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CORSO DI LAUREA IN: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

NUTRITIONAL QUALITY OF STRAWBERRY FRUITS OBTAINED WITH REDUCED WATER AND NITROGEN SUPPLY

Qualità nutrizionale di fragole ottenute da un apporto ridotto di acqua e azoto

TIPO TESI: SPERIMENTALE

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Le caratteristiche qualitative della fragola e le performance produttive sono influenzate da molteplici aspetti tra cui genotipo, ambiente e tecniche di coltivazione. Questa ricerca nasce per acquisire e comprendere la risposta delle piante di fragola alla riduzione di input (idrici e nutrizionali), attraverso l'analisi dei vari parametri qualitativi di 3 varietà di fragole rifiorenti: Albion, Monterey e San Andreas. Le tre varietà sono state coltivate in un'azienda agricola sperimentale del centro Italia, a regimi differenti di irrigazione (60%, 80%, 100%) e fertilizzazione azotata (60%, 80%, 100%). I parametri qualitativi presi in analisi sono stati: contenuto in acido ascorbico, folati, polifenoli, antociani, acidi fenolici e capacità antiossidante. Lo studio ha rilevato che una riduzione di percentuale idrica e azotata può essere applicata, senza influenzare negativamente la qualità del frutto. Valutando la capacità antiossidante tuttavia, si è notata una certa correlazione tra la riduzione di acqua d'irrigazione e l'aumento delle componenti antiossidanti nel frutto. Si è visto inoltre, come il genotipo della pianta influenzi anche la resa qualitativa finale: la varietà Monterey è risultata, nella maggior parte dei casi studio, qualitativamente più performante delle altre due.

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1 Introduction

The modification of lifestyle as part of today's society, has highlighted new requirements from the nutritional point of view. The food, in fact, is no longer seen only as a means of support, but as a basic element from which the individual's health depends. The growing interest in these foods with strong nutritional properties has stimulated scientific interest in those biologically active molecules of plant origin that appear to play an important role in the prevention of various diseases. In this context, the fruits and vegetables are the richest foods in bioactive compounds, and therefore showed the best effects on human health.

Among the fruits, the strawberry in particular has received increasing attention in recent years and an increasing number of studies have shown that more or less prolonged intake of this fruit can bring several benefits to consumers. The strawberry fruit nutritional properties are closely related to the high content of bioactive compounds and antioxidants molecula. This quality characteristic and its productive performance is influenced by several factors including genotype, environment and cultivation techniques. The agronomic quality of strawberry plant, as good adaptability, resistance to biotic and abiotic stress and high yield, represents the main important aspect to the farmers. The development and the improvement of new cultivation techniques and the variety innovation have allowed the increase of the plant yield. Overall demographic increase, over the years, has created a big food demand and the agricultural sector is committed to satisfy it. An intensive economic growth has determined an imbalance between the resources' exploitation and their natural regeneration capacity, consequently, the agricultural sector needs to increase the quantity and the quality of the products, reducing the inputs (water, fertilizers, plants protection) for environmental sustainability.

1.1 Strawberry: botanical description

Strawberry is a perennial herbaceous plant belonging to the *Rosaceae* family, *Fragaria* genus, and including many species (e.g. cultivated: *Fragaria* × *ananassa*, wild: *F. virginiana*). The propagation takes place both by seeds (sexually) and runners (vegetative). The plant consists of a root system, a primary stem (rhizome or crown), runners and foliage (Figure 1).

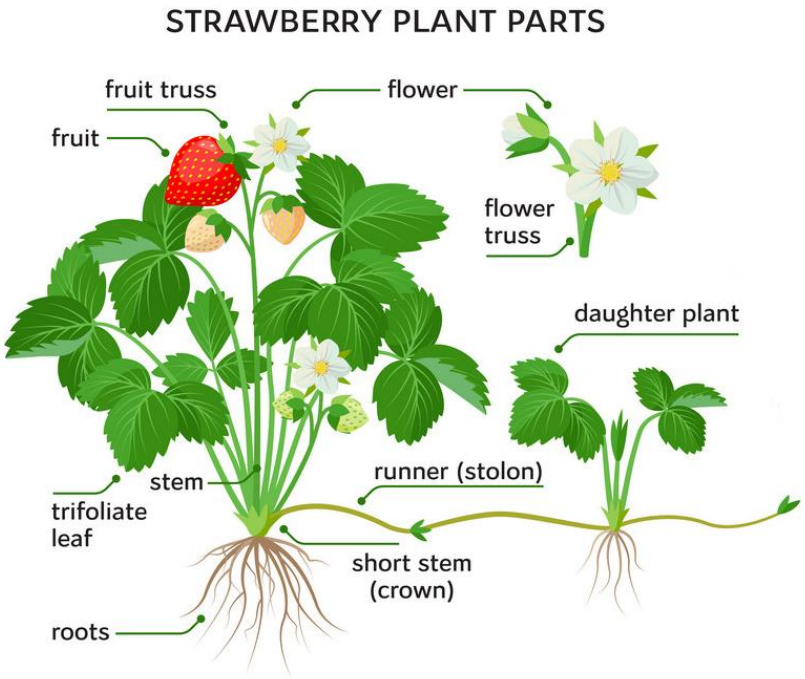

Figure 1: Strawberry plant parts, botanical drawings.

