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Wheat and low–input agriculture:
agronomic, nutritional and
nutraceutical implications

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ABSTRACT

Recently, the increasing interest in organic food products and environmental friendly practices has emphasized the importance of selecting crop varieties suitable for the low-input systems. Additionally, in recent years the relationship between diet and human health has gained much attention among consumers, favoring the investigations on food nutraceutical properties. Among cereals, wheat plays an important role in human nutrition around the world and contributes to the daily intake of essential nutrients such as starch and protein. Moreover, whole grain contains several bioactive compounds that confer to wheat-derived products unique nutraceutical properties (dietary fibre, antioxidants). The present research provided interesting insights for the selection of wheat genotypes suitable for low-input systems and the development of specific breeding programs dedicated to organic farming. The investigation involved 5 old not dwarf genotypes (Andriolo, Frassineto, Gentil rosso, Inallettabile, Verna) and 1 modern dwarf variety (Palesio), grown under biodynamic management, over two consecutive growing seasons (2009/2010, 2010/2011). Results evidenced that under low-input farming some investigated old wheat genotypes (Frassineto, Inallettabile) were comparable to the modern cultivar in terms of whole agronomic performance. As regards the nutritional and nutraceutical properties, some old genotypes (Andriolo, Gentil rosso, Verna) emerged for their relevant content of several investigated phytochemicals (such as insoluble dietary fibre, polyphenols, flavonoids, *in vitro* antioxidant activity) and nutrients (protein, lipid, minerals). Despite of the low technological features, the six wheat varieties grown under low-input management may efficiently provide raw material for the preparation of traditionally processed bread with valuable sensory and nutritional properties. Results highlighted that old wheat varieties have peculiar phytochemical composition and may be a valuable source of nutraceutical compounds. Some of the genetic material involved in the present study may be used in breeding programs aimed at selecting varieties suitable for low-input farming and rich in health-promoting compounds.

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Section 1:

INTRODUCTION

1.1. CONVENTIONAL AND LOW–INPUT FARMING SYSTEMS

Sustainability is a complex and wide–ranging concept. According to a dictionary definition sustainability refers to “keeping an effort going continuously, the ability to last out and keep from falling”. In the context of agriculture, sustainability can be basically considered as the capacity to remain productive while maintaining the resource base. The sustainable agronomic practices may contribute to long–term welfare by providing foods economically viable, environmentally sound and socially acceptable. The contrast between conventional and sustainable agriculture is mainly related to the environmental problems created by the growth in output of the intensive system (i.e. homogenization of landscape, decline in biodiversity, increasing amount of N–input and pesticides, soil erosion).

1.1.1. Conventional agriculture

Conventional agriculture is part of an economic and social model of development that currently share common objectives with several other human activities: increase profits while reducing workforce. Therefore in the last decades, a progressive raise of productivity (yield per unit area) and mechanized operations occurred till the development of the present–day intensive system. This agricultural management allowed the achievement of good results in terms of productivity, determining a significant increase of food production. The recorded productivity increase is not due to an expansion of arable land but derives from the use of selected high–yield cultivars (Meadows et al. 2006). Modern crop production technology has considerably raised output but has created problems of land degradation, pesticide residues in both environment and farm products, gene erosion, and atmosphere and water pollution. Soil depletion is one of the major concern worldwide. The United Nations Environment Programme (UNEP) has estimated that, in the last 1000 years, human agricultural practices caused the degradation of 2 milliards hectares of soil, equivalent to 15 percent of the Earth’s land area, as shown in Figure 1.1. Soil erosion is a major factor in land degradation and has serious effects on soil ability to act as buffer and filter pollutants, on its role in the hydrological and nitrogen cycle and on the overall soil fertility. In most western countries the agriculture has nowadays become an highly intensive practice, based on the use of increasing amounts of external inputs such as chemical fertilizers and pesticides.

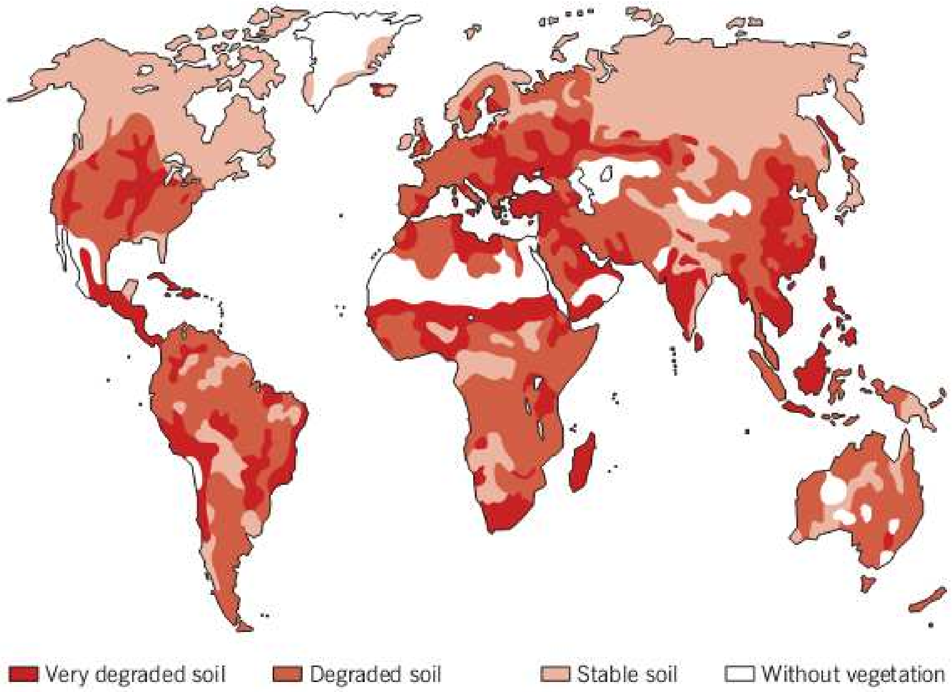


Figure 1.1. Map of the extent of degraded soil area in the world.
Source: UNEP 1992.

In addition to the negative collateral effects of these chemicals on the agroecosystem (soil and water pollution, damages to non target organisms, resistant induction in weeds, biodiversity reduction), the economic aspects are not less important. Indeed, to obtain high yields, farmers have to cover elevated costs to support chemical treatments that are often not compensated by the sell of their own agricultural products. The paradox of this economic investment render the intensive agricultural system unsustainable from both an environmental and economic long–term point of view.

1.1.2. Integrated farming

The debate surrounding sustainability of farming systems often tends to be characterized in terms of the contrast between intensive and organic agriculture. However there is a range of other agricultural managements that fall between these categories. One emerging farming system is, for instance, the integrated farming that encompasses elements belonging to both conventional and organic agriculture. Integrated farming generally involves the use of locally available resources (i.e. feeds, wastes and other internal outputs) and high level of nutrient recycling, thus maximizing the improvement of resource use. Synthetic chemicals for fertilizing and pest control are allowed but limited to the maximum extent possible, with an estimated 20–30% reduction of treatment number compared to conventional agriculture. Moreover, the integrated practices exclude any treatments in the stage close to harvest, and preferably use biological pest control strategies by the utilization of natural antagonist organisms.

1.1.3. Organic agriculture

The organic agriculture was firstly born in Germany, Austria and Switzerland in the 1950s and successively reached several countries in Europe and all over the world. At the beginning, the low appealing aspect and high price of organic products did not provide good receipts but in 1990s the success of these food products increased among consumers that started to firmly consider them safer and healthier than conventional ones. Organic agriculture represent an important economic opportunity in Italy, which is the second country in Europe taking into account the extension of organic certified area (>1 million hectares) and the number of involved operators (47000) (Figure 1.2). Considering the global scale, Italy is the fourth country for organic certified area and the first for the percentage of land converted. Moreover at world level, the Italian organic agriculture is among the first countries for the organic sales, which approximately accounts for 800 million of Euros (Paull, 2011). As set by the International Federation of Organic Agriculture Movements, the organic agriculture is defined as "a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than use of inputs with adverse effects." The focal point of organic farming is to take care the health of soil, which is considered as a vital element rather than an inert one, due to the role of physical, chemical and biological soil components in the organic matter cycle. Another important aspect is the fertilization, which aims at providing nutrients not directly to plants but to the soil microorganisms that elaborate and make available the nutrient substances needed for plant growth and development. The maintenance of good soil fertility results from several agronomic practices routinely applied in the organic systems, such as crop rotation, alternating soil–exhausting (i.e. gramineous plants) with soil–restoring crops (i.e. leguminous plants) plants to avoid soil depletion. Tillage operations are reduced and generally interest the most superficial layers to avoid mechanical disturbance of soil structure and promote the achievement of humus balanced content. Finally, the pest control management is often carried out using beneficial organisms which control the presence of pathogens and reduce their damages on crops. The forbidden use of chemical pesticides in the organic agriculture assure food products free from chemical residues and therefore commonly considered healthier among consumers. Contrasting results are reported in literature as regards the nutrient and phytochemical composition of organic foods compared to conventional ones. Worthington (2001) indicated that organic products contain significant higher vitamin C, magnesium, iron and phosphorus levels and significant lower nitrate content compared to conventional products. Further studies have demonstrated a greater accumulation of polyphenols in organic products, as plants produce these compounds as defense response against abiotic and biotic stresses (Carbonaro, 2002). However, other authors reported that the effects of agricultural system highly depend on the involved crop and the organic management is not necessarily correlated with an increase of secondary metabolite accumulation (Lotter, 2003).

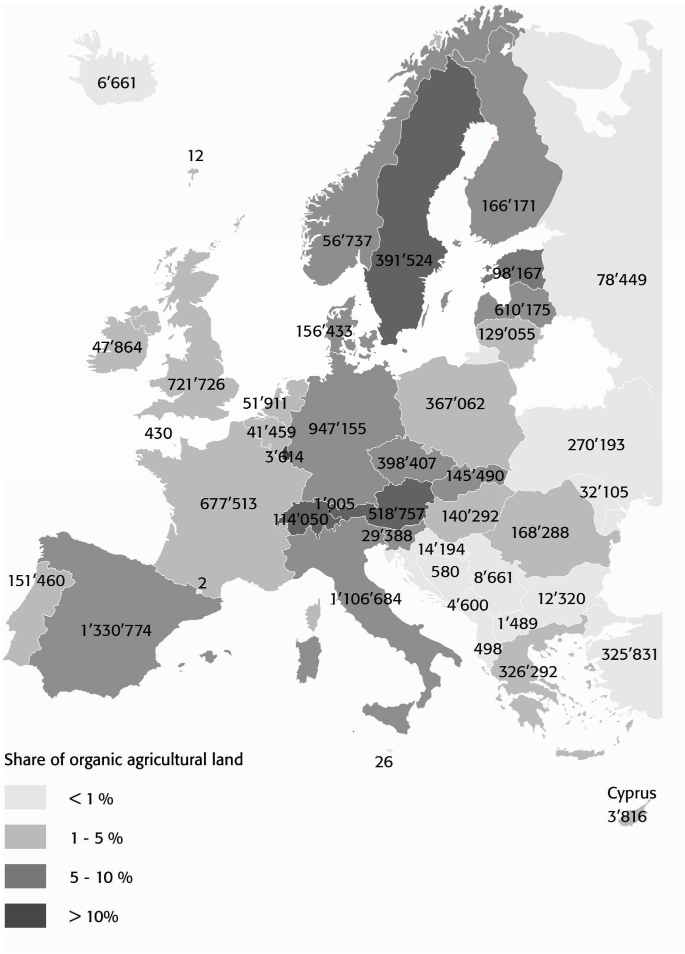


Figure 1.2. Organic agriculture land in Europe (hectares).
Source: FiBL and IFOAM 2011

1.1.4. Biodynamic agriculture

The principles of biodynamic agriculture are based on an ecological and sustainable approach delivered in 1924 by an Austrian scientist and philosopher, Rudolf Steiner. The biodynamic agriculture was the first form of organic farming system and it was developed in response to the depletion of soil fertility caused by the use of chemical fertilizers at the turn of the century, in addition to a decrease of the health and quality of crops. The basic principle of the biodynamic system is that the farm as a whole is considered as a living organism and a self–contained entity, governed by the natural kingdom laws. The biodynamic farm is a closed self–sustaining system and emphasis is placed on the integration of crops and livestock, recycling of nutrients, maintenance of soil quality and health of crops and animals. A central aspect of biodynamic farming is to time the agricultural practices according to lunar and astrological cycles. In fact, the heavenly body are recognized to influence plant growth and vitality, affecting biological systems. On the basis of moon and planetary positions, different calendars are elaborated each year to suggest the most favourable times for planting root, leaf, flowering and fruiting crops (Kollerstrom, 1993). Therefore, the biodynamic farming can be considered as a spiritual and holistic approach to alternative agriculture, associated with non–physical forces in nature such as vitality and life force. The biodynamic practices include a series of well–known organic farming techniques to improve soil fertility (i.e. rotations) and total exclusion of any kinds of chemical fertilizers and pesticides. A typical characteristic of biodynamic farming is the use of different biodynamic preparations to enhance soil quality and stimulate plant life. They consist of mineral, plant or animal manure materials treated or fermented with animal organs, water and/or soil. The biodynamic preparations, numbered 500–508, are applied in highly–diluted form to compost, soil or directly onto plants, after a stirring procedure called *dynamisations*. The 500 biodynamic preparation (horn–manure) is made from cow manure (fermented in a cow horn that is buried in the soil for six months through autumn and winter) and is applied to the soil to stimulate root growth and humus formation. The 501 biodynamic preparation (horn–silica) is made form powedered quartz (packed inside a cow horn and buried in the soil for six months through spring and summer) and used as foliar spray to stimulate and regulate plant growth. The biodynamic preparations numbered from 502 to 507 are added to the compost to improve the microorganism fermentative process and either enhance vital energies. They are made from 6 different officinal plants such *as Achillea* *millefolium*, *Chamomilla officinalis*, *Urtica dioica*, *Quercus robur*, *Taraxacum officinale* and *Valeriana officinalis*. The 502–506 preparations are strategically placed 5–7 feet apart inside the compost pile, while the 507 preparation (liquid valerian) is applied to the outside layer by spraying. The compost is traditionally constructed using a static pile method: the organic materials are first inoculated with the biodynamic preparations, than covered with straw and left undisturbed for 6 months to one year prior to use. The addition of biodynamic preparations to the compost has been shown to increase temperatures and nitrate content, compared to compost piles inoculated with field soil (Carpenter–Boggs et al., 2000). These effects are related to the improvement of fermentative processes, although the biodynamic preparations are applied at high–diluted concentrations (homeopathic quantities). Moreover, further investigation on soil quality evidenced that biodynamically managed soils had increased capacity to support heterotrophic microflora activity and higher microorganism biodiversity than conventionally managed soils (Carpenter–Boggs et al., 2000). In addition to the use of biodynamic compost, several agronomic practices, such as cover crops and crop rotation, are commonly applied to maintain good soil fertility and quality. Cover crops generally provide good accumulation of nutrients, nematode control and soil building, in addition to soil protection and nitrogen fixation. Moreover the typical biodynamic practices include the green manure, with soil incorporation of forage crops. The biodynamic farming strongly relies on crop rotation and companion planting. The latter is a specialized form of crop rotation based on the benefits (pest control, higher yield, increasing biodiversity) related to the association of two or more plant species cropped in close proximity (Koepf et al., 1976). Nowadays biodynamic agriculture promptly spread around the world and is widely practiced in Europe, North America, Asia and Australia. (Paull, 2011) According to the certifier Demeter–International, an area of 142,482 hectares across 47 countries is cropped under biodynamic conditions. Germany accounts for 45.1% of the total biodynamic areas, followed by Italy and India. The leading three countries totally account for 56.3% of the world’s biodynamic hectares (Paull, 2011).

1.2. OLD WHEAT VARIETIES

Before the beginning of modern breeding and the implementation of seed laws, farmers used to reproduce their own seed that was then utilised and/or exchanged amongst farmer communities (Brush, 2004). As a result, farmers developed the knowledge of the adaptability of the different varieties and species. The genetic material utilised was then adapted to the specific farming environment. Nowadays modern breeding has resulted in the development of new varieties under high–input, standard conditions, with selection criteria set for conventional agricultural systems. Therefore, all the varieties have been adapted to same environment with a decrease in their diversity. Moreover, varieties have lost the capability to adapt to different conditions and there has been a reduction in the frequencies of those alleles, which are important for the rusticity and yield production. A modern variety, in order to be effective, should be cultivated under the same environmental conditions in which it has been bred. Hence to ensure the success of the variety under conventional farming, the farmer should modify the environment, using tools such as chemical fertilisation, irrigation, weed control by herbicides, and pests control by pesticides. As stated by Ceccarelli (1994) these new varieties are not "widely" adapted, they are actually specifically adapted to conditions which are at or near the optimum for crop growth. In summary, the environment has been modified to adapt it to the plant and non vice versa. That is the reason why, changing farming systems (i.e. organic or low input agriculture) or in non optimal conditions these varieties do not perform well. Moreover adapting the environment to the variety has an enormous ecological cost in terms of environmental pollution. Regarding the low–input agricultural systems described in the previous paragraph, one of the main problems encountered is the lack of suitable varieties. As recently reviewed by Lammerts van Bueren *et al.* (2011), more than 95% of organic production is based on crop varieties that were bred for the conventional high–input sector. Indeed, very few breeding programs have addressed the development of varieties specifically adapted to low–input agricultural systems. As a consequence, the choice of available varieties is much lower in comparison to that for cultivation in conventional agriculture. In summary, the varieties available for organic farmers are characterised by elevated genetic uniformity and are selected in conventional agriculture, which traditionally requires a high–energy input both in terms of fertilizers and chemical weed agents. Additionally, these varieties are often selected in environments that never reflect the conditions of the local cultivation environment on the farm level. Up to now, the most important strategies undertaken in wheat genetic improvement programmes have been those involved in increasing yield and the improvement of technological characteristics, better adapted to processing procedures as defined by industry. As such, selection strategies for improved nutritional, functional and digestibility properties, as well as allergenic potential have been largely neglected. Breeding programmes had actually selected against the presence of secondary metabolites (a large part of the functional and bioactive products form part of the classes of secondary metabolites) believing them to be a useless waste of investment to the detriment of primary metabolites (proteins, starch, lipids). Therefore, the necessity in identifying new parameters for consideration in breeding programs, that also address the improvement of the nutritional quality of common wheat in a sustainable environment, is evident. Aside from the beneficial functional properties found in wheat, the flour is also a source of potential problems. Wheat is responsible of three clinical forms of IgE–mediated allergy: a) baker's asthma, an occupational disease which is often caused by the inhalation of wheat flour, b) food allergy, occurring after ingestion of wheat flour or wheat–containing food, and c) wheat –dependent, exercise–induced anaphylaxis (WDEIA), which is a particular form of food allergy in which symptoms appear only in connection with physical strain. Soluble proteins belonging to the seed–specific protein family of the alpha–amylase inhibitors are considered to be important allergens for baker's asthma, whereas the insoluble proteins (gluten proteins) gliadin and low molecular weight subunits of glutenin (LMW–GS) are reported to be involved in food allergy, with a 65 Kda omega–5 gliadin appearing to be a major causal agent for the WDEIA type allergy (Palosuo, 2003). Besides the allergies, increasingly greater numbers of people are complaining about intolerance reactions (intestinal swelling, colic, diarrhoea/constipation and other forms of disturbances). The factors hypothesised to be responsible for these adverse reactions include an excessive daily consumption of cereals in the form of bread, pasta, pizza and baked products, the decline in certain quality aspects such as the digestibility and functional components, especially in modern varieties, and technological processes involved in the production of foods.

1.3 WHEAT PRODUCTION IN ITALY

Nowadays the cereals cultivated area in the world exceeds 650 million hectares and in the 2000–2005 years this area reached an average of 674 million hectares. The most widely cultivated cereals are common wheat and durum wheat (32%), followed by rice (22%), corn (21%), barley (8%) and oats (2%). In Italy, the cereal production hold a prominent place both in terms of annual consumption and industrial processing. In particular as regards durum and common wheat, the area of production accounts for 1.5 and 0.7 million hectares respectively, with an annual production that exceeds 4.2 and 3.3 million tons respectively (Eurostat database). Despite the technological gap between the Italian productive system and the emerging countries, Italian ability to compete is severely limited by workforce costs by 10 to 50 times higher than that of emerging countries. As a consequence, over the last ten years, a significant reduction of the Italian wheat production was recorded, accompanied by an increase of wheat imports. The post–war period in Italy has been characterized by the involvement of few selected high–yield varieties for an high–energy input agriculture. This resulted in increasing yield per unit from 2.0 t/ha ('50s) to 3.7 t/ha (nowadays), However a more restricted varietal heterogeneity has caused the loss of many useful genes and these cultivars often resulted unsuitable for the low–input sector (i.e. organic agriculture). The organic agriculture represent an important economic opportunity in Italy and the converted certified area is constantly increasing, nowadays accounting for 71.1 and 18.7 thousand hectares for durum and common wheat respectively. The world common wheat grain production exceeds 556 million tones, with China, India, United States, Russia, France, Germany and Australia producing about 60% of the total world production. The wheat annual production in the EU account for 113.5 million tons, equivalent to over 20% of world production. Among the most producing countries, France, Germany, England and Poland together supply 70 percent of the European production, while Italy contribute for approximately 3% (3.3 million tones). Additionally, Italy produces 177.2 thousand tones of organic certified cereals per year, among which the most produced specie is durum wheat (71.1 thousand tones, 40% of the organic cereal production), followed by common wheat (36.3 thousand tones, 21% of the Italian organic cereal production), maize (16%), barley (10%), oat (9%) and rice (4%) (Eurostat database).

1.4. NUTRITIONAL ASPECTS OF WHEAT

The nutritional value of wheat is related to the high carbohydrate content and contemporarily the noticeable protein levels. Most of the nutrients present in the wheat caryopsis are accumulated in the endosperm, which constitutes the energy reserve of the seed for germination. Wheat endosperm contains starch (70–80%), proteins (10–15%), sugars (glucose, hemicelluloses, fructans) and vitamins. The wheat germ is rich lipids (essential fatty acids) and vitamins (vitamin E), while the bran is rich in minerals.

1.4.1. Proteins and gluten

Wheat proteins have been classified in four groups based on their solubility in water, salt or alcohol solutions. Albumins (water soluble) and globulins (soluble in salt solutions) account for 12% and 8% of the total proteins respectively, and are mainly present in the germ and aleurone layer. Gliadins (alcohol soluble) and glutenins (soluble in acid or alkaline solutions) are present in approximately equal amounts in the endosperm, accounting for about 80% of the total storage proteins of the wheat kernel. Beyond their nutritional importance, grain protein composition determine the flour baking quality, especially as regards the gluten fraction (gliadins and glutenins). Due to the viscoelastic properties of the gluten proteins, one of the main target of breeding programs was to improve the gluten strength and elasticity, allowing dough to tolerate high technological stress during mixing and baking processes. The variation in dough rheology and bread making performance among wheat varieties is largely determined by the differences in specific high–molecular weight glutenin subunits (HMW–GS) and gliadin subunits (MachRitchie, 1992; Gupta et al., 1993; Shewry et al., 2001; Sliwinski et al., 2004). The gluten viscoelasticity is directly related to the gluten content and to the structural arrangement of HMW and gliadin subunits. As outlined in the previous paragraph, some allelic variants of the gluten proteins are known for their toxicity, as these peptides are responsible for the celiac disease, allergies and gluten sensitivity pathologies (Silano et al., 2007; van den Broeck et al., 2009; Vincentini et al. 2009; van den Broeck et al., 2011). In the last 60 years the breeding programs of wheat varieties have been focused on the improvement of rheological properties by selecting wheat genotypes for their qualitative and quantitative gluten content rather than for their nutritional value in relation to gluten related pathologies (Wolfe et al., 2008). The wide spread of the mentioned pathologies highly remark the need to address the improvement of common wheat towards new parameters, taking into account the nutritional and healthy properties of the wheat genotypes.

1.4.2. Starch

Wheat represent the major complex carbohydrate source in the diet as more than 80% of the endosperm is made of starch, which provides available glucose energy for the human body processes. In wheat kernel, starch is accumulated in granules containing linear and branched polymers of glucose, referred as amylose and amylopectin respectively (Figure 1.3). Amylose is a linear chain of α–1,4–D–glucopyranosyl residues, which has a right handed helix formation with hydrophobic and lipophilic interiors capable of forming complexes. Amylopectin is a branched polymer with a backbone of α–1,4–D–glucopyranosyl residues, with branches of glucose units linked by an α–1,6–D bond (Whistler and BeMiller, 1997). Starch is the only natural polysaccharide that can be hydrolyzed by the enzymes α–amylases, glucoamylase and sucrose–isomaltase present in the gastrointestinal tract, providing free glucose that can be easily assimilated (Lehman and Robin, 2007). Starch may possess different digestibility properties, varying from readily to slow digestible fractions, depending on the extent of polymerization and branching and the ratio of amylose to amylopectin (Slattery et al., 2000). Indeed, the structural arrangement of the starch polymers causes amylopectin to be more vulnerable to enzymatic attacks. However, there is a portion of starch that can not be digested and absorbed by the small intestine referred as resistant starch (RS). RS is defined as the starch fraction which is not hydrolyzed to D–glucose in the small intestine within 120 min of being consumed, but which is fermented in the colon (Fuentes–Zaragoza et al., 2010). On the basis of the starch characteristics causing the slow digestibility, RS can be distinguished in four types (RS1, RS2, RS3, RS4) (Fuentes–Zaragoza et al., 2010). RS1 is a physically inaccessible starch due to a compact molecular structure that limits the accessibility of digestive enzymes. RS2 consists of high–amylose starch granules which are structured which are structured in a way which prevents the digestive enzymes from breaking them down. Starch can also undergo modifications after heating and successive cooling down processes, for instance during cooking operation. When heated in presence of water, starch granules are disrupted in a process known as gelatinization, which renders the molecules highly accessible to digestive enzymes. After re–cooling, starch may form crystals known as “retrograded starch” (RS3) that is resistant to digestion. Finally, the fourth class of resistant starch (RS4) includes molecules that have been chemically modified by etherisation, esterisation or cross–bonding and therefore prevents digestion by blocking enzyme access. The slow digestibility of resistant starch–rich foods allows glucose to be released slowly but constantly into the bloodstream, therefore reducing the postprandial glycemic and insulinemic responses, compared to high glycemic foods, and also increases the lasting feeling of satiety (Haralampu, 2000). The fermentation of resistant starch by colonic bacteria generates short chain fatty acids, particularly high levels of butyrates (Scheppach et al., 1988). Butyrate is the primary energy substrates of colonic mucosa and prevent colon cancer by promoting cell differentiation, cell–cycle arrest and apoptosis of transformed colonocytes, decreasing the proliferation of abnormal cells (Wong et al., 2006). In addition, RS has been shown to exert high prebiotic activity, as it is not degraded by human digestive enzymes in the small intestine and passes into the colon where it constitutes an optimal fermentative substrate for the resident microflora, promoting the growth of beneficial organisms.

a) Amylose

b) Amylopectin

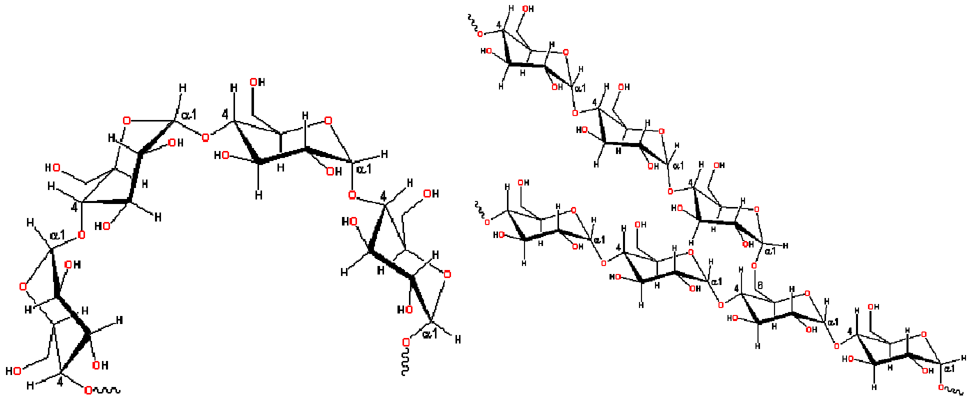


Figure 1.3. Structural formulae of amylose and amylopectin starch components.

1.4.3. Lipids

Lipid fraction is a minor component of the wheat grain, constituting about 2% of the whole grain weight and mainly accumulated in the germ fraction. More than 20 classes of lipids are present in wheat; triglycerides, phospholipids and essential fatty acids represent the majority of the total lipid fraction. Among essential fatty acids, tocopherols and tocotrienols are potent antioxidants known as vitamin E.

They consist of a polar chromanol ring and a hydrophobic 16–carbon side chain attached to the ring via the C–2 atom; tocopherols have saturated phytyl side chains while tocotrienols have isoprenyl side chains with three double bonds. Despite the high content of tocopherols and tocotrienols in cereal kernel, their function in plants is still unknown. A study on barley cultivars showed that tocopherols are accumulated for 80% in the vital part of the grain (germ), while tocotrienols are localized in the endosperm and pericarp fractions (Falk, 2004).

1.5. NUTRACEUTICAL ASPECTS OF WHEAT

The term functional foods was first introduced in Japan in the mid–1980s and refers to processed foods containing ingredients that provide benefits to human health beyond its nutritional value. The beneficial ingredients, known as "phytochemicals", are chemical compounds that occur naturally in plants, characterized by specific biological properties (antioxidant, anticancer, hormone similar and cardioprotective activities).

Several researches confirmed that whole grains and whole–grain–based products, in the context of a balanced diet, may have a protective effect on humans and the ability to enhance health beyond the simple provision of energy and nutrients. In the specific case of wheat, these important functions are due to macro–, micro–nutrients and phytochemicals present in whole wheat grain. Since most phytochemicals are accumulated in the bran and germ fractions, most of these compounds are unfortunately lost during the refining processes.

1.5.1. Dietary fibre components

The American Association of Cereal Chemists defines dietary fibre (DF) as the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. DF includes polysaccharides, oligosaccharides, lignin and associated cell wall components. From an analytical point of view, the fibre can be subdivided into two groups according to the solubility in water: soluble dietary fibre (SDF) and insoluble dietary fibre (IDF). Structural polysaccharides such as cellulose and lignin constitute the IDF fraction that remain largely unfermented and increase faecal bulk (Alabaster et al., 1996; Charalampopoulos et al., 2002; Scheppach et al., 2004). On the other hand, resistant starch (RS) and non–starch polysaccharides (NSPs) such as β–glucan and arabinoxylan compose SDF fraction that may be fermented in the colon from the resident microbial flora, with principal effects on glucose and lipid absorption, gut bacterial composition and anticancer activity (Macfarlane et al., 2006; Roberfroid et al., 2010; Marotti et al., 2012). β–glucans (BG) are linear polysaccharides made up of (1 → 4) and (1 → 3) linked β–D–glucopyranosyl units (Sidhu et al., 2007) and located mainly in the aleurone layer and bran fractions, whereas narrow amounts are accumulated in the endosperm (0.3% of dry matter) (Henry et al. 1997; Barron et al., 2007). They are grouped in water–soluble BG and water–insoluble BG, however the insoluble form is primarily represented in wheat grain, as BG are entrapped in the matrix of a cross–linked ferulic acid–arabinoxylan complex (Cui et al., 2000). Wheat is recognized as a limited source of β–glucans (1% of dry weight), while the highest BG levels are found in barley (3–11% and 3–7% on a dry basis) and oat (3–7% of dry weight) (Genc et al., 2001; Gebruers et al., 2008). However, BG is an important wheat dietary fibre fraction due to its widely demonstrated health–promoting properties. In the small intestine BG form a viscous solution that reduces the absorption of cholesterol, fatty acids, bile acids and glucose, resulting in lower risk of diabetes and cardiovascular diseases (Arnoldi, 2004). In the colon BG act as a prebiotic stimulating the proliferation and growth of beneficial bacteria (Kontula et al., 1998). Arabinoxylans (AX) are the major dietary fibre component in wheat kernel and account for about 50% of the total fibre content (Marotti et al., 2012). From a chemical point of view, AX are hemicelluloses that consist of α–L–arabinofuranose residues attached as branch–points to β–(1 → 4) linked D–xylopyranose polymeric backbone chains; some arabinose residues can additionally be substituted with ferulic acid moieties. AX are principally present in the germ and outer layers of the caryopsis. There are two types of AX, classified in water–extractable and water–unextractable due to their differences in chemical structures (degree of arabinose substitution, ferulic acid cross linking and degree of xylan polymerization) (Courtin and Delcour, 2002; Sorensen et al 2007). AX represent a new class of potential prebiotics: they are selectively degraded in the colon by further bacteria belonging to the intestinal microflora, possessing selective AX–degrading endoxylanases (Vardakou et al., 2008). Furthermore AX play a prominent role in the breadmaking process, due to the ability to form highly viscous solutions and to absorb water and swell of soluble and insoluble AX respectively (Courtin and Delcour, 2002).

1.5.2. Antioxidant compounds

Phenolic compounds are secondary metabolites that constitute the major group of phytochemicals found in plants. The most common phenolic compounds in wholegrain cereals are phenolic acids and flavonoids. In wheat kernel they are mainly located in the outer layer of the caryopsis. The interest in phenolic compounds is due to their high antioxidant activity acting as radical scavengers; several researches demonstrated their role in the prevention of degenerative pathologies such as cancer and heart disease (Carter et al., 2006; Fardet, 2010). Indeed, the consumption of antioxidant–rich foods may efficiently contribute to reduce the risk of oxidative stress pathologies (Renaud et al., 1998; Temple, 2000). Polyphenols are the main antioxidant compounds in wheat grains and they can efficiently react with free radicals by inactivating and converting them in more stable molecules. In wheat kernel, phenolic compounds occur in soluble (free) and insoluble (bound) forms and both fractions have been shown to possess valuable health–promoting properties. The bound forms, cross–linked with cell wall macromolecules, may resist upper digestive process and reach the colon where they are liberated by the intestinal microflora. The digested phenolic directly exert their health benefits reducing the incidence of colon cancer; indeed, colonic endothelial cells may absorb the liberated phenolics and gain powerful antioxidant protection (Kroon et al., 1997). Organically produced wheat is expected to accumulate higher concentrations of phenolic compounds with respect to conventionally grown varieties. The explanation relies on a change of plant metabolism toward carbon–containing compounds (including non–nitrogen secondary metabolites such as polyphenols and flavonoids). According to the carbon/nutrient balance hypothesis, the low nitrogen availability induces an activation of the phenylpropanoid pathway enhancing the phenolic biosynthesis instead of proteins and other nitrogen–containing compounds (Kovacik et al., 2007).

