±0.29	0.48±0.09	0.52±0.07	10.9±2.9
Regir	2.29±0.89	0.32±0.01	2.20±0.14	0.57±0.10	0.33±0.04	9.0±3.5

Table 5 - Proximate composition of soybean seeds (g/100g DM; Different letters indicate significant differences at P=0.05).

	Protein (%)	Ash (%)	Fat (%)	Total dietary fiber (%)
Atlantic	33.9	6.4	22.0	27.9
Cresir	34.1	6.0	22.1	27.3
Pr91m10	35.6	6.0	21.2	27.6
Regir	32.0	6.2	22.5	31.4
Significance		ns	ns	
[LSD P=0.05]	[0.35]			[1.5]

Table 6 - Daily and cumulative values of plant water consumption and nitric acid used for pH adjustment in the recirculating nutrient solution, in the four selected cultivars of soybean.

	Daily water consumption (ml plant^{-1} d^{-1})	Cumulative water consumption (l plant^{-1})	Daily nitric acid supply (mmol plant^{-1} d^{-1})	Cumulative nitric acid supply (mmol plant^{-1})
Atlantic	63.0	8.4	0.33	44.03
Cresir	63.2	7.2	0.37	41.79
Pr91m10	60.6	8.1	0.24	32.42
Regir	59.5	7.9	0.29	37.95
Significance	ns	ns	ns	ns

Table 7 - Biomass accumulation rates and efficiency indexes of the four selected cultivars of soybean (Mean values; ns = not significant; * = significant at P≤0.05).

	AR Total DM (g plant^{-1} d^{-1})	AR DM seeds (g plant^{-1} d^{-1})	HI (% DM_{seeds}/DM_{plant})	WUE_{seeds} (g l^{-1})	RUE_{seeds} (g mol^{-1})	AUE_{seeds} (g mmol^{-1})
Atlantic	0.17	0.07	0.44	1.16	0.21	0.21
Cresir	0.17	0.09	0.55	1.46	0.26	0.30
Pr91m10	0.15	0.08	0.57	1.36	0.24	0.31
Regir	0.15	0.08	0.56	1.38	0.24	0.27
Significance	ns	ns	*	ns	ns	ns
[LSD P=0.05]			[0.08]			

Figure 1 - Layout of NFT system and climate control devices, double gully and nutrient solution reservoir with the submerged pump.

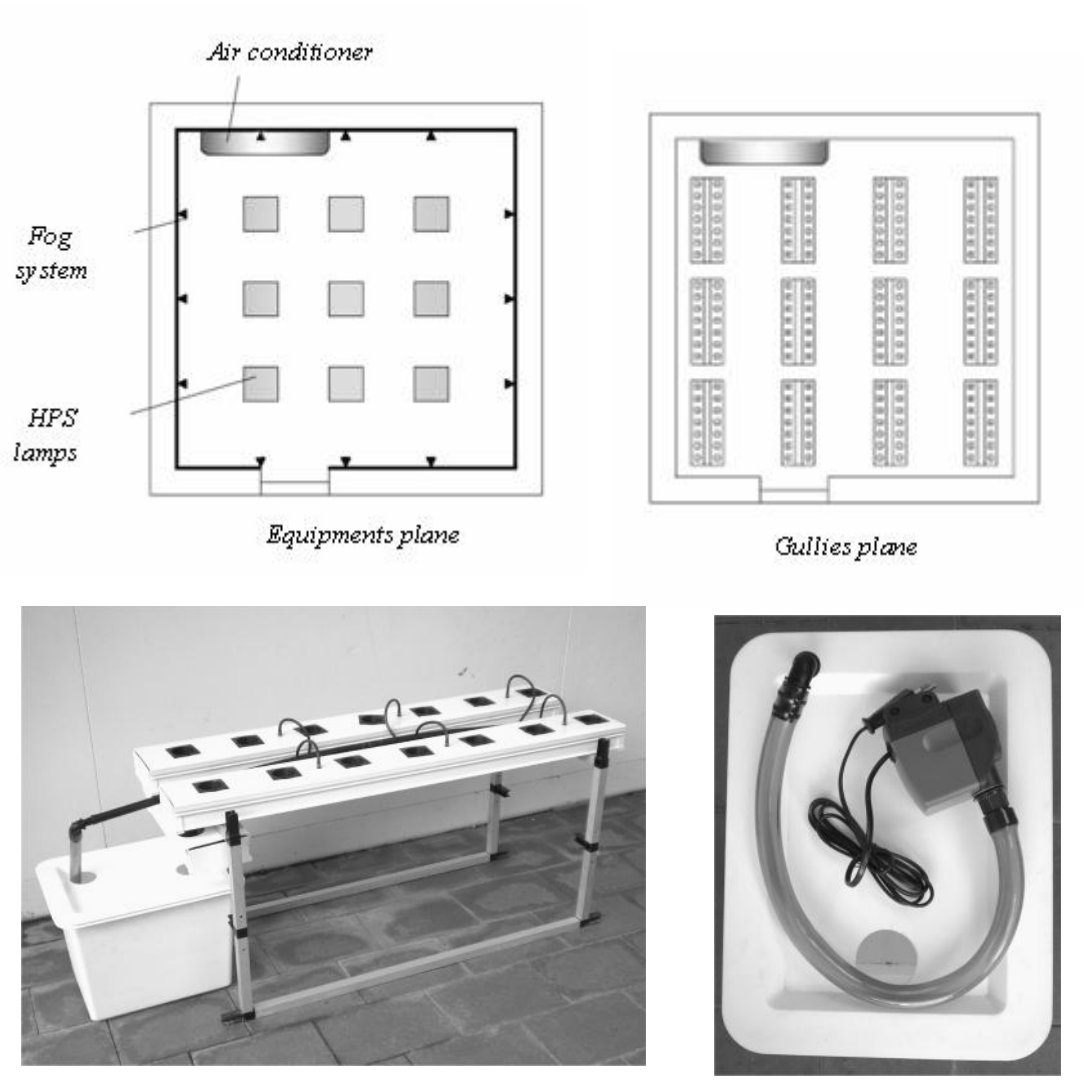


Figure 2 - Average values of temperature and relative humidity in the growth chamber during the experiment (Average ± Standard Error; n = 133 days).

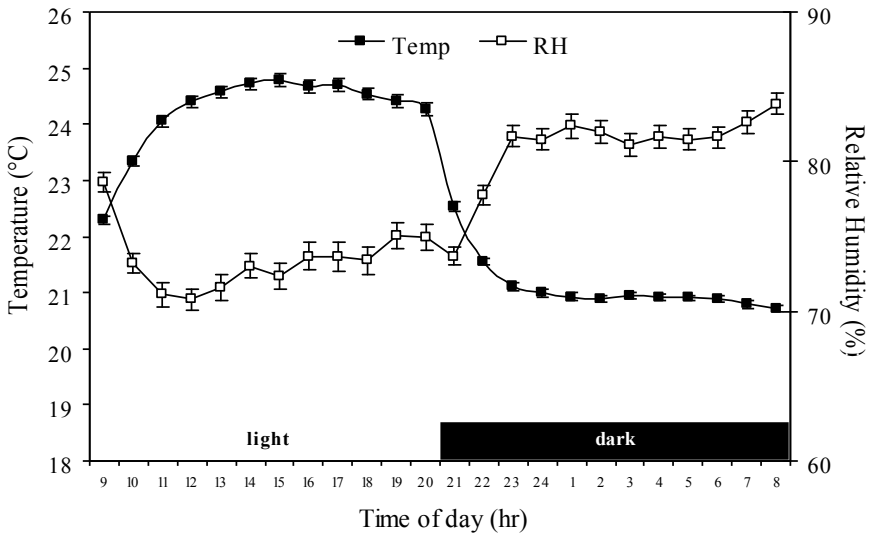


Figure 3 - pH and EC values of recirculating nutrient solution for the soybean cultivars ‘Atlantic’, ‘Cresir’, ‘Pr91m10’, ‘Regir’, before and after the adjustment to the target values (pH 5.5; EC 2.0 dS/m).