Source: https://www.vectorstock.com/royalty-free-vector/strawberry-plant-parts-botanical-drawings-vector-31087617

The crown is the primary stem: the leaves grow in rosette at the top, while roots and stolons develop at the base of the crown. Some of the axillary buds can become branch crowns or long internodes called runners. The runners generate a new rosette, with a node having small roots. The plants obtained by runners have an identical genetic characteristic compared to the mother plant. The vegetative development (e.g.: the formation of

runners and floral initiation) is influenced by the environmental factors (temperature, nutrition, water) and their interactions. The root system originates from the rhizome. The roots are responsible for the absorption and transport of nutrients and water to the crown and for the storage of reserve substances The foliar structure of the strawberry plant consists of petioles supporting leaves in groups of three separate leaflets, called trifoliate leaves. The size of the petioles is usually utilized as a plant vigor parameter, as an indicator of plant response to environmental conditions. The inflorescences are ramified and represent the terminal structures of the development of the crown axis (Anderson and Guttridge, 1975). The stamens correspond to the male part of the flower, containing the pollen to fertilize the pistils, the female part placed on the receptacle. After fecundation, the achenes, commonly called seeds, are generated, and represent the true fruit. The edible fruit is actually a false fruit formed by the enlargement of the receptacle tissue (Figure 2).

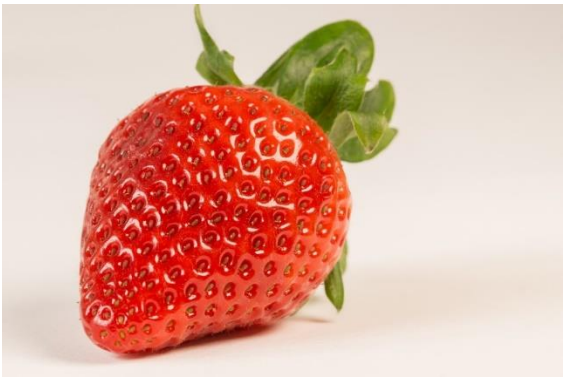

Figure 2: Strawberry fruit.

Source: https://www.pexels.com/it-it/foto/cibo-delizioso-dieta-dolce-934066/

1.2 Strawberry: classification criteria for cultivars

The classification of the available strawberry cultivars keeps in consideration some pomological and behavioural characteristics of the plant. One of the classification criteria is how different cultivars react to

the photoperiod and so they can be classified as perpetual flowering (PF), classifying different types of plants identified also as remontant or recurrent, ever-bearing, day-neutral plants or long day) and seasonal flowering (SF), classifying different types of plants identified also as non-remontants, short day or June-bearing.
These two groups of cultivars are distinguished by the following characteristics:

Perpetual flowering → they produce fruits more times per year, due to their different sensitivity to day length in relation to the temperature for flower induction. The long-day cultivars differentiate flowers with a light period longer than 14 hours and produce more berries from spring until autumn. They have a good spread on an industrial scale, but they are used almost exclusively at familiar level for their slow reproduction. The day-neutral cultivars differentiate flower buds with various lighting conditions, provided that the thermoperiod is respected.

Seasonal flowering → they provide only one harvest in spring-summer, as a result of flower induction that took place in the previous late summer-autumn, when their termophotoperiodic requirements for flower initiation were satisfied by short-days (less than 11-16 h) or low temperatures (9-21°C, optimal 15-18°C). A minimum number (7-14) of short-day cycles is required for flower induction, according to cultivar, temperature and day length. Under long-day condition, the terminal apex of the crown remains vegetative and many runners develop from the axillary buds.

1.3 Strawberries: cultivation techniques

Strawberry is a particular fruit species that can grow in different soils; it prefers generally sandy soils with a good and proper drainage. It also

prefers sub-acid or acid soils with a pH between 5.5 and 7. The presence of pH higher than 7 can cause some disorders to the plant: for instance, the strawberry plant may hardly absorb iron, and other damage may be related to less marked yellowing of leaves, also known as chlorosis.

The first step of strawberry cultivation is the preparation of soil: the species requires a careful preparation, in order to avoid water logging that can lead to roots diseases. It is necessary to plow and level the ground (Figure 3), mainly in those soils that have never hosted strawberry, and arrange the drainage network. A second and more superficial processing at a depth of 20-25 cm is helpful, for burying the organic substance (possibly manure) and organic fertilizers useful for the development of the plant.

Figure 3: Leveling the ground.

Source: https://aziendaagricolasantoro.wordpress.com/2016/07/10/in-tenuta-da-lavoro/spianatura-del-terreno/

The rotation is an important process to set up between different cultivation seasons. It is necessary to avoid stunted development problems, stress or collapse of the plant. The objective of rotation is to improve soil structure, maintaining chemical fertility and reducing the presence of pathogens. Other important aspect is related to the crop's rotation choices: for

strawberry is recommended the rotation with pea and bean, that improve both structure and fertility of the soil. If the rotation is not possible, the sterilization of the soil is an important and widespread practice and represent an important aspect that should be taken in account before strawberry cultivation. The solarization is one of the most used sterilization methods (Figure 4). To obtain a proper solarization action it is necessary to cover the soil with green or transparent polyethylene films (PE), for 4-8 weeks. The proper solarization and the combination time/temperature should guarantee an optimal effectiveness against various telluric pathogens.