Section 2:

AGRONOMIC PERFORMANCES AND COMMERCIAL QUALITY OF WHEAT VARIETIES

2.1. SECTION OBJECTIVES

In recent years, the increase of public awareness towards environmental pollution risks has focused the attention on the use of eco–friendly agronomic practices, other than conventional agricultural management. To date, plant breeders have supplied several top–yield wheat varieties that are strictly dependent on artificial fertilizer and agro–chemical input typical of the conventional agriculture. Conversely, the low–input farming system, where synthetic crop protection and chemical fertilization are not allowed, is typically characterized by unfavourable growing conditions such as low soil nutrient status and pressures deriving from weeds, pest and diseases. One of the major challenge of organic farming systems is to provide high yields and excellent quality products utilizing agronomic practices with acceptable environmental impacts. The low–input agriculture has long been criticized for its lower yielding capacity compared to conventional production. The unavailability of crop varieties specific suitable for organic farming has contributed to the existing yield gap between organic and conventional systems, since the available cultivars lack traits of crucial interest such as increased competitiveness against weeds and resistance to diseases. Indeed, the introduction of semi–dwarf genes into cereals resulted in reduced development of root systems, low nutrient–use efficiency and decreased competitiveness against weeds. All these varietal characteristics are not useful for the low–input sector and additionally showed some negative side–effects (i.e. greater susceptibility to diseases). The organic system mainly aims at stimulating the internal self–regulation of crop varieties through the functional agrobiodiversity effects in and above the soil, instead of external regulation through chemical protectants. Several small–scale low–input farmers rely on the use of local varieties and landraces rather than modern cultivars. Indeed, in organic and low–input agriculture the agrobiodiversity play a key role and the genetic variation is the primary mechanism for buffering environmental fluctuations and maintaining the required traits. It is essential therefore to intensify investigation concerning the comparison of existing varieties for the agronomic performance and physiological responses under low–input growing conditions. As reviewed by Wolfe and co–workers (2008), the range of characters needed for organic agriculture includes the efficient use of a wide range of nutrients and water, weed competition, disease and pest resistance, quality for end use as well as yield and yield stability. The relatively low nutrient availability in the organic and biodynamic systems may become limiting factors for yield and quality. For instance, considering that N–

mineralization after anthesis is limited, the translocation of nitrogen adsorbed in pre–anthesis stages may favor the wheat varieties with more vegetative tissues and higher biomass (i.e. not dwarf genotypes). Moreover, an extended root system may enhance the exploration of the soil and provide an efficient nutrient uptake. In order to limit yield loss, an adequate weed management is essential to suppress undesirable weeds. Plant competitiveness is thoroughly required in low–input systems but competitive traits have unlikely received sufficient attention in conventional plant breeding. Old genotypes are often considered as more competitive than recent introductions due to a series of desirable characteristics (i.e. plant height, shading ability). As regards disease resistance, it is a major issue in cereal breeding for both conventional and low–input systems. In particular, the organic and biodynamic agriculture generally aims at using broader healthy plants that combine morphological and physiological traits ensuring overall plant vigor instead of specific resistance. On the basis of these considerations, the investigation of ancient wheat genotypes may provide interesting insights for future selection of varieties suitable for low–input farming.

The broad–spectrum scope of the present research was to develop an experimental plan to improve low–input cereal production. In particular, the investigation aimed at providing a complete characterization of six wheat varieties (including one modern and five old genotypes), by considering their agronomic performance under biodynamic management. To assess the specific adaptation of wheat varieties to different environments, the field trials were carried out in three different biodynamic farms and the interaction between genotypes and growing locations evaluated. The research aimed at contributing to the comprehension of wheat adaptability to organic and biodynamic farming and to provide innovative insights for the selection of wheat genotypes for specific breeding programs dedicated to low–input agriculture.

2.2. MATERIAL AND METHODS

2.2.1. Wheat varieties and location description

Wheat varieties involved in the present study included five old, not dwarf and unregistered Italian genotypes (Andriolo, Frassineto, Gentil rosso, Inallettabile, Verna) and one dwarf registered cultivar (Palesio) of common wheat (*Triticum aestivum* L.). A brief description of each variety is reported in Table 2.1. Trials were carried out at three biodynamic farms (namely Cenacchi, Collina and Ferri farms) of the Emilia Romagna region, Italy, over two consecutive growing seasons (2009/2010, 2010/2011) (Figure 2.1). All the three farms are certified and converted to organic production from at least ten years. Cenacchi (latitude 44° 39′ 57′′ N, longitude 11° 19′ 43′′ E, 25 m a.s.l.) and Ferri (latitude 44° 53′ 98′′ N, longitude 11° 18′ 63′′ E, 38 m a.s.l.) farms are typical locations of the South Po River Valley, with clay soil. The Collina farm (latitude 44° 41′ 16′′N, longitude 10° 33′ 59″E, 65 m a.s.l.) is located in the hill countryside and has a sand silty soil. In each farm, a meteorological station was placed near experimental fields and data on temperature, rainfall, humidity, dewpoint, wind speed and direction were recorded.

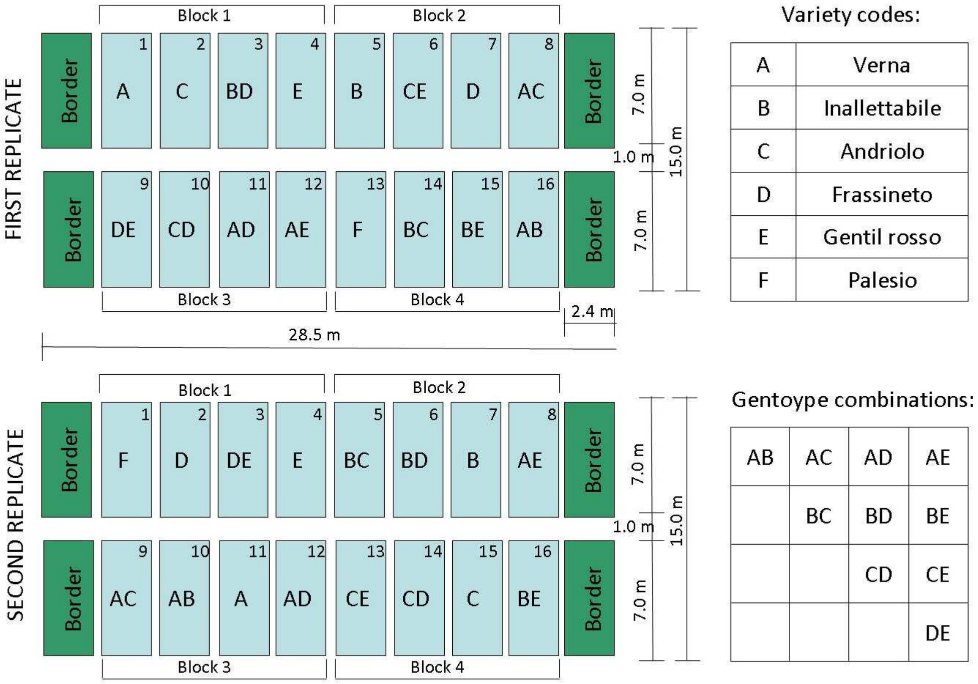
Table 2.1. Principal morpho–phenological characteristics of the investigated wheat varieties.

Variety	Code	Date of release	Grain colour	Habitus	Earliness
Verna	A	≈ 1945	Red	Tall	Late
Inallettabile	B	≈ 1920	White	Tall	Late
Andriolo	C	≈ 1945	Red	Medium–tall	Medium–late
Frassineto	D	≈ 1930	White	Tall	Medium–late
Gentil rosso	E	≈ 1900	Red	Tall	Late
Palesio	F	2003	Red	Semi–dwarf	Early

2.2.2. Experimental design

The field experiments were carried out adopting a simple lattice design with four blocks replicated two times (Figure 2.2). Wheat grains were sown with a density of 200 kg/ha in 2.4 x 7 m plots. Genotypes were sown individually and, as regards the old varieties, in binary combinations. The binary mixtures of old wheat genotypes are aimed at testing the effects of plant competition on yield and other agronomic and nutritional features. The grain samples used for mixture plots were prepared by

Figure 2.1. Locations of the three involved biodynamic farms (A, Cenacchi; B, Ferri; C, Collina).



Variety codes:

A	Verna
B	Inallettabile
C	Andriolo
D	Frassineto
E	Gentil rosso
F	Palesio

Gentoype combinations:

AB	AC	AD	AE
	BC	BD	BE
		CD	CE
			DE

Figure 2.2. Experimental design (simple lattice) adopted at the three biodynamic farms in 2009/2010 and 2010/2011. The border plots were sown with Palesio (F).

considering the specific kernel weight of each variety, to obtain a balanced density of both genotypes. Wheat varieties were managed under strict low–input conditions, following the typical biodynamic management. The experimental plots were treated with compost and biodynamic preparations (500, 501, 508). To improve soil fertility and organic matter, green manure (with forage crops) was grown in all the experimental fields during the previous agronomic year.

2.2.3. Agronomic traits

During both crop cycles (2009/2010, 2010/2011), the phenological growth for each genotype was recorded (BBCH scale – Biologische Bundesanstalt, Bundessortenamt and CHemical Industry) (Stauss, 1994). The phenological development was elaborated on the basis of the sum of degree days (SDD). At the harvest, grain and straw yield (t/ha), Harvest Index (ratio of grain yield to grain–plus–straw yield), yield components (number of spikes per square meter, number of kernels per spike, single kernel weight), 1000–kernel weight (g) and test weight (kg/hl) were determined. Moreover, for each pure genotype and mixture of old genotypes different morphological traits (plant height, plant weight, spike length, spike weight) were recorded.

2.2.4. Mycotoxin assay

Whole wheat grains harvested from each plots were ground in a stone mill and the whole flours were analyzed for the mycotoxin content. The deoxynivalenol (DON) levels were determined by using the AgraQuant®DON assay based on the direct competitive enzyme–linked immunosorbent assay (ELISA) (Romer Labs Inc., Union, MO, USA). The DON content was expressed in ppm and determined as mean value of three replicates.

2.2.6. Statistical analysis

A General Linear Model (GLM) was used to assess the variance significance among the main (genotype) and the random (location, year) factors, as well as their interactions. The analysis of variance was performed using Systat v. 9.0 (SPSS Inc. 1998). Significance between means was determined by least significant difference values for $P < 0.05$. The STATISTICA Software v. 7.1 (StatSoft, Tulsa, Oklahoma, USA) was used for performing the multiple regression analysis, via a forward stepwise model (inclusion criterion $F > 1.0$). Significance for each included independent variables was assessed at $P < 0.05$.

2.3. RESULTS AND DISCUSSION

2.3.1. Meteorological and phenological data

In order to illustrate the main differences of mean temperatures and precipitation among locations and growing seasons, in Figure 2.3 the heat and precipitation maps are reported. The meteorological data, expressed as monthly mean temperature and cumulative rainfall recorded in each farm during both growing seasons, are reported in the *Annex Section* (Tables I and II). As regards the mean temperature of the whole growing season (from November to July), the first year (2009/2010) was slightly colder than the second one (2010/2011) at Cenacchi and Ferri farms, while the opposite trend was observed at the Collina farm (*Annex Section*, Table I). During the two considered years relevant seasonal differences in temperature levels were found. In general, in November and December (corresponding to the phenological stages of leaf development and early tillering) the highest monthly mean temperatures were recorded during the first growing season (2009/2010). On the contrary, in the January–May period (corresponding to the phenological stages of late tillering, stem

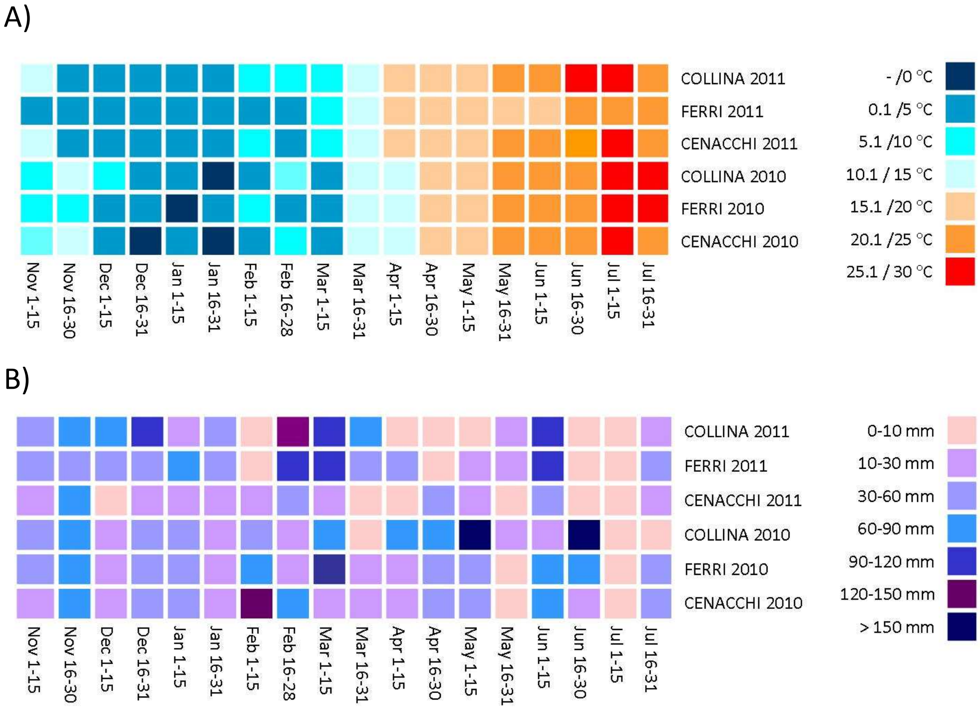


Figure 2.3. Heat maps showing the mean temperature (A) and total precipitation (B) recorded for 15 day periods at the three biodynamic farms in both growing seasons (2009/2010, 2010/2011).

elongation, booting and early heading) the second year (2010/2011) was characterized by higher monthly mean temperatures than the first year (2009/2010). Finally, in the June–July period (corresponding to the phenological stages of full heading, flowering and seed maturation) the first year (2009/2010) was markedly warmer than the second one (2010/2011). In particular, in July of the first year (2009/2010) the mean temperature recorded at the Cenacchi, Ferri and Collina farms was respectively 2.8, 2.6 and 4.2 °C higher than that observed in the second year (2010/2011). At the whole, the three experimental farms were characterized by similar thermometric trend during the two growing seasons (from November to July), with a mean temperature ranging from 13.1 and 11.0 °C (*Annex Section*, Table I), in agreement with the mean temperature values recorded in the South Po Valley. As regards the cumulative rainfall during the wheat growing season (from November to July), the first year (2009/2010) was markedly more rainy than the second one (2010/2011). In particular, during the first growing season (2009/2010) the total precipitation recorded at the Cenacchi, Ferri and Collina farms was respectively 356, 66 and 514 mm higher than that observed in the second growing season (2010/2011) (*Annex Section*, Table II). Substantial differences of the precipitation regime among the two years were observed. At the Cenacchi farm in each month of the second growing season the decrease of the cumulative precipitation was ranging from approximately 5 to 132 mm. At the Ferri and Collina farms in the November–March period (corresponding to the phenological stage of leaf development, tillering and early stem elongation) of the second year (2009/2010) the increase of cumulative rainfall with respect to the second year (2010/2011) was respectively equal to 108 and 277 mm. An opposite trend was observed for the April–July period (corresponding to the phenologicical stages of booting, heading, flowering and seed maturation) of the second year (2010/2011): at the Ferri and Collina farms the decrease of total precipitation was respectively equal to approximately 174 and 791 mm. Independently from the observed differences in total rainfall due to the year effect, within each growing season a similar precipitation ranking among cultivation sites was found: the highest and the lowest cumulative precipitation was observed at the Collina and Cenacchi farm, respectively, while an intermediate value was recorded at the Ferri farm. In conclusion, on the basis of the two main climate determinants (temperature, precipitation) the two considered growing seasons were not comparable and relevant differences in pluviometric regimes were observed in the three farms.

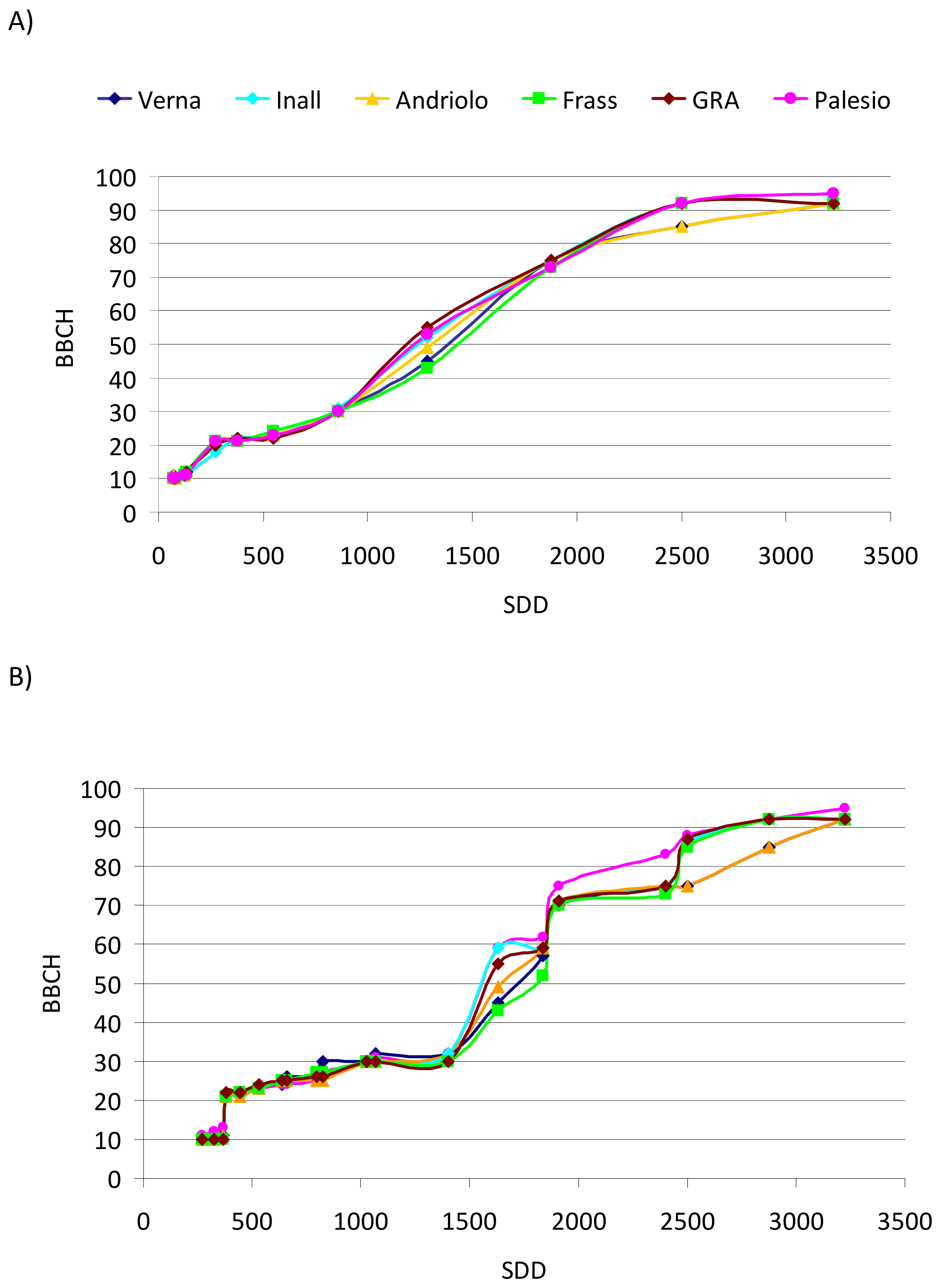


Figure 2.4. Phenology of the six investigated genotypes observed at the Cenacchi farm during 2009/2010 (A) and 2010/2011 (B). The phenological growth is expressed using the BBCH scale as a function of the sum of degree days (SDD).

Wheat phenology is strongly dependent by the genetic framework of cultivars due to genotype differences in vernalization requirement, photoperiod response and earliness. The knowledge of phenological development of different wheat varieties is an essential matter for investigating genotype adaptation to specific environments and agricultural management. The phenological growth stages for each genotype was recorded using the BBCH scale (Stauss, 1994) and elaborated on the basis of the sum of degree days (SDD). On the basis of the phenological observations carried out at each cultivation site, no significant differences among wheat genotypes as a function of growth location were observed (data not reported). As a consequence, the phenological growth of the six investigated genotypes only recorded at the Cenacchi farm during both growing seasons is reported (Figure 2.4). During the first phenological stages (BBCH 10–30) no differences were detected among genotypes, that developed almost simultaneously during the tillering period in both growing seasons. The modern cultivar Palesio and the old varieties Inallettabile and Gentil rosso were shown to be more earliness in reaching the booting (BBCH 45) and heading (BBCH 60) phases compared to Andriolo, Frassineto and Verna. The flowering stage (BBCH 69) occurred at the same time for all genotypes but differences were observed as regards the extent of flowering period. In particular, during the second year, all the old varieties showed an extended spike flowering while Palesio rapidly passed to the maturity stages (BBCH 71–89). This trend was not clearly observed during the first growing season and may be related to an increase of maturity rate of Palesio grains in 2010/2011, due to warmer environmental conditions at the end of the crop cycle which had favored the typical earliness of the modern cultivar. After the milky maturity stage (BBCH 73), the old genotypes Inallettabile, Frassineto and Gentil rosso aligned with the phenological growth of Palesio, whereas Andriolo and Verna presented a slower grain filling development.

2.3.2. Agronomic performances

At the harvest, data on agronomic performance (grain yield, straw yield, Harvest Index, yield components) and commercial quality (humidity, 1000–kernel weight, test weight) were recorded for the six pure genotypes and for the varieties grown in mixture. In Table 2.2, results of each agronomic parameter are reported as mean values for locations, year and genotypes. Yield varied as a function of the growing locations, with the Collina Farm having the highest grain and straw yield and Harvest Index (HI) values with respect to the other two farms. The high productivity at this location may be related to the superior quality of the soil; in fact the over–20–year organic and biodynamic management at this location provided reliable fertility and

Table 2.2. Grain yield, straw yield and Harvest Index of the investigated wheat varieties grown in single–genotype and mixture plots.

		Grain yield (t/ha)	Straw yield (t/ha)	Harvest Index
Location	Cenacchi	2.3(c)	5.0(b)	0.32(b)
	Ferri	3.0(b)	6.7(a)	0.32(b)
	Collina	4.0(a)	7.2(a)	0.36(a)
Year	2009/2010	3.0 ns	6.4 ns	0.32(b)
	2010/2011	3.3 ns	6.2 ns	0.35(a)
Genotype	A	3.0(bc)	6.2(a)	0.34(b)
	B	3.2(a–c)	6.3(a)	0.34(b)
	C	2.8(c)	6.2(a)	0.31(b)
	D	3.1(a–c)	7.0(a)	0.32(b)
	E	3.0(bc)	6.5(a)	0.32(b)
	F	3.5(a)	4.6(b)	0.43(a)
	AB	3.1(a–c)	6.5(a)	0.33(b)
	AC	3.1(a–c)	6.6(a)	0.32(b)
	AD	3.2(a–c)	6.2(a)	0.34(b)
	AE	3.1(a–c)	6.7(a)	0.32(b)
	BC	3.2(a–c)	6.7(a)	0.32(b)
	BD	3.4(ab)	6.5(a)	0.34(b)
	BE	3.0(bc)	5.9(a)	0.33(b)
	CD	3.1(a–c)	6.1(a)	0.34(b)
	CE	3.0(bc)	6.5(a)	0.31(b)
	DE	3.1(a–c)	6.3(a)	0.34(b)
Interaction	LxY	**	**	*
	LxG	**	*	**
	GxY	**	ns	ns

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; L, location; Y, year; G, genotype; ns, not significant.

Means followed by different letters are statistically different at $P<0.05$.
* = significant interaction at $P<0.05$.
** = significant interaction at $P<0.01$.

organic matter content of the soil (data not shown). Long–term experiments showed that the application of cattle manure compost exerts certain positive effects on the organic matter content and soil microbial activity (Fliebach et al. 2007); additionally, the combination of these factors with the influences of biodynamic preparations on soil fertility and compost quality can efficiently guarantee the maintenance of soil health (Carpenter–Boggs et al. 2000; Zaller and Kopke, 2004). Among wheat varieties, the highest grain yield was obtained for the dwarf cultivar Palesio, equal to 3.5 t/ha (Table 2.2). Some of the old not dwarf genotypes, such as Frassineto and Inallettabile and their mixture combination, presented grain yield values not statistically different from that of Palesio. The lowest yield was obtained for the old varieties Verna and Gentil rosso (3.0 t/ha) (Table 2.2). The gap between dwarf and not dwarf genotype yields is consistent under conventional farming however these results remarked that, under low–input management, differences in grain yield are strongly reduced (Guarda et al. 2004; Hilderman et al., 2009). The obtained grain yields are aligned with data previously reported for old and modern wheat genotypes grown under organic/biodynamic management (Hilderman et al. 2009) and low N–rate applications (Guarda et al., 2004). Moreover, the mean value of grain yield agrees with those reported for organic wheat production in Italy in the last 10 years (3.2 t/ha) (Eurostat database). The higher straw yields, ranging from 6.0 to 7.0 t/ha, were obtained for all the old varieties due to their tall *habitus*, whereas Palesio showed a mean straw yield value of 4.6 t/ha (Table 2.2). In the conventional farming, straw has been long considered as an useless agricultural waste but, in recent years, it gained much attention for its utilization as biomasses sources in the biofuel production (Blaschek et al. 2010). Moreover, considering the closed self–sustained organization of biodynamic farms, straw may represent a precious resource due to its use as an essential component of the compost preparation and also as soil amendment. As regards Harvest Index, the highest value (0.43) was obtained for the modern cultivar Palesio while old varieties showed statistically significant lower values (0.31–0.34) (Table 2.2). As expected, Harvest Index was strongly divergent between the dwarf variety and the not dwarf genotypes. Previous investigations described an increase of Harvest Index values in modern wheat varieties compared to the old ones, mainly due to higher spike fertility and dwarf *habitus* of modern cultivars, resulting in improved partitioning of biomass to the grain (Di Silvestro et al., 2012; Guarda et al. 2004). As mentioned above, the second year (2010/2011) was characterized by high temperatures and very low cumulative precipitation from heading to harvest (April–July) (Figure 2.3; *Annex Section*, Tables I and II). These weather conditions may cause heat and/or drought stress to the crop, affecting the number of grains per spike and grain weight and thus resulting in lower spike fertility (Fischer, 1985; Acevedo et al., 1991). However, an opposite trend was observed in this study with an increase of Harvest Index values during the second growing season (Table 2.2). These results may be related to the strong and abundant rainfall (over 160 mm) occurred during the first year at the end of crop cycle (May–June 2010), that caused the lodging of most of the not dwarf

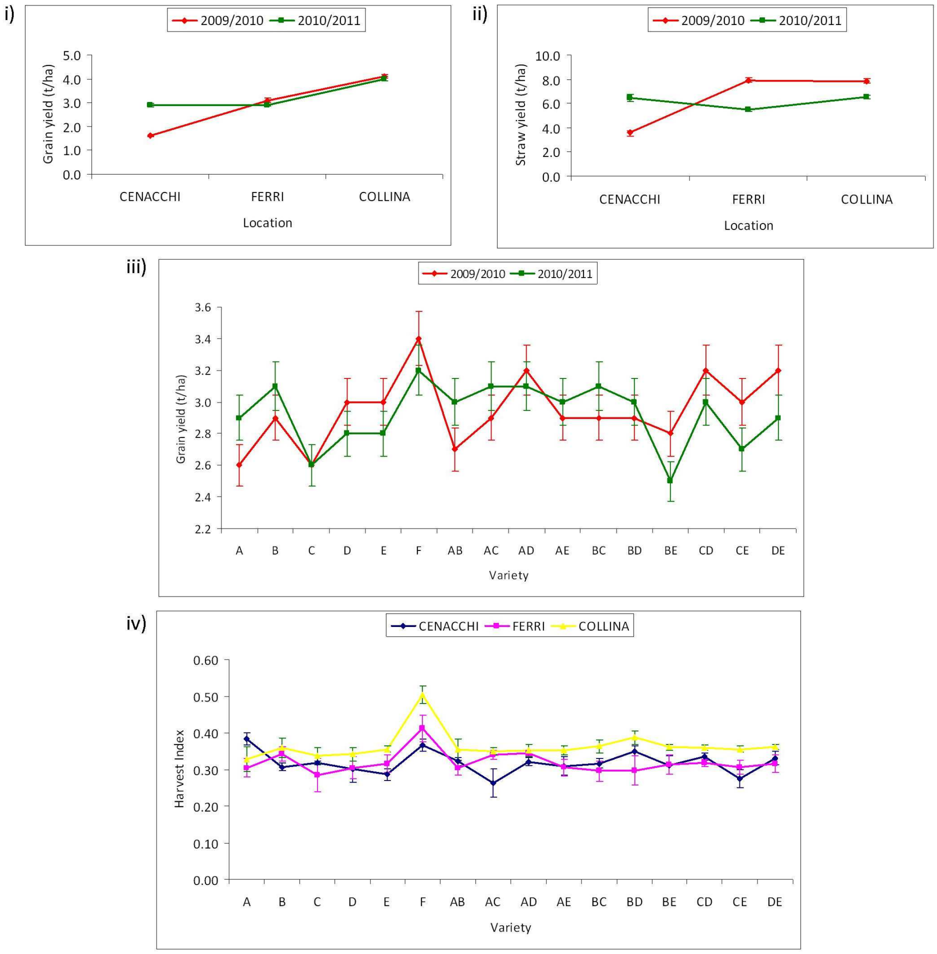


Figure 2.5. Significant interactions observed for grain yield (i, L x Y; iii, G x Y), straw yield (ii, L x Y) and Harvest Index (iv, G x Y).

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

varieties and may have differently penalized the grain and straw harvest. Grain and straw yield were significantly affected by the interaction between location and year (LxY). Figure 2.5 clearly shows that grain (i) and straw (ii) yields resulted in very low values at the Cenacchi farm during the first year. On the contrary, straw yield decreased during the second year at the Ferri and Collina farms while grain yield did not vary. The lower yield at this location may be related to the adverse soil conditions during the first year. In particular, the excessive soil compaction during the sowing caused problems to the crop emergence and consequently yield loss. Additionally, grain yield resulted affected by the interaction between genotype and year (GxY). As showed in Figure 2.5 (iii), wheat genotypes and their mixtures differently responded to changes in environmental conditions between 2009/2010 and 2010/2011. For instance, an increase of grain yield was recorded for the old varieties Verna and Inallettabile during the second year, whereas Frassineto, Gentil rosso and Palesio showed an opposite trend. On the other hand, the old genotype Andriolo did not seem to be affected by weather conditions for grain yield. This finding suggested possible different physiological response of wheat genotypes to environmental factors. The interaction genotype x location significantly influenced the obtained Harvest Index for each wheat sample (Figure 2.5). Among genotypes, the modern cultivar Palesio presented the highest Harvest Index values at the Ferri and Collina farms but not at the Cenacchi location, where the highest HI was observed for Verna. Moreover, very similar values were obtained for all the old genotypes at the Collina and Ferri farms while higher variability was observed for the Cenacchi field, as a result of the not ideal growing conditions during the first year.

2.3.3. Lodging

One relevant problem concerning old varieties is the lodging. In the last sixty years, the trend in breeding programs was to strongly reduce the plant *habitus* to maximize the nitrogen uptake avoiding lodging problems. The old wheat varieties were not subjected to this drastic selection pressure and are characterized by a tall *habitus*, over 100 centimeters (Table 2.3). On the other hand, the modern cultivar Palesio showed a dwarf *habitus* with a mean plant height of 62.1 centimeters. To evaluate the entity of lodging problems, a lodging index was calculated and expressed as percentage of plot acreage. The lodging was significantly affected by the location: the highest mean value was observed at the Collina farm (0.77), while the lowest one was recorded at the Cenacchi farm (0.07) (Table 2.3). In general, the most relevant environmental factors in determining this phenomenon are the precipitation regime and the wind speed. After the anthesis, during the spike development, the most frequent event inducing the lodging are rain events of medium–high intensity (>10 mm) associated with wind speed higher than 7 in the Beaufort scale (14–17 m/s). The highest lodging was observed in the most windy (data not shown) and rainy farm (Collina), while the lowest mean lodging was associate with the most calm (data not shown) and dry farm (Cenacchi). A relevant variability was observed among the old genotypes for the lodging score, with Gentil rosso being the most prone to lodging (0.82) (Table 2.3). Among old varieties, Inallettabile showed the lowest lodging scores

Table 2.3. Plant height, Lodging Index, 1000–kernel weight and Test weight of the investigated wheat varieties grown in single–genotype and mixture plots.

		Plant height (cm)	Lodging Index	1000–kernel weight (g)	Test weight (kg/hL)
Location	Cenacchi	98.1(b)	0.07(c)	47.2 ns	77.6(a)
	Ferri	106.6(b)	0.41(b)	46.5 ns	76.7(b)
	Collina	121.1(a)	0.77(a)	47.4 ns	76.3(b)
Year	2009/2010	107.9 ns	0.47 ns	44.9(b)	78.7(a)
	2010/2011	109.3 ns	0.36 ns	49.1(a)	75.1(b)
Genotype	A	109.3(b–d)	0.32(c–e)	42.5(e–g)	74.8(f)
	B	109.6(b–d)	0.17(de)	49.9(a–d)	76.7(b–d)
	C	102.4(d)	0.33(c–e)	40.6(g)	78.8(a)
	D	112.9(b–d)	0.43(b–d)	54.2(a)	77.0(bc)
	E	126.3(a)	0.82(a)	47.8(a–f)	76.5(b–e)
	F	62.1(e)	0.00(e)	42.1(e–g)	77.2(b)
	AB	105.8(d)	0.18(de)	46.2(b–g)	75.9(e)
	AC	105.7(d)	0.32(c–e)	41.1(fg)	77.0(b–d)
	AD	110.3(b–d)	0.51(a–d)	50.4(a–c)	76.3(c–e)
	AE	118.2(a–c)	0.76(ab)	45.9(b–g)	76.2(de)
	BC	108.0(cd)	0.31(c–e)	43.22(d–g)	78.0(a)
	BD	111.1(b–d)	0.24(de)	51.3(ab)	77.0(bc)
	BE	110.4(b–d)	0.55(a–d)	49.1(a–e)	76.7(b–e)
	CD	106.8(d)	0.29(de)	47.5(a–d)	78.1(a)
	CE	119.6(ab)	0.70(a–c)	43.4(c–g)	77.1(b)
	DE	119.0(ab)	0.72(ab)	51.2(ab)	76.5(b–e)
Interaction	LxY	**	*	ns	**
	LxG	ns	**	ns	**
	GxY	ns	**	ns	**

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; L, location; Y, year; G, genotype; ns, not significant.