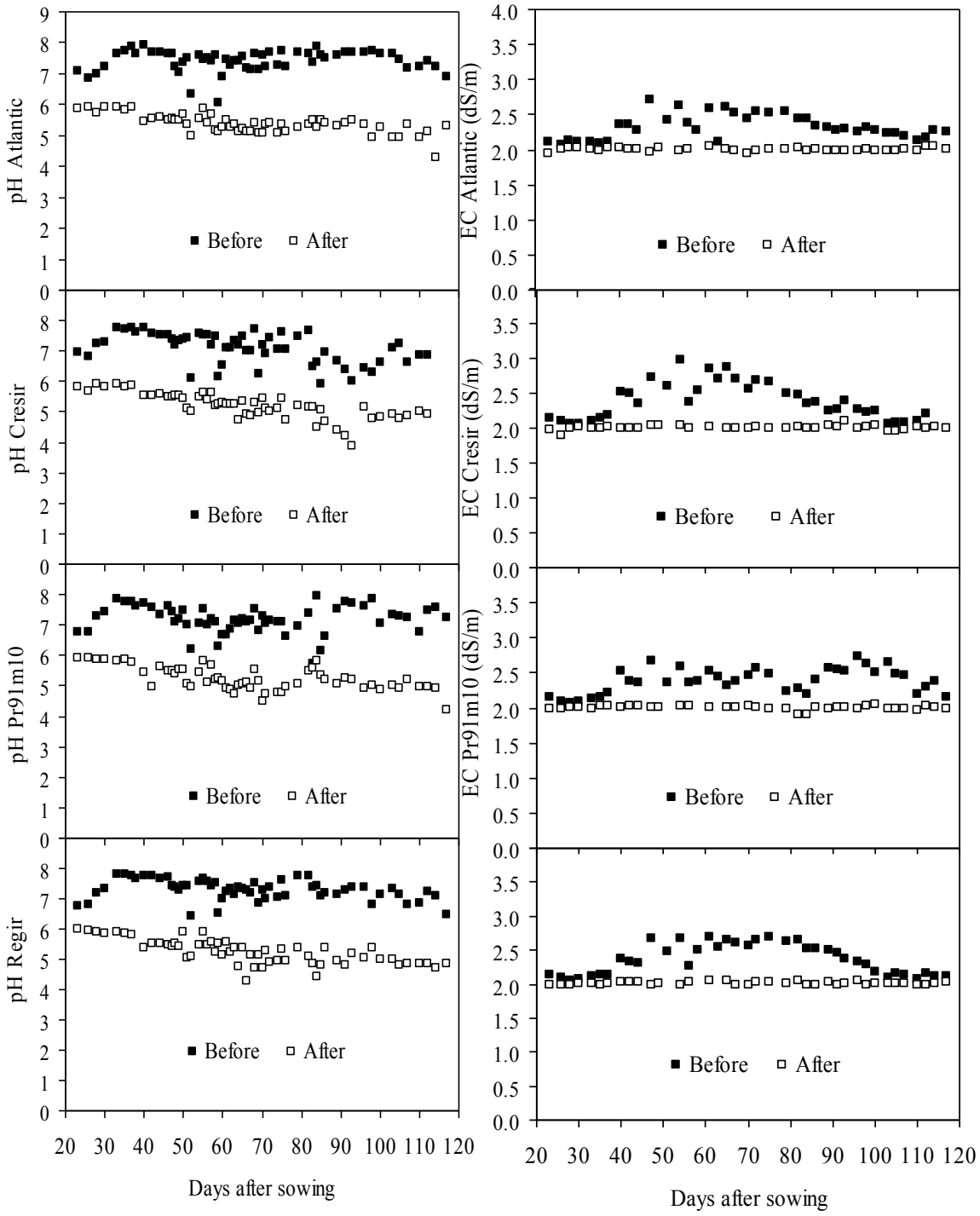


Figure 4 - Time evolution of plant height (A), plant leaf area (B) and daily water consumption (C) in the four selected cultivars of soybean (Mean values ± Standard Error; n=6). R2 of the polynomial equations is reported.

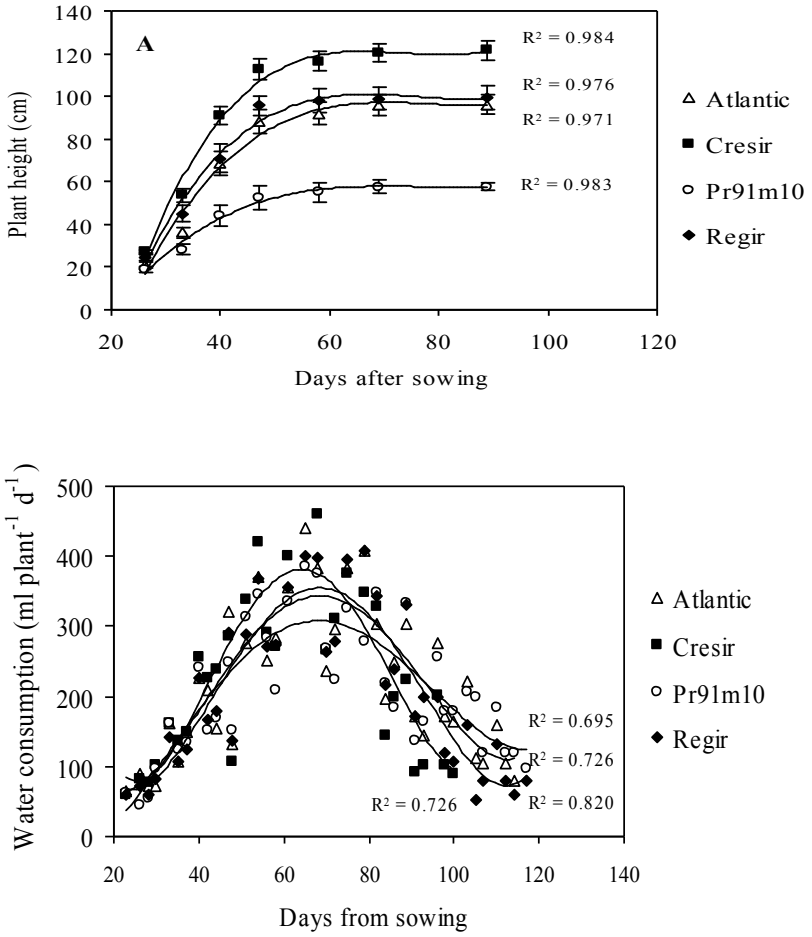
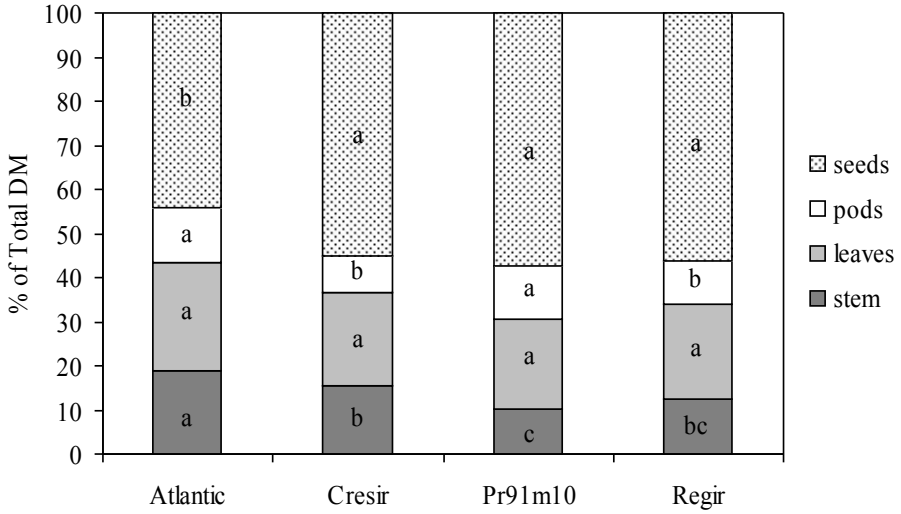


Figure 5 - Dry matter partitioning in different plant parts of the four selected cultivars of soybean at the harvest (% of the total dry weight). Different letters indicate significant differences within the same plant part (P=0.05).