Figure 4: Transparent polyethylene film is applied to solarize a field.
Source: http://calag.ucanr.edu/Archive/?article=ca.v054n06p42

The strawberry field cultivation is traditionally carried out on well baulate row, with a height from 10 to 30 cm variable depending on the texture of the soil (Figure 5). This plantation maintains the plant in a drier microclimate, avoiding dangerous water logging, and limits the danger of fungal infections on the fruits. The mulching film is a dark polyethylene plastic film with holes usually placed at a distance of 30-40 cm.

Figure 5: Strawberries mulching plastic film.

Source: https://www.indiamart.com/proddetail/strawberry-mulching-paper-21261013662.html

The plantation period depends on the area and type of cultivation adopted. For instance, in mountain regions the planting begins in early June, in the northern plains environment the plantation begins in mid-June. In the southern regions the plantation operations begin in mid-August. The harvesting represents the last step of the entire cultivation. It is also the most delicate operation, and it requires special care. The fruits shall be harvested when they reach the red color on the overall surface. The detachment of the fruits shall be made manually for fruits destined to fresh consumption, while it may be done mechanically only for the products intended to industry.

The most common cultivated strawberry plants are the Frigo plants, the Bare root fresh plants, the Fresh plug plants and the Waiting bed. The Frigo plants are cold stored plants (-2°C), useful for a production flow immediately after planting, exploiting the flower buds differentiated in nursery, for autumn-spring programmed crops in soil and outside soil. The Frigo plants are distinguished in A- (collar diameter: 7-8 mm) and A+ (collar diameter: >16 mm). The Bare root fresh plants are cultivated in

nursery, on fertile and sandy soils with a good water availability. These plants are mechanically cut just above the collar, usually at the beginning of October, and placed in wooden boxes in plastic film bags. The Fresh plug plants are large in size plants on peat substrate, in alveolate plastic containers. They are derived from young runners, with small roots. The Waiting bed are large in size plants originated by Frigo plant (A-), Bare root plants or Plug plants produced in special waiting beds. The plants are finally planted towards the end of June and early August.

1.3.1 Influence of water in strawberry performance

The strawberry plant has an extremely shallow root system; the roots have an extension of about 20-25 cm deep in soils. The depth of soil with available water changes according to the soil type: for example, it is 10 mm in sandy soils and 24 mm in medium-textured soils. The susceptibility to water stress is mainly due to the limited depth and poor efficiency of the root system. Water deficiency can be defined an insufficient availability of water that leads to the reduction of numerous physiological and biochemical process in all plant organs. This condition leads to stunted growth, difficulty in the formation of reproductive organs and in the accumulation of reverse substances (Singer et al., 2002). The insufficient amount of water causes a limited starch accumulation in the roots and the reduction in the buds' number, that turns in a yield loss greater than 25%. On the other hand, a water excess can cause root asphyxia or plant collar rot, that brings significant losses in production. The marked deterioration in the fruit sensory qualities (a reduction in sugar content, an increase in acidity) is determined by excessive water application. The determination of correct irrigation requirements and appropriate irrigation system choice are the prerequisite to obtain good productive results in strawberries, especially in protected system, where the only water input is the irrigation. Therefore, the irrigation management must be carried out in the most

effective way, in order to save water and maximize its utilization (Fereres and Sorano, 2007; Xiloyannis et al., 2012). The results reported in Krüger et al. (2000) show that maximum plant yield is obtained in slight water deficit (-20 kpa). Fruit firmness decreases in case of lower water supply. Furthermore, Kirnak et al. (2003) demonstrated that there is a loss of fruits weight and a minor production in severe drought conditions, while a little reduction of water does not generate any effect on yield. High water reduction causes a decrease of fruit number and weight and of total production (Davies and Albrigo, 1983; Gehrmann, 1984; Yuan et al., 2004; Johnson et al., 2008). Bordonaba and Terry (2008) explains that an irrigation system focused on reducing water consumption between flowering and harvest phases can be a right compromise for increasing fruit quality in “Elsanta”, “Sonata”, “Simphony” and not have a negative influence on berry size in “Christine” and “Florence”. Adak at al., (2017) explains that the nutritional parameters (Total Antioxidant Capacity, Total Phenolic Content) are positively influenced by water stress. Water stress in strawberries also influences the fruit biochemical properties as well as yield and quality (Bordonaba et al., 2010). In recent studies, it has been observed that, in Elsanta cultivar, water stress increased some taste- and health-related compounds (Terry et al., 2007), raised the sugar and acid rate as a fruit taste indicator (Bordonaba et al. 2008), without any influence on the vitamin C and protein content and increased total soluble carbohydrates and proline content (Ghaderi et al., 2011). In addition, there are variations among strawberry cultivars with respect to sugar content (Davik J. et al 2006; Wang S.Y. et al, 2002). Under water stress conditions, glucose and fructose concentrations were determined to be 1.2-fold higher than the control treatment (Bordonaba et al., 2010). Saied et al. (2005) reported that total soluble solid content decreased under stress conditions. Hence, the screening of cultivars to stress conditions should be evaluated according to region. The irrigation method could influence the water amount optimization: the surface drip irrigation positively stands out, unlike other methods. In geographical regions characterized by water