Means followed by different letters are statistically different at *P*<0.05.
* = significant interaction at *P*<0.05.
** = significant interaction at *P*<0.01.

when grown both in mixture (0.17–0.31) and as pure genotype (0.17). To determine the main contribution affecting the variety lodging, a multiple regression analysis was carried out, including separately the pure genotypes and the binary mix of old varieties. The plant height and other morphological traits such as the ratio of spike to whole plant weight, the whole plant weight, the grain weight, the spike length, the ratio of spike to whole plant length and the number of spikes per square meter were included in both models. As expected, the lodging resulted mainly affected by the

Table 2.4. Partial and cumulated R^2 values and statistical significance (*P*) of each morphological trait as obtained by multiple regression analysis, for wheat varieties grown in single–genotype (a) and mixture (b) plots.

a)

Variables	Partial R^2	Cumulated R^2	*P*	Coefficient
Plant height	0.640	0.640	***	+
Spike/whole plant (weight)	0.076	0.716	***	+
Whole plant weight	0.043	0.759	***	–
Grain weight	0.034	0.793	**	–
Spike length	0.019	0.813	*	+

b)

Variables	Partial R^2	Cumulated R^2	*P*	Coefficient
Plant height	0.638	0.638	***	+
Spikes / m2	0.050	0.726	***	+
Spike/whole plant (length)	0.038	0.676	**	+
Grain weight	0.020	0.746	**	–

plant height, which contributed to the 79% and 85% of the total cumulated square R values for old variety mixtures and pure genotypes, respectively (Table 2.4). A different significant contribution of the other morphologic characteristics to the lodging was observed. For instance, the ratio of spike to whole plant weight resulted the second factor to positively contribute to the lodging of varieties grown in the pure genotype plots. Differently, the spike density and the spike/whole plant length were shown to be the secondary factors that positively affected the lodging of varieties grown in mixture. The multiple regression analysis highlighted that even if these morphological features had a limited contribution to the main factor, totally lower than 15% (Table 2.4), the competition between the genotypes grown in mixture caused significant modifications of the morphological factors determining the plant lodging. Since the old genotype Inallettabile had not the lowest plant height but showed the lowest tendency to the lodging, the anatomy of the stem was observed at the optical microscope, in order to investigate the main factors conferring to Inallettabile resistance to lodging. 20 samples for each old genotype were collected at the phonological stage of 45–47 BBCH scale. The stems were cut using a microtome at the second internode level to obtain the cross sections. The obtained images showed that Andriolo have a typical oval–shaped stem, with parenchymatic tissues in the central part of the section. Frassineto and Verna cross sections showed an oval shape similar to that of Andriolo, but with hollow stems (Figure 2.6). Gentil rosso presented a stem profile oval–shaped with a slight central constriction whereas Inallettabile had a peculiar shape with three lobes. The stem sections were analyzed for their dimensional parameters (*Annex Section*, Figure I). Gentil rosso, Inallettabile and Verna showed the highest values of perimeter and area of the stem section, in addition to the highest number of total vessels (68, 61 and 59, respectively). Moreover, the analysis of vessel dimensions (perimeter, area) highlighted that Inallettabile had the smallest vessels compared to the remaining old varieties (*Annex Section*, Figure II). The morphological analysis suggested that the resistance of the old variety Inallettabile to the lodging may be related to the peculiar stem morphology, characterized by a three–lobe shape and high density of small vessels.

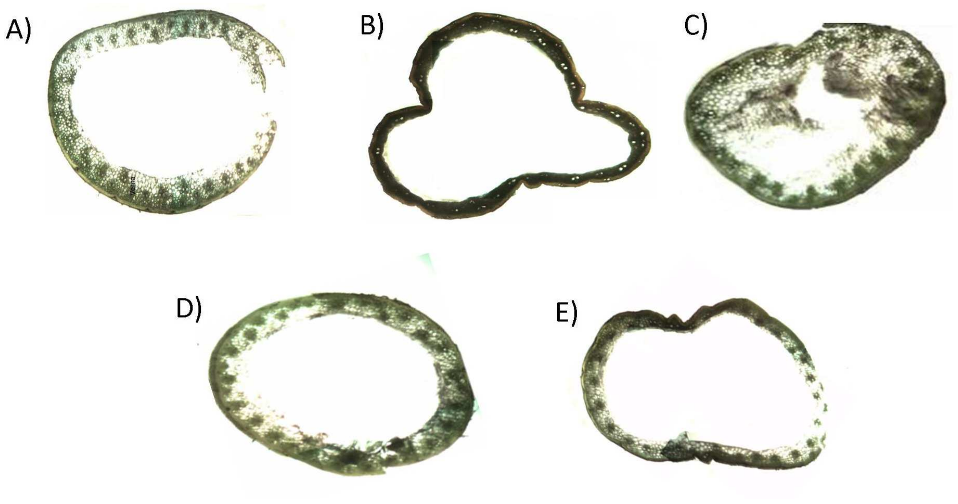


Figure 2.6. Stem cross sections of the investigated old varieties (A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso) as observed at the optical microscope.

2.3.4. Yield components

The main yield components (number of spikes per square meter, number of grains per spike, single grain weight) were determined for each grain sample, including single varieties and genotypes grown in mixture. In Table 2.5, data of sum of squares and statistical significance obtained by the General Linear Model analysis for each yield component are reported. Results highlighted that the main factor (genotype) did not affect the number of spikes per square meter and the number of kernels per spike, whereas the kernel weight was mainly determined by the wheat variety and the

Table 2.5. Sum of squares and statistical significance obtained by the General Linear Model analysis for each yield component (spike per square meter, kernel per spike, kernel weight).

Source of variability	G.L.	Spikes per square meter	Kernels per spike	Kernel weight
Year	1	30,983,379.0 ns	459,929 *	827,535 ns
Location	2	89,628,865.0 ns	4,868,145 **	31,703 ns
Year x Location	2	39,404,589.0 ns	523,308 ns	472,300 ns
Error A	3	36,289,825.0	133,360	265,097 ns
Genotype	15	54,973,381.0 ns	648,232 ns	2,997,380 **
Genotype x Location	30	109,444,483.0 **	807,196 ns	1,370,854 **
Genotype x Year	15	37,230,081.0 ns	555,364 ns	904,114 ns
Error B	123	325,337,502.0	3,231,246	5,328,453

* = significant at $P<0.05$.
** = significant at $P<0.01$.

interaction genotype x location. This finding was expected as single kernel weight is strictly related to genetic traits setting the size and shape of the kernel (length, width, perimeter, etc.). The random factors (year and location) significantly affected the number of kernels per spike; in particular, high variability was observed among the three cropping locations, that contributed to 43% of the total variability. In order to evaluate which yield component mostly contributed to the grain yield, a multiple regression analysis was carried out. Data on partial and cumulated R^2 coefficients for each variety and mixture of old varieties are reported in the *Annex Section* (Tables III and IV). Firstly, the multiple regression analysis was applied to samples grown as single genotypes and it showed that yield component contribution to grain yield varied as a function of the wheat varieties (Figure 2.7). Gentil rosso and Palesio yields resulted largely affected by the number of grains per spike, while no significant contribution was observed for the single grain weight. On the contrary, the single grain weight contributed for almost 50% to the grain yield of Andriolo. As regards the old varieties Verna, Inallettabile and Frassineto, yields were mostly determined by the number of spikes per square meter. Based on data obtained for single varieties, a simple additive model was used to calculate a prediction for each combination of old genotypes (Figure 2.8).

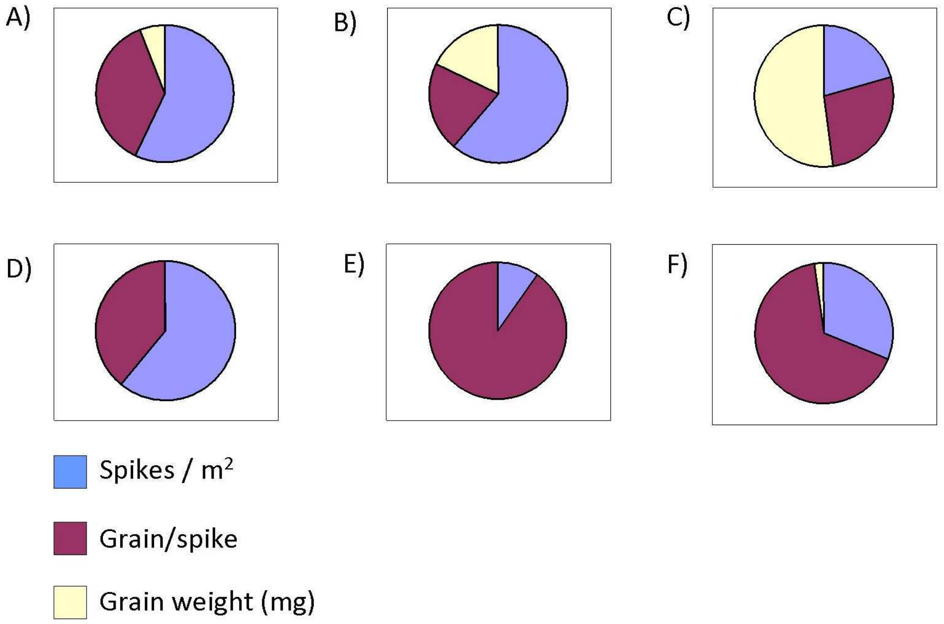
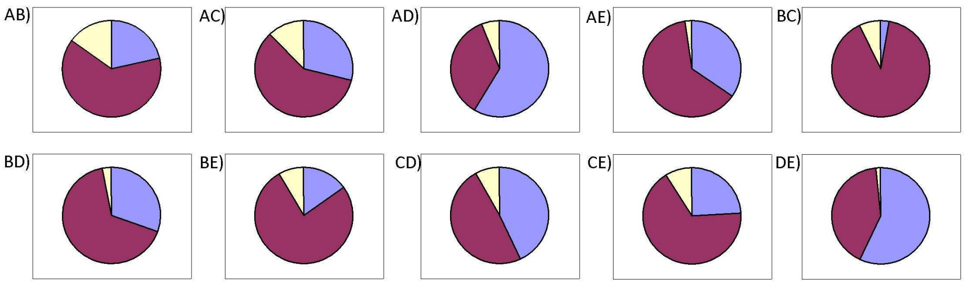


Figure 2.7. Yield components (spikes/m^2, grain/spike, grain/weight) of the investigated wheat varieties grown in plots as single genotypes

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio

The comparison between the obtained and the predicted results showed that some mixtures (such as AD, AE, DE) highly matched the predicted models. However, in few cases (BE, CE, namely Verna–Gentil rosso and Andriolo–Gentil rosso) the genotype effects were shown to be not typically additive, suggesting that the competition between the two genotypes resulted in a variation of yield components. In particular, in both cases (BE and CE mixtures), yield was mainly determined by the number of grains per spike as previous observed for Gentil rosso grown as single genotype (Figure 2.7 and 2.8). These remarks suggest that some old genotypes grown in mixture showed a balanced development although under reciprocal competition. In contrast, the old variety Gentil rosso exerted a major intra–specific competition on plants having lower plant height, due to its tall–straw *habitus*.

1) Observed



2) Predicted

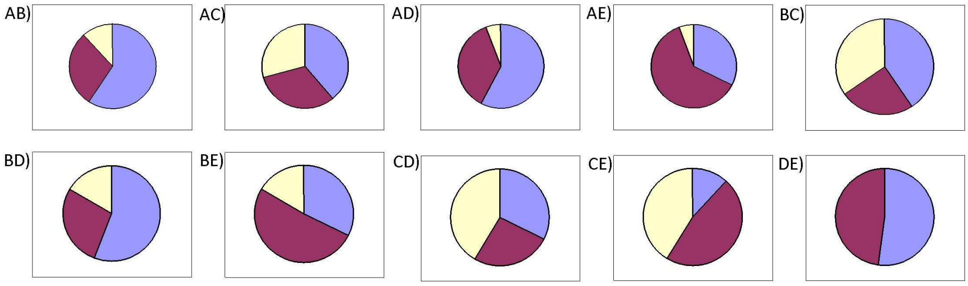
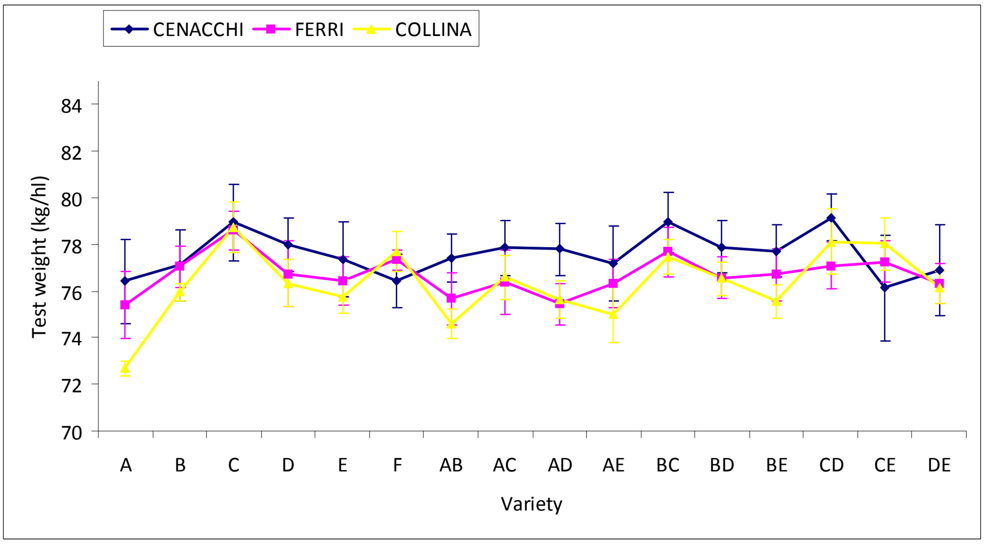


Figure 2.8. Observed and predicted yield components (spikes/m^2, grain/spike, grain/weight) of the investigated wheat varieties grown in plots as mixtures.

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso.

2.3.5. Commercial quality traits

Commercial quality includes kernel characteristics (i.e test weight, 1000–kernel weight) that provide information on milling performance and potential flour extraction of the wheat samples. Both test and kernel weight are inherited traits influenced by genetic variety differences and additionally they are often affected by environmental factors and the interaction genotype x environment (Houshmand et al., 2004). Test weight is an important parameters for assessing the commercial quality of wheat kernels and expresses the grain–filling rate. Low values of test weight can cause price deduction or rejection of the materials, as it induces a low flour yield. In the present study, all test weights resulted equal or higher than the recommended threshold value established for wheat flour trade in Italy (>75 kg/hL) (Cocchi et al. 2005), indicating a well–formed grain development. Test weight varied as a function of the wheat genotype and ranged from 74.8 kg/hL (Verna) to 78.8 kg/hL (Andriolo). A test weight decrease may occur as a consequence of abiotic and biotic stresses, such as adverse weather conditions, insect damage, defoliation, heat stress, lodging or delayed harvesting. The test weight of wheat grain additionally depends on the grain size, shape and endosperm density. An association between test weight and kernel weight has long been reported in literature (Simmonds, 1989). However, few investigations on kernel morphological parameters, showed that test weight was highly significantly correlated with morphological traits such as grain length, perimeter and width rather than kernel weight (Troccoli and Di Fonzo, 1998). Among the morphological traits, the ratio of grain length to width has been shown to be the most important parameter determining test weight values (Merkle et al. 1969; Pushman and Bingham, 1975). In the present study, the mentioned correlation between test weight and 1000–kernel weight was not observed. For instance, the old variety Andriolo showed the highest test weight value (78.8 kg/hL) in both growing seasons even if it had one of the lowest 1000–kernel weight (40.6 g) (Table 2.3). These results suggested that the variation of test weight among genotypes were mainly due to varietal characteristic linked to kernel dimensions. Although grain size and plumpness can be strongly influenced by environmental conditions, different varieties may have inherently large or small kernels and therefore their ultimate potential for these quality traits is genetically determined (Simmonds, 1989). The obtained 1000–kernel weights, ranging from 40.6 g to 54.2 g (Table 2.3), agreed with those formerly reported for old and modern wheat varieties grown under conventional and low–input farming (Guarda et al., 2004; Kindred et al., 2008; Hilderman et al., 2008; Di Silvestro et al., 2012). Previous investigations highlighted that high temperature at the final maturity stage have negative effects on grain quality by irreversible reducing the grain filling and consequently test weight values (Gan et al., 2000; Rharrabti et al., 2003). In the present study, the higher temperatures and the low precipitation of the second year during the final stages of grain development may have caused the decrease of test weight observed between the two growing seasons (Table 2.3). Interestingly, the significant interactions genotype x location and genotype x year evidenced that wheat genotypes differently responded to environmental stresses occurred during the second year. In fact, as reported in Figure 2.9 some old varieties (Inallettabile, Verna) were not influenced by heat/drought stress and presented similar test weight values in both growing seasons. On the contrary, a 1–2% reduction of test weight values was observed for the remaining investigated genotypes. Moreover, the interaction between genotype and location evidenced that Andriolo and Palesio had stable test weight values among the three farms, whereas the remaining old varieties showed the lowest values when grown at the Collina farm (Figure 2.9). As regards the 1000–kernel i)



ii)

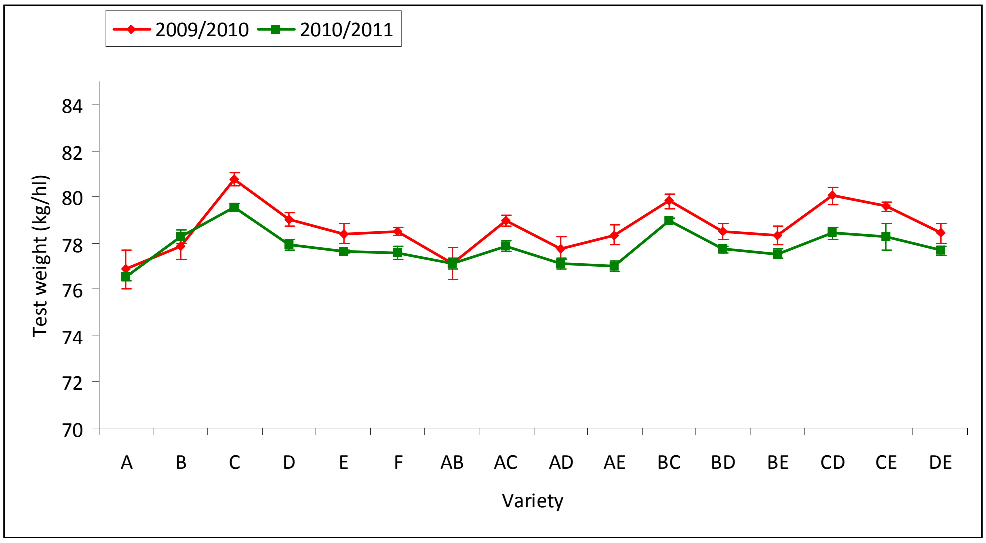


Figure 2.9. Significant interactions observed between genotype and location (i) and genotype and year (ii) for test weight.

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

significant differences were detected among the three biodynamic farms and no significant interactions were observed (Table 2.3). Unlike the test weight decrease during the second year, an unexpected increase of kernel weight was observed in the second growing season (Table 2.3). This remark may be related to different response of kernel morphological traits to environmental factors; as previously reported in literature by Troccoli and Di Fonzo (1998) weather conditions significantly affect kernel weight, perimeter and area, but no effect was observed on kernel weight, thickness, width and volume. Finally, the location by year, genotype by year and genotype by location interactions were not significant for 1000–kernel weight, confirming the high inheritability if this trait.

2.3.6. Mycotoxin content

The mycotoxin contamination of flours is an important concern of the organic sector, in which no fungicide can be applied to the crop. The most common toxin occurring in wheat flours is deoxynivalenol (DON) that belongs to the trichothecene class and is produced by several fungi of the *Fusarium* genus (mainly *F. culmorum* and *F. graminearum*) (Edwards, 2009). Mycotoxins cause major safety problems in cereal production and are known to have adverse and chronic health impacts on humans and animals (Murphy et al., 2006). The DON levels are regulated with a decree issued by the European Commission (EC N. 1126/2007) that sets the maximum allowed threshold values for unprocessed cereal and derived products (*Annex Section*, Table V). The accumulation of mycotoxins in wheat grain depends on fungi development that in turn is strongly affected by weather conditions (Champeil et al. 2004). The first growing season (2009/2010) presented quite favorable environmental features for head blight *Fusarium* development during the anthesis stage (April–May), with wet and middle–warm conditions (Figure 2.3; *Annex Section*, Tables I and II). On the contrary, in the second year (2010/2011) the drier conditions occurred during springtime limited the fungi infection. As reported in Table 2.6, grain samples obtained from the second growing season showed an ideal hygienic–sanitary quality, with DON values under the limit of detection (lower than 0.02 ppm). Higher mycotoxin content was observed for the samples harvested in the first year. Considering the mean values over the different locations, all DON amounts resulted lower than the legislative limits set for all the food categories indicated in the EU regulation. The statistical analysis highlighted that the DON levels did not vary as a function of the wheat genotypes but

Table 2.6. Deoxynivalenol (DON) content detected in the whole flours of the investigated varieties obtained from the first (i) and the second (ii) growing season. Results are expressed in mg per kg of wholeflour (ppm).

i)

Variety	Cenacchi	Ferri	Collina	Mean
A	0.09	0.07	0.18	0.11
AB	0.13	0.29	n.d.	0.17
AC	0.15	0.21	0.22	0.18
AD	0.18	0.39	0.15	0.19
AE	0.19	0.18	0.12	0.16
B	0.22	0.26	0.12	0.19
BC	0.17	0.28	0.02	0.15
BD	0.24	0.24	0.06	0.14
BE	0.07	0.22	0.31	0.15
C	0.11	0.21	0.27	0.16
CD	0.13	0.19	0.23	0.16
CE	0.15	0.17	0.21	0.16
D	0.23	0.14	0.19	0.18
DE	0.05	0.23	0.22	0.16
E	0.14	0.22	0.22	0.19
F	0.29	0.41	0.27	0.26
Mean	0.16(a)	0.23(b)	0.19(a)	

ii)

Variety	Cenacchi	Ferri	Collina	Mean
A	0.01	0.02	0.02	0.02
AB	0.02	0.00	0.00	0.01
AC	0.01	0.01	0.01	0.01
AD	0.02	0.01	0.02	0.02
AE	0.02	0.07	0.00	0.03
B	0.01	0.02	0.02	0.02
BC	0.02	0.01	0.00	0.01
BD	0.02	0.01	0.01	0.01
BE	0.01	0.03	0.01	0.02
C	0.02	0.02	0.00	0.01
CD	0.01	0.01	0.01	0.01
CE	0.01	0.03	0.02	0.02
D	0.01	0.01	0.02	0.01
DE	0.02	0.02	0.02	0.02
E	0.02	0.01	0.02	0.02
CD	0.01	0.01	0.01	0.01
CE	0.01	0.03	0.02	0.02
F	0.01	0.01	0.01	0.01
Mean	0.20	0.20	0.20	

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

Means followed by different letters are statistically different at *P*<0.05.

the Ferri farm (0.23 ppm), followed by Collina (0.19 ppm) and Cenacchi (0.16 ppm) farms (Table 2.6). The differences observed among the three locations may not be related to weather conditions but to a minor severity of the fungal pathologies at the Cenacchi and Collina farms. Soil preparation plays a key role in the control of pest development, including appropriate soil cultivation and rotations. Previous investigations reported on the increasing levels of contamination for flours grown under no tillage management, due to high disease severity (Teich and Hamilton, 1985; Dill–Macky and Jones, 2000; Champeil et al. 2004). The accurate soil preparation may be the cause of limited DON accumulation in wheat grains grown under organic management, in which the protection by fungicide treatments is not allowed.

2.4. AT A GLANCE

- ✓ The two growing seasons (2009/2010; 2010/2011) were non comparable as characterized by different thermo–pluviometric features;
- ✓ The three sites were characterized by different micro–climatic conditions: the Collina farm is the most wet environment, the Cenacchi farm is the most dry location, while the Ferri farm exhibits an intermediate condition;
- ✓ The most remarkable phonological differences among the investigated genotypes were observed during flowering and seed maturity. In general, the not–dwarf varieties show a slightly prolonged flowering and seed maturation with respect to the dwarf Palesio cultivar;
- ✓ Under low input cropping system, some ancient varieties (Inallettabile, Frassineto), grown pure or in binary mixture, assured the same grain yield of the Palesio modern cultivar;
- ✓ The analysis of the main yield components evidences that, except for Gentil rosso, all remaining old not–dwarf wheat genotypes can be grown in binary mixture without detrimental effects of intra–specific competition;
- ✓ The old varieties assure a straw production approximately double with respect to the Palesio dwarf cultivar. Considering the closed self–sustained organization of several low–input farming systems, straw represents a precious resource (compost preparation, soil amendment, bio–energy);
- ✓ Old not dwarf wheat varieties are prone to lodging, but significant differences are observed as a function of cropping location and genotype. Due to its

peculiar stem morphology, the old genotype Inallettabile shows a remarkable resistance to the lodging;

- ✓ No significant differences among genotypes (grown pure or in binary mixture) for the 1000–kernel weight and test weight are observed. The observed commercial quality traits are acceptable for low–input production systems;
- ✓ Despite of the interdiction in using fungicides, for all tested genotypes the mycotoxin content was lower than the legislative limits set for all the food categories , as indicated in the current EU regulation.

Section 3:

NUTRIENT CONTENT OF WHEAT VARIETIES

3.1. SECTION OBJECTIVES

Cereals are the basis of human diet all over the world and, among them, wheat and its derived products are the most consumed staple food. The nutritional value of wheat is related to the high carbohydrate content and contemporarily the noticeable protein levels. Indeed, wheat represent the major complex carbohydrate source in the diet as more than 80% of the endosperm is made of starch, which provides available glucose energy for the human body processes. In addition, unrefined wheat represents an excellent source of complex carbohydrates, and 25 percent of the total carbohydrates is dietary fibre. This source of carbohydrates is ideal for diabetics because it takes longer to digest and absorb sugar into the bloodstream. The wheat protein fraction constitutes about 20 to 25 percent of the caloric content: it is mainly made up of gluten proteins (gliadins and glutenins), which are incomplete proteins (lacking for some essential amino acids). Beyond their nutritional importance, wheat proteins play a significant role in the determination of flour baking quality, especially as regards the gluten fraction. Hence, one of the main target of breeding programs was to improve the gluten strength and viscoelasticity, allowing dough to tolerate high technological stress during mixing and leavening processes. Considering the negative correlation between grain yield and protein concentration, one of the main breeding focus was to select for high gluten technological quality accompanied with increasing fertilizer distribution over the crop cycle. In turn, the modern wheat varieties may result quite unsuitable for the low–input sector, as they often lead to low yield and scarce protein quality that does not fulfill the industry baking requirements. In the last 50 years the worldwide spread of celiac disease and other gluten sensitivity pathologies has induced to pay attention on the presence of toxic or allergenic peptides in the gluten fraction. This is in contrast with the post–"Green Revolution" breeding programs primarily focused on enhancing wheat technological properties rather than the nutrition values in relation to gluten related pathologies. If starch and proteins are mostly accumulated in the endosperm, lipids (accounting for approximately 2–3%) and micronutrients, such as mineral elements, are mainly concentrated in the germ and bran, respectively. Flour lipids have long been considered undesirable compounds due to their negative influence on the shelf life of flours. From a nutritional point of view noticeable is the presence of tocopherols and tocotrienols (vitamin E) that approximately contribute to 25% of the total lipid content of grains. As regards micronutrients, wholegrain derived products provide a plenty of mineral elements: the most abundant are phosphorous (P), potassium (K), magnesium (Mg) and calcium (Ca) which may significantly contribute to the achievement of the recommended dietary intakes. The accumulation of mineral elements in wheat grains is known to be strongly affected by genetic traits, as it highly depends on the nutrient uptake and translocation efficiency of the wheat varieties. The modern wheat varieties have been shown to have lower ability to accumulate grain microelements, probably due to their limited root system development, compared to not dwarf wheat genotypes. Therefore, old varieties may represent an interesting raw material for the selection of wheat genotypes with improved nutritional value. In literature, relatively few investigations are focused on the nutrient composition of different wheat varieties as a function of the genotype, environment and agricultural management. The aim of the present research was to describe the complete nutrient profile of the investigated modern and old wheat genotypes through the determination of total protein, starch, lipid and mineral element content. Additionally, the gluten protein composition of investigated varieties was comparatively evaluated by gel electrophoresis.

3.2. MATERIAL AND METHODS

Grain samples of modern (Palesio) and old (Andriolo, Frassineto, Gentil rosso, Inallettabile, Verna) varieties grown in pure genotype plots from the field trial described in *Section 2* were used for nutrient determinations. Wheat grains harvested at the three involved locations (Cenacchi, Ferri and Collina farms), from both growing seasons (2009/2010, 2010/2011), were stone milled and the nutrient composition was analyzed as outlined below. Results are expressed on whole flour dry weight basis.

3.2.1. Protein and gluten content

The grain protein content was measured according to the AACC official method 46–12 (AACC, 1983). The method is based on the total nitrogen determination according to the Kjeldahl procedure, using 5.7 as nitrogen–to–protein conversion factor. The gluten amount was determined using the method previously described (Kieffer *et al.*, 2007; Di Silvestro et al., 2012). Briefly, flour was mixed with NaCl solution (0.4 M), washed by hand until a cohesive mass was obtained and successively freeze dried and weighted to obtain the total gluten content.

3.2.2. Gluten protein extraction and electrophoretic analysis

The gliadin and glutenin fractions were extracted as previously described by Singh *et al.* (1991) and van den Broeck *et al.* (2009). Briefly, 200 mg of whole wheat flour was extracted by the addition of 1 ml of 50% aqueous isopropanol (v/v) under continuous mixing for 30 min at room temperature, followed by centrifugation (2500 x *g*, 15 min), to obtain the “first gliadin extract”. The residue was extracted twice with 50% (v/v) aqueous isopropanol and the recovered supernatants referred as “second” and “third gliadin extracts”. Subsequently, the pellet was treated with 1 ml of 50% (v/v) aqueous isopropanol, 50 mM Tris–HCl (pH 7.5) containing 1% (w/v) DTT for 30 min at 60°C, mixing every 10 min, and than centrifuged at 10000 x *g* for 10 min to obtain the glutenin supernatant. The 2nd gliadin extracts and the glutenin extracts were separated on SDS–PAGE gel (10%) as previously described (Laemmli, 1970), using the Mini–PROTEAN Tetra Cell for Mini Precast Gels vertical electrophoresis system (Bio Rad Laboratories S.r.l., Milan, Italy), followed by staining with PageBlue™ (Fermentas International Inc., Canada).

3.2.3. Starch and lipid determination

Total starch was measured according to the method described by McCleary and Monaghan (2002) using a Megazyme assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The procedure was based on α–amylase and amyloglucosidase overnight digestion of flour and removal of soluble starch with 95% and 50% ethanol consecutive washes. The obtained extracts were than spectrophotometrically quantified using a glucose oxidase–peroxidase (GOPOD) reagent. Lipid analysis was carried out according to standardized methods (AOAC, 1990). Briefly, 500 mg of whole flour was treated with 10 ml of chloroform–methanol (2:1, v/v) under continuous shaking for 20 min and centrifuged (10000 rpm, 20 min). The supernatant was collected and extraction repeated once. Finally, supernatants were pooled, evaporated to dryness and the residue weighted to quantify the lipid content.

3.2.4. Ash and mineral element analysis

Ash content was determined using the standard method AACC 08–01 (AACC, 1983) by incinerating 1.5–2 g of flour at gradually increasing temperature from 425 °C to 550°C until light grey ash was obtained (5 hours). Whole flours were analyzed for mineral element composition by using an inductively coupled plasma optical emission spectrometer (ICP–OES IRIS Intrepid II XSP Radial, Thermo Fisher Scientific, Waltham, MA, USA) after mineralizing the samples in 5 ml concentrated HNO_3 and 2 ml concentrated H_2O_2, using a closed microwave system digestion (MarsExpress CEM Corp.). Macro (Ca, K, Mg, Na, P) and micro mineral elements (Al, As, B, Ba, Be, Bi, Cd, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, Pb, Se, Sr, Ti, Tl, V, Zn) were quantified by comparison with multi–element standards (Analytika, Prague, Czech Republic) and expressed in milligrams per kilograms of flours (ppm).

3.2.5. Statistical analysis

A General Linear Model (GLM) was used to assess the variance significance for the main (genotype) and the random (location, year) factors, as well as their interactions, for all measured variables (except for mineral elements). One–way ANOVA (Tukey's test) was performed to test the variance significance of genotypes harvested in the first growing season (2009/2010) for mineral content. Significance between means was determined by least significant difference values for $P < 0.05$. The analysis of variance was performed using Systat v. 9.0 (SPSS Inc. 1998). Cluster analysis

(Unweighted Pair Group Average Method with arithmetic averaging – UPGMA) was carried out on gluten subunit data obtained by vertical electrophoresis and on Manhattan distance matrix to seek for hierarchical association among the wheat varieties, using STATISTICA Software v. 7.1 (StatSoft, Tulsa, Oklahoma, USA).

3.3. RESULTS AND DISCUSSION

3.3.1. Protein content

In organic wheat production, one of the major concern is the grain quality and particularly the protein content. The protein accumulation in wheat kernel is strictly dependent on the nitrogen (N) availability. In low–input agricultural systems, the N supply relies on symbiotic N–fixation and application of organic fertilizers (i.e. manure, biodynamic compost). Generally, the mineralization of manure and crop residues results in high N availability at the beginning of crop cycle (when the crop uptake is low) and limited N supply in later growth stages when the plant demand is greater (Wolfe et al. 2008). To assure a medium–high protein content under low–input management, the selection of wheat varieties with improved N uptake into the grain should be favored. The nitrogen absorption is directly related to (i) the total uptake from the soil, (ii) the translocation capacity from the vegetative tissues to the grain, (iii) the direct transfer from the soil to the grain after anthesis and (iv) the losses of nitrogen already absorbed (Wolfe et al., 2008). The genetic background strongly influences the efficiency of N accumulation, especially by determining the root system development (as a high root extension may enhance nitrate uptake in N–limited conditions) and the nitrogen transfer efficiency during post–anthesis stages (Laperche et al., 2006; Vaccari et al., 2007). In the present study, the protein content varied as a function of the wheat genotype with the modern cultivar showing the lowest value (12.0 g/100g) (Table 3.1). All the old varieties presented higher protein contents equal to 13.1 g/100g (Inallettabile, Andriolo, Gentil rosso), 14.0 g/100g (Verna) and 14.1 g/100g (Frassineto) (Table 3.1). These results are in agreement with previous investigations reporting on the inability of modern dwarf wheat cultivars to fully express their potentialities under low–input management (Lammerts van Bueren et al., 2011; Di Silvestro et al., 2012). Indeed old wheat varieties have shown a more efficient nutrient use in low–N environments compared to the modern ones, which are strictly dependent on high nitrogen inputs (Lammerts van Bueren et al., 2011). As outlined above, the high N uptake efficiency of old varieties is probably related to their tall *habitus*, which implies a more extended root system compared to dwarf genotypes. Among cropping locations, the Ferri farm showed the highest protein content (14.3 g/100g), followed by the Collina (13.8 g/100g) and Cenacchi (11.7 g/100g) farm (Table 3.1).

Table 3.1. Protein, gluten, starch, lipid and ash content of the investigated wheat genotypes.

		Protein g/100g	Gluten %	Starch g/100g	Lipid g/100g	Ash g/100g
Location	Cenacchi	11.7(c)	67.6(b)	73.9(a)	4.1(a)	2.1 ns
	Ferri	14.3(a)	72.7(a)	70.5(b)	4.4(a)	2.3 ns
	Collina	13.8(b)	69.4(b)	67.3(c)	3.5(b)	2.1 ns
Year	2009/2010	15.1(a)	69.1(b)	70.1 ns	4.1 ns	2.4(a)
	2010/2011	11.4(b)	70.6(a)	71.1 ns	3.9 ns	1.9(b)
Variety	A	14.0(a)	72.4(abc)	68.7 ns	4.6(a)	2.2(b)
	B	13.1(b)	73.4(a)	71.4 ns	4.2(b)	2.1(c)
	C	13.1(b)	68.9(c)	71.4 ns	3.9(cd)	2.1(c)
	D	14.1(a)	70.5(bc)	70.1 ns	4.1(bc)	2.2(b)
	E	13.1(b)	72.2(ab)	71.1 ns	3.9(d)	2.4(a)
	F	12.0(c)	61.8(d)	70.9 ns	3.3(e)	1.9(c)
Interaction	LxY	ns	ns	ns	ns	*
	LxG	ns	*	ns	ns	ns
	GxY	ns	ns	ns	ns	ns

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; L, location; Y, year; G, genotype; ns, not significant.