SEASONAL AND MULTIANNUAL EFFECTS OF SALINIZATION ON TOMATO YIELD AND FRUIT QUALITY

Authors: Stefania De Pascale, Francesco Orsini, Rosanna Caputo, Mariantonella Palermo, Giancarlo Barbieri, Albino Maggio

Research highlights:

- Multiannual salinization affects tomato yield not only via ionic and osmotic stress
- Multiannual salinization affects soil physical-chemical properties
- Tomato plants growing in short- and long-term salinized soils have distinctive metabolic and physiological responses
- Plant's ability to withstand high pH and anoxia could contribute to salt stress adaptation

Keywords: short- and long-term salinization, water relations, stomatal conductance, lipophilic and hydrophilic antioxidant capacity.

ABSTRACT

Salinity is one of the most critical abiotic stresses affecting agricultural productions. In most agricultural systems, field salinization is not an abrupt event, yet it evolves over time. While there is an overwhelming literature on plant adaptation to short-term salinization, crop response to long-term salinized soils has rarely been the focus of salinity research. We hypothesized that multiannual salinization may cause yield reductions and physiological imbalances that cannot be solely attributed to hyperosmotic and ionic stresses, as generally observed in short-term and/or seasonal salinization experiments. By comparing plant growth on a soil exposed to *one-season salinization* (ST=Short-Term) *vs*. growth on a soil exposed to *>20 years salinization* (LT=Long-Term) we were able to highlight remarkable differences in tomato responses to salinity. These were associated to substantial modifications of the soil physical-chemical characteristics in the root-zone, including deteriorated structure, reduced infiltration properties and increased pH. Fresh yield, fruit number and fruit weight - a yield component highly responsive to salinity - were similarly affected by ST and LT salinization. In contrast, the marketable yield was significantly lower in the LT salinized soil, a response that could be caused by a nutritional imbalance due to the relatively higher pH of this soil. As reported for plants growing under oxygen deprivation stress, LT salinized plants had high hydrophilic antioxidant capacity. Therefore under multiannual salinization, plant's ability to withstand high pH and anoxia may be important traits that should be considered, in addition to the most studied control of ion homeostasis and osmotic adjustment, to improve plant salt tolerance.

1. Introduction

The need to improve crop productivity in sub-optimal agricultural lands is pivotal to meet the food demand of a growing world population and has generated an unprecedented interest towards saline agriculture (Wild, 2003; Rozema and Flowers, 2008). Salinization is a phenomenon of expanding dimensions, mostly associated with common irrigation practices. It is estimated that of over 270 million hectares of irrigated land, nearly 20% is salt affected (Flowers and Flowers, 2005). In addition, intensive cultivation schemes combined with an increasing competition for fresh water in agricultural, domestic and industrial uses is further exacerbating the progression of salinization (Rozema and Flowers, 2008).

Accumulation of salt in the root zone may cause transitory metabolic disturbances that plants may overcome without impairing yield and product quality. This may occur if the salt concentration in the root zone does not reach species-specific toxicity levels during the growth season or if a critical salt concentration is not achieved in concomitance with sensitive developmental stages (Lutts et al., 1995; Maas and Grattan, 1999; Hasegawa et al., 2000). At advanced soil salinization and/or permanently high salinity, plants activate physiological stress-adaptation responses that interfere with plant growth and ultimately yield (Maggio et al., 2004). These two possibilities (*transitory* vs. *permanent* salinization) can be framed in concrete agricultural contexts. In Mediterranean environments, when saline irrigation is performed in non-salinized soils, spring-summer crops such as tomato cultivations may encounter problems if salts accumulate in the root zone - beyond its physiological tolerance threshold - because of inefficient irrigation practices and/or scarce seasonal rainfall (Cuartero and Fernàndez-Muñoz, 1999; De Pascale et al., 2005). Plants ability to overcome such seasonal stress depends on their responsiveness to external stimuli (salinity) and promptness in re-establishing water/ion homeostasis and growth in an unfavorable environment (Maggio et al., 2006; 2007). The overall outcome in terms of growth and final yield/product quality depends on a combination of biological and environmental variables, including timing/overlapping of toxicity levels reached in the soil with critical phenological phases (i.e. in tomato this could be the stage of fruit on-set and development) (De

Pascale et al., 2005). In contrast, soils which have been repeatedly exposed to spring/summer saline irrigation and to insufficient autumn/winter rainfall will gradually undergo to significant modifications of the soil physical-chemical properties due to salt accumulation (Katerji et al., 1992; De Pascale et al., 2003). These events add a further level of complexity respect to seasonal salinization. Long-term salinized soils often present reduced permeability due to the formation of surface seals and higher water content because plants growing in these soils transpire less and use less water throughout the growth season (Rhoades et al., 1992; De Pascale and Barbieri, 1995). Salinized clay-loam soils typically have decreased structure index, poor root zone aeration, surface crusting and reduced water infiltration rate, which are often associated to prevalence of sodium on the adsorption complex and consequent deflocculation of the clay particles (Hachicha et al., 2000; De Pascale et al., 2005). It is intuitive that plants adaptation either to seasonal and multiannual salinization - hereafter short-term (ST) and long-term (LT) salinization, respectively - may share common responses, such as those controlling ion/water homeostasis and detoxification of reactive oxygen species (Hasegawa et al., 2000). However, specific *physiological needs* may be also associated with different aspects of short- vs. long-term salinization (Maggio et al., 2004; 2011). In the first case, plants have to cope mainly with seasonal fluctuations of soil water potentials (e.g. spring/summer rains that leach out the salt brought about by irrigation), which may require rapid stomatal responses to minimize water stress during the hottest hours of the day (Horchani et al., 2010). Conversely, plants growing in multiannual salinized soils may have to cope also with anoxia phenomena, due to poor soil water infiltration, as well as pH alterations determined by permanent salinization (Maggio et al., 2004).

While these specificities suggest that it is necessary to understand which physiological traits are more critical to overcome salt stress under given saline conditions (Flowers and Flowers, 2005), they also clearly indicate that short- and long-term *salinities* may have a different impact on yield and product quality. Since most published literature refers to short-term salinized soils (Gonzales-Fernandez and Cuartero, 1993; Plaut, 1997; Cuartero and Fernandez-Munoz, 1999; Sakamoto et al., 1999) there is a true need to complement current information on crop response to salinity with quantitative data on long-term effects of salinization. In this manuscript, we present the results of a comparative analysis of physiological and growth responses of tomato to short-term vs. long-term salinization.

2. Materials e methods

2.1 Experimental design

This research was carried out in Southern Italy at the University of Naples Federico II experimental farm "Torre Lama" (latitude 43° 31 N; longitude 14° 58 E) during three growth seasons (2007, 2008 and 2009) on processing tomato (*Lycopersicon lycopersicon* Mill.). The long-term salinized field used for the experiment was a clay-loam soil (clay-loam soil: 42% sand, 27% silt, 31% clay, and trace amounts of lime) that had been irrigated since 1988 with saline water (Maggio et al., 2004). The short-term salinized plots were adjacent to the long-term salinized ones but had not been previously irrigated with saline water.

Three irrigation/salinity treatments were imposed: a non salinized control (NSC, EC_w = 0.5 dSm^{-1} at 25 °C), and two saline concentrations, SW1 and SW2, corresponding to EC_w = 2.3 dSm^{-1} at 25 °C and EC_w = 4.4 dSm^{-1} at 25 °C respectively. The saline water was obtained by adding commercial sea salt (Na^+ 12.3 mol kg^{-1}, K^+ 3.8 mol kg^{-1}, Ca^{2+} 0.02 mol kg^{-1}, Mg^{2+} 0.04 mol kg^{-1}, Cl^- 14.4 mol kg^{-1}, SO_4^{2-} 0.03 mol kg^{-1}) to the irrigation water (Na^+ 0.53 mol m^{-3}, K^+ 0.05 mol m^{-3}, Ca^{2+} 1.55 mol m^{-3}, Mg^{2+} 0.84 mol m^{-3}, Cl^- 0.38 mol m^{-3}, SO_4^{2-} 0.15 mol m^{-3}, HCO_3^- 4.73 mol m^{-3}) to a final concentration of 0.125% (w/v) (SW1) and 0.25% (w/v) (SW2). No salt was added to the irrigation water of non-salinized control plants (NSC). The experimental design was a split-plot with three replications. The *field* treatments (long- vs. short-term salinization) were assigned to the main plots whereas the *salinity* treatments were assigned to the sub plot. It should be pointed out that in 1988, when the soil salinization was begun in the LT salinized soil, the salinity treatments and relative controls were arranged in a randomized block design replicated three times. However, to assess cumulative effects of salinization over the years, the salinity treatments, which had been randomly assigned within each block in 1988, had to be reassigned to the same experimental field plots in each of the following years. Consequently, since 1988, each plot of the long-term experimental field used in this study has received irrigation water with the same EC_w.