deficiency, the use of irrigation allows to counteract this scarcity and to achieve a rational strawberry production. Weber et al. (2016) explains that deficit irrigation significantly increased the content of sugars (from 1.1- to 1.3 fold), organic acids (from 1.1- to 1.3-fold), their ratio (from 1.1- to 1.2-fold) and the content of most identified phenolics in cv. 'Flamenco'. Conversely, higher amounts of total sugars and organic acids (1.7- to 1.8-fold) were detected in 'Eva's Delight' strawberries at upper limit of field capacity (UFC) and lower limit of field capacity (LFC) irrigation. Deficit irrigation generally decreased strawberry yield of cv. 'Eva's Delight'. In Tunc et al. (2019), different watering amounts (full irrigation until 75% deficit irrigation) were applied with different irrigation systems. Surface drip irrigation with black polyethylene mulch (MD) resulted the best system for plant productivity and fruit quality; mild stress (-25% irrigation amount than control) with MD showed high plant yield and fruit quality similar to full irrigation. Fruit firmness, soluble solids content, and acidity had higher values in the lowest irrigation trial. Therefore, the mild stress has been tested as a method to reduce the water use, but the qualitative and productive performances depend on many variables (genotype, irrigation system, crop cycle phase, stress duration).

1.3.2 Influence of nitrogen in strawberry performance

The strawberry plants, subjected to different fertilizing techniques and products, are able to produce better productive and qualitative results, due to a balanced ratio between supply (fertilizers) and plant demand (nutritional requirements) (Tagliavini et al., 2004). Knowledge of the plant's nutritional requirements is a fundamental aspect for environmental and economic sustainability. However, fertilizers should be applied efficiently, taking seasonal conditions into account. This means to apply the proper amount of nutrients for good crop growth without providing excess nutrients, that may be lost into groundwater or surface waterways. Nitrogen is an essential nutrient for plant development and for a good qualitative and quantitative production. The runners and shoot formation are stimulated by nitrogen

availability; an excess of this element, due to fertilization, accelerates their development at the expense of flower induction (Fujimoto, 1972; Furuya et al., 1988). Therefore, the reduction of nutrients can determine a retarded plant growth and a stimulation of the floral induction (Guttridge, 1985). The excessive supply of nitrogen can lead to low fruit firmness and plant yield, to fruits rot and malformation, to a retarded maturation and to pests and diseases increase (Voth et al., 1967; May and Pritts, 1993). Nitrogen deficiency can manifest symptoms such as undersized fruits and yellowish-green foliage. There is also a reduction in vegetative growth and runner production (Johanson, 1980; Pritts et al., 1998). The increase of N level in the nutrient solution (40, 80, 120 and 160 mg L-1) has a significant impact on increment of runner number and on decrease of fruits soluble solid content (Trejo-Tellez and Gomez-Merino, 2014). Faedi et al. (2006) show that the effects of nitrogen fertilization depend on the doses and administration periods.

1.4 Sensorial quality and nutritional quality of strawberry fruits

The sensory quality of strawberry is the result of a complex balance among sweetness, aroma, texture, and appearance. Some authors have investigated the relationship between sensory quality attributes and instrumental analysis (Hoberg and Ulrich, 2000; Pelayo et al., 2003; Pelayo-Zaldívar et al., 2005; Schulbach et al., 2004; Shamaila et al., 1992; Ulrich et al., 1997; Wozniak et al., 1997). Sugar and volatile contents were found to be important biochemical components that influence consumer acceptance. The influence of volatile compounds on the flavor quality of the strawberry was widely studied, and among the hundreds of volatiles identified in fresh strawberry, only a small portion contribute to aroma (Dirinck et al., 1981; Larsen and Poll, 1992; Pérez et al., 1992; Ulrich et al., 1997). Esters

are known to be important contributors to typical strawberry aroma (Gomes da Silva and Chaves das Neves, 1999; Larsen and Poll, 1992; Pérez et al., 1992; Schieberle and Hofmann, 1997). Among them, methyl and ethyl butanoates, methyl and ethyl hexanoates, and ethyl 2-methyl butanoate are often mentioned as active aroma compounds in strawberry. Furanones (furaneol and mesifurane) are also contributors to aroma providing sweet caramel-like notes (Larsen and Poll, 1992; Pérez et al., 1996; Sanz et al., 1994). Additionally, aldehydes and alcohols such as n-hexanal, (*Z*)-3-hexenal, and (*E*)-2-hexenal are responsible for green and pungent notes (Schieberle and Hofmann, 1997; Schulbach et al., 2004).

Strawberries represent a healthy food choice. They are low in total calories, with a 100 g serving providing only 32 kcal, and their sweet flavour makes them a delicious snack alternative to processed foods. At the same time, they are an important dietary source of bioactive compounds, most of which are natural antioxidants that contribute to the high nutritional quality of the fruit. Strawberries contain fat-soluble vitamins (i.e. vitamin A and tocopherol) and carotenoids (i.e. lutein and zeaxanthin), but one of the aspects of major nutritional relevance is the extremely high content of vitamin C, even higher than citrus fruits. A handful of strawberries are sufficient to cover the vitamin C recommended daily allowance (Carr & Frei, 1999). Together with vitamin C, folate plays a crucial role in emphasising the nutritional quality of strawberry when considering that, among fruit, it is one of the richest natural sources of this essential micronutrient and that folate is an important factor in health promotion and disease prevention (Tulipani, Mezzetti, & Battino, 2009; Tulipani, Mezzetti, et al., 2008; Tulipani, Romandini, et al., 2008). Moreover, both its dietary fibre and fructose contents may contribute to regulate blood sugar levels by retarding the digestion, while the fibre content may control calorie intake by its satiating effect. Finally, strawberries are also found to be an excellent source of manganese, so that a serving of strawberries may provide more than 20% of the daily adequate intake (AI) for this mineral.