Means followed by different letters are statistically different at *P*<0.05.
* = significant interaction at *P*<0.05.

The protein content observed at the Cenacchi farm is in agreement with the results reported in a long–term trial involving old wheat varieties grown under biodynamic management (Hilderman et al., 2009). These results are also aligned with previous investigations reporting a protein content ranging from 11.2 to 12.6 g/100g for wheat varieties grown in other forms of organic and low–input agricultural systems (Wang et al., 2008; Zuchowski et al., 2011). Additional determinations on soil organic matter content at the three cropping sites are in progress with the main aim to justify the higher protein values observed at the Ferri and Collina farms. A noticeable reduction of the protein content occurred during the second year (2010/2011), accounting for a 3.7 % decrease (Table 3.1). The relationship between environmental conditions and grain protein accumulation was deeply demonstrated in literature (Graybosch et al., 1995; Daniel and Triboi, 2000; Dupont and Altenbach, 2003; Altenbach, 2012). The protein content is directly related to the duration and rate of the grain filling period. High temperatures during the final stages of the crop cycle can accelerate the grain development and, additionally, limited precipitations may induce a drought stress for the plant, reducing or arresting the grain nutrient accumulation (Dupont and Altenbach, 2003). The meteorological data recorded during the two growing seasons indicated a significant reduction of the cumulative rainfall during the second year, from flowering to harvest, suggesting a possible correlation with the observed low protein content (Figure 2.3; *Annex Section*, Table II). Although the protein content was closely influenced by environmental factors such as cropping locations and different weather conditions, no significant interaction was observed between the genotype and the random factors (location and year) (Table 3.1). Indeed, the variation range among wheat varieties was approximately constant in all the involved locations and over the two growing seasons, suggesting a strong influence of the genetic traits on the grain protein accumulation and a similar physiological response of each variety to the changing environmental conditions.

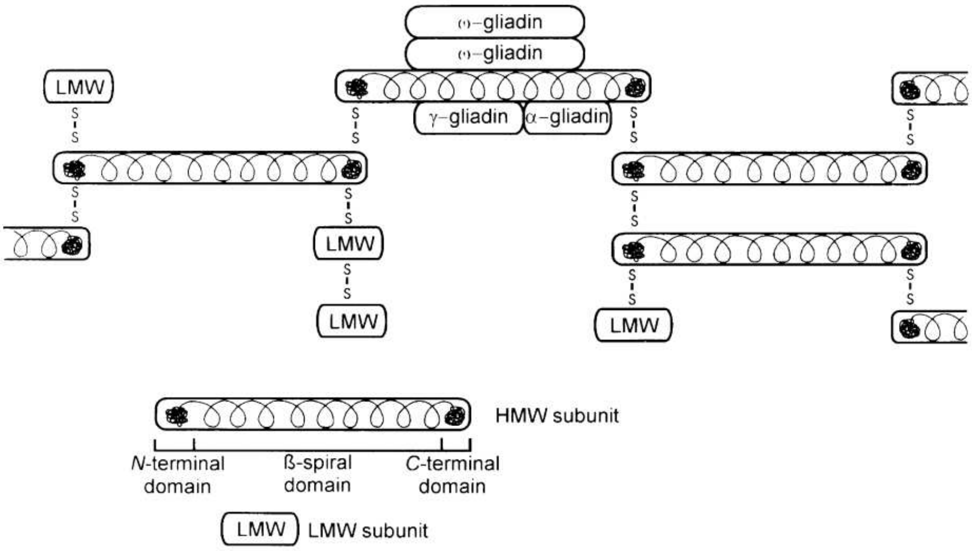


Figure 3.1. Structural model of wheat gluten in which HMW subunits provide disulphide–bonded backbone and interact with other gluten proteins by disulphide bonds (LMW–GS) and non–covalent linkages (gliadins) (Shewry et al., 2001).

3.3.2. Gluten storage proteins and electrophoretic patterns

The gluten storage proteins constitute up to 80% of the total grain protein. The gluten fraction includes gliadin and glutenin subunits, which are present in approximately equal amounts. Gliadins are 30–60 kDa monomeric proteins, alcohol soluble, that can be divided in α, β, γ and ω subunits. In wheat endosperm, gliadins are structured in large complexes constituted of subunits linked by disulphide bonds (Dupont and Altenbach, 2003). Glutenins are polymeric proteins highly different for their molecular size, which include either low molecular weight subunits (LMW–GS) (30–50 kDa) and high molecular weight glutenin subunits (HMW–GS) (75–100 kDa) (Ciaffi et al., 1999; Gu et al., 2010). The gluten proteins play a crucial role in determining the end–use quality of common wheat, by influencing the viscoelastic properties of dough. The variation in dough rheology and bread making performance among wheat varieties is largely determined by the differences in both protein content and composition, depending on the presence of specific HMW–GS and gliadin subunits (MachRitchie, 1992; Gupta et al., 1993; Shewry et al., 2001; Sliwinski et al., 2004). The gluten viscoelasticity is directly related to the gluten content and to the structural arrangement of HMW subunits. As shown in Figure 3.1, the high molecular weight glutenin polymers provide an elastic backbone which interacts with other gluten subunits by inter–chain disulphide bonds, forming the LMW–GS branches (Shewry et al., 2001). The disulphide bonds are widely considered to be essential for guaranteeing the gluten viscoelasticity. Gliadins may also interact with the glutenin polymers by non–covalent linkages, contributing mostly to gluten viscosity rather than elasticity (Shewry et al., 2001). The presence of specific allelic variants may confer different viscoelastic properties to the gluten arrangement, thus conferring different technological properties. Consequently, in the last 60 years the breeding programs of wheat varieties have been focused on the improvement of rheological properties by selecting wheat genotypes for their qualitative and quantitative gluten content rather than for their nutritional value in relation to gluten related pathologies (Wolfe et al., 2008). In most cases the use of modern varieties in organic and low–input farming led to low yield and protein quality that did not fulfill the baking requirements, as evidenced by the observed rheological properties of the modern cultivar(Palesio) used in the present study (see *Section 5*). Gluten proteins (gliadins and glutenins) are rich in proline and glutamine occurring in specific allergenic motifs (epitopes) which can induce the typical autoimmune response of the celiac disease (CD). Worldwide CD is one of the most spread food allergy and it is estimated that 0.5–1% of the Western population suffers for this pathology (van den Broeck et al., 2009). In the last 50 years an increasing awareness about the presence of toxic peptides in the wheat gluten fraction occurred among scientists and consumers, as a consequence of the observed wide spread of several forms of gluten sensitivity and allergies (Silano et al., 2007; van den Broeck et al., 2009; Vincentini et al. 2009; van den Broeck et al., 2011).

An *in vitro* investigation on prolamins extracted from several spelt landraces (*Triticum turgidum* ssp. *dicoccum*) evidenced different proliferative responses on T–cell lymphocytes (Vincentini et al., 2009). Vicentini *et al.* (2009) highlighted that the differential T–cell immunological activation is mainly due to the wide variation in prolamin composition of the investigated spelt genotypes. This study confirmed previous investigations on the variable immunological response to cereal flours as a function of the gluten composition.

In the present study the six wheat varieties were investigated for their gluten content and composition. As regards the total gluten, the highest content was observed at the Ferri farm (72.7 % on total protein), whereas at the Collina and Cenacchi farms the gluten accounted for 69.4 and 67.6 % of total protein, respectively (Table 3.1). No significant effect of weather conditions on gluten content was recorded over the two years, suggesting that the heat/drought stressing conditions during the second cropping year (2010/2011) (Figure 2.3) equally influenced all the protein fractions (albumin, globulin, gliadin, glutenin) and did not alter their relative percentage (Table 3.1).

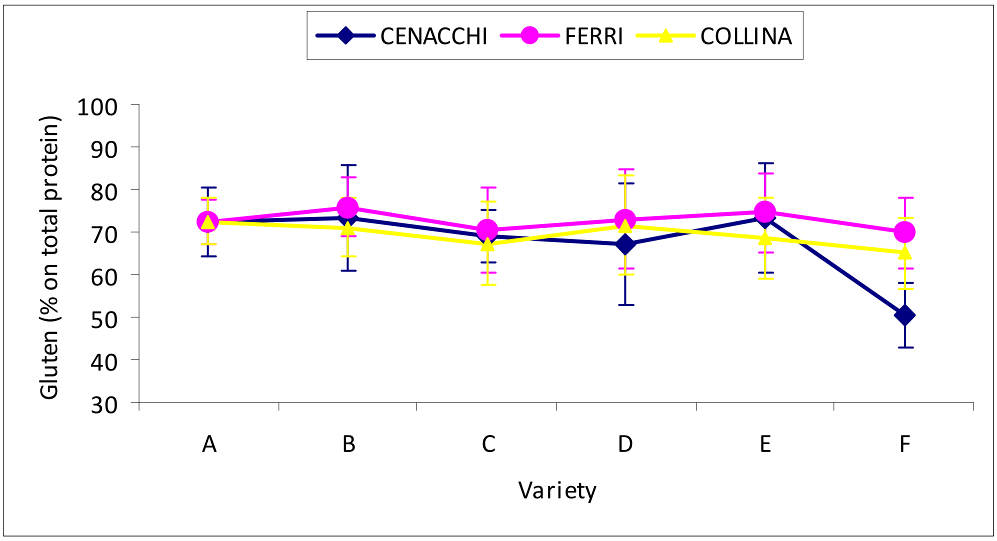


Figure 3.2. Significant interaction between genotype and location (GxL) observed for gluten content (mean values ± standard error).
Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F = Palesio

The gluten content varied as a function of the wheat genotype and ranged between 61.8 and 73.4 % of total protein (Table 3.1). The lowest value was found for the modern cultivar Palesio (61.8 % of total protein), whereas Inallettabile presented the highest one (73.4 % of total protein), being additionally the poorest and the richest in protein content, respectively. The observed significant interaction between genotype and location was mainly due to the low amount of gluten obtained for Palesio at the Cenacchi farm in both growing seasons, while the old varieties showed similar values at all the locations (Figure 3.2).

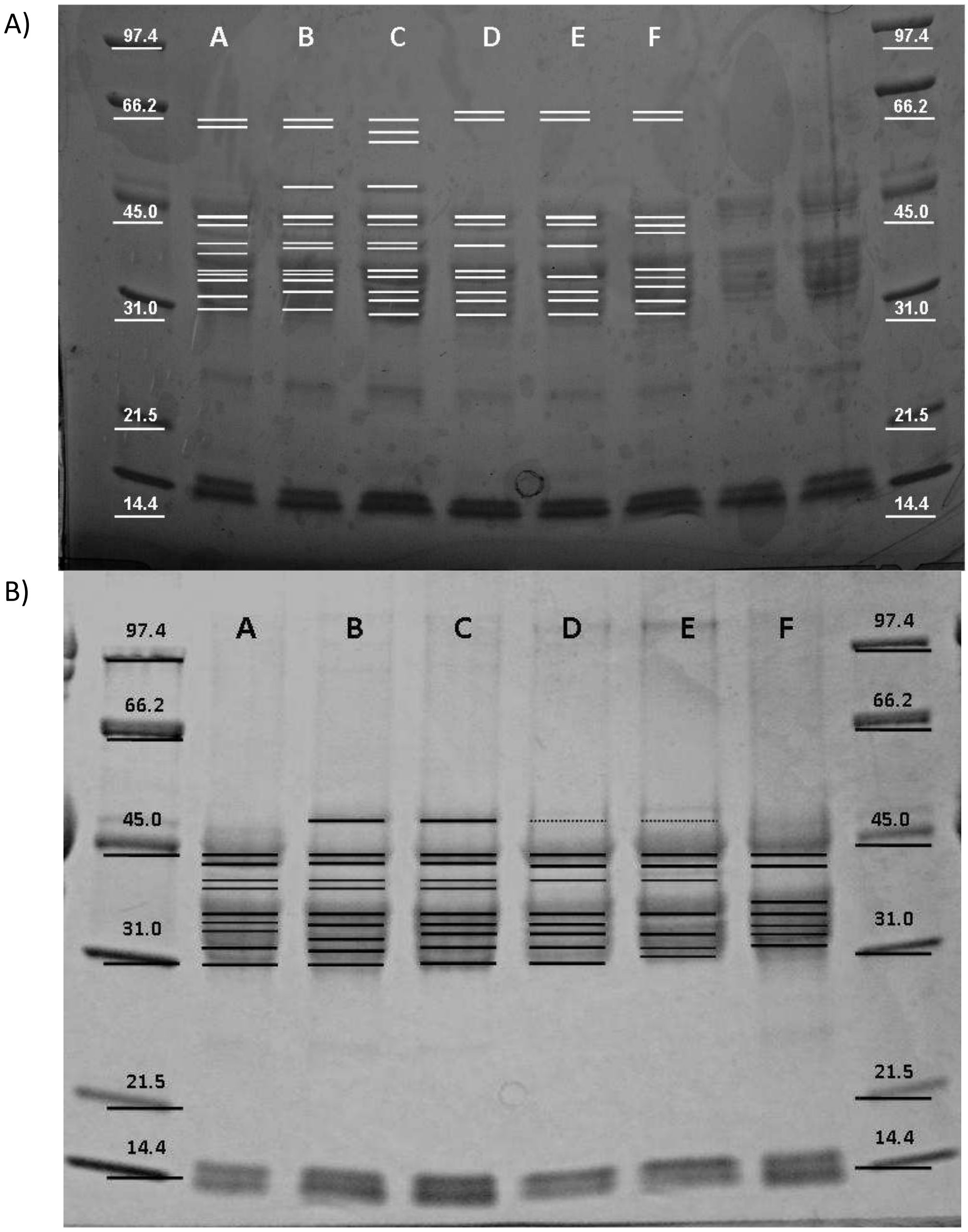


Figure 3.3. Gliadin electrophoretic patterns of the investigated wheat varieties grown in the first (A) and second (B) growing season.
Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

These results again remark the failure of modern cultivars to fully express their potential quality in the absence of high nitrogen input by providing low gluten quality (see *Section 5*) and content. The observed gluten quantities are in general agreement with those previously reported for old and modern wheat varieties grown under low–input management (values ranging from 63 to 74% of total protein content) (Sliwinski et al., 2004; De Vita et al., 2007; Di Silvestro et al., 2012). In order to evaluate the gliadin and glutenin variability among the investigated genotypes, the two gluten subunits were fractionated by vertical electrophoresis. Figure 3.3 shows the electrophoretic pattern of the gliadin fraction extracted from each genotype (as a bulk sample of the three locations) harvested in the first (A) and second (B) year. Data were elaborated as presence/absence of each gluten subunit and computed to obtain a similarity dendrogram (Figure 3.4).

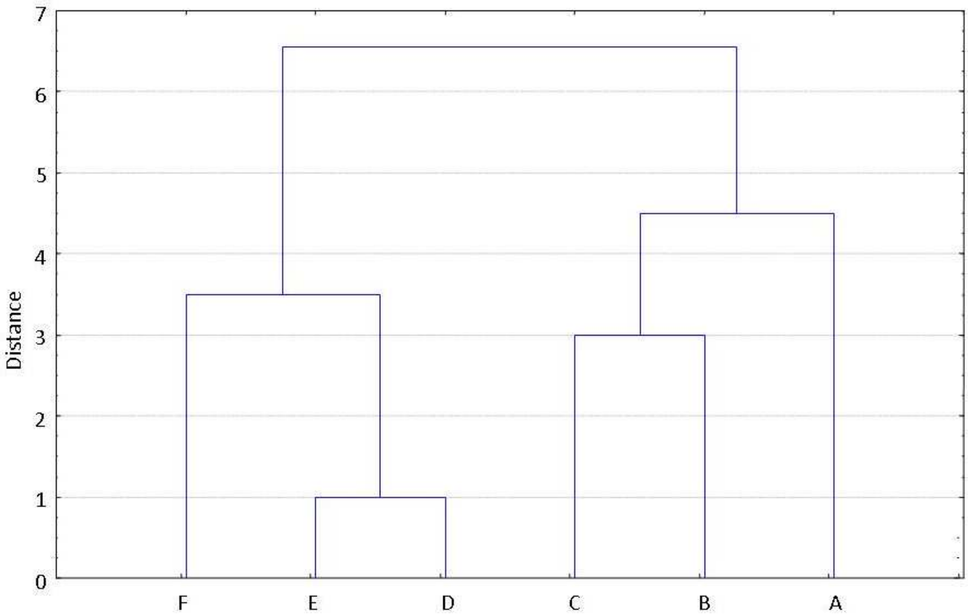


Figure 3.4. Dendrogram obtained using data of the gliadin electrophoretic patterns for the investigated wheat varieties over two growing seasons.
Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F = Palesio

In the six genotypes a total of 16 gliadin subunits (with molecular weight ranging between 31 and 68 kDa) were observed. The old varieties Frassineto and Gentil rosso showed similar gliadin profiles, sharing certain similarities with the pattern of Palesio. For instance, the three genotypes showed the common presence of 66 kDa and 68 kDa subunits, identified as ω–gliadins on the basis of available literature (van den Broeck et al., 2009). The old genotypes Andriolo, Inallettabile and Verna formed a highly divergent cluster, presenting higher variability in their gliadin composition and differing from the first group mainly for the presence of 40 and 60 kDa subunits (belonging to the α/β– and γ–gliadins). Andriolo and Inallettabile are separated from Verna for the unique presence of a 50 kDa gliadin fraction. Similarly, the dendrogram presented in Figure 3.6 was computed on the basis of data obtained from the HMW and LMW glutenin electrophoretic patterns (Figure 3.5).

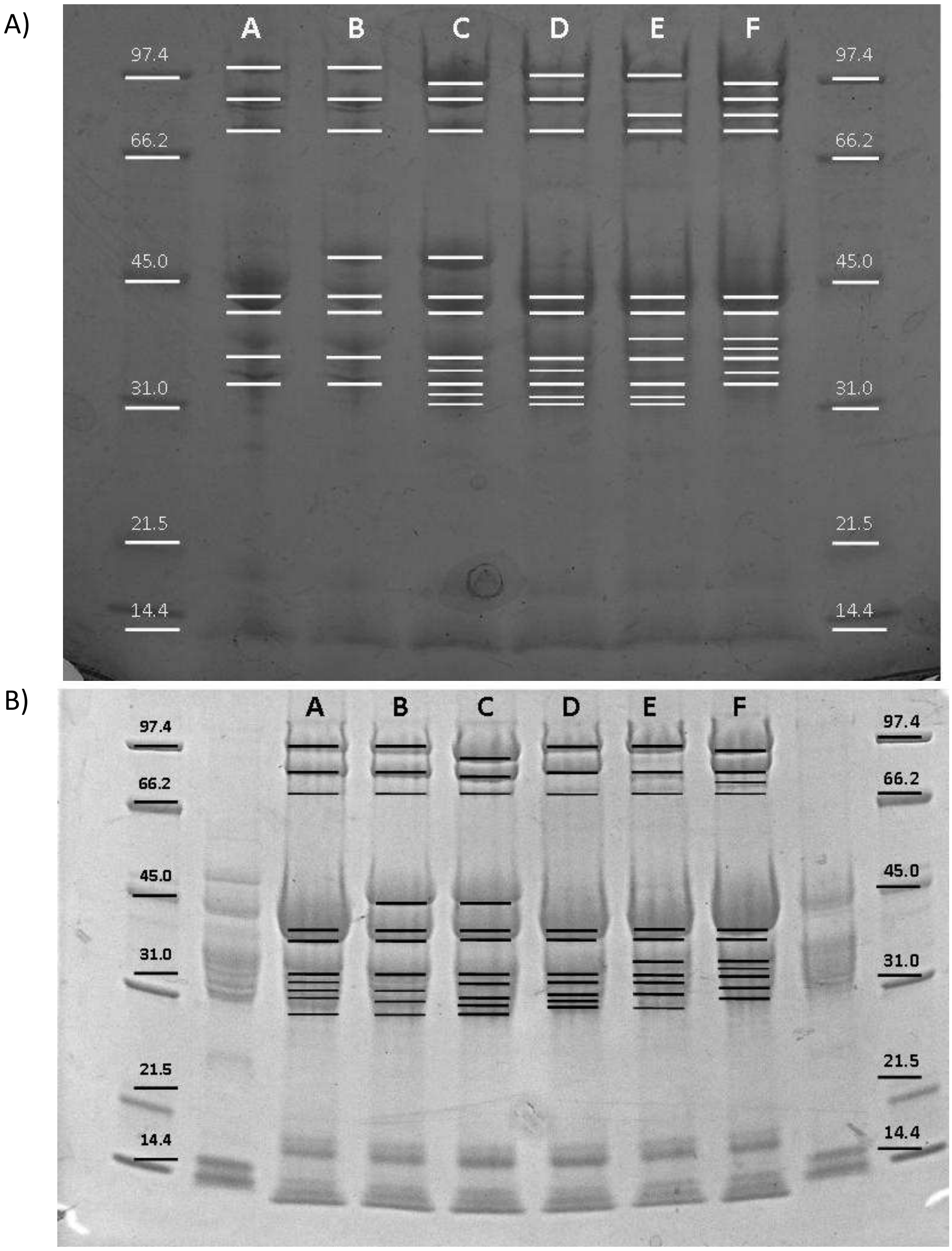


Figure 3.5. Glutenin electrophoretic patterns of the investigated wheat varieties grown in the first (A) and second (B) growing season. *Abbreviations:* A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

The modern cultivar Palesio showed a glutenin profile highly divergent from all the ancient wheat varieties. Considering the HMW–GS, Palesio was the sole genotype presenting four subunits with a molecular weight ranging from 76 to 90 kDa. Verna and Inallettabile shared the same HMW–GS profile, with three subunits (76, 86 and 97 kDa), while the remaining old varieties showed higher variability. As regards the LMW–GS, Verna and Inallettabile sampled in the first cropping year (2009/2010) presented the poorest electrophoretic pattern given by the presence of 33, 35, 42 and 44 kDa fractions which are common to all the investigated genotypes. In addition, Inallettabile and Andriolo showed the unique presence of 50 kDa glutenin fraction. High variability in LMW glutenin composition was observed among old genotypes also comparing the two growing seasons, whereas Palesio showed a divergent and stable electrophoretic pattern apparently not affected by environmental conditions. The differences between modern and old genotypes and the observed variability among the old varieties evidenced the effects of breeding programs on protein composition of the wheat genotypes.

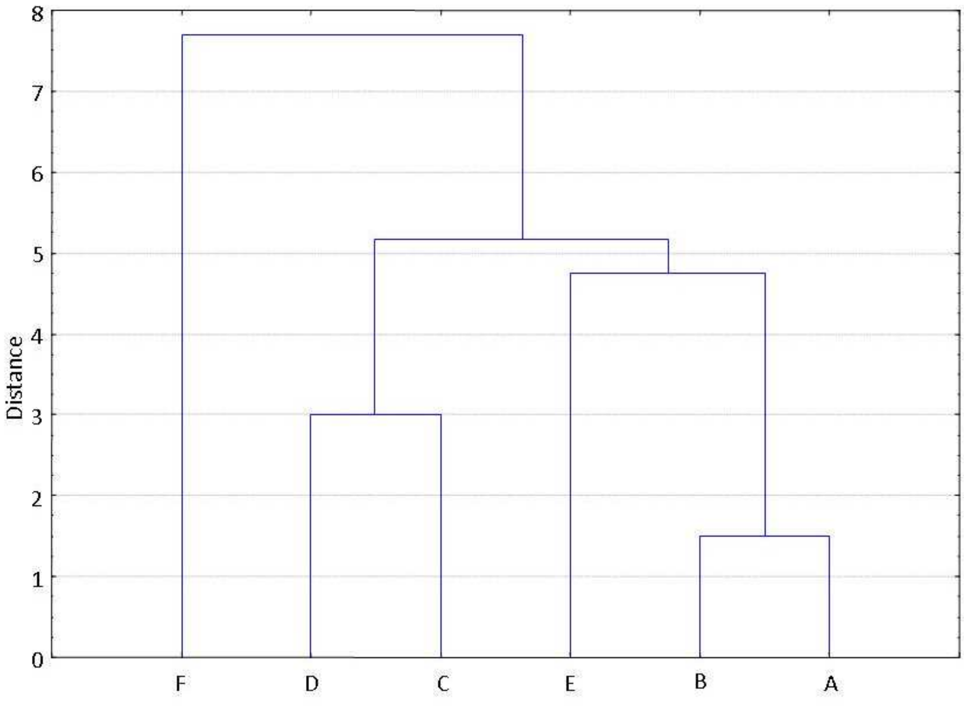


Figure 3.6. Dendrogram obtained using data of the glutenin electrophoretic patterns for the investigated wheat varieties over two growing seasons.
Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F = Palesio

3.3.3. Starch content

Starch represents the major carbohydrate source in the human diet, accounts for 65–75% of the wheat kernel, and it is mainly accumulated in the endosperm (Dupont and Altenbach, 2003). Starch consists of two glucose polymers, amylose and amylopectin, characterized by a linear and a branched molecular structure, respectively. On the basis of their diameter, the wheat starch granules can be distinguished in A– (>15 µm), B– (5–15 µm) and C–type (<5 µm) (Wilson et al., 2006). They present a complex and highly ordered structure in which amylopectin and amylose form alternative layers of crystalline and amorphous regions (Lehmann and Robin, 2007). The amylose/amylopectin ratio is one of the major factors affecting starch digestibility, due to the higher assimilability of the amylopectin with respect to amylose (Maningat et al., 2009). The ready digestible starch (rich in amylopectin) is the main glucose source in the diet and provide promptly available energy to the human body, even if it causes a fast and high peak of blood glucose levels as well as a fast decline. On the contrary, the slowly digestible starch (rich in amylose) decreases the digestibility of food, but offers the advantage of a slow increase of postprandial blood glucose levels and moderate the food Glycemic Index (GI) (Shewry et al., 2001). In wheat, the starch content is determined by both genetic and environmental factors (Kindred et al., 2008). In the present study, the genetic variability did not affect the starch accumulation and thus no significant differences were observed among wheat genotypes, showing a starch content ranging from 68.7 (Verna) to 71.4 g/100g (Andriolo, Inallettabile) (Table 3.1). In wheat kernel, the starch content is inversely correlated to the protein content but the environmental conditions are known to affect the starch biosynthesis with a smaller extent compared to protein accumulation (Kindred et al. 2008; Maningat et al., 2009). Taking into account the decrease of protein yield in 2010/2011, the expected increase of starch content during the second year did not occur and comparable amounts were observed in both growing seasons (Table 3.1). The negative correlation between protein and starch content was confirmed exclusively comparing mean values recorded at the three different locations. In fact, samples harvested at the Cenacchi farm, which presented the lowest protein yield, showed the highest starch amount (73.9 g/100g) (Table 3.1). Moreover, no significant G x L and G x Y interactions were observed for starch content highlighting the high stability of this trait.

3.3.4. Lipid content

Wheat kernel contains very low amount of lipid compounds, accounting for about 2–4% of its total weight (Simmonds, 1989). They are mainly accumulated in the embryo and in the outer layers of the caryopsis, constituting up to 15% of the germ fraction and 6% of the bran (Pomeranz et al., 1966). In wheat kernel lipids may occur as membrane components (phospholipids), spherosomes (triglycerides) and other lipid forms (high molecular waxes, hydrocarbons, sterols). Lipid membrane components constitute most of the lipids of the endosperm fraction, while germ and pericarp are rich in spherosomes (Simmonds, 1989). Althoug cereals are a modest source of lipids, whole–grain derived products are a good source of vitamin E, which include lipid compounds such as tocopherols and tocotrienols (tocols). The total concentration of tocols in wheat kernel has been reported to be 63–75 mg/kg, therefore approximately contributing to 25% of the total lipid amount (Yu, 2008; Moore and Hao, 2012). Previous investigations demonstrated that wheat tocopherols possess higher bioaccessibility with respect to other vitamin E food sources, underlining the important role of whole–grain consumption in meeting the recommended dietary allowance (RDA) (15 mg per day) (Leenhardt et al., 2008; Reboul et al., 2008). Tocols occur in wheat grain as eight different vitamers, namely α–, β–, δ– and γ–tocopherols and –tocotrienols, which posses a relevant antioxidant activity (Yu, 2008; Moore and Hao, 2012). In wheat, lipid content is known to be a varietal characteristic and is roughly affected by environmental factors (Simmonds, 1989). Indeed, in the present study no significant differences between 2009/2010 and 2010/2011 growing seasons were observed, while significant differences were observed among wheat genotypes (Table 3.1). The lipid amount ranged from 3.3 to 4.6 g/100g, with the old genotype Verna and the modern cultivar Palesio showing the highest and the lowest values, respectively (Table 3.1). Data obtained in the present research for lipid content are in general agreement with previous investigations reporting a high variability among wheat genotypes, with a crude fat amount ranging between 2.7 and 4.2 g/100g (Akhtar et al., 2008; Di Silvestro et al., 2012). The not significant interactions genotype x location and genotype x year furthermore confirmed the predominance of genetic factors in determining grain lipid content.

3.3.5. Ash and mineral element content

Ash is composed of inorganic compounds (mineral elements) predominantly located in the outer layers of the wheat kernel. The aleurone layer is particularly rich in minerals, which are mainly stored in phytin granules (Simmonds, 1989). The ash content is commonly used as a quality parameter to estimate the bran contamination of white flours: it is generally considered detrimental to baking performance, since bran and some of its components can adversely affect the bread–making quality. However, mineral elements are involved in several biological processes and therefore are an essential component of the human diet. Minerals are generally classified as macro– (i.e. Ca, K, Mg, Na, P,) and micro– (i.e. Fe, Cu, Co, Zn, Mn, Mo, Cr, Se) elements on the basis of their recommended daily intake (more than 100 mg/dl and less than 100 mg/dl, respectively). Cereal products are an important source of dietary minerals and significantly contribute to the achievement of recommended daily intakes (Fan et al., 2008). However it is important to underline that the bioavailability of most minerals in wheat flours is strongly reduced by the presence of phytic acid (inositol–phosphate) (Reddy and Sathe, 2001). Several nutritionally important minerals such as calcium, magnesium, copper, iron, zinc, cobalt and manganese may be chelating by the phytates forming an insoluble complex. Since in human gut the intestinal phytase degrading the chelating complexes can be a limiting factor, an excessive phytate diet may compromise the micronutrient absorption. Nevertheless, for wholegrain derived foods the mineral bio–accessibility is a limited problem, as the leavening process efficiently decreases the phytate levels (Reddy and Sathe, 2001). In wheat, the ash content is dependent on both genetic and environmental factors. In particular, the ash content is in relation with the mineral status of the soil and the uptake and translocation efficiency of the wheat varieties (Flan et al., 2008). In the present study, the ash content did not vary among locations whereas a significant decrease was recorded during the second year (Table 3.1). This reduction is probably associated with the observed decrease of protein content in the second cropping year (2010/2011) , as a positive correlation between protein and ash has been previously reported for wheat grain (Anglani, 1998). The significant interaction between growing location and year showed that the reduction in ash content occurred exclusively at the Cenacchi and Collina farms, whereas no year dependent variation was observed at the Ferri farm (Figure 3.7). Further investigations on the soil mineral composition are in progress with the aim to justify the differences observed among experimental farms.

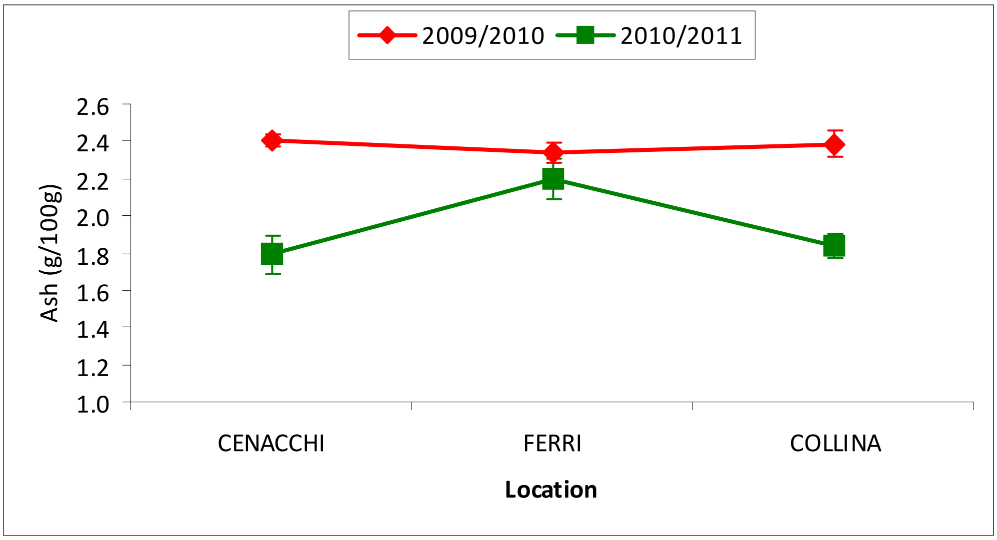


Figure 3.7. Significant interaction between location and year (LxY) observed for ash content (mean values ± standard error).

Statistically significant differences were observed among genotypes, with the old variety Gentil rosso presenting the highest ash content (2.4 g/100g), while the modern wheat cultivar Palesio exhibited the lowest amount (1.9 g/100g): the remaining genotypes showed intermediate values (Table 3.1). Fan *et al.* (2008) reported a decrease of mineral content in wheat grains over the last 160 years. In particular, the study associated the decreasing amounts of Zn, Cu, Fe and Mg with the introduction of semi–dwarf, high–yielding cultivars in the late 1950s. This finding was successively confirmed by other investigations carried out with different common and durum wheat varieties (Ficco et al., 2009; Zhao et al., 2009; Rodriguez et al., 2011). Additionally, the analysis of mineral elements on the grain samples harvested in the first year (2009/2010) gave additional insights on the genotype variability for mineral content (Table 3.2). On the whole, the mineral element analysis confirmed the variability among wheat genotypes, as already observed for the ash content. Considering the total amount of micronutrients, the old varieties Verna, Inallettabile, Frassineto and Gentil rosso showed significant higher contents than Andriolo and Palesio. In particular, Verna (11044.19 mg/kg) presented a mineral content 11% higher than that observed for Palesio (9879.52 mg/kg). The most abundant minerals were phosphorous (P), potassium (K), magnesium (Mg) and calcium (Ca), whereas the remaining 18 detected elements were present in lower amounts (<50 mg/kg) (Table 3.2). Potassium (K) and phosphorus (P) were the most abundant minerals, accounting for 39 and 44 % of the total mineral content, respectively. P amounts significantly varied among the investigated genotypes, ranging from 4224.00 to 4894.50 mg/kg, with Andriolo and Palesio varieties showing the lower amounts (Table 3.2). From a nutritional point of view, phosphorous is used by the human body mainly as a bone component (85% of the total body P). Additionally, P has an important structural function in the cell membrane, occurring as phospholipids. Cereals are an important source of dietary P and the consumption of 100 g of wholewheat flours has been estimated to contribute to 33.5% of the recommended dietary intake (RDI) (Food and Nutrition Board, 1997). High variability was observed among varieties for the potassium (K) content, that varied from 3587.67 (Andriolo) to 4315.83 mg/kg (Verna) (Table 3.2). Although K is one of the most abundant minerals in wheat, it has been estimated that 100 g of wholewheat flour have a very low contribution to the adequate intake of this mineral element (9%) (Food and Nutrition Board, 2004). The nutritional relevance of K is given by its presence as major intracellular cation in the human body, required for normal cellular function. The high intracellular concentration of K is maintained via the activity of the Na^+/K^+–ATPase pump. As a direct consequence, the balance between K and sodium (Na) should be controlled by not exceeding the maximum level of Na intake (above 6.9 g/day) which may cause K loss. In the investigated whole flours, the ratio Na/K was very low (0.003), which is of special interest in the prevention and treatment of hypertension and associated cardiovascular diseases (Rodriguez et al., 2011). Indeed, Na was present in very low amount in all the analyzed genotypes ranging from 10.33 and 15.58 mg/kg (Table 3.2). The magnesium (Mg) content varied within the interval 1242.67–1351.83 mg/kg and no significant differences were observed among genotypes (Table 3.2). More than 50% of Mg total body resides in human bone. Moreover, Mg is present in the extracellular fluids and it plays several physiological roles as cofactor for enzyme systems, as part of the Mg–ATP complex or directly as enzyme activator (Food and Nutrition Board, 1997). The consumption of 100 g of wholewheat flours may assure 27.7% of the recommended dietary intake (RDI) (Food and Nutrition Board, 1997). It is estimated that the net absorption of dietary magnesium is approximately half of the total Mg amount, especially in wholewheat products due to the high fibre and phytate content which may decrease intestinal magnesium bioavailability (Wisker et al., 1991). As regards calcium (Ca), the lowest Ca amount was detected for the modern cultivar Palesio (305.73 mg/kg) while Andriolo and Frassineto showed the significantly highest values (377.00 and 369.73 mg/kg, respectively) (Table 3.2).