2.2 Soil analyses

Soil samples were taken monthly during the crop cycle to a depth of 0.40 m to measure the electrical conductivity of the saturation extract (EC_e) at 25 °C and the pH. For each treatment, 3 individual soil samples (one per each replication) were taken monthly from transplanting until harvest. Soil aggregate stability was also determined on soil samples collected in the 0-0.40 m soil layer before transplanting and after harvesting. Aggregate stability was determined using the wet sieve method developed by Kemper and Rosenau (1986) and expressed as the mean weight diameter (MDW) of the water-stable aggregates. At the same time, soil

bulk density (Bd) and soil porosity (Sp) were measured on undisturbed soil core collected by using 100 cm^3 metal cylinder samplers (Blake and Hartge, 1986a and b).

2.3. Plant culture

Tomato plantlets (TAMPICO F1) were transplanted from the nursery into the field on 23 April (2007), 26 May (2008) and 14 May (2009) at a density of 4.3 plants/m^2 (0.25 m apart within rows and 0.7 m between rows). Every year, prior to transplanting, 54 kg ha^{-1} of N [$(NH_4)_2SO_4$], and 138 kg ha^{-1} of P (*di*-ammonium phosphate 18-46-0) were applied to the soil. Subsequently, plants were fertilized with additional applications of 33 kg ha^{-1} of N, 66 kg of P_2O_5 ha^{-1} and 48 of kg K_2O ha^{-1} (as NPK fertilizer 11-22-16) three weeks after transplanting. The total amounts of nutrient applied via fertigation every year from the beginning of the saline treatments were: 103 kg N ha^{-1}, 87 kg K_2O ha^{-1} and 93 kg CaO ha^{-1} in 2007, 105 kg N ha^{-1}, 90 kg K_2O ha^{-1} and 99 kg CaO ha^{-1} in 2008 and 107 kg N ha^{-1}, 84 kg K_2O ha^{-1} and 89 kg CaO ha^{-1} in 2009.

To ensure the establishment of the plantlets, 30 mm (2007), 33 mm (2008) and 30 mm (2009) of non-salinized water were applied from transplanting to the beginning of the treatments. Saline irrigation was initiated on 22 May (2007) (29 Days After Transplanting, DAT), 23 June (2008) (28 DAT) and 9 June (2009) (26 DAT). Plots were irrigated at 4-day intervals, using a drip irrigation system with 2 l h^{-1} emitters (5 emitters m^{-2}).

The amount of water applied at each irrigation was equal to the net evaporation between two irrigation events using a Class "A" pan evaporimeter. A pan coefficient of 1 was used for the entire growth season to include a leaching fraction in the total volume of water applied. The estimated water consumption was based on a non-stressed crop, so that all treatments received the same amount of water. All treatments received 355 mm (2007), 360 mm (2008) and 330 mm (2009) of water from beginning of the saline treatment to harvest, distributed in eighteen irrigation events.

2.4. Plant growth and water relations

Plant leaf number, leaf area, and dry matter were measured at the beginning of the salinity treatments and subsequently at approximately 30-day intervals on nine plants per treatment (three plants per each replication). The leaf area was measured using a Li-Cor 3000 leaf area meter (Li-Cor, Lincoln, NE). Leaf, stem and fruit dry weights were determined upon dehydration in an oven at 60 ^oC (until steady weight). In coincidence with the plant growth measurements, stomatal resistance, leaf total and osmotic potentials and turgor pressure were measured between 12:00AM and 1:00PM on the youngest fully expanded leaf. The stomatal resistance was measured with a diffusion porometer (AP-4, Delta-T Devices, Cambridge, UK). Water potentials (Ψ_t) were measured on tissue discs punched from the first, uppermost, fully expanded, healthy and sun-exposed leaf (Slavik, 1974) using a dew-point psychrometer (WP4, Decagon Devices, Pullman, WA). The osmotic potential (Ψ_π) was measured after freezing at -20 ^oC and thawing leaf samples and the pressure potential (Ψ_p) was estimated as the difference between Ψ_t and Ψ_π, assuming a matric potential equal to zero (Hsiao, 1973).

2.5. Harvest

Fruits were harvested on August 6, 2007 (105 DAT), on September 5, 2008 (102 DAT) and on August 18, 2009 (96 DAT). Fruits were counted, weighed and ranked for their marketability (the non-marketable yield included fruits with mechanical, physiologic - e.g. blossom-end rot - and/or phytosanitary damages, yellow fruits and fruits having lesions). Total and marketable yield, average dry weight (after drying them at 60^o C) and qualitative characteristics of the fruits (titratable acidity, total soluble solids, pH, EC, antioxidant activities, reducing sugars, nitrates and lycopene contents) were measured. The qualitative parameters were determined in a sample of 5 fruits per replication. Titratable acidity was determined on homogenized fresh and filtrated samples titrated to pH 8.1 with 0.1 N NaOH and expressed as grams of citric acid per 100 g of fresh weight. Total soluble solids were measured on tomato juice samples with a refractometer (ATAGO Palette PR-32, Atago Co., Ltd., Tokyo, Japan) and expressed as °Brix. The pH was determined using a pH meter at 25^o C (Mettler Toledo MP 220, Mettler-Toledo, Schwezenbach, Switzerland) and the electric conductivity (EC) was measured with a conductimeter at 25 ^oC (Conductimeter BASIC 30, Crison Instruments SA, Barcelona, Spain). The antioxidant activity was measured on the water-soluble fraction using the *N*,*N*-dimethyl*p*-phenylenediamine (DMPD) method (Fogliano et al., 1999). The antioxidant activity of the water soluble fraction was expressed as equivalent millimolar of ascorbic acid for 100 g of tissue fresh weight. The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method performed as described by Pellegrini et al. (1999) was used to assess the antioxidant activity of water-insoluble fractions. The antioxidative activity of the lipophilic fraction was expressed in millimoles of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) present in 100 g of fresh tomato. The concentration of reducing sugars (fructose and glucose) was determined using Fehling's method. Briefly, 50 ml of fruit pulp was cleared by the addition of a mixture of 20 ml saturated basic lead acetate and 20 ml saturated sodium

phosphate and filtered. The two stock Fehling solutions A and B were mixed in equal quantities immediately before use and diluted with 40 ml distilled water, and this solution was used to titrate the fruit filtrate, using two drops of methylene blue solution as an indicator (AOAC, 2002). Nitrates were measured spectrophotometrically on dried and ground fruit tissue sub-samples after cadmium reduction (Sah, 1994). Lycopene quantification was performed by spectrophotometric analysis (Fish et al., 2002).

Data were analyzed by ANOVA and means were compared by Duncan's Multiple Range Test.

3. Results

3.1 Meteorological conditions and soil parameters

The growth season was warmer and dryer compared to historical trends for the 3 years considered in this study, as indicated by rainfall and air temperature values (Table 1). Mean temperatures during the experiments were slightly higher in 2008 (24.3 ^{o}C) compared to 2009 (23.8 ^{o}C) and 2007 (23.4 ^{o}C). The hottest months were July and August with mean temperatures of 24.7-25.5 ^{o}C. The total rainfall during the experiments was 40.2 mm in 2007, 41.7 mm in 2008 and 65.4 mm in 2009. With the exception of the 1st decade of June 2008, the 3rd decade of June and the 1st of July 2009, the rainfall was below the 50-year average in July and August (in coincidence with the vegetative and fruit enlargement stages).

Main physical-chemical differences between ST and LT salinized soils are reported in Table 2. In general EC_e, pH and Bd increased with salinity, whereas Sp and MDW decreased. However, while the differences in EC_e and pH were apparent at 0.125 and 0.25% NaCl, the stability of soil aggregates (MDW) was reduced more in the LT salinized soil only at the 0.25% treatment (Fig. 1). Overall, LT salinized soils were more compact (increased Bd) and with a significant reduced porosity (-12%) respect to ST salinized soils.