The same amount of strawberries are able to provide about 5% of the AI for potassium, and has been qualified as a good source of iodine, magnesium, copper, iron and phosphorus (Giampieri et al., 2012).

1.5 Ascorbic acid

Ascorbic Acid, also known as Vitamin C has the chemical formula C6H8O6 (Figure 6). Vitamin C is a water-soluble vitamin essential for humans, and it is a potent reducing agent.

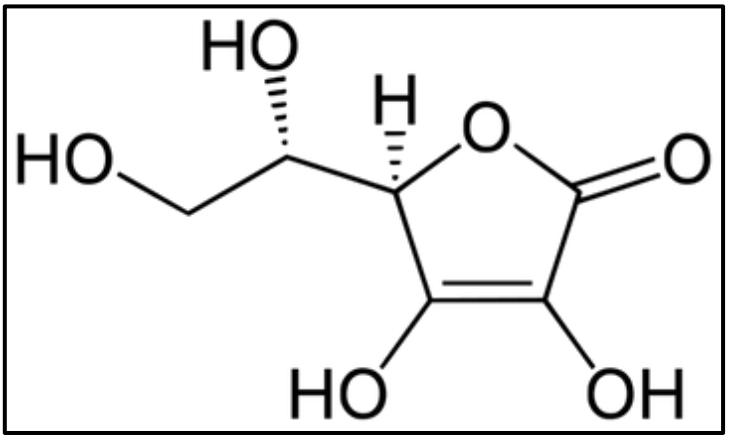

Figure 6: Ascorbic acid.

Source: https://www.chimica-online.it/composti-organici/acido-ascorbico.htm

Although it can be found in all plant cell compartments and tissues, the level of ascorbic acid varies among them (Davey et al. 2000; Lorence et al. 2004). This vitamin, as others, may not be synthetized by human-body, for this reason it shall be taken from diet. Several biosynthesis pathways for vitamin C are being discussed but are still not completely understood (Davey et al. 2000; Cruz-Rus et al. 2011; Smirnoff and Wheeler 2000). Its biosynthesis in plants depends on various factors and can vary between organs. Of course, fruits and vegetables are the main sources of this kind of vitamin. Strawberries average vitamin C content is 54 mg/100g, this

value makes that fruit one with the highest vitamin C content (US Department of Agriculture 2017). Only guava, kiwi and papaya have higher amount of vitamin C. Instead agrumes, like orange, lemon and clementine provide a similar, even slightly lower amount of vitamin C (~ 50 mg/100 g). The main function of vitamin C on human-body is to ensure the proper collagen biosynthesis. Collagen is the main protein that maintains the integrity of fibrous tissues as connective tissue, bone matrix, tendons and skin. Vitamin C is an important antioxidant for the organism, as it acts as electron donor to free radicals. It prevents oxidative damage in tissues by preventing oxidation of LDL (Tappi, 2018). It also enhances the bioavailability of iron in the body; among the several functions it exerts, they are to be underlined also stimulation of the immune system and fighting against infections. A proper vitamin C intake showed to reduce risk of cardiovascular diseases and cancer (Li and Schelhorn 2007; Hemilä 2017; Locato et al. 2013; Giampieri et al. 2012; Carr et al. 2012). The absorption of vitamin C occurs in the small intestine with Na+ dependent transport. Absorption is almost total at low doses, while decreasing up to 16% at higher doses. If the intake of vitamin C excesses the normal daily doses, it can be eliminated with urine or sweat. This vitamin can be stored in the body (liver and supernal glands), the storing is useful, giving that it can be used whenever the body requires this vitamin (Tappi, 2018).

1.6 Folates

Folates (Figure 7) is a group of water-soluble vitamins, also known as vitamin B9; the name folate usually outlines a class of compounds with chemical structures related to pteroylmonoglutamic acid and generally recognized as folic acid (FA, vitamin M, B9 or B11) (Deconinck et al, 2011). Folate is a vitamin that has only recently been appreciated for its importance beyond its essential role in normal metabolism, especially for

its relevance to the etiologies of chronic diseases and birth defects. Folates occur in a wide variety of foods of both plant and animal origin. Liver, mushrooms, and green leafy vegetables are rich sources of folate in human diets, while oilseed meals (e.g., soybean meal) and animal by-products are important sources of folate in animal feeds. The folates in foods and feed-stuffs are almost exclusively in reduced form as polyglutamyl derivatives of tetrahydrofolic acid (FH4). Very little free folate (folyl monoglutamate) is found in foods or feedstuffs.