Table 3.2. Mineral element composition of the investigated wheat varieties harvested in 2009/2010, over three growing locations, expressed in mg/kg of wholewheat flour.

Mineral elements	Variety					
	A	B	C	D	E	F
Al	3.13 (b)	2.39 (b)	7.53 (a)	3.42 (b)	5.22 (b)	4.37 (b)
B	0.75	0.37	0.64	0.45	0.46	0.59
Ba	1.33	1.39	2.06	1.82	1.72	1.37
Ca	328.45 (ab)	343.30 (ab)	377.00 (a)	369.73 (a)	341.60 (ab)	305.73 (b)
Cd	0.04	0.04	0.05	0.05	0.03	0.04
Co	0.11	0.25	0.23	0.16	0.34	0.07
Cr	0.19	0.12	0.15	0.11	0.18	0.07
Cu	4.89 (ab)	5.13 (a)	4.23 (ab)	4.85 (a)	4.22 (ab)	4.42 (b)
Fe	45.60 (a)	36.25 (ab)	39.89 (ab)	41.40 (ab)	38.99 (ab)	37.24 (b)
K	4315.83 (a)	3946.00 (bc)	3587.67 (d)	4283.33 (ab)	3938.00 (ab)	3824.33 (cd)
Li	0.06	n.d.	0.04	0.03	0.04	0.04
Mg	1351.83	1242.67	1252.33	1335.67	1244.67	1271.33
Mn	31.12	32.51	31.29	33.93	33.09	31.41
Mo	1.54	0.73	0.86	1.42	1.06	1.29
Na	13.83	10.33	15.58	10.45	13.28	14.83
Ni	0.15	0.18	0.16	0.14	0.08	0.13
P	4894.50 (a)	4618.33 (a)	4224.00 (b)	4570.33 (a)	4442.67 (a)	4338.67 (b)
Pb	0.07	0.25	0.09	0.24	0.25	n.d.
Se	1.93	2.58	1.71	1.06	1.80	2.47
Sr	1.35	1.25	1.77	1.76	1.47	1.47
V	0.48	0.60	0.59	0.98	0.61	0.35
Zn	47.03 (a)	43.74 (a)	42.02 (a)	40.26 (a)	40.15 (a)	39.28 (b)
Total	11044.19 (a)	10288.42 (b)	9589.90 (c)	10701.61 (ab)	10109.92 (ab)	9879.52 (c)

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; n.d., not detected.

Means followed by different letters are statistically different at $P<0.05$.

Although the contribution of 100g of wholewheat flour to the Ca intake is quite low (about 3.5%) (Food and Nutrition Board, 1997), the presence of this mineral element is of particular nutritional interest, as Ca is an essential constituent of teeth and bones (over 99% of the total body amount). Among the 17 detected microelements, zinc (Zn), iron (Fe) and manganese (Mn) were present in the highest amounts (above 30 mg/kg) (Table 3.2). The old genotype Verna showed the highest Zn and Fe content (47.03 and 45.60 mg/kg, respectively), while Palesio had the lowest levels (39.28 and 37.24 mg/kg, respectively). No significant difference was observed for the Mn content, with values ranging from 31.1 to 33.93 mg/kg (Table 3.2). Data obtained for Ca, Fe, K, P and Zn confirmed the abovementioned reduction of mineral content in dwarf high–yielding varieties, as Palesio presented in all cases the lowest amounts. The remaining 14 trace microelements were detected in amounts lower than 5 mg/kg and a narrow variability was observed among genotypes. Data obtained for mineral content generally agrees with those previously reported for several wheat varieties grown under conventional, low–input and organic managements (Fan et al., 2008; Hussain et al., 2010; Rodriguez et al., 2011).

3.4. AT A GLANCE

- ✓ The old wheat varieties show a protein and gluten content significantly higher than modern cultivar Palesio which in turn fails to express its potentialities under low–input management;
- ✓ The grain protein accumulation in the six investigated genotypes is mainly influenced by genetic traits;
- ✓ A relevant diversity among wheat varieties in the gliadin and glutenin pattern profiles is observed;
- ✓ The modern cultivar Palesio shows a whole gluten profile highly divergent from old wheat varieties;
- ✓ The investigated wheat varieties present a similar starch content;
- ✓ The mineral content of old wheat varieties is statistically higher than that detected in modern wheat Palesio;
- ✓ The not–dwarf wheat variety Verna presents peculiar nutritional properties (high protein, lipid and mineral content).

Section 4:

PHYTOCHEMICAL PROPERTIES OF WHEAT VARIETIES

4.1. SECTION OBJECTIVES

Among cereals, wheat is one of the most consumed staple foods all over the world. In addition to the essential nutrients, such as protein, starch, lipid and mineral elements, whole grain contains several bioactive compounds that confer to wheat–derived products unique health promoting properties. Recently, an increasing awareness of the relationship between diet and human health has been widely diffused not only among researchers, but also among consumers, regulatory agencies and food producers, focusing the attention on the functional food sector. Several epidemiological evidence correlated whole grain consumption with beneficial effects on human health by preventing chronic and cardiovascular diseases and lowering cancer incidence. The overall health benefits of wheat grains have been largely attributed to their unique phytochemical profile, which includes dietary fibre (DF) components (insoluble and soluble dietary fibre, resistant starch, arabinoxilans, β–glucans) and antioxidant compounds (polyphenols, flavonoids). As regards dietary fibre, whole wheat was found to be the most important DF source in the human diet, thus significantly contributing to the achievement of the recommended daily intake. The health benefits of dietary fibre have been largely demonstrated in literature and, based on their physiological behavior, the DF components may exert different health–promoting activities. The insoluble fraction has direct effects on the colon by preventing constipation and cancer, while the soluble components are known to modulate blood glucose and insulin levels and have a high prebiotic activity. Indeed, the soluble fibre polysaccharides resist to digestion and act as fermentative substrates for colon bacteria, promoting the growth of beneficial microorganisms and consequently enhancing human well–being and health. A number of chronic diseases including cancer and cardiovascular pathologies are also induced by oxidative damage of cellular components operated by free radicals (i.e. reactive oxygen species, ROS), commonly present in the human body. In order to arrest the oxidative chain incepted by free radicals, the consumption of antioxidant–rich foods constitutes the primary and efficient prevention of oxidative stress–associated diseases. Whole grains possess significant amounts of antioxidants and, due to their daily regular consumption in the diet, highly contribute to provide dietary antioxidant protection. The most representative antioxidant compounds in wheat are polyphenols and, among them, flavonoids. Their antiricadical capacity derives from the presence of hydroxyl groups linked to the aromatic rings that can efficiently react and stabilize free radicals. Since most of the above described phytochemicals are concentrated in the outer layers of the caryopsis (aleurone, bran and germ fractions), the choice of whole wheat products instead of refined flour–based foods may assure the greatest health–promoting intake. To date, the availability of literature studies concerning the complete nutraceutical description of wheat is quite scarce, especially as regards comparative evaluation of different wheat varieties. Additionally, most literature data pointing on wheat phytochemical determinations do not give details about field agronomic conditions and growing locations, neither about the involved wheat varieties. As a consequence the knowledge on the wheat grain nutraceutical properties has to be improved and deepened, in order to individuate the most promising wheat genotypes with high levels of health–promoting compounds. The present research aimed at screening the phytochemical composition of old and modern wheat varieties by considering also the genotype responses to different environmental factors (growing location, year) and the agricultural management (biodynamic agriculture). The final scope of the research was to provide a complete description of the raw material and eventually promoting them for the selection of wheat varieties characterized by valuable health–promoting properties and suitable for the low–input agricultural sector.

4.2. MATERIAL AND METHODS

Grain samples of modern (Palesio) and old (Andriolo, Frassineto, Gentil rosso, Inallettabile, Verna) varieties grown in pure genotype plots from the field trial described in *Section 2* were used for phytochemical determinations. Wheat grains harvested at the three involved locations (Cenacchi, Ferri and Collina farms), from both growing seasons (2009/2010, 2010/2011), were stone milled and the nutraceutical composition was analyzed as outlined below. Results were expressed on whole flour dry weight basis.

4.2.1. Total dietary fibre determination

The total dietary fibre determination, including the extraction of the soluble and insoluble fibres, was performed according to the enzymatic–gravimetric procedure previously described by Prosky *et al.* (1998), using a Megazyme assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). Briefly, 1 g of whole flour was subjected to sequential enzymatic digestion by heat–stable α–amylase, protease and amyloglucosidase to remove starch and protein. Sample solution was then filtered to obtain the insoluble dietary fibre (IDF) residue and the filtrate was treated with 95% heated ethanol to precipitate the soluble dietary fibre (SDF). IDF and SDF residues were dried and corrected for protein, ash and blank for final calculation of dietary fibre content.

4.2.2. Resistant starch determination

Resistant starch was analyzed according to McCleary and Monaghan (2002) using a Megazyme assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). After overnight α–amylase and amyloglucosidase digestion of 100 mg of whole flours, soluble starch was removed with 95% and 50% ethanol consecutive washes. The pellet was extracted with 2 mol/L KOH to dissolve resistant starch, hydrolysed with amyloglucosidase and spectrophotometrically quantified using a glucose oxidase/peroxidase reagent (GOPOD).

4.2.3. Arabinoxylan determination

Arabinoxylan (AX) content was determined using the enzymatic assay kit D–Xylose (Megazyme International Ireland Ltd, Wicklow, Ireland). Firstly, 100 mg of whole flour were deproteinized using 85 mM potassium hexacyanoferrate and 0.45 M zinc sulphate and successively subjected to acid and alkaline hydrolyses. The obtained extracts were incubated with xylose mutarotase to convert α–D–xylose in the isomeric form β–D–xylose. β–D–xylose was then reacted with NAD^+ in the presence of β–xylose dehydrogenase to form D–xylonic acid and NADH. The corresponding increase in NADH, which is stoichiometric with the amount of D–xylose, was measured at 340 nm. The AX concentration was calculated by considering the estimated D–xylose percentage content of the sample, which is equal to 62 % for wheat flour.

4.2.4. β–glucan determination

β–glucan content was determined according to a streamlined procedure based on the official methods AOAC 995.16, AACC 32–23 and ICC 166 (McCleary and Codd, 1991), using the Megazyme Mixed–Linkage β–Glucan assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The procedure included a targeted enzymatic digestion of the sample with lichenase and β–glucosidase. Subsequent colorimetric measurement of the hydrolyzed glucose was performed using a glucose oxidase/peroxidase reagent (GOPOD).

4.2.5. Prebiotic activity assay

The prebiotic activity of soluble dietary fibre (SDF) extracted for each investigated genotype was assessed using the *Bacillus pseudocatenulatum* B7003 and *Lactobacillus plantarum* L12 strains from the BUSCoB strain collection (Bologna University Scardovi Collection of Bifidobacteria) of the Department of Agroenvironmental Science and Technology, University of Bologna, Italy. The probiotic strains were maintained at –80°C in 120 g/L sterile reconstituted skim milk supplemented with D–glucose (10 g/L), yeast extract (0.5 g/L) and glycerol (400 ml/L) and activated as described by Marotti *et al.* (2012). the assay was performed by adding 10 ml/L of an overnight–incubated culture of each probiotic strain to separate tubes containing modified MRS medium for L12 and modified TPY broth for B7003, supplemented with 20 g/L glucose or 20 g/L prebiotic fibre (SDF) as the sole carbon source (Marotti et al., 2012). Strains were also grown on basal medium with no added carbon source to verify that negligible growth occurred from the use of indigenous carbon sources present in the medium. After 0, 6, 24, 30 and 48 h of incubation at 37°C in anaerobic atmosphere, samples were enumerated using the serial dilution method on either TPY or MRS agar plates and calculated as colony–forming units (CFU) per ml of culture. In addition, a mixture of 1:1:1 (v/v/v) enteric strains (*Escherichia coli* ATCC25645, *Klebsiella pneumoniae* GC 23a, *Enterobacter cloacae* GC 6a) was added at 10 ml/L to separate tubes containing M9 medium with 20 g/L glucose or 20 g/L SDF. The cultures were incubated at 37°C under anaerobic conditions and enumerated on plate count agar after 0, 6, 24, 30 and 48 h of incubation. All the assays were performed with three replicates. The ability of the gut bacteria to ferment SDF fractions from each investigated genotype was expressed using a Prebiotic Index based on the ability of a given substrate to support the growth of probiotic strains but not other intestinal bacteria (Huebner et al., 2007; Marotti et al., 2012). The prebiotic activity score was determined according to Huebner *et al.* (2007) as follows: [(probiotic log CFU/ml on prebiotic at 0h) / (probiotic log CFU/ml on glucose at 24 h – probiotic log CFU/ml on glucose at 0 h)] – [(enteric log CFU/ml on prebiotic at 24 h – enteric log CFU/ml on prebiotic at 0 h) / (enteric log CFU/ml on glucose at 24 h – enteric log CFU/ml on glucose at 0 h)].

4.2.6. Polyphenol and flavonoid extraction

Free and bound phenolics were extracted according to the method previously described (Dinelli et al., 2011; Di Silvestro et al., 2012). Briefly, 1 g of whole wheat flour was treated with 20 ml of cold 80% ethanol by continuous shaking for 15 min. After centrifugation at 5000 g for 20 min , the supernatant containing the free soluble compounds was recovered and extraction was repeated once. Supernatants were pooled, evaporated to dryness and reconstituted in 10 ml of 80% methanol. The residue from the free phenolic extraction was subjected to alkaline and acid hydrolysis to recover the bound phenolic compounds, as reported by Mattila *et al.* (2005) with some modifications. Briefly, after an overnight (16 h) shaking with 12 ml of distilled water and 5 ml of 10M NaOH, the liberated phenolics were extracted three times with 15 ml of ethyl acetate by manually shaking and centrifuging. After the above alkaline hydrolysis was completed, an acid hydrolysis was carried out by adding 2.5 ml of concentrated HCl to the residue and incubating the test tube at 85°C for 30 min. the sample was than allowed to cool and the ethyl acetate extraction performed as described for alkaline hydrolysis. The ethyl acetate layers from both hydrolyses were pooled, evaporated to dryness and reconstituted in 10 ml of methanol. Free and bound phenolic extracts were filtered through a 0.20 µm filter and stored at –20°C until use for the subsequent analyses of polyphenol and flavonoid content, antioxidant activity and mass spectrometry determination.

4.2.7. Determination of polyphenol and flavonoid content

Free and bound polyphenol content of each wheat sample was determined using the Folin–Ciocalteu procedure described by Singleton *et al.* (1999). Gallic acid was used as standard and polyphenol content expressed as milligrams of gallic acid equivalent (GAE) per 100 g of dry weight. Free and bound flavonoid content was determined according to the colorimetric method described by Adom *et al.* (2003). Briefly, appropriate dilutions of methanolic extracts were reacted with sodium nitrite, followed by reaction with aluminum chloride to form a flavonoid–aluminium complex. Solution absorbance at 510 nm was immediately measured and compared to that of catechin standards. Flavonoid content was expressed as milligrams of catechin equivalent (CE) per 100 g of dry weight.

4.2.8. HPLC–ESI–TOF–MS experimental conditions

Phenolic extracts obtained from the grain samples harvest in the first growing season (2009/2010) were used for the determination of phenolic profiles by high performance liquid chromatography coupled with mass spectrometry. HPLC analysis was performed using an Agilent 1200–RRLC system (Agilent Technologies, CA, USA) consisting of a vacuum degasser, autosampler, a binary pump and a UV–vis detector. Phenolic compounds were separated using a RP C18 analytical column (4.6 mm × 150 mm, 1.8 µm particle size) from Agilent ZOR–BAX Eclipse plus. The mobile phases and gradient program used were as previously described (Dinelli et al., 2011). The gradient elution was performed with mobile phases consisting of water with acetic acid (0.5% acetic acid v/v) (A) and acetonitrile (B) as follows: from 5% to 10% B in 5 min; from 10% to 35% B in 35 min; from 35% to 70% B in 20 min; from 70% to 95% B in 2 min; from 95% to 5% B in 2 min. An 8 min re–equilibration time was used after each analyses. The flow rate was set at 0.50 mL/min throughout the gradient. The effluent from the HPLC column was splitted using a T–type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Thus in this study the flow which arrived into the MS detector was 0.125 mL/min. The column temperature was maintained at 40°C and the injection volume was 10 µL. The HPLC system was coupled to a microTOF (Bruker Daltonics, Bremen, Germany), an orthogonal–accelerated TOF mass spectrometer (oaTOFMS), equipped with an ESI interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50 to 1000. The optimum values of the ESI–MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190°C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 2 bar. The accurate mass data of the molecular ions were processed through the newest software Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formula by using the Smart Formula Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring–plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm. We also have to say that even with very high mass accuracy (<1 ppm) many chemically possible formulae are obtained depending on the mass regions considered. So, high mass accuracy (<1 ppm) alone is not enough to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes > 95% of false candidates. This orthogonal filter can condense several thousand candidates down to only a small number of molecular formulae. During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). Using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the microTOF, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

4.2.9. Statistical analysis

A General Linear Model (GLM) was used to assess the variance significance for the main (genotype) and the random (location, year) factors, as well as their interactions, for all measured variables (except mineral elements). One–way ANOVA (Tukey’s test) was performed to test the variance significance of genotypes harvested in first growing season for mineral content. Significance between means was determined by least significant difference values for $P < 0.05$. The analysis of variance was carried out using Systat v. 9.0 (SPSS Inc. 1998). Cluster analysis (Unweighted Pair Group Average Method with arithmetic averaging – UPGMA) was carried out on phenolic profiles obtained by HPLC–ESI–TOF–MS and on Manhattan distance matrix to seek for hierarchical association among the wheat varieties, using STATISTICA Software v. 7.1 (StatSoft, Tulsa, Oklahoma, USA). A linear discriminant analysis (LDA) was performed using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA). The supervised technique was applied to the standardized data matrix of the nutrient (*Section 3*) and phytochemical (*Section 4*) content of the investigated varieties from both growing seasons. LDA allowed to score the cases as a function of the first two roots (canonical discriminant functions) to visualize similarities among the wheat genotypes.

4.3. RESULTS AND DISCUSSION

4.3.1. Total dietary fibre content

The health benefits of wholegrain consumption have been widely demonstrated in literature and include reduced risk of type 2 diabetes, gastrointestinal cancers and cardiovascular diseases (Slavin, 2004; Anderson et al., 2009). The health–promoting properties of wholegrain are mainly related to the rich fibre content of bran, highly contributing to the achievement of the recommended fibre daily intake (30–35 g/day) (Harris and Smith, 2006). In recent years, consumers have increased their awareness on the relationship between food and health, raising the consumption of wholegrain products as major source of fibre in the human diet. The physiological effects of dietary fibre components depend on their physico–chemical properties, which are influenced by degree of polymerization, arabinose/xylose ratio, distribution of side chains, degree of cross–linking as well as the extent of degradation in the gut (Napolitano et al., 2009). Dietary fibres are the most important phytochemicals in wheat grain and can be divided into two categories according to water solubility. The insoluble dietary fibre (IDF) is the major fraction in wheat kernel and consists of structural polysaccharides such as lignin, cellulose and hemicellulose (water–unextractable arabinoxylan) (Sidhu et al., 2007). IDF has direct beneficial effects by preventing constipation and cancer; indeed IDF remains largely unfermented and have a bulking effect, which results in increased faecal output (Alabaster et al., 1996; Charalampopoulos et al., 2002; Scheppach et al., 2004). The soluble dietary fibre (SDF) includes non–starch polysaccharides, β–glucans and pentosans (i.e. water–extractable arabinoxylan). SDF is known to modulate blood glucose and insulin levels and additionally exerts high prebiotic activity (Macfarlane et al., 2006; Roberfroid et al., 2010; Marotti et al., 2012). Moreover, dietary fibre may act as satiety ingredient, prolonging the secretion of appetite regulating hormones from the small intestine, and furthermore the viscous aspect of SDF delays gastric emptying and blunt nutrient uptake reducing postprandial glycemia (Chaudhri et al., 2008; Kristensen et al., 2010). Few studies investigated the extent to which wheat varieties may vary in their content and composition of dietary fibre and little is known on the effects of environmental factors on fibre accumulation (Gebruers et al., 2008; Shewry et al., 2010). In the present study, the IDF and SDF contents did not vary among the three involved farms and on average accounted for 16.3 g/100g and 4.3 g/100g, respectively (Table 4.1).

Table 4.1. Insoluble diteray fibre (IDF), soluble dietary fibre (SDF), resistant starch (RS), arabinoxylan (AX) and β–glucan (BG) contents of the investigated wheat genotypes.

		IDF g/100g	SDF g/100g	RS g/100g	AX g/100g	BG g/100g
Location	Cenacchi	16.6 ns	4.0 ns	0.8 ns	3.9 ns	0.58(a)
	Ferri	16.3 ns	4.5 ns	0.9 ns	4.0 ns	0.52(b)
	Collina	16.1 ns	4.4 ns	1.0 ns	3.9 ns	0.47(c)
Year	2009/2010	17.6(a)	3.8(b)	0.7(b)	3.8(b)	0.51(b)
	2010/2011	15.1(b)	4.8(a)	1.1(a)	4.0(a)	0.53(a)
Variety	A	17.2(a)	4.3 ns	0.8 ns	4.2(a)	0.44(d)
	B	16.5(a)	3.8 ns	1.1 ns	3.7(b)	0.51(c)
	C	16.8(a)	4.2 ns	0.9 ns	3.8(b)	0.56(a)
	D	15.2(b)	4.3 ns	0.9 ns	3.8(b)	0.52(bc)
	E	17.2(a)	5.2 ns	1.0 ns	4.3(a)	0.53(b)
	F	15.1(b)	4.1 ns	0.9 ns	3.7(b)	0.58(a)
Interaction	LxY	ns	ns	**	ns	ns
	LxG	ns	ns	ns	ns	ns
	GxY	ns	ns	ns	ns	ns

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; L, location; Y, year; G, genotype; ns, not significant.

Means followed by different letters are statistically different at *P*<0.05.
** = significant interaction at *P*<0.01.

These results remark that the variability of growing conditions given by the different locations did not affect the accumulation of fibre polysaccharides in the wheat grains. However, an effect of the changing weather conditions on the fibre amounts was observed between the two cropping years (Table 4.1). In particular, a decrease of IDF from 17.6 to 15.1 g/100g and an opposite increase of SDF from 3.8 to 4.8 g/100g was recorded during the second year (2010/2011). The warmer and drier conditions occurred during 2011 from heading to harvest may have somehow affected the proportional amounts of each kernel tissue (endorsperm, aleurone, pericarp) and the grain size (as showed in *Section 2*, Table 2.3), which are strongly related to the dietary fibre accumulation. The fibre content is commonly considered as an inherent characteristic of the wheat variety and indeed high variability was observed among the wheat genotypes for the insoluble fraction (Table 4.1). The lowest IDF contents were obtained for Palesio (15.1 g/100g) and Frassineto (15.2 g/100g), while the remaining old varieties showed significantly higher values within the interval 16.5–17.2 g/100g. On the other hand, no significant differences were observed among wheat varieties for the soluble fibre content that ranged from 4.1 to 5.2 g/100g (Table 4.1). The obtained total dietary fibre amounts resulted quite higher compared to literature data (Charalampopoulos et al., 2002; Gebruers et al., 2008). Gebruers and co–workers (2008) investigated the fibre content of 131 winter wheat varieties, showing high variability among genotypes and values comprised within the interval 11.5–18.3 g/100g of dry flours. However, in their study an indirect approach was used for fibre determination by calculating the difference between total wholemeal sample weight and the sum of analytes such as protein, ash, lipids, available starch and free sugars. The present results aligned with those previously determined by a more accurate analytical method for old and modern wheat varieties grown under low–input management (Di Silvestro et al., 2012). No significant genotype x location and genotype x year interactions were observed for both insoluble and soluble fibre fractions, highlighting the low influence of environmental factors and the high stability and heritability of these traits.

4.3.2. Resistant starch content

Resistant starch (RS) is the fraction of starch that escape enzymatic digestion in the small intestine and thus reaches the human colon where it is digested by the resident microflora (Lehmann and Robin, 2007). RS can be ascribed to the dietary fibre components as its general behavior is physiologically similar to that of soluble fermentable fibre (Jenkins and Kendall, 2000). Therefore, the health benefits of RS include positive impacts on the colon by providing nourishment to the colonic mucosa through colonic fermentation and high production of short fatty acids (butyrates). Moreover, RS induces lowered pH and ammonia concentration in the colon and increases fecal bulk, diluting possible carcinogens (i.e. bile acids) (Muir et al., 2004). All these benefits have been related to a reduced risk for colon cancer and inflammatory bowel disease (Jenkins and Kendall, 2000). Food sources of RS comprise tubers such as potatoes, fruits as bananas, legumes and cereal grains (Caballero, 2009). Wheat kernel may have different resistant starch levels, depending on starch granule size and composition. Therefore, RS content is known to be a varietal characteristic, but above all, it relies on environmental and processing factors affecting the starch molecule structural organization. In the present study, no significant differences were observed among the three biodynamic farms in terms of RS content (Table 4.1). Conversely, a 0.4 percent raise in RS amount was obtained for the samples grown during the second year (2010/2011), as compared to grains of the first growing season (2009/2010) (Table 4.1). The difference between the two growing season for RS content may be a result of the strongly diverse weather conditions recorded from heading to harvest growth stages. Although no information is reported in literature as regards the environmental effects on RS accumulation, a previous study reported a significant influence of growing conditions, such as water availability, on the size and distribution of starch granules in wheat grains as well as on starch and amylose content (Zhong–Min et al., 2008). Therefore, it is likely to hypothesize that, even if the total starch amount did not differ between years (Table 3.1), environmental changes probably induced modification of the starch composition as well as starch granule size, causing a major fraction to be structurally resistant to digestion. The significant interaction location–by–year shown in Figure 4.1 highlighted that the differences on RS content between 2009/2010 and 2010/2011 are the result of the variation occurred at the Ferri and Collina farms. Grain samples grown at the Cenacchi farm presented similar RS levels in both years (0.8 and 0.9 g/100g DW), whereas an increase of 0.3 and 0.7 g/100g DW was recorded for Ferri and Collina locations, respectively (Table 4.1). Considering the wheat genotypes, no significant differences were observed and RS contents ranged from 0.8 to 1.1 g/100g (Table 4.1). To date, only few studies investigated the resistant starch content of cereal raw material as whole flours and the available literature data on resistant starch content generally aligned with those obtained in the present study (Ragaee et al., 2006; Di Silvestro et al., 2012).

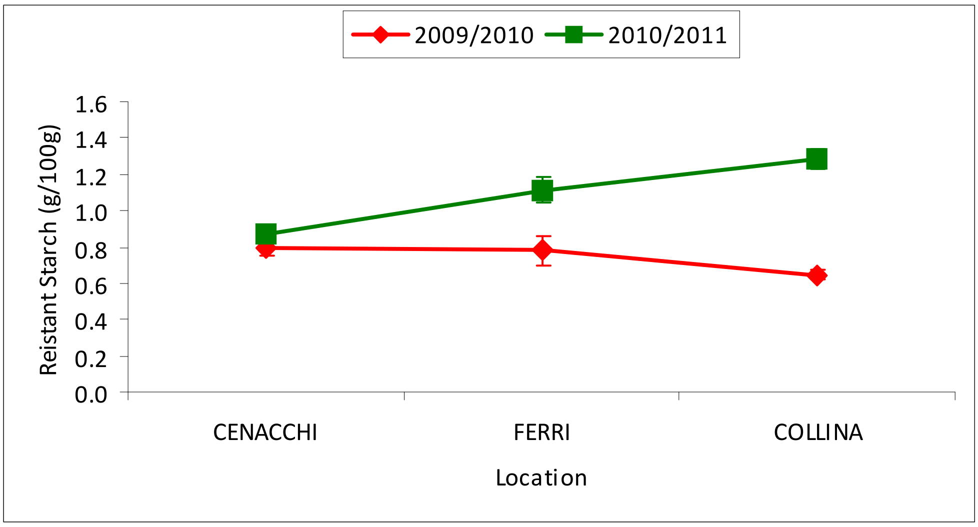


Figure 4.1. Significant interaction observed between location and year for resistant starch (RS) content . RS values are expressed in g per 100 g of dry flours.

4.3.3. Arabinoxylan content

Arabinoxylans (AX) are the most important dietary fibre component in wheat kernel. They account for about 50% of the total fibre content and occur mostly in the bran and aleurone fractions (Marotti et al., 2012). AX are hemicelluloses presenting a xylose backbone with arabinose side chains, in which the arabinose residues can be substituted with ferulic acid moieties. They are mainly present in the outer layers and in the germ of the caryopsis and present different chemical structures conferring water–extractability or water–unextractability characteristics (Courtin and Delcour, 2002). Apart from their nutritional relevance as dietary fibre component, wheat AX were widely investigated in the last 50 years for their role in the breadmaking process, mainly deriving from the ability of soluble AX to form highly viscous solutions and of insoluble AX to absorb water and swell (Courtin and Delcour, 2002). In biotechnological applications, a variety of bacteria endoxylanases (AX–degrading enzymes) are frequently used to modify the functionality of AX. For instance, the GHF11 endoxylanase produced by *Bacillus subtilis* has been shown to hydrolyze water–unextractable AX and improve bread loaf volume (Courtin et al., 2001). Additionally, selective AX–degrading endoxylanases are possessed by further bacteria belonging to the intestinal microflora, thus conferring to arabinoxylans high prebiotic properties (Vardakou et al., 2008).

The AX amount in wheat grain is highly variable and depends on genetic varietal differences in addition to the environmental factors. Whole grains investigated in this study presented an AX content between 3.7 g/100g DW (Palesio, Inallettabile) and 4.3 g/100g DW (Gentil rosso). Along with Gentil rosso, the old variety Verna showed high AX content (4.2 g/100g DW) while the remainders had significant lower values (Table 4.1). The obtained amounts aligned with the 3.1–4.7 g/100g DW range reported by Li *et al.* (2009) for 50 common wheat varieties. In a study involving more than 130 wheat varieties, significantly lower AX levels were observed by Gebruers *et al.* (2008); the highly divergent results may be explained by the use of refined flours rather than wholegrain, as AX are mainly present in the bran fraction. The abovementioned studies also reported on the environmental effects on total arabinoxylan content of several common wheat varieties. High variability was observed among the wheat samples grown at different locations, showing that the environmental contribution to the variation in AX content often was largely greater than the genotype influence (Gebruers et al., 2008; Li et al., 2009). Gebruers and coworkers additionally indicated that mean temperature and precipitation from heading to harvest may respectively have negative and positive influence on the water–extractable arabinoxylan amount. The observed relationship was explained as an higher xylanase activity in wheat grain grown under cool wet conditions (Dornez et al., 2008). On the basis of these considerations, the low value observed for the samples grown in the first year (3.8 g/100g DW) may be related to the lower temperatures and higher rainfall amounts recorded during the first growing season (Table 4.1). The environmental effects on AX content were only related to weather conditions, since no influence of the growing locations was observed among the biodynamic farms (Table 4.1). Moreover, it should be underlined that the variation between the two growing seasons accounted only for 0.2 percent decrease, thus highlighting the high inheritability of this fibre component, as additionally confirmed by the absence of significant interaction between genotype and random factors (year, locations).

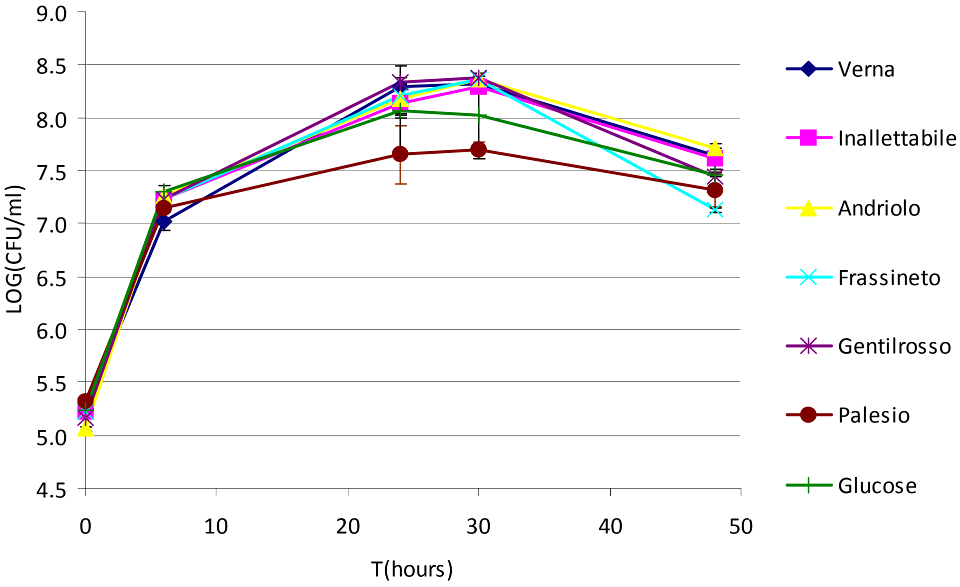
4.3.4. β–glucan content

β–glucans (BG) are unbranched polysaccharides made up of (1 → 4) and (1 → 3) linked β–D–glucopyranosyl units (Sidhu et al., 2007). Only a minor part of wheat BG are water–soluble; BG are mainly constituted of insoluble forms as they are entrapped in the matrix of a cross–linked ferulic acid–arabinoxylan complex (Cui et al., 2000). Additionally, wheat BG have been shown to have a cellotriosyl/cellotetraosyl composition that confers poor solubility and fast gelation (Li et al., 2006). Most β–glucans are found in the aleurone layer, accounting for about 23% of cell wall polysaccharide fresh weight, and bran fractions whereas narrow amounts are accumulated in the endosperm (0.3% of dry matter) (Henry et al. 1987; Barron et al., 2007). Among cereals, the highest BG levels are found in barley and oat, covering the ranges of 3–11% and 3–7% on a dry basis respectively, while wheat is recognized as a limited source of β–glucans which usually account for 1% of dry weight (Genc et al., 2001; Gebruers et al., 2008). However, BG constitute an important dietary fibre component in wheat due to their widely demonstrated health–promoting properties. They have been reported to form a viscous solution in the small intestine that reduces the absorption of cholesterol, fatty acids, bile acids and glucose, resulting in lower risk of diabetes and cardiovascular diseases (Arnoldi, 2004). Moreover, BG act as a prebiotic in the colon, stimulating the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* strains (Kontula et al., 1998). Along with the other dietary fibre components, BG grain content varies as a function of genotype and environmental conditions. Grain samples harvested at the three biodynamic farms presented significantly different BG levels, comprised between 0.47 g/100g DW (Collina) and 0.58 g/100g DW (Cenacchi). Furthermore, considering the mean values obtained for the two growing seasons, a small significant increase of BG content was recorded during the second year and this may be related to drier conditions occurred in 2011 (Table 4.1). Several studies investigated the effects of genotype and environment on the BG levels in cereals such as barley and oats (Henry, 1986; Peterson, 1991; Asp et al., 1992;); however, no clear information are available for environmental influence on wheat grains BG. Gebruers *et al.* (2008) reported on the variability of β–glucan content among several wheat varieties grown at different locations, but no significant correlation with temperature and rainfall were highlighted. Further investigations would be necessary to understand how varietal and environmental factors may influence the BG metabolism and accumulation in wheat.