3.2 Plant growth and yield

Salinity had detrimental effects on plant biomass and fruit production with a few specificities (Table 3). Plant growth in the LT salinized field was impaired to a greater extent compared to ST salinization. Leaf number and leaf area were 15% and 27% smaller in LT respect to ST plants with a concomitant reduction of the overall plant dry weight (-24% in LT *vs.* ST), and a concurrent increase in dry matter percentage (+15% in LT *vs.* ST) (Table 4). However, in 2008 the differences in terms of leaf number per plant were reduced and the plant dry weight was similar in LT and ST salinized soils (data not shown).

A remarkable effect of the year was observed on major yield components, including total and marketable yield and fruit production per plant (Table 3; Figure 2), indicating that other growth/yield determinants (meteorological factors) may have prevailed on the soil conditions, including the level of salinization, to determine the final yield. Salinity considerably affected all yield components causing, at the highest salinization, 25% and 20% reductions in terms of marketable yield and average fruit weight, respectively (Table 2). Although major differences in total yield were not observed between ST (57.9 t ha $^{-1}$) and LT (56.8 t ha^{-1}) plots, LT salinization had a significant impact on the marketable *vs.* non-marketable fractions (waste). Under LT salinization, the marketable yield was 3% lower compared to ST salinization, with a concomitant increase in the percentage of waste (Table 2). The significant interaction Year x Field once again indicated that higher temperatures in 2008 may have exacerbated the differences between ST and LT salinization in terms of yield per plant. In contrast, the exceptional June-July rainfall in 2009 may have been responsible for the increased percentage of non-marketable fruits in LT plots (+30% respect to ST) (Fig.3). The effect of salinity on the average fruit weight was reduced in the second year of experimentation (2008), due to an overlapping of abiotic stresses (high temperature and salinity), which nullified the difference between non-salinized control and 0.125% NaCl treated plants (Fig. 4).

3.3 Fruit quality

Low (0.125% NaCl) and high (0.25% NaCl) salinization both improved major quality parameters of tomato fruits (Table 5) by increasing TSS (+18% at 0.25% NaCl), reducing sugars (+12% at 0.25% NaCl), LAC (+50% at 0.25% NaCl) and lycopene (+13% at 0.25% NaCl). However, salinity also increased nitrates levels (+30% at 0.25% NaCl) (Table 5). The concentration of reducing sugars increased from 2007 to 2009 and with salinity. However this parameter was similar in LT and ST salinized soils, indicating that other differences observed in plants growing in these two soils were unlikely due to the level of salinization. LAC and HAC, which are generally associated to various stress responses, did not seem to respond to the same environmental factors. LAC was mostly affected by salinity whereas HAC seemed to be more responsive to the overall soil conditions, although a tendency to increase with salinity was also observed (Table 5, Fig. 5). These results suggest that different pathways of the antioxidant response may have been activated by different environmental stimuli. Moreover, the significant interactions between Y x F, Y x S and F x S for TSS, reducing sugars, HAC and lycopene (Table 5) also confirmed that variations in quality parameters are largely sensitive to combinations of environmental stressors that may lead to the accumulation of different metabolites in response to specific adaptation needs.

3.4 Plant water relations

Higher stress was also experienced by plants growing in LT salinized soil (Table 6). This was probably due to a combined effect of soil salinity and other soil variables that may have differentiated ST *vs*. LT salinized soils, including a reduced aeration and high soil pH that may have further affected plants water relations and growth (Table 2). Salinity reduced the stomatal conductance and leaf water potentials (Table 6). Lower stomatal conductance, Ψ_t and Ψ_π measured in plants growing in LT respect to the ST salinized field (-17%, -14% and -11%, respectively at 0, 0.125 and 0.25%) were consistent with higher stress conditions experienced by those plants. Nevertheless, both ST and LT salinized plants were able to adjust to the external environment as indicated by similar turgor pressure (Ψ_p) detected in plants growing in the two fields under assessment (Table 6). The adjustment to stressful environmental conditions was mediated by a quite remarkable regulation of the cellular Ψ_π that was very responsive to salinity, soil modifications and environmental parameters (Table 6).

4. Discussion

4.1 Soil salinization

Understanding the progression of salinization in agricultural systems, as well as crop responses to salinity is essential to quantify the deleterious effect of salinity and to define strategies to improve plant salt tolerance in specific agricultural contexts (Maggio et al., 2011; Pardo, 2010). Despite the multifaceted complexity of salinization, most research on plant responses to salinity has been conducted in experimental systems that often oversimplify such complexity. Our data indicate that salinization substantially modifies the physical-chemical characteristics of the soil in the root-zone, an occurrence that may further exacerbate the effect of salinization (Table 2, Fig. 1). Barrett-Lennard (2003) has reported that at advanced salinization many soils present reduced infiltration, water stagnation and consequent depressive effects on plant growth due to hypoxia. In a previous experiment we have found an increase of the soil water content in the soil exposed to multiannual saline irrigation (Maggio et al, 2004), which was caused by a reduced soil permeability (Rhoades et al., 1992; De Pascale and Barbieri, 1995). These results were indirectly confirmed in this 3-years experiment where increased soil bulk density (Bd) and reduced soil porosity in LT salinized plots were also found.

The remarkable increase of pH in the LT salinized soil may have reduced mobility and uptake of micro-elements with a consequent nutritional imbalance that may have affected fruit quality. Consistent with this possibility, we found a significant increase in fruit waste in LT *vs.* ST salinized plants (Table 3, Fig. 3) (Cuartero and Fernàndez-Muñoz,, 1999). Furthermore, in Mediterranean environments, overlapping of multiple stresses such as daily water stress and salinity may have further enhanced the differences between LT and ST salinized soils, where modifications of the matric and osmotic potentials may contribute to a different degree (Rengasamy, 2002). It is therefore critical to consider plant response to salinity in specific cultural and agro-environmental conditions since these specificities may have important implications to develop reliable prediction models and identify critical traits that could be potentiated to improve plant stress tolerance.

4.2 Physiological responses and agronomic performance

A reduction of stomatal conductance in response to salinity has been reported for tomato (Maggio et al., 2007; Orsini et al., 2010) and most crop plants and has been recognized as a fundamental adaptive response to water deficit. The reduction of leaf water potential (Ψ_t) observed under salinization was also symptomatic of a soil-plant-atmosphere disequilibrium typically caused by hyper-osmotic stress (Maggio et al., 2004). Tomato plants responded to these unfavorable conditions by reducing the osmotic potential (Ψ_π), a physiological mechanism that, conjointly with a reduced stomatal conductance, consented to partially regain their tissue turgor pressure (Ψ_p) (Table 6). Plants growing in LT salinized plots were relatively more stressed compared to those growing in ST salinized plots since they had significant lower Ψ_t and stomatal conductance. Nevertheless these plants were able to osmotically adjust quite effectively so that no difference in terms of Ψ_p were observed between ST and LT plants. Although the EC_e of the LT salinized soil was slightly higher than ST plots (2.85 *vs*. 2.20), at comparable levels of saline irrigation, the extra stress experienced by LT plants could not be attributed only to salinity. As previously discussed, destructured long-term salinized soils had significantly reduced infiltration properties. Consequently, LT plants have likely experienced oxygen deprivation due to water stagnation and consequently may have manifested additional stress symptoms. The deleterious effects of hypoxia on stomatal conductance have been reported for a large number of species (Sojka, 1992). A 20% further reduction of stomatal conductance has been documented in tomato when anoxia was combined with salt stress (Horchani et al., 2008 and 2010). Hypoxia also affects root hydraulic conductivity (Ehlert et al., 2009) and consequently impairs tissue hydration under stress conditions, which could also explain the reduced Ψ_t observed in LT plants (Table 6). In line with this

possibility, we also found that the increase of the hydrophilic antioxidant capacity, a frequent response under oxygen deprivation stress (Blokhina et al., 2003), was higher in LT compared to ST salinized plants.