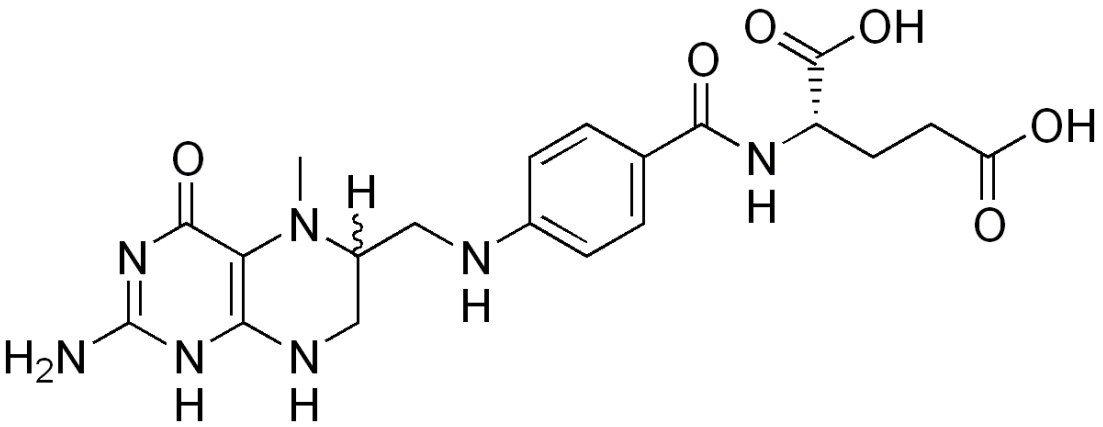

Figure 7: L-5-methyltetrahydrofolate.
Source: https://en.wikipedia.org/wiki/Levomefolic_acid

The biological availability of folates in foods has been difficult to assess quantitatively. Estimates are variable among foods, but generally indicate bioavailability of about half that of folic acid; a recent study found a relatively high (80%) aggregate bioavailability of a mixed diet. In general, folates appear to be less well utilized from plant-derived foods than from animal products. Several factors affect the biologic availability of food folates:

- Anti-folates in the diet. Folates can bind to the food matrices; many foods contain inhibitors of the intestinal brush border folate conjugase and/or folate transport;
- Inherent characteristics of various folates. Folate vitamers vary in biopotency;
- Nutritional status of the host. Deficiencies of iron and vitamin C status are associated with impaired utilization of dietary folate. Vitamin C has also been shown to enhance the utilization of 5-methyl-FH4 by preventing its oxidative degradation to 5-methy-FH2, which does not enter the folate metabolic pool (Gerald F. Combs, Jr, 2012).

1.7 Polyphenols

As stated above, strawberry is a fresh fruit with many nutritional compounds as vitamins (mainly vitamin C followed by vitamin B1, B2, B3, B6, B9, A, E and K) (Strålsjö et al. 2003; Tulipani et al. 2008b). But, besides the numerous micro and macro-nutrients, it is possible to find many non-nutrients components with very interesting properties for human health. The most represented category of the non-nutritional compounds in strawberries are called polyphenols. The term “non-nutritional” means that those compounds are not strictly necessary for the health of the humans, and their absence in the diet did not lead to any disease. However, their assumption with the diet is absolutely suggested, giving that they possess many interesting healthy properties, and they can significantly help the human body in the prevention of many diseases (Balasundram et al. 2006; Giampieri et al. 2012; Tulipani et al. 2009b; Alvarez-Suarez et al. 2014). Polyphenolic compounds are the main responsible of the antioxidant properties of strawberries. In plants and vegetables, they constitute secondary metabolites, and they are responsible for coloring, tastes and

flavors, but the main characteristic is to act as radical scavengers. Furthermore, they can also interact with proteins and protect the plant from an excessive UV radiation. Finally, polyphenols may help the host from other stress situations, e.g. pathogens attacks, leading role for chemical defense mechanism (Ozcan et al. 2014; Dixon and Paiva 1995; Bravo 1998). Polyphenols are present in three different classes (Figure 8): flavonoids (e.g. anthocyanins), non-flavonoids (e.g. stilbens, lignans and tannins) and phenolic acids (Tappi,2018).

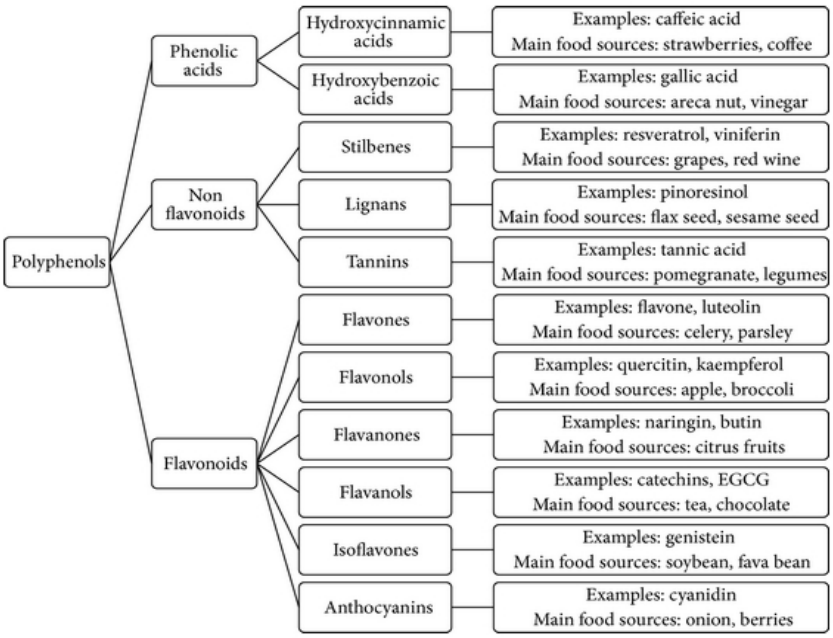

Figure 8: Polyphenols.