4.3.5. Prebiotic activity of wheat soluble dietary fibre

A prebiotic is defined as 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well–being and health' (Gibson et al., 2004). The commensal microbiota of the human gut is populated by an array of bacterial species belonging to the genera *Bifidobacterium* and *Lactobacillus*. Almost any carbohydrate that reaches the large bowel provide a selective substrate for beneficial bacteria and stimulate their growth and metabolomic activities (Macfarlane et al., 2006). Therefore, the dietary fibre polysaccharides, that resist to digestion in the small intestine, may exhibit high prebiotic properties being fermented by the gut microbiota. In general, both soluble and insoluble dietary fibre can be degraded by intestinal bacteria, but soluble fibre is more easily and completely fermented than the insoluble fraction (Bach Knudsen and Hansen, 1991). The dietary fibre composition of the investigated wheat varieties were described in the previous paragraphs as regards the quantification of each fibre component. Additionally, the wheat fibre prebiotic activity was tested by investigating the ability of two potentially probiotic strains, *Lactobacillus plantarum* L12 and *Bifidobacterium pseudocatenulatum* B7003, to ferment the soluble fibre fractions extracted from each genotype.

A)



B)

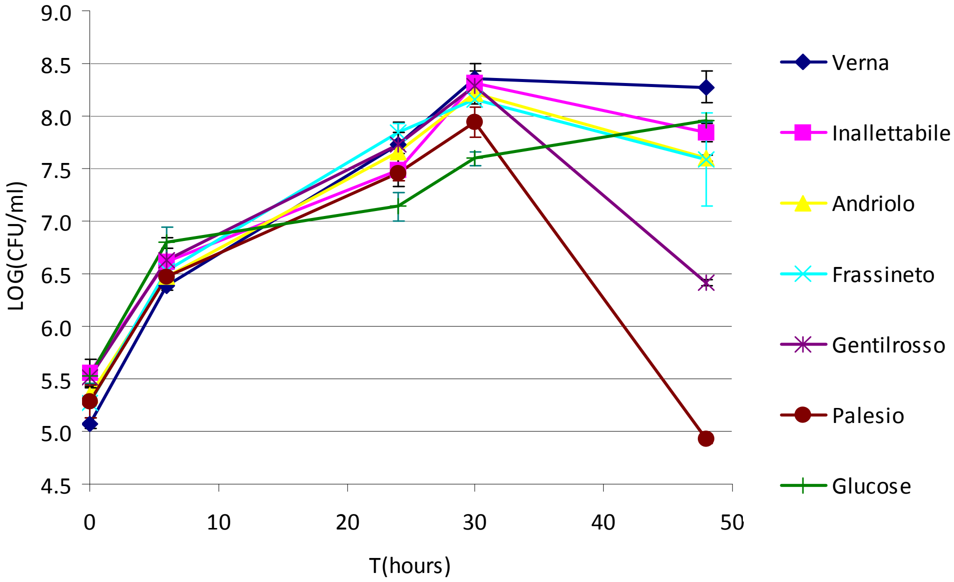


Figure 4.2. Growth curves for *Lactobacillus plantarum* L12 (A) and *Bifidobacterium pseudocatenulatum* B7003 (B) grown on basal medium (modified MRS and TPY respectively) supplemented with soluble dietary fibres from the investigated wheat varieties.

Figure 4.3 shows the growth curves of *L. plantarum* L12 and *B. pseudocatenulatum* B7003 after 30 h of incubation on media supplemented with either 20 g/L glucose or 20 g/L SDF, expressed as $\log_{10}$ colony–forming unit (CFU) per ml culture. The increases in cell density for both probiotic strains and for the enteric mixture are also reported in Table 4.2. Of the six carbohydrate sources and one control substrate (glucose) tested for L12, the maximal growth occurred in all media supplemented with SDFs from the old wheat varieties. It is interesting to note that the lowest cell yield for L12 was achieved during growth on SDFs extracted from the modern cultivar Palesio (2.37 $\log_{10}$ CFU/ml after 30 h incubation) (Table 4.2). Among the old genotypes, the highest ability to promote the *Lactobacillus* growth was recorded for Andriolo (3.30 $\log_{10}$ CFU/ml) and Gentil rosso (3.22 $\log_{10}$ CFU/ml), while the remainder varieties showed intermediate values. The growth curve of *B. pseudocatenulatum* B7003 evidenced that the SDFs extracted from all the investigated varieties supported a good growth of this beneficial bacterium strain, with cell density increases significantly higher compared to glucose–supplemented medium. After 48 h of incubation, a drastic reduction of cell density was observed for Gentil rosso and Palesio, showing a possible exhaustion of the fermentable substrate. Differently, the SDFs from Verna, Inallettabile, Andriolo and Frassineto, as well as the glucose, continued supporting the vitality of B7003 also after 48 h of incubation. An essential characteristic of a prebiotic substrate is the selective support of probiotic bacteria growth and, at the same time, the restricted fermentability by other commensal microorganisms (i.e. coliforms).

Table 4.2. Increase in cell density between 0 and 30 h ($\log_{10}$ CFU/ml) for bacterial cultures grown as wheat soluble dietary fibre as carbohydrate sources.

	Bifidobacterium pseudocatenulatum B7003	*Lactobacillus plantarum* L12	Enteric mixture
Glucose	2.07 (c)	2.79 (b)	4.25 (a)
A	3.28 (a)	3.07 (ab)	2.43 (c)
B	2.76 (b)	3.07 (ab)	3.37 (b)
C	2.86 (b)	3.30 (a)	3.30 (b)
D	2.88 (b)	3.15 (ab)	2.52 (c)
E	2.77 (b)	3.22 (a)	2.40 (c)
F	2.66 (b)	2.37 (c)	3.40 (b)

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

Means followed by different letters are statistically different at $P<0.05$.

In the present study a mixture of three enteric strains (*E. coli*, *K. pneumoniae*, *E. cloacae*) was chosen as representative of the gut commensal flora. As shown in Table 4.2, the SDFs from all the investigated wheat varieties supported a limited growth of enteric bacteria and presented cell density increases significantly lower than those observed for glucose, suggesting the prebiotic potential of these fibre fractions. The Prebiotic Index was obtained for each variety by considering the growth stimulation of *Bifidobacterium* and *Lactobacillus* strains and corrected for the enteric bacterium growth. On the whole, B7003 achieved higher prebiotic activity scores (mean value 0.71) than L12 (mean value 0.41) (Figure 4.3). This finding confirms the differences in the metabolic capacity of B7003 and L12 previously reported by Marotti *et al.* (2012), which compared the ability of the two strains to ferment SDFs extracted form durum wheat genotypes. The highest prebiotic activity scores were obtained for the old genotype Verna for both microorganisms, along with Frassineto and Gentil rosso. It is interesting to underline that, as regards the *Lactobacillus* strain, the fibre extracted from the modern cultivar Palesio presented Prebiotic Index values close to zero, showing a narrow ability to selectively stimulate the growth of L12. The variability observed among the investigated SDF sources for their *in vitro* prebiotic activity suggested that wheat varieties may differ for their qualitative dietary fibre composition, differently affecting the prebiotic properties of these substrates.

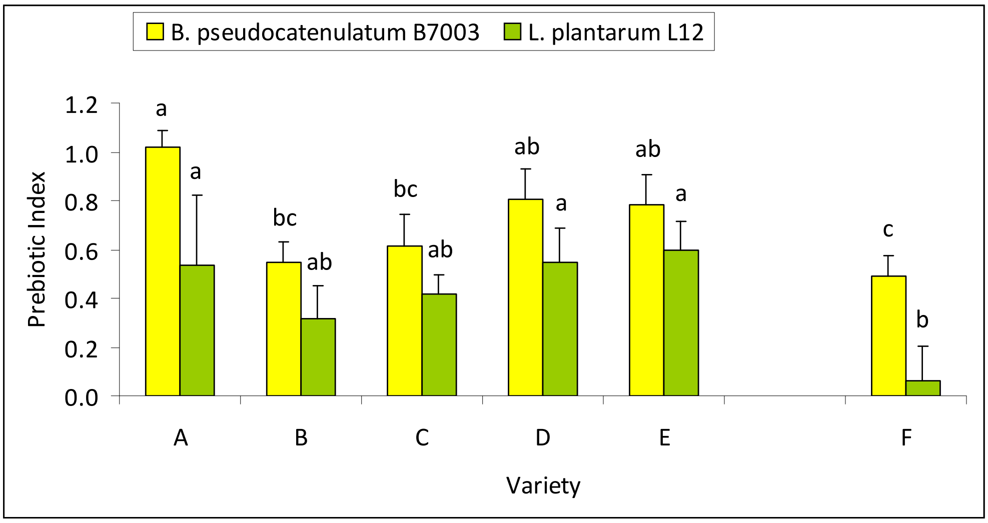


Figure 4.3. Prebiotic Index scores of *Bifidobacterium pseudocatenulatum* B7003 and *Lactobacillus plantarum* L12 grown on soluble fibre fractions of investigated wheat varieties. Values are means of three different replications (± standard deviation). Means followed by different letters are statistically different at $P<0.05$.

4.3.6. Polyphenol and flavonoid content

Polyphenols and flavonoids are the most representative class of antioxidants in wheat and exert high antioxidant activity acting as radical scavengers. Moreover, their role in the prevention of cancer and several chronic diseases were widely demonstrated in literature (Carter et al., 2006; Fardet, 2010). In wheat kernel, phenolic compounds are mainly concentrated in the outer layer of the caryopsis and they exist in the soluble (free) and insoluble (bound) forms. Both phenolic fractions have been shown to possess valuable health–promoting properties, acting as radical scavengers and preventing several chronic disease. Additionally, the bound phenolics, which are crosslinked with cell wall components (i.e. arabinoxylans), may resist upper digestive process and reach the colon where they are liberated by the intestinal microflora. The digested phenolics directly exert their health benefits reducing the incidence of colon cancer; indeed, colonic endothelial cells may absorb the liberated phenolics and gain powerful antioxidant protection (Kroon et al., 1997). During kernel development, the accumulation of phenolic compounds may vary as a function of both genetic and environmental characteristics. Previous investigations reported on the high variability of phenolic content among several common wheat varieties and different cropping location, especially in relation to environmental conditions and agricultural management. Organically produced wheat is expected to accumulate higher concentrations of phenolic compounds with respect to conventionally grown varieties. The explanation relies on a change of plant metabolism toward carbon–containing compounds (including non–nitrogen secondary metabolites such as polyphenols and flavonoids). According to the carbon/nutrient balance hypothesis, the low nitrogen availability induces an activation of the phenylpropanoid pathway enhancing the phenolic biosynthesis instead of proteins and other nitrogen–containing compounds (Kovacik et al., 2007). However, contrasting results are reported in literature comparative evaluations of wheat varieties grown under conventional and organic management. For instance, Zuchowski *et al.* (2011) indicated higher phenolic acid concentrations for organically produced wheat varieties compared to the conventional ones. This theory was not supported by previous investigation showing similar phenolic content in wheat varieties grown under both agricultural managements (organic/conventional) over three years (Stracke et al., 2009). Nevertheless, the relationships between different management methods were strongly affected by

Table 4.3. Polyphenol and flavonoid content (free and bound fractions) of the investigated wheat varieties.

		FP mg/100g	BP mg/100g	FF mg/100g	BF mg/100g
Location	Cenacchi	110.5(b)	181.3(b)	33.4(c)	21.7(a)
	Ferri	104.6(b)	153.4(c)	36.0(b)	16.8(c)
	Collina	136.5(a)	198.3(a)	41.2(a)	18.3(b)
Year	2009/2010	108.0(b)	176.0 ns	30.0(b)	18.8 ns
	2010/2011	126.5(a)	179.3 ns	43.8(a)	19.0 ns
Variety	A	125.6(a)	186.7(b)	37.1 ns	20.5(ab)
	B	129.5(a)	176.6(c)	35.3 ns	18.5(b)
	C	113.8(b)	207.2(a)	38.5 ns	25.2(a)
	D	114.8(b)	151.5(d)	38.5 ns	14.8(c)
	E	121.3(ab)	185.4(bc)	36.2 ns	19.3(ab)
	F	98.4(c)	158.3(d)	35.7 ns	15.1(b)
Interaction	LxY	**	**	ns	ns
	LxG	ns	ns	ns	ns
	GxY	ns	ns	ns	ns

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; L, location; Y, year; G, genotype; ns, not significant; FP, free polyphenols; BP, bound polyphenols; FF, free flavonoids; BF, bound flavonoide.

Means followed by different letters are statistically different at $P<0.05$.
** = significant interaction at $P<0.01$.

varietal differences, as well as cropping years, pointing out that the climate variations have a greater impact on the phenolic accumulation in wheat grains than the production method. In the present study, the polyphenol and flavonoid contents varied as a function of the growing locations (Table 4.3). Samples harvested at the Collina farm showed the highest free and bound polyphenol content (136.5 and 198.3 g/100g, respectively). The highest free flavonoid amount was also recorded at this location (41.2 g/100g), whereas the highest bound flavonoid content was obtained at the Cenacchi farm (21.7 g/100g). An increase of free polyphenol and flavonoid amounts was observed during the second year, while no significant differences were obtained for the bound phenolic compounds (Table 4.3). The observed variability may be related to the highly divergent weather conditions occurred during the two cropping years. Growing conditions as solar radiation and number of hours exceeding 32°C during grain–filling period are associated with free radical formation and result in increasing oxidative stress and consequently biosynthesis of antioxidants for self–

defense against environmental stress (Yu et al., 2003). Additionally, phenolic biosynthesis can be induced by various biotic stress and therefore the observed variability among locations may also be related to plant pathologies present at different levels of severity (Dixon and Paiva, 1995). As mentioned above, the phenolic content of wheat grains is also a varietal characteristic as indicated by several studies involving both old and modern genotypes (Adom et al., 2005; Moore et al., 2005; Okarter et al., 2010; Heimler et al., 2010; Zuchowski et al., 2011; Di Silvestro et al., 2012). Grain samples investigated in the present study showed high variability among genotypes for all phenolic fractions, with the only exception of the free flavonoid content that did not vary as a function of the wheat genotype (Table 4.3). Noticeable are the results obtained for the red coloured grain varieties such as Verna, Andriolo and Gentil rosso that presented the higher free and bound polyphenol amounts as well the highest bound flavonoid content. This finding was expected as several phenolic compounds (i.e. anthocyanins) confer coloured features to plant tissues. Liu *et al.* (2010) showed significant differences in total phenolic contents of purple, red, yellow and white grain wheat, with the first two grains having the highest antioxidant amounts. Even if the modern cultivar Palesio is characterized by red grains, it did not seem to keep up with this trend and conversely showed the lowest phenolic content (Table 4.3). The obtained polyphenol and flavonoid amounts resulted generally higher than data previously reported (Adom et al., 2003; Moore et al., 2005; Okarter et al., 2010) but almost comparable with the phenolic contents indicated for old wheat genotypes grown under low–input management (Heimler et al., 2010; Dinelli et al., 2011; Di Silvestro et al., 2012). A significant location by year interaction was observed considering both free and bound polyphenol fractions. As evidenced by the graphs in Figure 4.4, an increase of the free polyphenol content occurred during the second year at the Ferri and Collina farms, while no significant differences were obtained at Cenacchi location and, as regards the bound fraction, the amount of polyphenols exclusively increased at the Ferri farm. The observed variability suggested that wheat genotypes grown in each experimental farm were subjected to changeable abiotic and biotic stresses, differently affecting the phenolic metabolism.

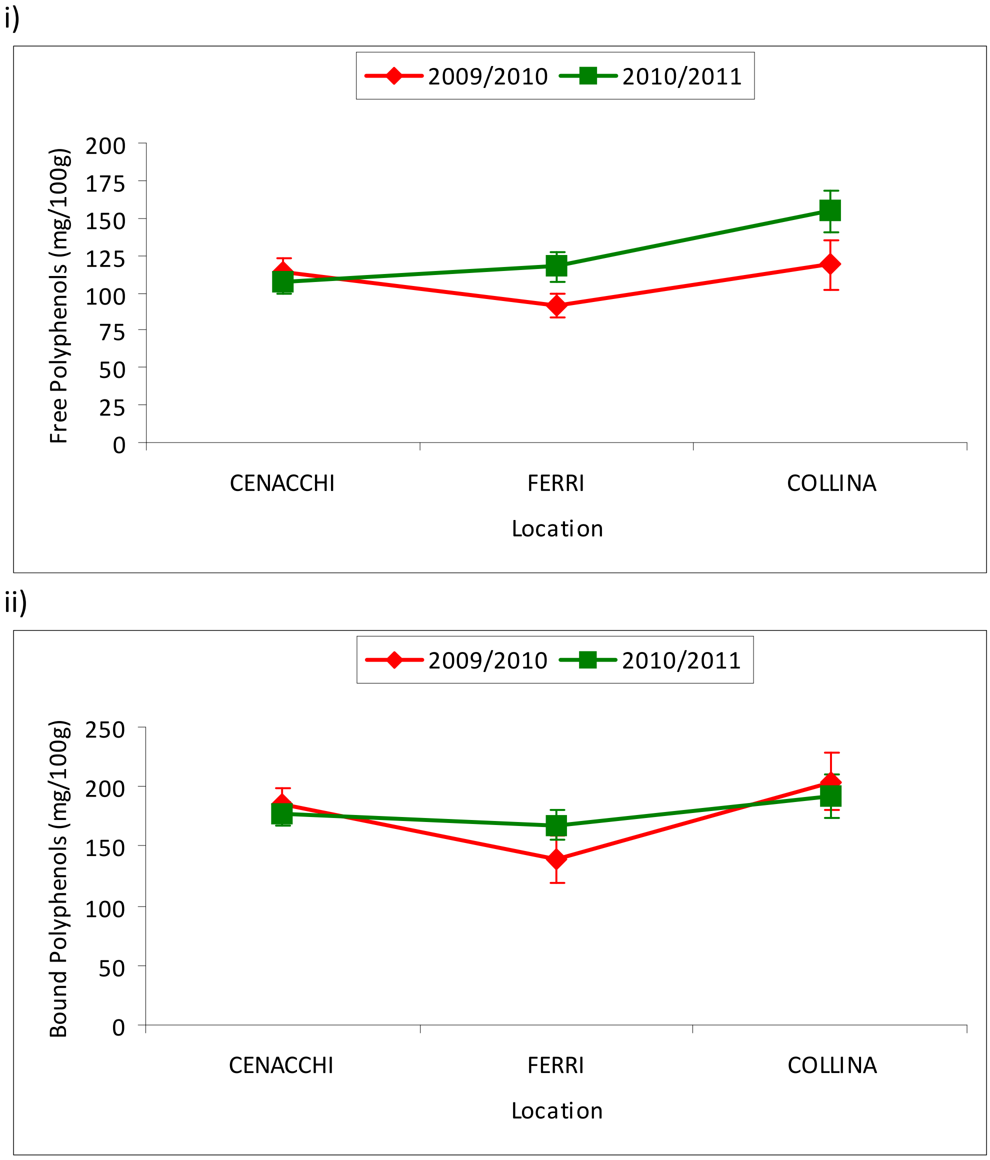


Figure 4.4. Significant interaction between location and year (LxY) for (i) free and (ii) bound polyphenol content.

4.3.7. Antioxidant activity

Several investigations correlated the incidence of pathologies such as cancer and cardiovascular diseases with the presence of highly reactive chemical species in the human body (free radicals) (Halliwell and Gutteridge, 1989; Chen et al., 1995; Benzie, 2000). The consumption of antioxidant–rich foods may efficiently contribute to reduce the risk of oxidative stress pathologies (Renaud et al., 1998; Temple, 2000). Free radicals are instable molecules with one or more unpaired electrons that can be inactivated by antioxidant compounds and converted in more stable molecules.

As outlined above, polyphenols are the main antioxidant compounds in wheat grains and they can act as reducing agents, hydrogen donators, singlet oxygen quencers and chelating agents of metal ions. These antioxidant properties derive from the presence of several hydroxyl groups linked to the aromatic structure and from their molecular geometry (Halliwell and Gutteridge, 1989). The antioxidant activity of the investigated wheat varieties were comparatively evaluated using two different *in vitro* tests (DPPH and FRAP assays). The DPPH assay allowed the assessment of antiradical potential through the proton exchange. DPPH (2,2–diphenyl–1–picrylhydrazyl) is a stable radical that simulate the activity of harmful free radicals commonly present in the human body (Brand–Williams et al., 1995; Yu et al., 2002). No differences were detected for radical scavenger activity among the biodynamic farms, which presented the same mean value (10.0 µmol TE/g) (data not shown). A significant increase of antioxidant activity was revealed by the DPPH assay for the samples harvested during the second year compared to the first one, passing from 8.3 to 11.7 µmol TE/g (data not shown), as a result of the increased amount of polyphenols discussed in the previous paragraph. The investigated wheat varieties showed different levels of radical scavengers, ranging from 8.5 µmol TE/g (Frassineto) to 10.9 µmol TE/g (Andriolo) (Figure 4.5). As observed for the phenolic content, the highest antioxidant power was obtained for the red grain varieties (Verna, Andriolo, Gentil rosso), while significant lower values were observed for white grain genotypes (Inallettabile, Frassineto) and the modern cultivar Palesio. The Ferric Reducing Antioxidant Power (FRAP) assay allowed the evaluation of antioxidant properties by measuring the reduction of ferrous ions Fe(III) to ferric ions Fe(II) operated by antioxidant compounds through the electron exchange (Benzie and Strain, 1996). No significant differences were observed for the antioxidant power assessed by FRAP assay among growing locations and cropping years (data not shown). Results obtained for each investigated variety partially confirmed the genetic differences detected with the DPPH test. Indeed, the lowest antioxidant power was observed for the old variety Frassineto (2.0 mmol/100g), followed by the modern cultivar Palesio (2.1 mmol/100g). The highest value was obtained for the old red grain varieties Andriolo and Gentil rosso (2.4 mmol/100g), whilst Verna and Inallettabile showed intermediate values (2.2 mmol/100g) (Figure 4.5). The observed antioxidant activity agrees with data previously reported for several common wheat varieties (Alvarez–Jubete et al., 2010; Dordevic et al., 2010; Liu et al., 2010).

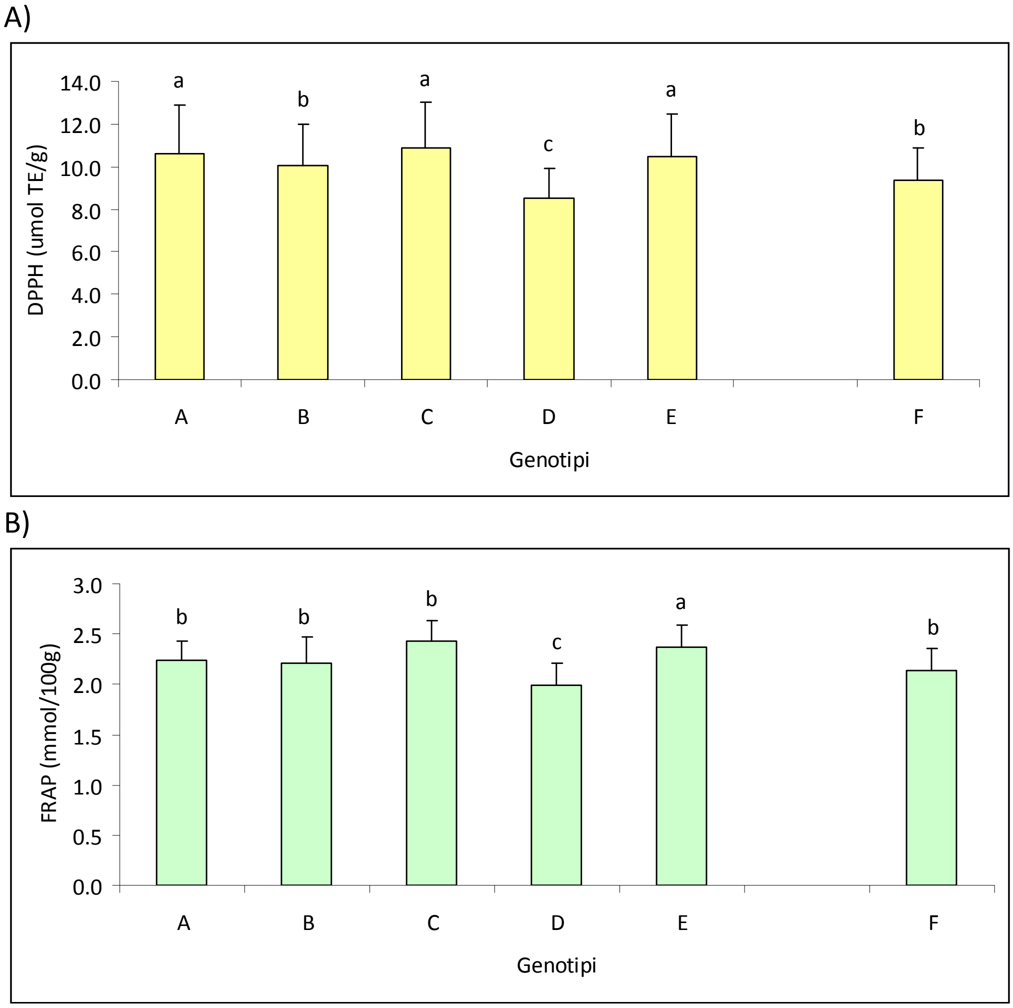
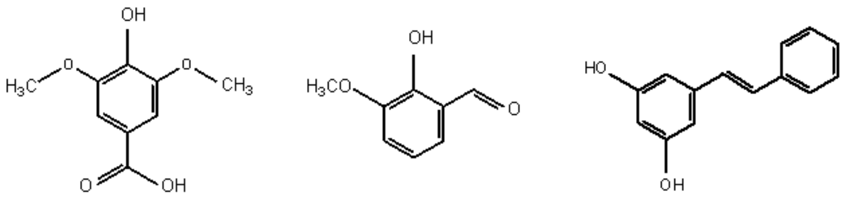


Figure 4.5. Antioxidant activity of the investigated wheat varieties grown at the three biodynamic farms over two cropping years (2009/2010, 2010/2011) as determined by the DPPH (A) and FRAP (B) assays. Mean values (± standard deviation) are expressed in micromoles of trolox equivalents (TE) per g of dry weight and in millimoles of Fe(II) per 100 g of dry weight, for DPPH and FRAP respectively.

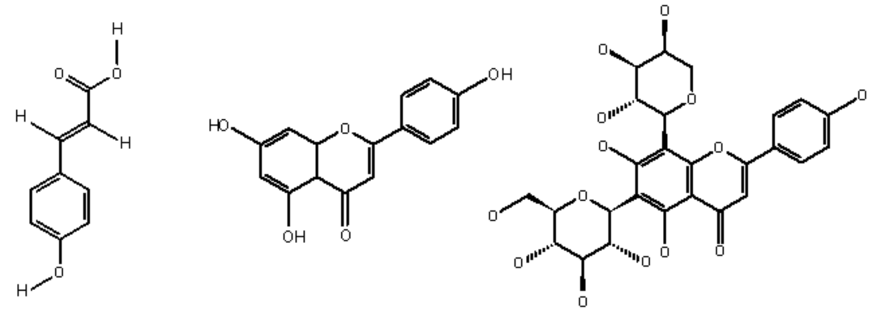
Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio

4.3.8. Determination of phenolic compounds using HPLC–ESI–TOF–MS

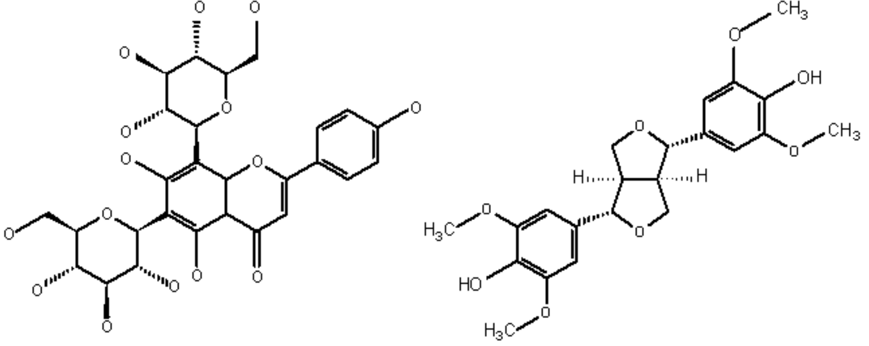
The optimization of chromatographic and MS conditions was carried out as previously described (Dinelli et al., 2011). Tentative characterization of free and bound phenolic compounds were generated based on elemental composition data determined by accurate mass measurements and comparison with literature data. The interpretation of the mass spectra allowed the identification of 19 phenolic compounds (43 including isomer forms) belonging to the phenolic acid, flavonoid, stilbene, proanthocyanidin and lignan classes (Figure 4.6). Phenolic compunds characterized in the free and bound extracts of each wheat genotype are listed in Table VI (*Annex Section*) and numbered according to retention time. The characterization by TOF–MS was carried out using the Generate Molecular Formula Editor, choosing a low tolerance value (5 ppm). After that, options with a low millisigma value (<50.0) and a low error (< 5 ppm) were taken into account and finally, the position of the molecular formula in the list of possible compounds was considered. Figure 4.7 shows the base peak chromatogram (BPC) obtained for the bound phenolic extract of the old variety Verna and the extracted ion chromatograms (EIC) for the main characterized compounds. Additionally, all polyphenols detected for Verna bound extract are summarized in Table 4.4.



a) Syringic acid b) Vanillin c) Pinosylvin



d) *p*-Coumaric acid e) Apigenin f) Schaftoside



g) Vicenin-2 (apigenin-6,8-di-*C*-glucoside) h) Syringaresinol

Figure 4.6. Structural formulae of representative phenolic compounds detected in the wheat genotypes.

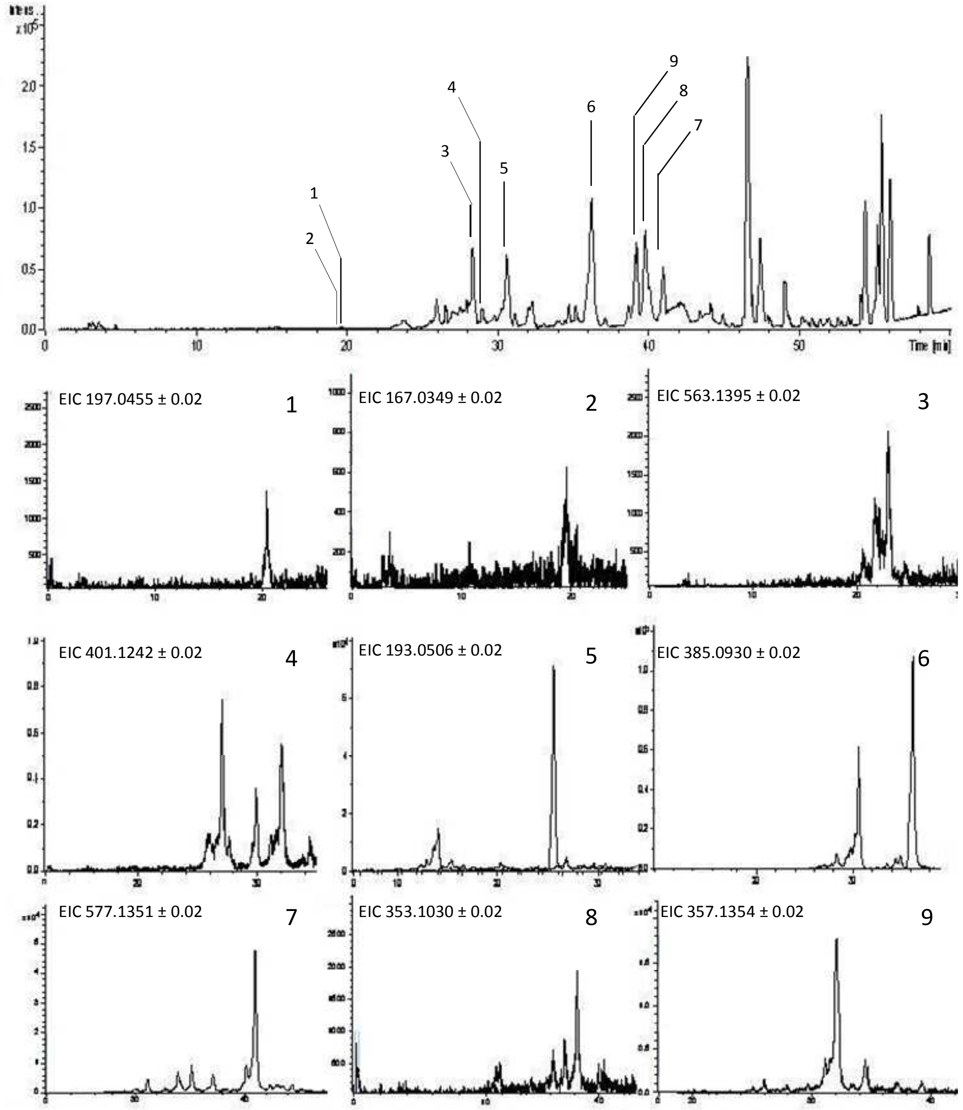


Figure 4.7. Base Peak Chromatogram (BPC) obtained by HPLC–ESI–TOF–MS for Verna free phenolic extract and Extracted Ion Chromatograms (EIC) ± 0.02 of well–known detected compounds: (1) syringic acid, (2) vanillic acid, (3) Apigenin–6–C–arabinoside–8–C–hexoside (Schaftoside/Isoschaftoside, (4) glycosylated pinosylvin, (5) ferulic acid, (6) dihydroferulic acid, (7) isovitexin–2''–O–rhamnoside, (8) hinokinin, (9) pinoresinol.

This table includes selected ion, tolerance (ppm), molecular formula, experimental and calculated *m/z*, error, sigma values, UV–absorption wavelength and retention time. All the characterized phenolic compounds were previously described in wheat in other reports (McCallum and Walker, 1989; Lachman et al., 2003; Asenstorfer et al., 2006; Hosseinian et al., 2008; Matus–Cadiz et al., 2008; Dinelli et al., 2011). Several studies investigated the phenolic acid composition of wheat grains and showed ferulic acid as the most predominant compound of the class, present in both free and bound forms.