With respect to yield, this 3-year experiment revealed an important *year* effect that seemed to be predominant on other variables, namely the salinity level and LT/ST salinization (Table 3). The significant effect of the year, and meteorological variables, on yield can be further appreciated by comparing the yield reduction at 0.25% NaCl over 3 years experiment with the results obtained in 1999 on the same experimental field, when total and marketable yield was not affected by salinity at 0.25% NaCl (Maggio et al., 2004). Although we could not exclude a cultivar effect (i.e. the hybrid *Cois HC01* used in 1999 could be more salt tolerant than *Tampico* used in this experiment), this could be explained by a significant reduced rainfall (below the 10-year average) in 2007 and 2008 and an average temperature in 2007-2008-2009 (23.8º C) higher than 1999 (22º C), which may have exposed plants to further stress. The general effect of salinity on tomato fruit yield was consistent with most published literature (Perez-Alfoces et al., 1996; Cuartero et al., 2006; Al-Busaidi et al., 2009; Hajer et al., 2006). In line with other assessments of tomato salinity responses, which highlighted an effect of salinity on fruit size rather than fruit number (Gonzales-Fernandez and Cuartero, 1993; Plaut, 1997; Cuartero and Fernandez-Munoz, 1999; Sakamoto et al., 1999) we found that the fruit weight was reduced more at high salinization (0.25%) compared to low salinization (0.125%), whereas the fruit number was similar at the two salinity levels considered. A reduced fruit enlargement could have been caused by high sensitivity to ionic and osmotic damages of tomato fruits during the exponential phase of growth (Adams and Ho, 1989).

Interestingly enough, fresh yield, fruit number and fruit weight, a yield component highly responsive to salinity (Cuartero et al., 2006), were similarly affected by ST and LT salinization, suggesting that further differences in terms of yield were most likely not only due to a *salinity effect* by itself. In 1999, based on the results obtained on the same experimental field we hypothesized that the higher salt-induced yield reduction observed in 1999 respect to 1988 (when the long term salinization project begun) was due to the long-term effects of salinization on the soil physical–chemical soil properties. By a direct comparison, here we demonstrate that ST and LT salinized soils may affect differently plant growth and development. Consequently, any assessment of yield reductions due to salinization should consider additional determinants that affect yield as a *consequence* (e.g. reduced permeability, anoxia, pH-induced nutritional imbalance) rather than direct effect (e.g. osmotic stress and ion toxicity) of salinization. These findings add new elements for the design of plants with improved salt tolerance (Pardo et al., 2010). The physiological responses associated to *seasonal* salinization have been addressed to a great extent (Cuartero et al., 2006; Hu and Schmidhalter, 2005). These studies should be complemented by a broader analysis of physiological traits that could be important for plant adaptation to long term salinization. These may include functional morphological modifications of root system architectures (Maggio et al., 2001; Rahnama et al., 2011) and improved tolerance to hypoxia conditions (Colmer and Flowers, 2008).

4.3 Consequences of salinization on fruit quality

An improved quality of tomato fruits grown in saline environments has been documented (Petersen et al., 1998) and often presented as a compensating factor for a concomitant reduced yield (De Pascale et al., 2001). In contrast to most published literature reporting an increase of fruits acidity in response to salinity in several solanaceae, including tomato (Savvas and Lenz, 1996; Feigin et al., 1987; De Pascale et al., 2001), we did not observe significant variations for this parameter. This could be explained by the moderate salinity levels used in this study or other interacting factors and/or climatic parameters that may have counteracted the specific response to salinity (Grattan and Grieve, 1999). Total soluble solids (TSS) and reducing sugars increased with salinity with an *year* effect that flattened down the differences between salt treatments during the most stressful seasons (2007 and partially 2008) (Table 5). Salinity generally increases reducing sugars and total soluble solids in tomato fruits, resulting in higher fruit quality (Plaut et al., 2004; Niedziela et al., 1993; Plaut, 1997; Cuartero and Fernandez-Munoz, 1999; Sakamoto et al., 1999; Dorais et al., 2001; Flores et al., 2003). An increase of sugars and TSS is caused by a concentration effect (stressed plants have lower water content) (Ehret and Ho, 1986; Willumsen et al., 1996), but also by an active accumulation of molecules (compatible solutes, sugars or amino, acids in the cytoplasm, and inorganic solutes in the vacuole) that could contribute to osmotic adjustment (Maggio et al., 2004). It has been demonstrated that photosynthetic rates in tomato plants are only moderately affected by salinity (Gao et al., 1998) and in some cases even enhanced (Xu et al., 1995), which would be consistent with a net accumulation of functional molecules necessary for stress adaptation (Plaut, 1997; Plaut et al., 2004).

With the exception of 2008, the level of reducing sugars for LT and ST salinized soils was similar indicating that additional modifications of the root-zone environment due to long term salinization did not affect this parameter. A distinctive response between LT and ST salinized fruits was a significant higher Hydrophilic Antioxidant Capacity (HAC) in the former. While the HAC has been reported either to increase or decrease in response to salinity as well drought (De Pascale et al., 2007), an increase of HAC has been

specifically associated to oxygen deprivation stress (Wallenweber-Ratzer and Crowford, 1994; Biemelt et al., 1998), suggesting that one possible specific effect of LT salinized soil is due to a reduced aeration of the root zone (Blokhina et al., 2003). Although the observed differences may be attributed to the slightly higher EC_e of LT plots, once again these findings tend to *uncouple* salt- vs. non-salt-induced responses in ST respect to LT salinized soils. The accumulation of lycopene in response to salinity was also in line with other reports (Dumas et al., 2003). However this parameter was not distinctive of LT *vs*. ST salinized plants.

The complexities of crop-salinity interactions here presented complement current knowledge on short term and/or seasonal responses to salt stress. Framing these responses in a dynamic and evolving field context is important to fully understand how plants can best adapt to salinization of agricultural areas. In the long run, improving plant's ability to control ion toxicity and osmotic adjustment, the main traits on which the biotechnology of salt stress tolerance has been focusing in the last decade (Pardo, 2010), may be not sufficient to maintain high and stable yield under advanced salinization.

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Table 1. Environmental conditions (minimum, maximum temperatures and rain) in the three years of experimentation.

Month	Decade	Mean 50 years			Deviation from the mean								
					2007			2008			2009		
		Tmin °C	Tmax °C	Rain mm	Tmin °C	Tmax °C	Rain mm	Tmin °C	Tmax °C	Rain mm	Tmin °C	Tmax °C	Rain mm
April	I	8.3	17.3	19.6	0.9	1.4	-3.9	0.2	1.3	-7.8	1.7	2.6	-16.3
	II	8.5	17.3	23.5	2.9	5.4	-23.2	1.3	1.9	-12.4	2.6	3.0	-16.9
	III	9.3	18.7	17.9	**2.7**	**4.8**	**3.2**	0.6	0.9	-9.3	2.5	1.1	9.6
May	I	11.1	20.5	20.5	**2.8**	**2.0**	**8.0**	0.5	3.1	-20.2	0.3	1.1	-18.0
	II	12.7	22.4	11.3	**0.7**	**0.9**	**-4.1**	1.8	1.7	-1.8	**3.9**	**3.2**	**-11.3**
	III	13.4	23.6	9.3	**1.4**	**1.5**	**12.4**	**2.3**	**3.5**	**2.9**	**3.6**	**2.9**	**5.1**
June	I	14.4	24.4	12.8	**0.7**	**0.4**	**5.7**	**1.4**	**1.3**	**20.5**	**1.7**	**2.2**	**-2.4**
	II	15.6	25.7	5.6	**2.7**	**2.5**	**-5.6**	**1.2**	**0.5**	**1.3**	**3.4**	**2.8**	**-5.3**
	III	16.9	27.1	6.2	**2.3**	**3.5**	**-6.2**	**3.2**	**3.8**	**-6.2**	**-0.4**	**-1.9**	**22.1**
July	I	17.4	27.8	4.0	**0.6**	**0.5**	**-4.0**	**2.8**	**2.3**	**-2.5**	**2.5**	**0.5**	**6.2**
	II	18.1	28.3	5.9	**0.0**	**1.6**	**-5.9**	**1.4**	**2.3**	**-5.9**	**1.4**	**1.2**	**-5.6**
	III	18.5	28.9	7.5	**3.2**	**3.0**	**-7.5**	**0.7**	**0.1**	**-7.5**	**2.0**	**2.5**	**-7.5**
August	I	18.5	29.1	4.2	**0.6**	**0.2**	**-4.2**	**2.8**	**2.1**	**-4.2**	**2.5**	**2.0**	**-4.2**
	II	18.2	28.9	11.7	0.9	-0.7	-11.7	**1.3**	**1.9**	**-11.7**	**3.1**	**4.1**	**-10.2**
	III	17.7	28.0	16.2	4.9	7.1	-16.0	**1.9**	**2.0**	**-16.2**	4.5	4.5	-14.2
September	I	16.6	26.9	16.0	-0.4	-0.7	-3.0	**3.7**	**4.4**	**-16.0**	2.9	2.8	-16.0
	II	15.6	26.2	22.4	1.0	1.3	-2.7	0.7	-1.2	39.3	2.4	0.3	41.3
	III	14.9	25.0	25.6	-0.7	0.2	51.2	-1.4	-2.6	-20.8	3.7	1.0	-23.3