Source: https://pubmed.ncbi.nlm.nih.gov/26180597/#&gid=article-figures&pid=figure-2-uid-1

1.8 Anthocyanins

Anthocyanins belong to flavonoids group, whom basic chemical formula consists of two aromatic C6 rings linked with a C3 structure. In strawberries, anthocyanins are the most represented compounds of the group of flavonoids, followed by flavonols (especially quercetin and

kaempferol derivatives) and flavanols in lower quantities (Tulipani et al. 2009b). In nature exist 23 different anthocyanidins, but most often glycosides of cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin are found in plants (Belitz et al. 2008). Pelargonidin-3-glucoside (pel-3-glu) is the quantitatively most important anthocyanin in strawberries, followed by pelargonidin-3-rutinoside (pel-3-rut) and cyanidin-3-glucoside (cya-3-glu). Altogether more than 25 different anthocyanins were detected in strawberries (Lopes-da-Silva et al. 2007). Many scientists, in last years, have focused their attention on the anthocyanins effect on human body. It is well known that these compounds have a good antioxidant action, not just in plants, but also in human body. Their main property is to scavenge free radicals that can lead to unwanted diseases. This is the reason why, foods or beverages with high anthocyanins content can be labelled as food with functional properties, due to their preventive effect on cardiovascular diseases, stroke and cancers insurgence (Algarra et al. 2014; Rimbach et al. 2015; Pandey and Rizvi 2009). Typically, anthocyanin contents found in strawberries are between 15 and 60 mg/100 g FW, but also higher values up to 80 mg/100 g FW were measured by some researchers (Giampieri et al. 2012).

1.9 Phenolic acids

Phenolic acids constitute another important group of beneficial compounds in strawberries belonging to phenolic compounds, and they can be divided in two subgroups. The first one is known as hydroxybenzoic acids: these acids content is generally very low in fruits and vegetables, with the exception of certain red fruits (strawberry), black radish and onion, which can have high concentration (several tens of milligrams per kilogram FW). Among these acids, the most common is gallic acid: for instance, tea is an important source of gallic acid, due to tea leaves that may contain up to 4.5

g/Kg FW. The second subgroup is recognized as hydroxycinnamic acids: these are more commonly present in many foods than hydroxybenzoic acids. They are: p-coumaric acid, caffeic acid, ferulic acid and sinapic acid. These acids are rarely found in the free form, except in processed food that has undergone freezing, sterilization, or fermentation (Giampieri et al. 2012, 2013; Määttä-Riihinen et al. 2004; Mattila et al. 2006; Tappi, 2018). Moreover, one of those acids (ellagic acid) is not so commonly find in foods. However, strawberries, and few other berry species, have a relevant quantity of this compound. Ellagic acid is a gallic acid dimer and is rarely present in its free form. More often, it occurs bound in ellagitannins, which belong to the phenolic group of hydrolysable tannins. Through acid hydrolysis, those complex compounds decay and ellagic acid is released (Koponen et al. 2007; Tulipani et al. 2008a; Nile and Park 2014).

The main characteristics of phenolic acids is their antioxidant and anti-inflammatory properties, but they can also exert other important activities, like anti-mutagenic, anti-carcinogenic and anti-allergy (Koponen et al. 2007; Giampieri et al. 2012, 2013). The biosynthesis of phenolic acids is the same of anthocyanins, because natural phenols in plants are derived from shikimate pathways. The polyphenolic substances derive from the biosynthetic pathway of shikimic acid and from the synthesis of the aromatic amino acids phenylalanine and tyrosine. Shikimic acid is the starting point for the synthesis of the chorismic acid, from which the aromatic amino acids derive (phenylalanine is the most important). From here, the phenylpropanoid pathway begins, through the production of cinnamic acid and para ceramic acid, precursor of all the following compounds.

1.10 Antioxidant capacity

The total antioxidant activity (TAC) is a common parameter used for determining nutritional quality as it is directly related to the quality of

bioactive compounds in the fruits. In strawberries, predominantly vitamin C, but also phenolic compounds are responsible for the TAC (Giampieri et al. 2013; Määttä-Riihinen et al. 2004; Tulipani et al. 2008a). Reactive oxygen species, such as peroxide or hydroxyl ions, cause oxidative stress in cells which leads to damages that can result in several diseases. Antioxidant compounds can donate an electron to those highly reactive free radicals which thereby become neutral again, consequently reducing the oxidative stress in cells and lowering the risk of disease (Tulipani et al. 2009b). Strawberries are among the fruit with the highest TAC levels, exceeding those of raspberries, apples, peaches, pear, grapefruit and even oranges and kiwis. Only a few fruits such as blackcurrant and blueberry show a higher TAC (Halvorsen et al. 2002; Wang et al. 1996; Proteggente et al. 2009; Wang and Lewers 2007; Wang and Lion 2000; Kalt et al. 1999).

2 Purpose of the thesis

The most important challenges of the agricultural production system are to reduce the use of precious resources like water and to limit the utilization of fertilizers, which could have negative effects on the ecosystem. The objective of the study was to test innovative cultivation strategies to manage the multiple and variable exogenous factors (climate-environmental changes, reduction of non-renewable resources), maintaining a high fruit quality level.