Table 4.4. Phenolic compound detected by HPLC–ESI–TOF–MS in the bound extract of Verna.

No.	Molecular formula	Selected ion	m/z exp	m/z calc	Error (ppm)	Sigma value	Tolerance (ppm)	Retention Time (min)	UV absorption (nm)	Classification order (number of possibilities)	Compound
1	$C_9H_{10}O_5$	$[M–H]^-$	197.0459	197.0455	5	8.6	–4.2	19.46	237	1 (2)	Syringic acid
2	$C_8H_8O_4$	$[M–H]^-$	167.0352	167.0350	5	50.2	–1.2	19.59	236	1 (1)	Vanillic acid
3	$C_{26}H_{28}O_{14}$	$[M–H]^-$	563.1399	563.1406	5	1.3	7.0	22.98	239 282	1 (9)	Apigenin–6–C–arabinoside–8–C–hexoside (schaftoside/isoschaftoside)
4	$C_{21}H_{22}O_8$	$[M–H]^-$	401.1217	401.1242	10	5.1	24.9	27.07	237 322	1 (5)	Glycosylated pinosylvin
5	$C_{10}H_{10}O_4$	$[M–H]^-$	193.0492	193.0506	5	4.2	13.4	17.64	236 321	1 (2)	Ferulic acid
6	$C_{20}H_{18}O_8$	$[M–H]^-$	385.0921	385.0930	5	2.0	7.5	36.19	246 312	1 (3)	Dihydroferulic acid
7	$C_{27}H_{30}O_{14}$	$[M–H]^-$	577.1546	577.1562	5	3.0	18.0	39.22	231	3 (10)	Isovitexin–2''–O–rhamnoside
8	$C_{20}H_{18}O_6$	$[M–H]^-$	353.1031	353.1030	5	–0.1	9.9	38.15	240 315	2 (5)	Hinokinin
9	$C_{20}H_{22}O_6$	$[M–H]^-$	357.1340	357.1344	5	1.1	19.2	45.54	241	2 (3)	Pinoresinol

In the present study, compound with molecular mass 194.0510 tentatively identifies as ferulic acid was detected in all the investigated genotypes, as well as the dihydroferulic acid (*m/z* 386.0930). Vanillic acid (*m/z* 168.0350) was also detected in all wheat extract, while compounds with mass 198.0460 and 164.0400, characterized as syringic acid and *p*–coumaric acid respectively, were found in the bound extracts of some investigated old wheat genotypes (Table VI). As outlined above, flavonoids are the most representative class of phenolic in wheat grains and possess valuable bioactivity as antioxidant, anticancer, antiallergic, anti–inflammatory, anticarcinogenic and gastroprotective agents (Harborne and Williams, 2000; Yao et al., 2004). The interpretation of the mass spectra showed that flavones was the most representative group of flavonoids in the wheat extracts, comprising of 1 aglyconic form (apigenin, *m/z* 270.0460, exclusively detected in the old varieties Inallettabile and Verna), 2 *O*–glycosylated and 6 *C*–glycosylated compounds. In common with previous studies, the glycosylated 3',4',5'–trihydroxy–3,7–dimethylflavone (*m/z* 492.1195) and the glycosylated and acetylated 3',4',5'–trihydroxy–3,7–dimethylflavone (*m/z* 534.1300) were detected in the free extracts of all the investigated wheat varieties. Compound with mass 594.1511 was characterized as Vicenin–2 (apigenin–6,8–di–C–glucoside) and detected in the free extracts of the wheat varieties, with Gentil rosso showing the highest number of isomer forms (5). The flavone–*C*–glycoside identified as schaftoside/isoschaftoside (*m/z* 564.1406) was present with the highest number of isomer forms in both free and bound extracts, with the old genotype Andriolo showing 9 isomers, followed by Verna (7) and Inallettabile (6), while the remaining wheat genotypes presented 5 schaftoside isomers. Moreover, lucenin–1/3 (luteolin–6/8–C–xyloside–8/6–C–glucoside), isovitexin–2''–O–rhamnoside, apigenin–6–C–B–galactosyl–8–C–B–glucosyl–O–glucuronopyranoside and methylisoorientin–2''–O–rhamnoside were detected in some of the investigated wheat varieties, both free and bound extracts.

Two phenolic compounds belonging to the stilbene class were found in most of the investigated wheat genotypes, namely glycosylated pinosylvin and its double glycosylated form (*m/z* 536.1821 and 402.1241 respectively). The importance of stilbene compounds is given by their physiological function as plant natural protective agents against pest and diseases. The glycosylated pinosylvin was detected in the bound fractions of all wheat varieties, except for Andriolo. However, Andriolo showed the double glycosylated form of pinosylvin in its free phenolic exctract, along with the bound fraction of Frassineto, Gentil rosso and Inallettabile. Proanthocyanidins are a class of colorless compounds constituted by catechin and epicatechin units. Compound with mass 578.1351 was assigned as procyanidin B–3 and detected in the bound extracts of almost investigated varieties, except for Inallettabile grains. Lignans are phenolics classified as phytoestrogens due to their structural similarities with human endogenous estrogens, and exhibit anticancerogenic, antioxidant, antiproliferative, pro–apoptotic and antiangiogenic properties (Webb and McCullough, 2005). Compound with mass 354.1030 was characterized as hinokinin and detected in the phenolic extracts of Andriolo, Gentil rosso, Inalletabile and Verna. Pinoresinol (*m/z* 358.1350) was exclusively found in the phenolic extracts of the old varieties Inallettabile and Verna. Data obtained from the interpretation of the mass spectra of the free and bound extracts were computed to obtain a dendrogram (Figure 4.8).

In Figure 4.8 the observed differences in phenolic compounds and isomer forms are also presented through a colorimetric map, ranging from white colour indicating the absence of phenolic compounds to black corresponding to the presence of more than 8 isomer forms. Two highly divergent clusters can be individuated among wheat genotypes: the first group includes the modern cultivar Palesio and the old genotypes Frassineto and Gentil rosso; the second cluster comprises the old varieties Andriolo, Inallettabile and Verna. Verna and Inallettabile presented the highest number of detected polyphenols (16 and 15 respectively) thus showing the richest phenolic composition. Additionally, these varieties shared the common presence of pinoresinol (compound 42) and apigenin (compound 43) exclusively detected in their phenolic extracts. The presence of Andriolo in the same cluster of Verna and Inallettabile is probably linked to the presence of elevated number of isomers, with the maximum value recorded for compound 9 (apigenin–6–C–arabinoside–8–C–hexoside) with 9 isomer forms detected in both free and bound fractions. It is not surprising that the red grain varieties Andriolo and Verna presented phenolic profiles with an high number of detected compounds (29 and 30 iso–forms, respectively), as they also showed the highest polyphenol and flavonoid content, along with the red grain genotype Gentil rosso. However, the interpretation of the mass spectra showed a lower number of detected polyphenols (13) in the Gentil rosso extracts and it clusterized with the old variety Frassineto and the modern cultivar Palesio. The latter showed the lowest number of detected polyphenols (11) and isomers (23), but its highly divergent position was also affected by the presence of apigenin–6–C–B–

galactosyl–8–C–B–glucosyl–O–glucuronopyranoside (compound 22), exclusively found in Palesio extracts. Despite the lower number of detected polyphenols, Gentil rosso presented a total number of isomers (27) higher than those observed for Frassineto(24) and Palesio (23), especially as regards flavone *C*–glycosylated compounds.

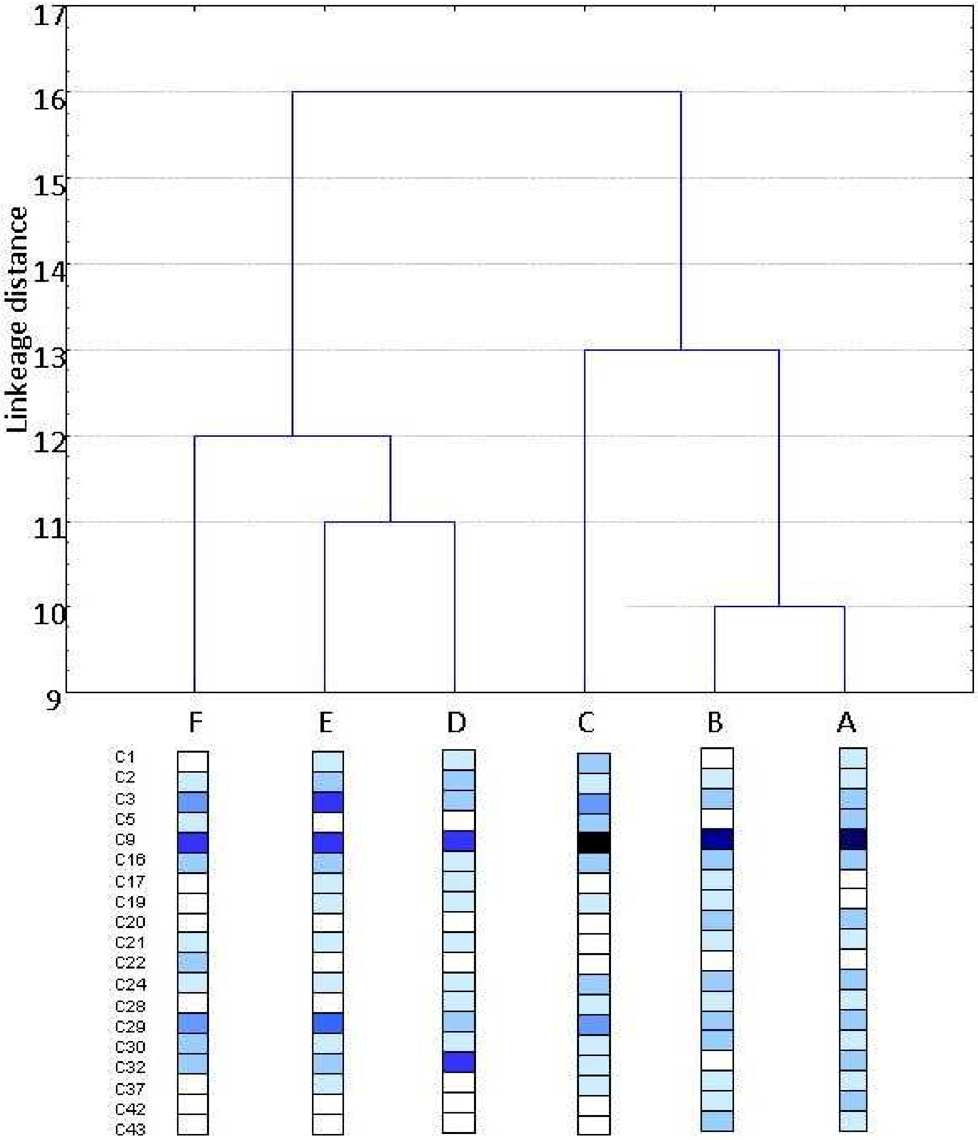


Figure 4.8. Dendrogram obtained from phenolic profiles of each investigated wheat variety determined by HPLC–ESI–TOF–MS. The color intensity is proportional to the number of isomers for each detected compound (white = 0 isomer; black = > 8).

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; C1–C43: phenolic compounds are numbered according to Table VI, *Annex Section*.

4.3.9. Discriminant analysis

The multivariate techniques of data analysis have been used to explain the observed variability concerning the nutrient content described in *Section 3* and the phytochemical composition of the investigated wheat genotypes, harvested at the three biodynamic farm in both growing seasons (2009/2010, 2010/2011). The LDA elaboration allowed to have an ultimate complete vision of the differences among genotypes and to obtain more information as regards the variables that mainly influence the sample similarities and differences. The plot of the wheat samples on the space defined by the first two canonical functions is presented in Figure 4.9. The calculated Wilks' lambda value of 0.0094 (significant at $P<0.00001$) is indicative of high discrimination power of the applied model. The score of cases as a function of the first two canonical functions (Root 1, Root 2) allowed the individuation of two clear separate groups of wheat genotypes along the Root 1.

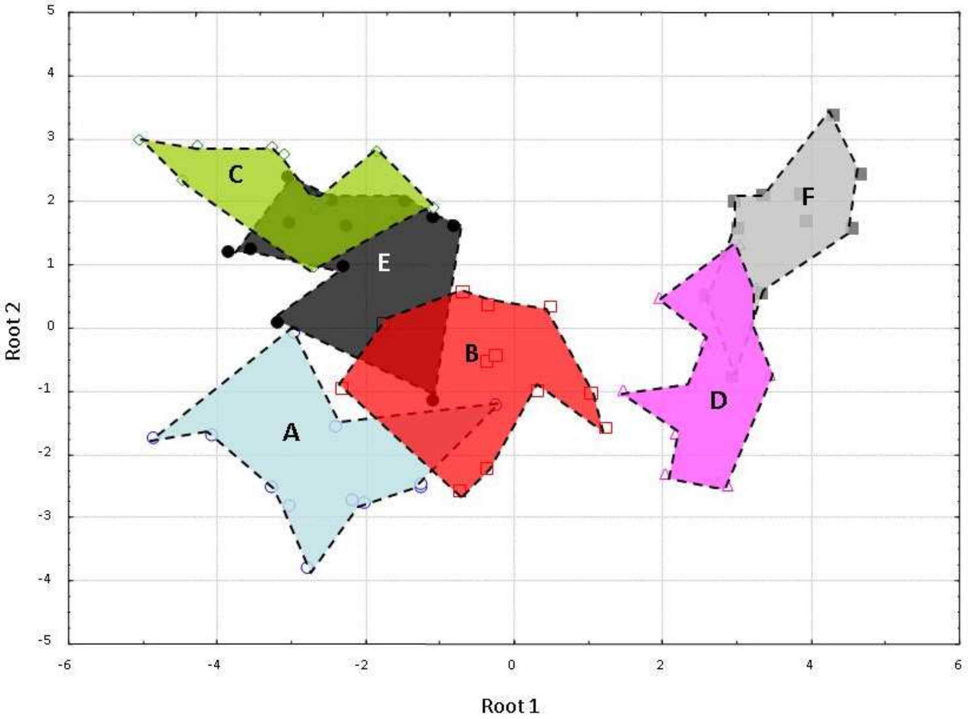


Figure 4.9. Scatterplot of the investigated wheat varieties from both growing seasons (2009/2010, 2010/2011) according to the nutrient and phytochemical content defined by the first two canonical functions (Root 1 and Root 2). A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.

The first group was scored along the negative part of the first root and included the old varieties Andriolo, Inallettabile, Gentil rosso and Verna. The second group is located in the positive part of the Root 1, completely divided from the first cluster, and included the old genotype Frassineto and the modern cultivar Palesio. Between the two described groups of varieties, Verna was shown to be the most divergent from Palesio, since they occupied opposite position in the plot, as similarly did Andriolo and Frassineto. The separation of the wheat samples was strongly affected by the phenolic content and the antioxidant activity, as revealed by the values of canonical functions standardized within variances for each variable on Root 1. Indeed, Frassineto and Palesio were scored on the positive part of Root 1 as they resulted the poorest in phenolic content and antioxidant activity. On the contrary, an increasing amount of polyhpenols and flavonoids characterizes the old varieties grouped on the left quadrant of the plot, with Andriolo and Verna having the highest antioxidant properties. Moreover, the score of the cases along the second root was strongly determined by the variability in β–glucan and lipid content, as revealed by the values of canonical functions standardized within variances for each variable on Root 2. The position in the plot of the old varieties Verna, Inallettabile and Frassineto was related to high lipid amount and low β–glucan content, and quite the opposite, Andriolo and Palesio were scored on the positive arm of the Root 2 as they presented limited lipid accumulation and high β–glucan content. Accordingly to the obtained results, the two–year investigation allowed the description of high variability among wheat genotypes in terms of nutrient and nutraceutical composition. Although the accumulation of several metabolites in the wheat grains was affected by environmental factors (location, year) as described in the previous paragraphs, the physiological response of each variety resulted in peculiar metabolite composition. Most of the observed total variability was related to the secondary metabolite concentration of wheat grains, while a limited contribution was shown for compounds deriving from primary metabolism. Among the investigated wheat varieties, Verna, Gentil rosso and Inallettabile were individuated as the most promising genotypes for the development of nutraceutical value–added wheat varieties.

4.4. AT A GLANCE

- ✓ The insoluble and soluble fibre accumulation in the grains of the six investigated genotypes is mainly influenced by genetic traits rather than environmental factors;
- ✓ No significant differences were observed among wheat varieties for the soluble fibre content. Palesio and Frassineto are characterized by the lowest insoluble fibre content with respect to the remaining old wheat varieties;
- ✓ On the basis of *in vitro* tests, the investigated wheat varieties differ for their qualitative dietary fibre composition, differently affecting the relative prebiotic properties;
- ✓ The old varieties Verna, Frassineto and Gentil rosso are characterized by the highest prebiotic activity scores;
- ✓ The resistant starch and arabinoxylans are stable parameters and only slight variations are observed as a function of different environmental conditions;
- ✓ The investigated wheat varieties present a similar resistant starch content, while Genitl rosso and Verna exhibit the highest arabinoxylan content;
- ✓ β–glucans are present in the grains of the six investigated wheat genotypes at low concentration levels (<0.5% of dry matter);
- ✓ The content of different polyphenol and flavonoid fractions is equally influenced by genetic and environmental factors;
- ✓ Except for Palesio, the remaining red coloured grain varieties such as Verna, Andriolo and Gentil rosso exhibit the higher free and bound polyphenol amounts as well the highest bound flavonoid content;
- ✓ The qualitative composition of the phenolic fractions extracted from the six investigated wheat genotypes is highly variable;
- ✓ Verna and Inallettabile show the highest number of phenolic compounds (16 and 15, respectively), while the modern cultivar Palesio exhibits the lowest one (11 compounds);
- ✓ Except for Palesio (23 isoforms), the remaining red grain varieties (Verna, Andriolo, Gentil rosso) show the highest number of phenolic isomers (30, 29 and 27 isoforms, respectively);
- ✓ For the six investigated genotypes, the trend of antioxidant activities (detected by *in vitro* DPPH and FRAP tests) is consistent with the chemically determined phenolic content;

- ✓ Except for Palesio, the remaining red coloured grain varieties (Verna, Andriolo and Gentil rosso) exhibit the highest *in vitro* antioxidant power;
- ✓ The present two–year investigation allows the description of high variability among investigated wheat genotypes in terms of nutrient and nutraceutical composition;
- ✓ Considering both primary nutrient fractions and phytochemical composition, the discriminant analysis permits the identification of two well–defined genotype clusters: in the first one the old varieties Andriolo, Inallettabile, Gentil rosso and Verna are included, while the old genotype Frassineto and the modern cultivar Palesio are placed in the second one;
- ✓ Among the investigated wheat varieties, Verna, Gentil rosso and Inallettabile are the most promising genotypes for the development of nutraceutical value–added wheat varieties.

Section 5:

RHEOLOGICAL PROPERTIES OF WHEAT VARIETIES AND SENSORY EVALUATION OF BREAD

5.1. SECTION OBJECTIVES

Bread is a staple foodstuff in most countries around the world. Over the centuries, bakers have developed a wide range of traditional bread products using their accumulated knowledge to make best use of raw materials and achieve the desired bread quality. The unique properties of wheat flour derive mainly from the protein composition as regards the gluten protein fraction. The gluten network is formed within the dough during the mixing process. Due to its viscoelastic properties, this gluten arrangement allows dough to deform, stretch, recover shape and trap gases during yeast fermentation and thus significantly determines the final quality of bread products. In many countries the nature of breadmaking has dramatically changed in recent years, due to the largely utilization nowadays of mechanized mixing and processing methods. The wide development of industrial breadmaking processes was accompanied by the selection of wheat varieties with improved gluten strength and viscoelasticity. Indeed, one of the main target of the post–Green Revolution breeding programs was to allow wheat flour to tolerate high technological stress during baking processes. The bread wheat cultivars characterized by an high–strength gluten are however strictly dependent on elevated nitrogen inputs to satisfy the technological requirements and, at the same time, provide high yield. As a consequence, the modern cultivars have been shown to be less adaptable to the low input sector, where they may failure in both yield and rheological performances. Recently, the increasing consumer awareness towards organic foods evidenced the need to improve the breadmaking process of organic products by considering the whole production chain. Besides the selection of raw material for the agronomic, nutritional and nutraceutical features, the mixing and baking processes should be taken into account. The use of appropriate breadmaking procedures may lead to the development of bread products with high sensory and organoleptic characteristics, in addition to unique nutritional and nutraceutical properties. The aim of the present *Section* was to determine the rheological properties of the investigated modern and old wheat varieties through the use of standardized methods (Chopin alveograph) and additionally assess the sensory evaluation of derived bread products as traditionally baked using sourdough fermentation. The research aimed at contributing to the development of breads with peculiar sensory and health–promoting properties.

5.2. MATERIAL AND METHODS

Grain samples of modern (Palesio) and old (Andriolo, Frassineto, Gentil rosso, Inallettabile, Verna) varieties grown in pure genotype plots from the field trial described in *Section 2* were used for rheological properties determination and breadmaking process. Wheat grains harvested at the three involved locations (Cenacchi, Ferri and Collina farms), from the first growing season (2009/2010), were stone milled, sifted to obtain refined flours and the rheological properties analyzed as outlined below. Semi–whole wheat flours were used for the breadmaking process and the subsequent sensory evaluation of bread products.

5.2.1. Rheological properties

Grain samples harvested in the first year (2009/2010) were stone milled, sifted to obtain white flours and analyzed for their baking quality. The rheological properties were assessed by using the Chopin alveograph according to the 54–30A method of the American Association of Cereal Chemists (1995). The dough prepared from each wheat flours was formed into disc–shaped pieces and inflated into bubbles. The pressure variation inside each bubble was recorded in graphical form as an "alveogram" (Borghi et al., 1997). The maximum height of the curve provides an estimate of dough tenacity (*P*, expressed in mm) and its length measures dough extensibility (*L*, expressed in mm) and swelling index (*G*, in ml). The area under the curve is proportional to the energy required to cause the dough bubble rupture (*W*, expressed in J x 10^{-4}). The ratio of the peak height to the length (*P/L*) was calculated to obtain an index commonly used to describe the gluten rheological properties (Preston and Williams, 2003).

5.2.2. Bread sensory evaluation

Flours obtained from each wheat genotypes grown during the first cropping year (2009/2010) were used for breadmaking process carried out by the bakery "Forno Baracca" at Nonantola (MO), Italy. All obtained breads were sourdough fermented, using the own traditional sourdough of the bakery "Forno Baracca". In order to determine the sensory profiles of bread obtained from each investigated wheat varieties, acceptance sensory test were carried out on freshly prepared breads. The sensory evaluation was performed by a panel of 370 not trained subjects, randomly selected among consumers, at the following locations in Italy: "Forno Baracca", Nonantola (MO); Agriturismo "Podere Santa Croce", Argelato (BO); La Collina Società

Cooperativa Agricola, Codemondo (RE); Cooperativa Agricola Biodiversi, Casalecchio di Reno (BO) (Figure 5.1); Az. Ferri, Anzola dell'Emilia (BO). The sensory evaluation form included the estimation of visual, olfactory and gustative characteristics expressed by numbers within the interval 1–9, going from the minimum (1) to maximum (9) level of sensory acceptance. All sensory evaluations were carried out as blinded tests.

5.2.3. Statistical analysis

One–way ANOVA (Tukey's test) was performed to test the variance significance of genotypes harvested in first growing season for each rheological parameter and sensory characteristic. Significance between means was determined by least significant difference values for $P < 0.05$. The analysis of variance was performed using Systat v. 9.0 (SPSS Inc. 1998).

Figure 5.1. Sensory test of breads carried out at the Cooperativa Agricola Biodiversi, Casalecchio di Reno (BO), Italy, on October 2011.

5.3. RESULTS AND DISCUSSION

5.3.1. Determination of flour rheological properties

The milling and baking quality were one of the main targets of breeding programs occurred after the “Green Revolution”. Though the requested traits varied depending on the final market use, the cultivar selection was frequently carried out for satisfying the industry requirements, thus obtaining flours able to tolerate high technological stress during the mixing and leavening processes (Cocchi et al., 2005). Two of the most important flour quality factors (mixing time and loaf volume) are known to be closely related to gluten components (glutenins, gliadins) affecting the viscoelastic properties of dough (Lookhart and Albers, 1988; Suchy et al., 2003). By reason of the negative correlation between yield and grain protein concentration, there has been a selection for higher gluten quality accompanied with increasing fertilizer distribution over the crop cycle (Wolfe et al., 2008). Consequently, in most cases the use of modern varieties in organic and low–input farming led to low yield and protein quality that did not fulfill the baking requirements. In the present study, the wheat flours obtained from the six investigated genotypes grown in the first year (2009/2010) were analyzed using the Chopin alveograph to assess their rheological properties. The W index measures the deformation energy and it is related to the gluten strength. W varied between 34.00 x 10^{-4} J (Inallettabile) and 88.33 x 10^{-4} J (Andriolo) (Table 5.1). The higher values were obtained for the old variety Andriolo and the modern cultivar Palesio, whereas the remaining old genotypes showed very poor gluten strength. Andriolo and Palesio presented also the higher P index values (43.67 and 41.00 mm, respectively), indicating the dough tenacity, namely the maximum over–pressure needed to blow the dough bubble. Andriolo and Palesio P values doubled those obtained for the remaining old varieties (Table 5.1). No significant differences were detected for the dough extensibility (L index) but the variability observed among genotypes for P are reflected in the P/L ratios, with Andriolo and Palesio showing the higher values (0.60 and 0.57, respectively). No significant difference was observed among wheat genotypes for the dough elasticity, as expressed by the swelling index (G) (mean value 18.23 ml) (Table 5.1). In Italy, flours are classified in four groups (improved wheat; superior bread making wheat; ordinary bread making wheat; wheat for biscuits and pastry) on the basis of their technological behavior during dough–making, as proposed by ASSINCER (Associazione Intersettoriale Cereali e altri seminativi), an important Italian cereal trade association (Cocchi et al. 2005). The classification is based on the Synthetic Index of Quality which includes alveographic indexes such as *W* and *P/L*. Results obtained in the present research were significantly lower than the threshold limits established for the last wheat class, equal to 110 x 10^{-4} J and 0.5 for *W* and *P/L*, respectively. The observed rheological properties resulted strongly poorer than those previously reported in literature for modern wheat cultivars grown under conventional and low–input farming (Lopez–Bellido et al., 1998; Guarda et al., 2004). Exclusively *W* index values obtained for Andriolo and Palesio aligned with those reported by Guarda *et al.* (2004) for old wheat varieties grown under low–input management. The swelling index (*G*) was the sole alveographic index in agreement with values previously reported in literature, ranging from 14.6 to 24.0 ml (Lopez–Bellido et al., 1998; D'Egidio et al., 2003). The alveographic analysis of the investigated wheat varieties highlighted that wheat genotypes grown under low–input management may supply raw material unsuitable for the industry breadmaking process, and suggested that the expected high gluten–strength cultivars (i.e. Palesio, commonly classified as "ordinary bread making wheat", W>140) provide flour with good technological properties exclusively when grown under high N–input management. Baking quality of flours is not exclusively related to the protein composition, but there are several additional factors that interact with gluten elasticity such as water absorption capacity and starch damage of flours, as well as processing methods (Stauffer, 1998; Ktenioudaki et al., 2010). Mixing is a crucial part of the breadmaking process and determines the development of the gluten net structure that will facilitate gas retention during the subsequent processing. During the mixing procedure, air is incorporated into the dough forming air bubbles that will

Table 5.1. Rheological properties of the investigated wheat varieties.

Variety	*W* (x 10^{-4} J)	*P* (mm)	*L* (mm)	*P/L*	*G* (ml)
A	42.00(b)	28.33(b)	67.00 ns	0.42(b)	18.23 ns
B	34.00(b)	22.00(b)	66.67 ns	0.34(b)	18.10 ns
C	88.33(a)	43.67(a)	73.33 ns	0.60(a)	19.07 ns
D	40.50(b)	26.50(b)	62.00 ns	0.43(b)	17.55 ns
E	36.67(b)	24.67(b)	60.33 ns	0.43(b)	17.20 ns
F	84.33(a)	41.00(a)	72.67 ns	0.57(a)	18.97 ns
Mean	54.61	31.11	67.33	0.46	18.23

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; ns, not significant.

grow during fermentation and the early stages of baking (Chin et al., 2004). Mixing attributes, i.e. intensity and time rate, cause different dough deformation and therefore significantly affect the dough development (Connelly and McIntier, 2008). The Chopin alveographic analysis provides standardized parameters for the evaluation of dough behavior, as regards gluten strength and elasticity under controlled conditions. However, the alveographic determination may not completely reflect the traditional breadmaking procedures typical, for instance, of the Italian bakers; on the contrary, it is more addressed to an industrialized processing system. In the organic and biodynamic farming contest, the use of different baking procedures belonging to the Italian breadmaking tradition, with higher mixing rate time and sourdough fermentation, probably represents the most efficient way to obtain a final product with high sensory and organoleptic characteristics, as outlined in the subsequent paragraph.

5.3.2. PANEL TEST: SENSORY EVALUATION OF BREAD

In contrast with the industry breadmaking system based on highly mechanized process, a substantial proportion of wheat bread is produced using sourdough leavening in many countries (Meuser and Valentin, 2004). Sourdough has been defined as the dough made from cereals, liquids and microbes (such as lactic acid bacteria and yeasts) in an active state. The industrial mechanized process highly relies on short mixing and leavening time, to maximize the bread production efficiency. On the contrary, sourdough bread production generally requires an extended leavening procedure (8–16 h or more). Both consumers and bakers well recognize that the use of sourdough provides beneficial effects on flavor, microbial shelf life and delays staling, thus generally improving the quality and palatability of wheat–derived products (Vermeulen et al., 2006). In order to evaluate the sensory acceptance of breads baked from the investigated wheat flours, a panel test was carried out involving 370 consumers (both males and females), between 18 and 65 years old (Figure 5.2). Figure 5.3 shows the obtained scores for visual (A), olfactory (B) and gustative (C) evaluations expressed as frequency percentage for each score within the interval values 1–9. The mean values obtained for the visual, olfactory and gustative evaluations for each investigated wheat varieties are presented in Figure 5.4. On the whole, all breads obtained a mean score value above the limit of sufficiency (>6), comprised between 6.86 (Verna) and 7.54 (Gentil rosso).

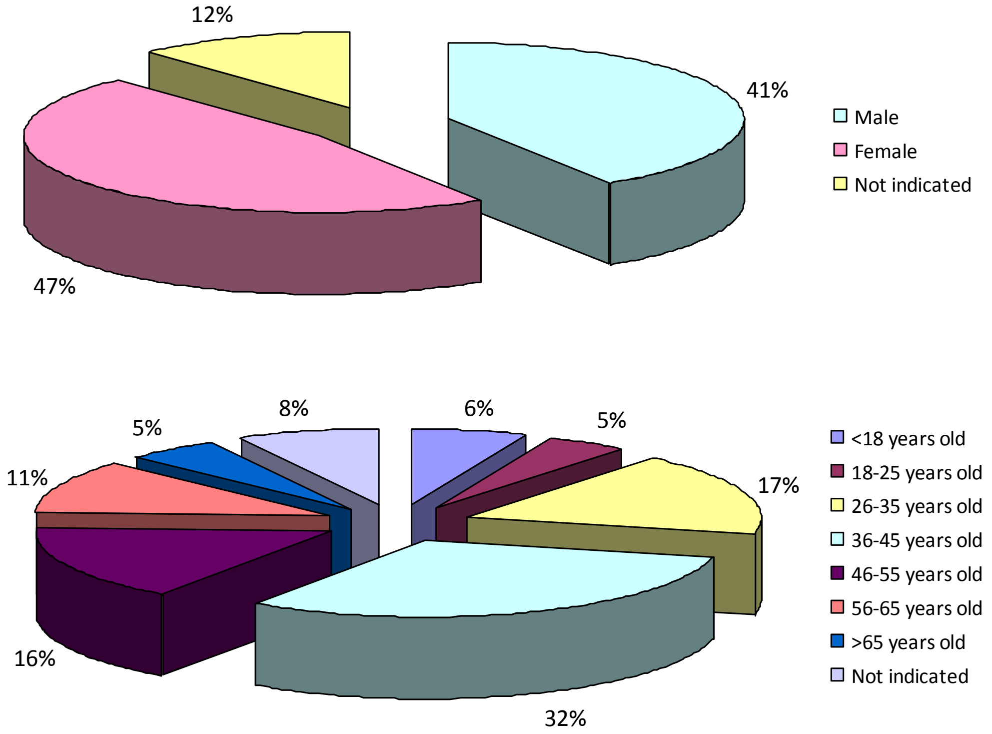


Figure 5.2. Gender and age of the 370 subjects involved in the sensory evaluation test.

The present finding suggests that all the involved wheat flours were able to provide a final product goodly appreciated by consumers. As regards the visual evaluation, the most appreciated breads resulted to be those produced using the old varieties Andriolo, Gentil rosso and Frassineto (7.81, 7.76 and 7.60, respectively), while Verna showed a significantly lower value (7.01). Inallettabile and Palesio obtained intermediate values, equivalent to 7.43 and 7.52 respectively. The olfactory evaluation highlighted that Palesio bread was the most appreciated for flavor characteristics (7.74) while Andriolo, Inallettabile and Verna presented lower evaluation scores, equal to 6.94, 7.00 and 7.09, respectively. As concerns the most important sensory parameter, Frassineto and Gentil rosso breads were evaluated as the most palatable for the gustative properties (mean values 7.49 and 7.54, respectively). The lowest gustative evaluation scores were obtained for Andriolo and Verna breads (6.88 and 6.63, respectively), while Palesio and Inallettabile showed intermediate values. As regards the frequency percentage of each evaluation scores, Figure 5.2 shows that almost 77% of the panel subjects assigned a sufficient (>6) score for the visual evaluation.