Table 2 – pH, ECe of the saturated soil extract, soil bulk density (Bd), soil porosity (Sp) and mean weight diameter of the water-stable aggregates (MDW) in response to shor- term (ST) and long-term (LT) salinization.

	pH	ECe (dS/m)	Bd (t/m^3)	Sp (%)	MWD (mm)
Year (Y)					
2007	7.15	2.71 a	1.35	47.23 a	1.28 a
2008	7.17	2.28 b	1.47	42.74 b	0.93 b
2009	7.22	2.59 c	1.47	42.79 b	0.91 b
	ns	**	ns	*	**
Field (F)					
ST	7.10	2.20	1.36	47.02	1.10
LT	7.26	2.85	1.50	41.48	0.98
	**	**	*	**	**
Salt (S)					
0	7.08 b	1.73 c	1.39 b	45.59 a	1.11 a
0.125	7.21 a	2.62 b	1.44 a	43.86 b	1.04 b
0.25	7.25 a	3.23 a	1.46 a	43.31 b	0.97 b
	**	**	**	*	**
Y x F	ns	ns	ns	ns	*
Y x S	ns	**	ns	ns	ns
F x S	**	**	ns	ns	*
Y x F x S	ns	ns	ns	ns	ns

Table 3. Survival rate, total and marketable yield, fruit waste percentage, weight, number and dry matter of processing tomato as affected by ST and LT salinization. Letters indicate significant differences at P≤0.05.

	Survival rate (plant m^{-2})	**Total Yield** (tonn ha^{-1})		**Mkt. Yield** (tonn ha^{-1})		**Waste** (%)		**Fruit weight** (g $fruit^{-1}$)		**Fruit number** (fruits $plant^{-1}$)		**Fresh yield** (kg $plant^{-1}$)		**Fruit dry matter** (%)	
Year (Y)															
2007	4.25	62.4	a	57.3	a	8.27	a	63.7		21.2	a	1.35	a	6.05	a
2008	4.23	49.6	b	45.9	b	7.43	a	62.1		17.6	b	1.09	b	6.52	b
2009	4.20	60.1	a	56.4	a	6.32	b	63.4		21.6	a	1.34	a	8.08	c
	ns	**		**		*		ns		**		**		**	
Field (F)															
ST	4.23	57.9		54.0		6.8		64.2	a	19.9		1.28		6.68	
LT	4.22	56.8		52.4		7.8		61.9	b	20.3		1.24		7.08	
	ns	ns		*		*		ns	c	ns		ns		ns	
Salt (S)															
0	4.26	65.0	a	60.9	a	6.3	a	69.1		21.7	a	1.43	a	6.18	a
0.125	4.24	57.1	b	53.0	b	7.2	b	64.5		19.6	b	1.25	b	6.81	b
0.25	4.18	50.0	c	45.8	c	8.5	c	55.5		19.2	b	1.10	c	7.65	c
	ns	**		**		**		**		**		**		**	
Y x F	ns	ns		ns		*		ns		ns		*		ns	
Y x S	ns	ns		ns		ns		*		ns		ns		ns	
F x S	ns	ns		ns		ns		ns		ns		ns		ns	
Y x F x S	ns	ns		ns		ns		ns		ns		ns		ns	

Table 4. Leaf number and area, plant dry weight and dry matter percentage of processing tomato as affected by ST and LT salinization,. Letters indicate significant differences at P≤0.05.

	Leaf number (n plant^{-1})		**Leaf area** (dm^2 plant^{-1})		**Plant dry weight** (g plant^{-1})		**Plant dry matter** (%)	
Year								
2007	63.4	a	40.3	a	75.3	a	7.8	a
2008	68.9	ab	47.2	a	91.1	b	8.2	a
2009	72.3	b	56.3	b	85.8	b	10.0	b
	*		*		**		**	
Field								
ST	73.7		55.3		95.5		8.1	
LT	62.7		40.6		72.6		9.3	
	**		**		**		**	
Salt								
0	82.6	a	61.4	a	101.7	a	8.1	a
0.125	73.1	b	50.2	b	87.3	b	8.4	a
0.25	48.9	c	32.2	c	63.2	c	9.5	b
	**		**		**		**	
Y x F	*		ns		**		ns	
Y x S	ns		ns		ns		ns	
F x S	ns		ns		ns		ns	
Y x F x S	ns		ns		ns		ns	

Table 5. pH, total soluble solids (TSS), reducing sugars, nitrate content, lipophilic and hydrophilic antioxidant capacities and lycopene concentration in fruits of processing tomato as affected by ST and LT salinization. Letters indicate significant differences at P≤0.05.

	pH		**TSS** (° Brix)		**Reducing sugars** ($g\ g^{-1}$)		**NO_3^-** ($g\ kg^{-1}$)		**LAC** (mM trolox kg^{-1} DW)		**HAC** (mM AsA kg^{-1} DW)		**Lycopene** ($mg\ kg^{-1}$ DW)	
Year														
2007	4.65	a	5.44		0.032	a	302		0.8		11.1	a	115.8	a
2008	4.37	b	5.54		0.036	b	275		0.9		5.0	b	161.0	c
2009	4.61	a	5.14		0.038	b	--		1.4		3.4	b	142.6	b
	*		ns		**		ns		ns		**		**	
Field														
ST	4.54		5.35		0.035		268		1.0		5.6		135.9	
LT	4.52		5.38		0.036		310		1.0		7.5		138.1	
	ns		ns		ns		*		ns		**		ns	
Salt														
0	4.53		4.93	a	0.034	a	257	a	0.8	a	5.9		127.3	a
0.125	4.53		5.35	b	0.035	a	276	ab	1.0	b	6.3		139.8	b
0.250	4.51		5.82	c	0.038	b	333	b	1.2	c	7.2		143.4	b
	ns		**		**		*		**		ns		**	
Y x F	ns		ns		*		ns		ns		ns		**	
Y x S	ns		**		ns		ns		ns		ns		ns	
F x S	ns		ns		*		ns		ns		*		ns	
Y x F x S	ns		ns		ns		ns		ns		ns		ns	

Table 6. Stomatal conductance (gs), water potential (Ψt), osmotic potential (Ψπ), and pressure potential (Ψp) in leaves of processing tomato as affected by ST and LT salinization. Letters indicate significant differences at P≤0.05.

	g_s		Ψ_t		Ψ_π		Ψ_p	
	(cm s^{-1})		(MPa)		(MPa)		(MPa)	
Year								
2007	0.89		-1.02	ab	-1.33	b	0.31	a
2008	0.90		-0.95	a	-1.23	a	0.28	b
2009	0.89		-1.09	b	-1.36	b	0.27	b
	ns		*		*		*	
Field								
ST	0.98		-0.95		-1.24		0.29	
LT	0.81		-1.09		-1.38		0.29	
	**		**		**		ns	
Salt								
0	1.14	a	-0.87	a	-1.18	a	0.31	a
0.125	0.87	b	-1.03	b	-1.31	b	0.28	b
0.25	0.67	c	-1.17	c	-1.45	c	0.28	b
	**		**		**		*	
Y x F	ns		ns		**		ns	
Y x S	ns		*		*		ns	
F x S	ns		ns		ns		ns	
Y x F x S	ns		ns		ns		ns	

Fig. 1 Effect of LT (black bars) and ST (white bars) salinization on electrical conductivity of the soil extract (ECe) pH and mean weight diameter of the water-stable aggregates (MDW). Within each salinity level letters indicate significant differences at P≤0.05.

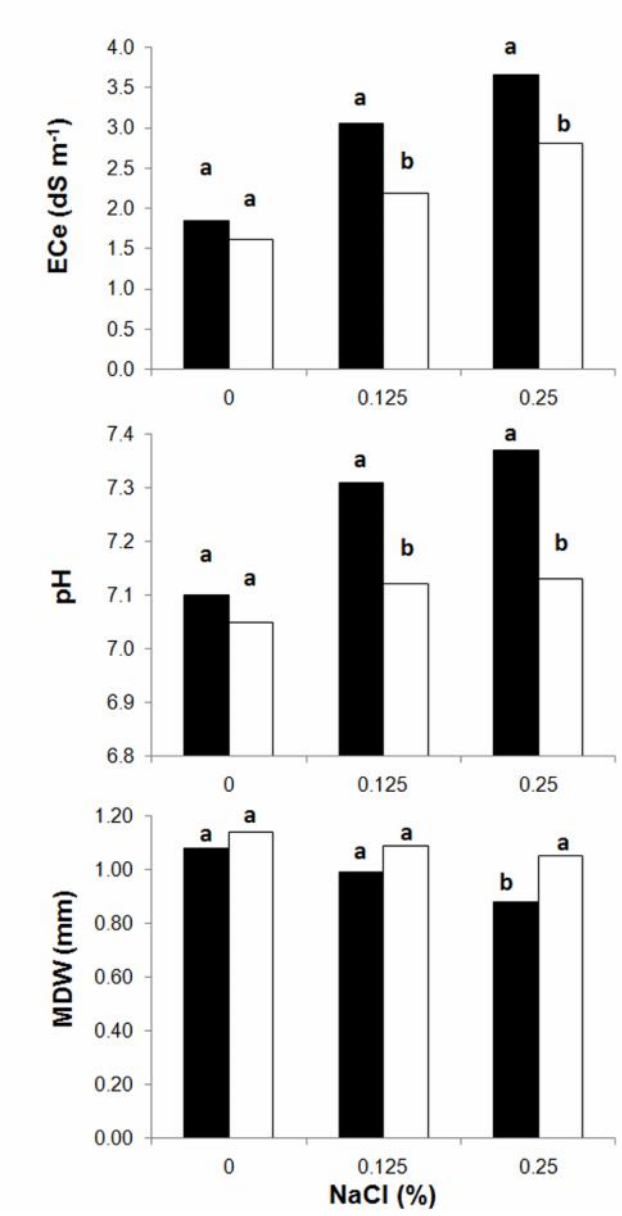

Figure 2. Effect of LT (black bars) and ST (white bars) salinization on the yield of processing-tomatoes in the three years of experimentation. Letters indicate significant differences at P≤0.05.

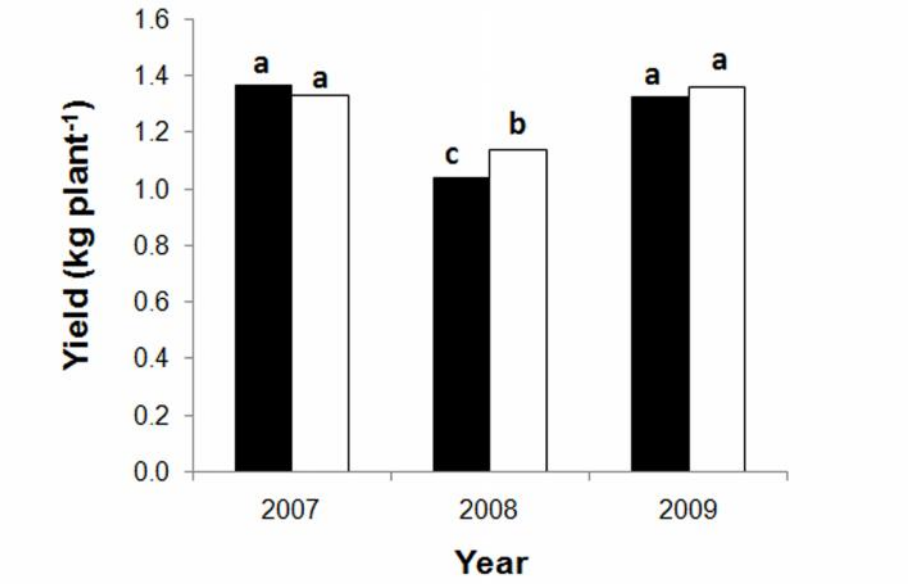

Figure 3. Effect of LT (black bars) and ST (white bars) salinization on waste percentage of processing-tomatoes in the three years of experimentation. Letters indicate significant differences at P≤0.05.

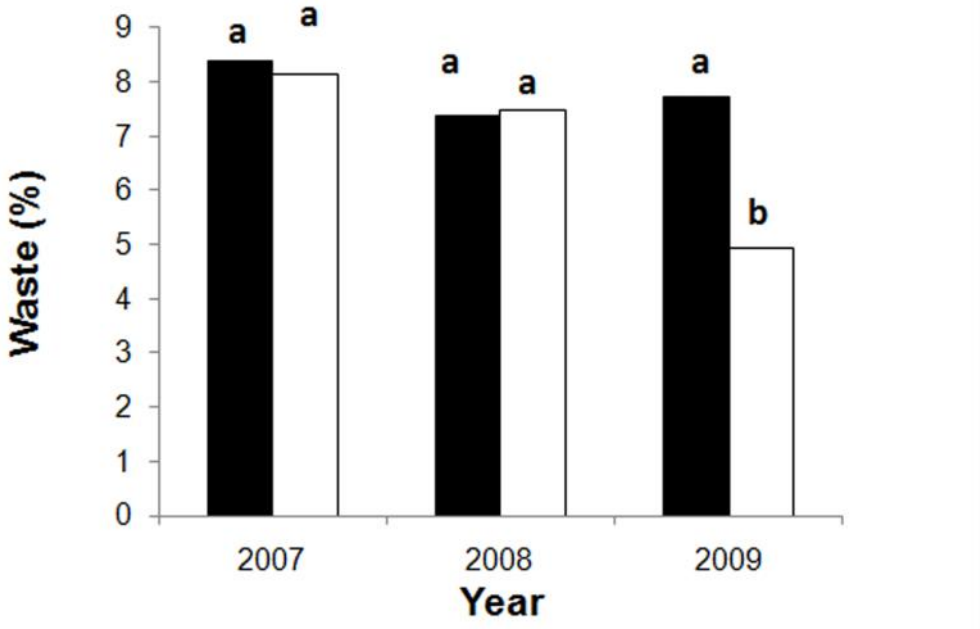

Figure 4. Effect of the growing season (2007, 2008 and 2009) and salinity (black bars, 0, grey bars, 0.125, and white bars, 0.250% NaCl) on the fruit weight of processing-tomatoes. Letters indicate significant differences at P≤0.05.

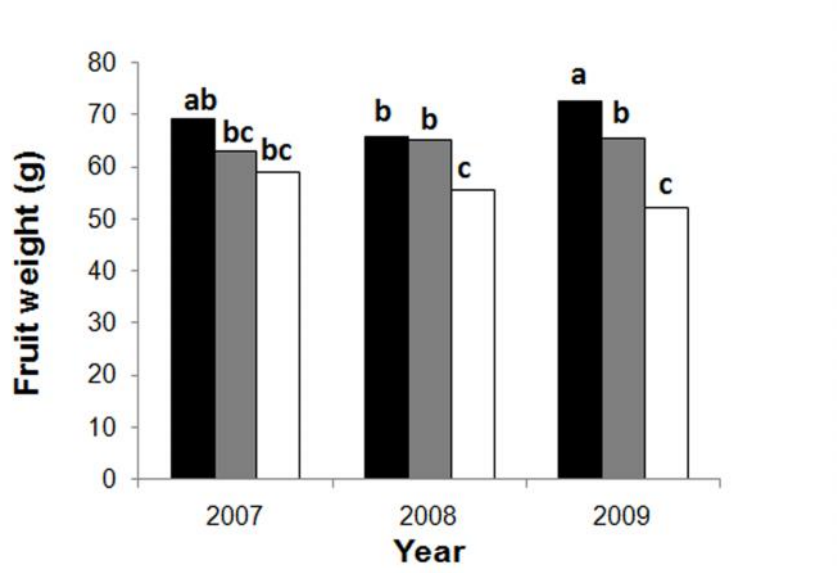

Figure 5. Effect of LT (black bars) and ST (white bars) salinization on fruit hydrophilic antioxidant capacity (HAC) as affected by salinity (0, 0.125 and 0.25% NaCl in the irrigation water). Letters indicate significant differences at P≤0.05.