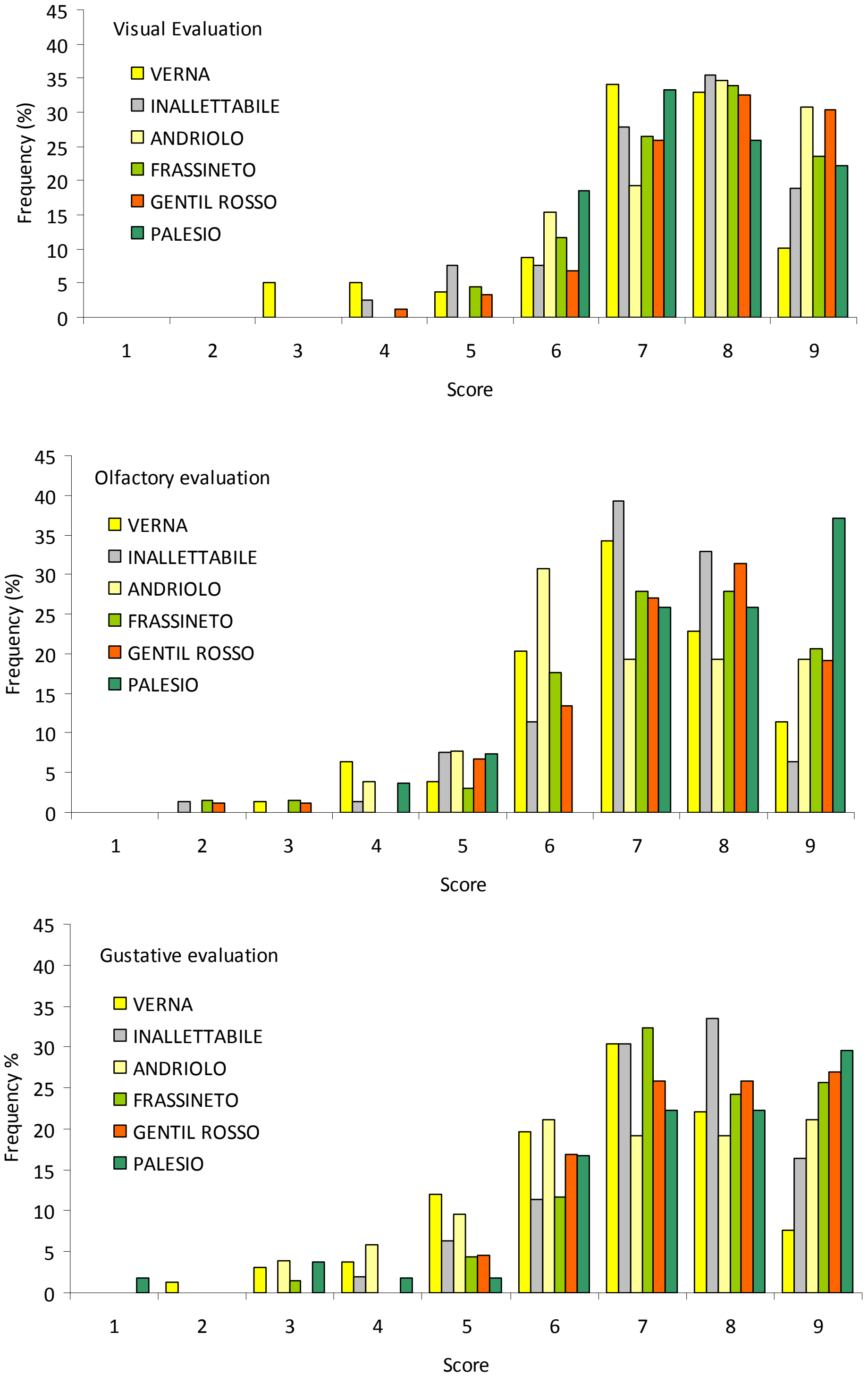


Figure 5.3. Frequency percentages of scores assigned to each tested bread for visual, olfactory and gustative evaluation.

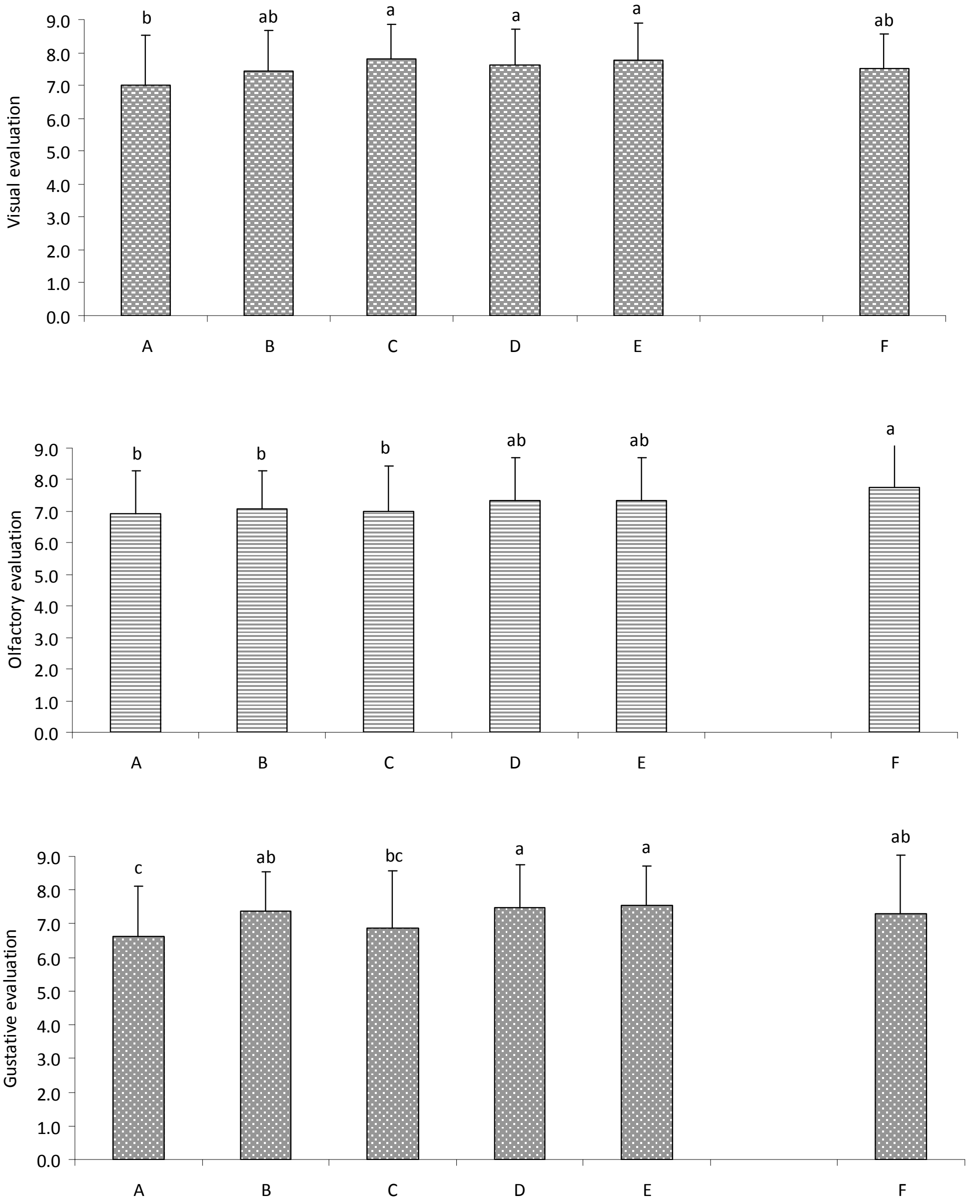


Figure 5.4. Mean values (± standard deviation) of the sensory scores obtained for each test bread for visual, olfactory and gustative evaluation.

In particular, Andriolo and Palesio breads did not received any negative (<5) acceptance. 68% of the consumers assigned sensory scores above the sufficient value for the olfactory evaluation. From the gustative point of view, most of the panel subjects (60%) highly appreciated the tasted breads, assigning evaluation scores between 7 and 9. The overall high sensory acceptance of breads among a broad spectrum of consumers evidenced that, despite the low quality indicated by alveographic parameters, the involved wheat flours provided a well appreciated final product with valuable organoleptic properties. Traditionally sourdough was one of the key methods for the production of bread with enhancement of flavor and texture, and it has been demonstrated that sourdough leavening can also modulate nutritional properties by increasing levels or bioavailability of bioactive compounds and starch digestibility (Katina et al., 2005). Accordingly, wheat varieties grown under low–input management may efficiently provide raw material for the development of traditionally processed bread with peculiar sensory and nutritional properties.

5.4. AT A GLANCE

- ✓ On the basis of Chopin alveograph determinations, the six investigated genotypes are characterized by extremely low *W* (gluten strength) values, ranging from 34 x 10^{-4} J (Inallettabile) and 88 x 10^{-4} J (Palesio, Andriolo);
- ✓ Palesio and Andriolo exhibit the highest *P* (dough tenacity) and *P/L* values;
- ✓ Considering the Italian classification of flour quality (Synthetic Index of Quality), the six wheat varieties, grown under low input management, present technological features largely below the threshold limit established for the last wheat class (FB) suitable for industrial processing (110 x 10^{-4} J and 0.5 for *W* and *P/L*, respectively);
- ✓ The expected high gluten–strength cultivar (i.e. Palesio, commonly classified as “ordinary bread making wheat”, *W*>140) does not provide flour with good technological properties when grown under low N–input management;
- ✓ Both modern and old wheat genotypes grown under low–input management may supply raw material unsuitable for the industry breadmaking process;
- ✓ Baking quality of flours is not exclusively related to the protein composition or alveographic parameters, as other additional factors can interact with gluten elasticity (water absorption capacity, starch damage of flours, processing methods);
- ✓ On the basis of the panel test results, the traditional transformation (stone milling, gentle mixing, sourdough leavening) of the investigated wheat flours provides a well appreciated final product with valuable organoleptic properties;
- ✓ Despite of the low quality indicated by alveographic parameters, the six wheat varieties grown under low–input management may efficiently provide raw material for the preparation of traditionally processed bread with peculiar sensory and nutritional properties.

Section 6:

CONCLUSIONS

The current and foreseeable expansion of the low input farming (i.e. organic and biodynamic agriculture) evidenced the need to select crop varieties that satisfy the low input sector requirements. One of the major challenge of organic farming system is to provide high yields and excellent quality products utilizing agronomic practices with acceptable environmental impacts. To date, the modern wheat cultivars supplied by plant breeders lack traits of crucial interest for the low input sector such as increased competitiveness against weeds, high nutrient uptake efficiency and resistance to diseases. The organic and biodynamic systems mainly aim at stimulating the internal self–regulation of crop varieties through the functional agrobiodiversity in and above the soil, instead of external regulation through chemical protectants. Consequently, one of the most important concern for the low input sector is to obtain varieties with performance and characteristics repeatable in time, specifically adapted to particular environments. The first requirement is therefore to intensify investigation of available wheat genotypes for the agronomic traits and the physiological response to low input growing conditions. In this contents, the present research involved the use of five old (Andriolo, Frassineto, Gentil rosso, Inallettabile, Verna) and one modern (Palesio) common wheat varieties grown at three biodynamic farms of the Emilia Romagna region (Italy), during two consecutive growing seasons (2009/2010, 2010/2011). The two growing seasons were not comparable as characterized by different thermo–pluviometric features and additionally the three sites presented different micro–climatic conditions. This allowed the evaluation of the environmental effects on genotype agronomic performance, as well as nutrient and phytochemical grain accumulation. Additionally, old wheat varieties were sown as pure genotypes and in binary mixture to assess the effect of intra–specific competition on agronomic traits. Considering the limited number of investigated genotypes, no definitive conclusions can be drawn on the adaptability of dwarf or not dwarf wheat genotypes to low–input farming. However, some interesting differences were observed among the involved wheat varieties.

First relevant conclusion: **the old wheat varieties Inallettabile and Frassineto, grown pure or in binary mixture, exhibit a whole agronomic performance near to that observed for the dwarf genotype Palesio**.

On the whole, the mean grain yield obtained from the field trials aligned with those reported for organic wheat production in Italy in the last 10 years (3.2 t/ha). The highest yield was observed for Palesio but some old wheat varieties (Inallettabile, Frassineto) assured comparable grain yield values. Inallettabile and Fassinetto showed high grain yield when grown both pure and in binary mixture, additionally evidencing no detrimental effects of the intra–specific competition. The present finding highlighted that the consistent gap between dwarf and not dwarf genotypes grown under conventional farming is strongly reduced when applying low–input (i.e. biodynamic) agronomic practices. The low–input farming systems are typically characterized by unfavourable growing conditions such as low soil nutrient status and pressures deriving from weeds, pest and diseases. The introduction of dwarf genes into cereals resulted in reduced development of root systems and decreased competitiveness against weeds. On the other hand, the tall–straw *habitus* of old wheat genotypes is more prone to lodging , but it may provide a major competitive capacity against weeds and a more efficient root system.

Second relevant conclusion: **due to its peculiar** *stem* **morphology, the old wheat variety Inallettabile shows a remarkable resistance to the lodging.**

Among the old wheat genotypes, Gentil rosso presented the tallest plant *habitus* that caused the lodging of 82% of the total cropped area. Besides to the lodging problem, Gentil rosso also exhibited high intra–specific competition when grown in binary mixture, as shown by the analysis of yield components. In addition to Gentil rosso, all the not–dwarf wheat varieties were prone to lodging to various extents, in relation to their plant height. Among them, Inallettabile showed a remarkable resistance to lodging, when grown pure and in binary mixture. The peculiar stem morphology of this genotype, given by a three–lobe shape of the stem section and the presence of high–density small vessels, probably confers to Inallettabile remarkable resistance to lodging.

Third relevant conclusion: **all the investigated wheat varieties show commercial quality traits acceptable for low–input production system and additionally provide flour with excellent hygienic–sanitary quality**.

Commercial quality includes kernel characteristics (i.e. test weight, 1000–kernel weight) that provide information on milling performance and potential flour extraction of the wheat samples. All the investigated wheat genotypes showed test weight and 1000–kernel weight values within the threshold limits established for wheat flour trade in Italy, indicating a well–formed grain development. Moreover, the determination of flour mycotoxin (DON) content highlighted that, for all tested genotypes, the mycotoxin content was lower than the legislative limits set for all the food categories, as indicated in the current EU regulation. Despite of the interdiction in using fungicides, the accurate soil preparation may be the cause of limited DON accumulation in wheat grains grown under biodynamic management.

Fourth relevant conclusion: **the old wheat variety Verna presents peculiar nutritional properties (high protein, lipid and mineral content).**

The nutritional values of wheat is related to the high carbohydrate content and contemporarily the noticeable protein levels, in addition to lipid and micronutrient compounds present in lower amounts. The modern wheat cultivars fail to express their potentiality under low–input management, where the low nitrogen availability limits the accumulation of proteins in the grains. Indeed, the grain protein accumulation was in the six investigated genotypes was mainly influenced by genetic traits, with the old varieties showing the highest protein content while Palesio presented the lowest amount. Relevant diversity was observed also for the gluten protein fraction as evidenced by the gliadin and glutenin profiles.

In addition to starch and protein which are the main nutrient components of the endosperm, whole wheat grain accumulates low amounts of lipid and micronutrients (mineral elements) in the germ and bran fraction. The consumption of whole–grain derived products significantly contribute to the achievement of the recommended dietary intakes of vitamin E (lipid compounds present at 63–75 mg/kg levels) and phosphorous, magnesium and calcium elements. The old variety Verna emerged among the investigated genotypes for its relevant lipid and mineral content, showing peculiar nutritional properties. Whole grain contains several bioactive compounds conferring to wheat–derived products unique health–promoting properties, as evidenced by epidemiological studies. The overall health benefits of wheat grains have been largely attributed to their dietary fibre (DF) and antioxidant composition. Whole grain products are the most important DF source in the human diet and, based on their physiological behavior, the DF components (soluble and insoluble DF, arabinoxylans, β–glucans, resistan starch) exert different health–promoting activities. While the insoluble DFs remain largely unfermented and have a bulking effect, resulting in increased faecal output, the soluble DFs act as fermentative substrates for colon bacteria, with principal effects on glucose and lipid absorption, gut bacterial composition and anticancer activity.

Fifth relevant conclusion: **the soluble fibre fractions extracted from the old varieties Frassineto, Gentil rosso and Verna exert high prebiotic activity.**

The characterization of dietary fibre content highlighted that the investigated wheat varieties did not differ for soluble DF accumulation, while Palesio and Frassineto showed the lowest insoluble fibre content compared to the remaining old wheat genotypes. On the basis of the *in vitro* tests, the investigated wheat genotypes additionally showed qualitative differences for their DF composition, differently affecting the relative prebiotic properties. Indeed, the highest ability to selectively promote the growth of beneficial human gut bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. was obtained for the fibre fractions extracted from the old genotypes Gentil rosso, Frassineto and Verna. The old varieties Gentil rosso and Verna also presented the highest content of arabinoxylans, which valuable prebiotic properties were deeply demonstrated in literature.

Sixth relevant conclusion: **except for Palesio, the remaining red grain varieties (Andriolo, Gentil rosso, Verna) have peculiar phenolic content and composition, as well as high *in vitro* antioxidant activity.**

Whole grain possess significant amounts of antioxidant compounds (polyphenols, flavonoids) mainly accumulated in the bran fraction. Phenolic compounds provide strong antioxidant protection and the consumption of antioxidant–rich foods constitutes the primary and efficient prevention of oxidative stress–associated pathologies (cancer, cardiovascular disease). The investigation of phenolic profiles highlighted high variance among the six genotypes both in the quantity and qualitative composition of the phenolic fractions. Except for Palesio, the remaining red grain varieties (Andriolo, Gentil rosso, Verna) exhibited the highest polyphenol content as well as the highest number of detected phenolic isomers. The peculiar polyphenols composition of the red–grain old varieties is consistent with strong antioxidant power, as revealed by the *in vitro* tests.

Recently, the increasing consumer awareness towards organic foods evidenced the need to improve the breadmaking process of organic products by considering the whole production chain. Indeed, both modern and old wheat genotypes grown under low–input management may supply raw material unsuitable for the industry breadmaking process. On the basis of Chopin alveograph determinations, the six investigated wheat varieties were characterized by technological features largely below the threshold limit established for industrial flour processing.

Seventh relevant conclusion: **despite of the low quality indicated by alveographic parameters, the six wheat varieties grown under low–input management may efficiently provide raw material for the preparation of traditionally processed bread with valuable sensory and nutritional properties.**

The baking quality of flours is not exclusively related to the alveographic parameters, as other additional factors can interact with gluten elasticity (water absorption capacity, starch damage of flours, processing methods). The traditional transformation (stone milling, gentle mixing, sourdough leavening) of the investigated wheat flours provided a well appreciated final product. According to the panel test results, all the investigated wheat varieties may provided raw material for the preparation of traditionally processed bread with valuable sensory and nutritional properties. The present two–year investigation allows the description of high variability among investigated wheat genotypes in terms of nutrient and nutraceutical composition. The research may contribute the development of breeding programs aimed at selecting wheat varieties suitable for low–input farming and rich in health–promoting compounds. Considering the primary nutrient fractions and the phytochemical composition, as well as the agronomic performance, Inallettabile, Gentil rosso and Verna are the most promising genotypes for the development of nutraceutical value–added wheat varieties.

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ANNEX

Table I. Monthly mean temperatures (°C) recorded at the three biodynamic farms (Cenacchi, Ferri, Collina) during the growing seasons 2009/2010 and 2010/2011.

	Mean Temperature (°C)					
	2009/2010			2010/2011		
	Cenacchi	Ferri	Collina	Cenacchi	Ferri	Collina
Nov	9.5	9.3	9.8	7.6	9.3	2.3
Dec	1.9	3.4	3.1	2.3	2.5	2.5
Jan	1.3	0.5	0.9	3.0	3.2	1.6
Feb	6.3	5.3	4.7	6.3	6.6	4.7
Mar	8.6	11.5	8.0	12.2	10.0	8.9
Apr	15.3	12.9	14.4	17.0	17.0	15.8
May	19.0	17.8	18.6	21.5	20.5	18.6
Jun	24.2	22.3	23.4	23.9	22.8	21.5
Jul	26.3	27.1	27.3	23.5	24.5	23.1
Mean	12.5	12.2	12.2	13.1	11.0	12.9

Table II. Monthly total rainfall (mm) recorded at the three biodynamic farms (Cenacchi, Ferri, Collina) during the growing seasons 2009/2010 and 2010/2011.

	Total Rainfall (mm)					
	2009/2010			2010/2011		
	Cenacchi	Ferri	Collina	Cenacchi	Ferri	Collina
Nov	103.4	100.8	124.0	98.1	83.0	124.0
Dec	59.8	53.4	67.8	20.4	73.8	179.1
Jan	80.0	50.4	65.0	25.2	105.4	54.0
Feb	201	83.5	66.0	69.0	116.0	147.3
Mar	38.1	138.0	84.0	24.9	156.0	179.7
Apr	55.2	63.8	144.6	47.4	34.2	9.0
May	61.5	126.6	394.8	15.9	35.2	16.2
Jun	86.4	151.9	411.6	42.9	99.2	115.2
Jul	43.8	48.6	5.6	29.0	48.6	25.2
Total	729.2	817.0	1363.	372.8	751.4	849.7

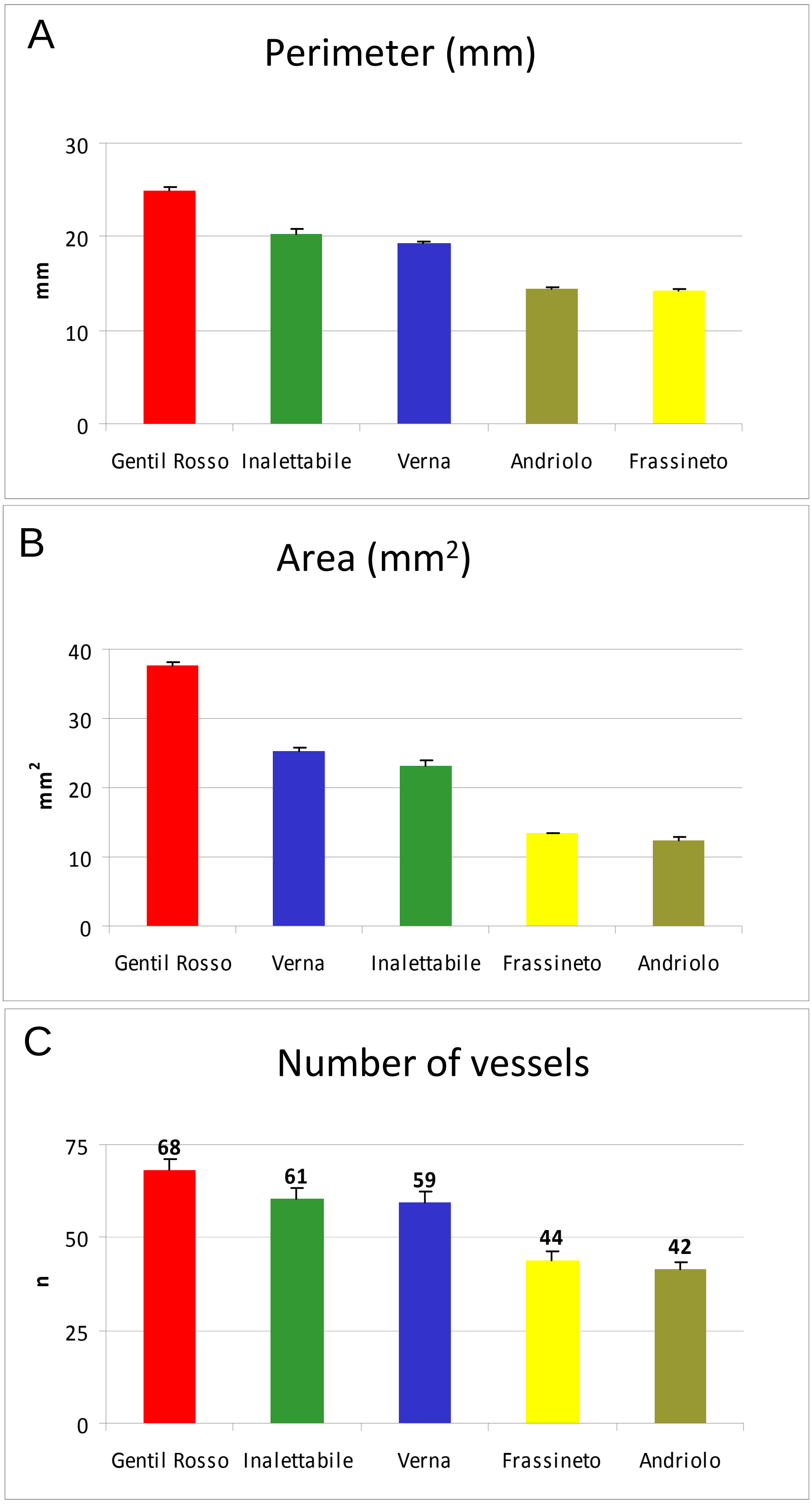

Figure I. Stem perimeter (A), area (B) and number of vessels (C) of the investigated old varieties.

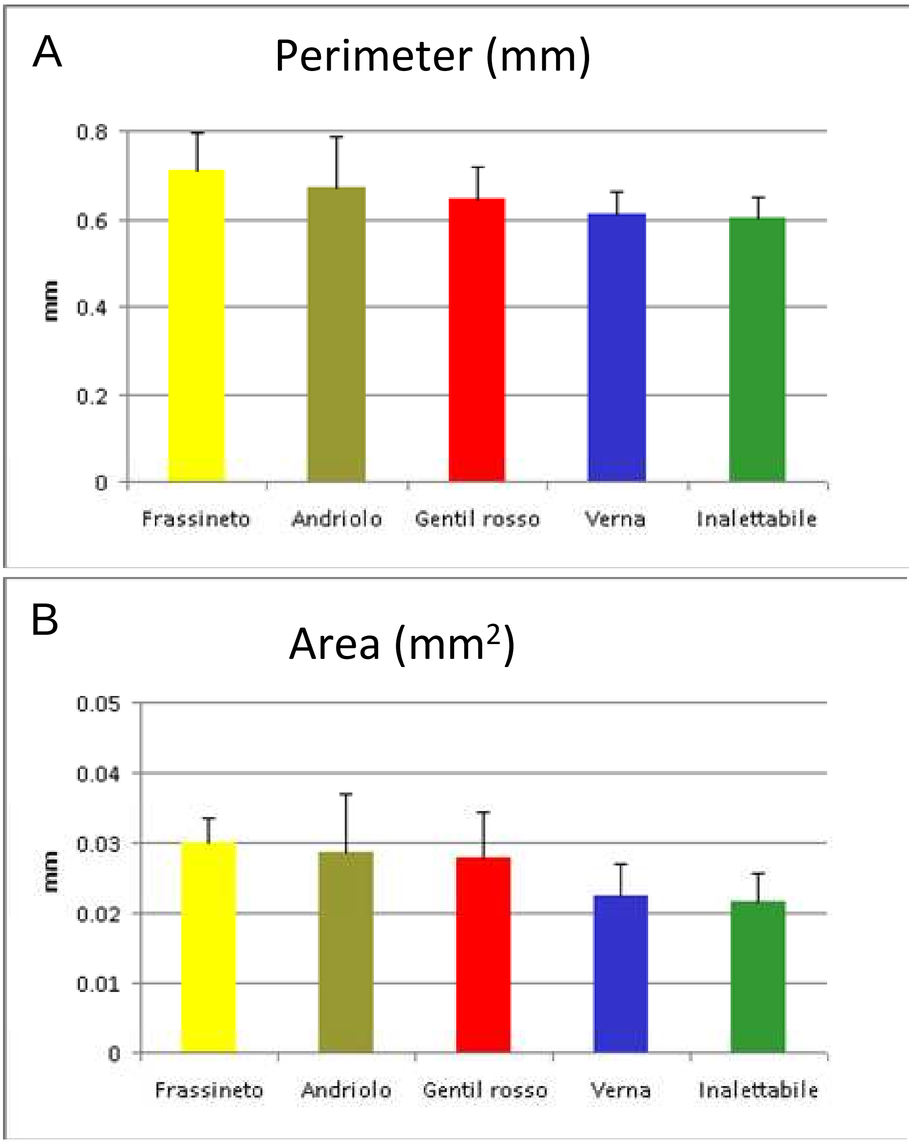

Figure II. Vessel perimeter (A) and area (B) of the investigated old varieties.

Table III. Partial and cumulated R^2 values and statistical significance (*P*) of each yield component as obtained by multiple regression analysis, for wheat varieties grown in single–genotype and mixture plots.

	Yield component	Partial R^2	Cumulated R^2	*P*
A	Spike/m^2	0.540	0.540	***
	Kernel/Spike	0.351	0.891	*
	Grain weight	0.055	0.947	*
B	Spike/m^2	0.596	0.596	**
	Kernel/Spike	0.211	0.807	*
	Grain weight	0.174	0.981	***
C	Spike/m^2	0.490	0.490	***
	Kernel/Spike	0.256	0.746	ns
	Grain weight	0.193	0.939	ns
D	Spike/m^2	0.576	0.576	***
	Kernel/Spike	0.375	0.950	*
	Grain weight			
E	Spike/m^2	0.845	0.845	***
	Kernel/Spike	0.091	0.935	*
	Grain weight			
F	Spike/m^2	0.600	0.600	**
	Kernel/Spike	0.280	0.880	**
	Grain weight	0.021	0.901	ns

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio; ns, not significant.

* = significant at $P<0.05$.
** = significant at $P<0.01$.
*** = significant at $P<0.001$

Table IV. Partial and cumulated R^2 values and statistical significance (*P*) of each yield component as obtained by multiple regression analysis, for old wheat varieties grown in mixture.

	Yield component	Partial R^2	Cumulated R^2	*P*
AB	Spike/m^2	0.545	0.545	**
	Kernel/Spike	0.183	0.728	*
	Grain weight	0.127	0.855	*
AC	Spike/m^2	0.551	0.551	**
	Kernel/Spike	0.275	0.827	**
	Grain weight	0.115	0.942	**
AD	Spike/m^2	0.522	0.522	*
	Kernel/Spike	0.317	0.839	**
	Grain weight	0.055	0.894	ns
AE	Spike/m^2	0.600	0.600	**
	Kernel/Spike	0.327	0.926	***
	Grain weight	0.021	0.948	ns
BC	Spike/m^2	0.865	0.865	***
	Kernel/Spike	0.068	0.933	*
	Grain weight	0.029	0.962	ns
BD	Spike/m^2	0.634	0.634	**
	Kernel/Spike	0.289	0.923	***
	Grain weight	0.025	0.948	ns
BE	Spike/m^2	0.678	0.678	***
	Kernel/Spike	0.134	0.813	*
	Grain weight	0.073	0.885	ns
CD	Spike/m^2	0.469	0.469	*
	Kernel/Spike	0.407	0.876	**
	Grain weight	0.078	0.954	*
CE	Spike/m^2	0.614	0.614	**
	Kernel/Spike	0.220	0.833	*
	Grain weight	0.081	0.914	*
DE	Spike/m^2	0.520	0.520	*
	Kernel/Spike	0.384	0.904	***
	Grain weight	0.013	0.917	ns

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; ns, not significant.

* = significant at $P<0.05$.
** = significant at $P<0.01$.
*** = significant at $P<0.001$

Table V. Maximum threshold limits (ppm) of deoxynivalenol (DON) in unprocessed and processed cereal products, as established by the Commission Regulation (EC) N. 1126/2007.

	ppm
Unprocessed cereals other than durum wheat, oats and maize	1.250
Unprocessed durum wheat and oats	1.750
Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	1.750
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs	0.750
Pasta (dry)	0.750
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	0.500
Processed cereal-based foods and baby foods for infants and young children	0.200

Table VI. Phenolic compounds detected by HPLC-ESI-TOF-MS in the free and bound extracts of wheat varieties.

Code	RT (min)	[M-H]-	Compound	Chemical Class	Free extract	Bound extract
C1	12.46	197.046	**Syringic acid**	Phenolic acid	C	A, C, D, E
C2	17.28	167.035	**Vanillic acid**	Phenolic acid	C, D, E, F	A, B, D, E
C3	19.17	593.151	Vicenin-2 (apigenin-6,8-di-C-glucoside)	Flavone-C-glycoside	E, F	
C4	19.94	593.151	**Vicenin-2 (apigenin-6,8-di-C-glucoside)**	Flavone-C-glycoside	A, B, D, E, F	
C5	20.05	579.136	**Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside)**	Flavone-C-glycoside	A, C, F	
C6	20.52	197.046	Syringic acid	Phenolic acid		A, C, D, E
C7	20.52	593.151	Vicenin-2 (apigenin-6,8-di-C-glucoside)	Flavone-C-glycoside	A, B, C, D, E, F	
C8	20.85	579.136	Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside)	Flavone-C-glycoside	A, C	
C9	21.09	563.14	**Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)**	Flavone-C-glycoside	A, B, C, D	C, F
C10	21.96	563.14	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)	Flavone-C-glycoside	A, B, C, F	A, B, C, D, E, F
C11	22.75	563.14	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)	Flavone-C-glycoside		C
C12	22.82	563.14	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)	Flavone-C-glycoside	A, B, C, D, F	A, B, C, D
C13	23.24	563.14	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)	Flavone-C-glycoside	A, B, C, F	A, C, D, E
C14	23.85	593.151	Vicenin-2 (apigenin-6,8-di-C-glucoside)	Flavone-C-glycoside	C, E	
C15	24.73	593.151	Vicenin-2 (apigenin-6,8-di-C-glucoside)	Flavone-C-glycoside	C, E	
C16	25.13	533.13	**Glycosylated and acetylated 3',4',5'-trihydroxy-3,7-dimethylflavone**	Flavone-O-glycoside	A, B, C, D, E, F	
C17	25.48	163.04	***p*-coumaric acid**	Phenolic acid		B, D, E
C18	25.98	533.13	Glycosylated and acetylated 3',4',5'-trihydroxy-3,7-dimethylflavone	Flavone-O-glycoside	A, B, C, E, F	
C19	26.05	535.182	**Pinosylvin (double glycosylation)**	Stilbenes	C	B, D, E
C20	26.68	577.156	**Isovitexin-2''-O-rhamnoside**	Flavone-C-glycoside	A, B	
C21	27.20	401.124	**Glycosylated pinosylvin**	Stilbenes		A, B, D, E, F
C22	27.40	769.183	**Apigenin-6-C-B-galactosyl-8- C-B-glucosyl- O-glucuronopyranoside**	Flavone-C-glycoside	F	

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio

(continued from Table VI)

Code	RT (min)	[M-H]-	Compound	Chemical Class	Free extract	Bound extract
C24	27.70	193.051	**Ferulic acid**	Phenolic acid	A, B, D	A, B, C, E, F
C25	27.90	193.051	Ferulic acid	Phenolic acid		C
C26	28.10	769.183	Apigenin-6-C-B-galactosyl-8- C-B-glucosyl- O-glucuronopyranoside	Flavone-C-glycoside	F	
C27	29.29	577.156	Isovitexin-2''-O-rhamnoside	Flavone-C-glycoside	A, B	
C28	29.84	607.167	**Methylisoorientin-2''-O-rhamnoside**	Flavone-C-glycoside	A, B, C, D	
C29	30.68	385.093	**Dihydroferulic acid**	Phenolic acid		A, B, C, D, E, F
C30	31.74	491.12	**Glycosylated 3',4',5'-trihydroxy-3,7-dimethylflavone**	Flavone-O-glycoside	A, B, C, D, E, F	F
C31	33.34	491.12	Glycosylated 3',4',5'-trihydroxy-3,7-dimethylflavone	Flavone-O-glycoside	B	
C32	33.95	577.135	**Procyanidin B-3**	Proanthocyanidin		B
C34	35.23	577.135	Procyanidin B-3	Proanthocyanidin		B
C35	36.28	385.093	Dihydroferulic acid	Phenolic acid		B, C, E, F
C36	37.18	577.135	Procyanidin B-3	Proanthocyanidin		B
C37	38.28	353.103	**Hinokinin**	Lignan	C	A, B, C, E
C38	39.22	385.093	Dihydroferulic acid	Phenolic acid		A, C, D, E, F
C39	39.50	385.093	Dihydroferulic acid	Phenolic acid		E
C40	40.22	577.135	Procyanidin B-3	Proanthocyanidin		A, B, E, F
C41	40.98	577.135	Procyanidin B-3	Proanthocyanidin		A, B, E, F
C42	45.55	357.135	**Pinoresionol**	Lignan	A, B	A
C43	46.07	269.046	**Apigenin**	Flavone	A, B	

Abbreviations: A, Verna; B, Inallettabile; C, Andriolo; D, Frassineto; E, Gentil rosso; F, Palesio.