In this thesis, the qualitative parameters of fruits from perpetual flowering cultivars (“Albion”, “S. Andreas”, “Monterey”) under different water and nitrogen fertilization regimes were evaluated. The quality of the strawberry was measured in terms of ascorbic acid, folate, polyphenols, anthocyanins, phenolic acid content and antioxidant capacity.

3 Material and methods

3.1 Plant materials

The experimental trials took place at the ASSAM (Agenzia Servizi al Settore Agroalimentare delle Marche) experimental farm in Petritoli, Marche region by using PF cultivars cultivated in one production cycle: the plants were planted on 24/04/2019 in soil, covered by a plastic tunnel, and the fruits were collected in summer 2019. The cultivars object of study were “Albion”, “San Andreas” and “Monterey”.

“San Andreas” is an early Remontant cultivar and produces high quality fruits. The production period is very similar to “Albion”. At the beginning of the season, the plant vigor is higher than “Albion” but the berry size throughout the fruiting season is similar to “Albion”. This variety has a good productivity, while the fruit color is slightly lighter than “Albion”. The plant is rustic, and resistant to diseases and stresses. The fruit taste is good. This variety produces few runners.

“Albion” is a Remontant cultivar. The fruit is long, conical and symmetrical. “Albion” produces consistently throughout the season. This variety produces a lot of runners and is necessary to cut them in order to increase plant yield.

“Monterey” is a Remontant cultivar, with slightly stronger flowering than “Albion”. The production is similar to “Albion”. Its plant is vigorous. The fruit is slightly larger but less firm than “Albion”, and quite sweet. “Monterey” is considered to be have good resistance to diseases. (https://research.ucdavis.edu/industry/ia/industry/strawberry/cultivars/).

3.2 Experimental design

The experimental trial was set up following a split-plot design, with 3 different levels of water supply and 3 different levels of nitrogen supply (6 main blocks), repeated for 3 cultivars (San Andreas, Albion, Monterey). Each cultivar represented a sub-block and was composed of three replicas called "plots", for a total of 54 plots in the experimental design (6 blocks x 3 cultivars x 3 replicas). Each plot was composed of 8 plants. Strawberry samples were harvested and collected in 6 different harvest dates: 15th July 2019, 22nd July 2019, 29th July 2019, 5th August 2019, 12th August 2019, and 19th August 2019. For each harvest date, ten full ripe and safe strawberry fruits were collected by the 6 central plants of each plot (Table 1 and Table 2).

Strawberry cultivar	**Block A**	**Block B**	**Block C**
Albion	1, 16, 22	2, 17, 23	3, 18, 24
Monterey	4, 10, 25	5, 11, 26,	6, 12, 27
S. Andreas	7, 13, 19	8, 14, 20	9, 15, 21

Block A	100% Nitrogen
Block B	80% Nitrogen
Block C	60% Nitrogen

Table 1: the organization of the strawberry fruits' plots, based on nitrogen supply. Plot numbers are reported.

Strawberry cultivar	**Block D**	**Block E**	**Block F**
Albion	28, 43, 49	29, 44, 50	30, 45, 51
Monteray	31, 37, 52	32, 38, 53	33, 39, 54
S. Andreas	34, 40, 46	35, 41, 47	36, 42, 48

Block D	100% Water
Block E	80% Water
Block F	60% Water

Table 2: the organization of the strawberry fruits' plots, based on water supply. Plot numbers are reported.

3.3 Fruit sampling

The fruits were sampled in six different days: 15 July 2019; 22 July 2019; 29 July 2019; 5 August 2019; 12 August 2019; 19 August 2019. Ten fruits were collected by six plants at the center of each plot, and fruits deriving from the three replicas of each cultivar were pooled together. The harvest of the strawberries follows some specific standards: the strawberries had to be sound, full ripen, not spoiled or injured and homogenous in size. The strawberries were stored in plastic bags at -18°C in laboratory freezers till the day of extraction. At that time, 5 strawberries were chosen from each bag, and each fruit was cut in 4 pieces: for the analysis were utilized only ½ of the fruit, deriving from opposite faces of the fruit, to avoid bias connected to the influence of sunlight during cultivation (Figure 9). The strawberry pieces were finely chopped and weighed: 10g for the methanolic extract suitable for the detection of polyphenols, anthocyanins, phenolic acids, and antioxidant capacity; 1g for the extraction of vitamin C; 2g for the extraction of folates (Figure 10).

Figure 9: Strawberries of the experimental field.

Figure 10: Chopped strawberries pieces.

Finally, after the extraction process, the fruit samples have been analyzed through two methods: HPLC, to detect vitamin C, folates and phenolic acids; spectrophotometry, to evaluate polyphenols, anthocyanins and antioxidant capacity.

3.4 Ascorbic acid analyses

3.4.1 Extraction

The 1g of strawberry samples were stored in falcon tubes at -18°C, till the beginning of the extraction. At the extraction day, 1 L of the extracting solution has been prepared (1L Milli-Q H_2O; 1µL DTPA; 5% Metaphosphoric acid) and 4 ml were added to each falcon tube. Vitamin C was extracted by grinding the strawberry/solution samples with ultraturrax-T 25 (Janke & Kunkel, IKA-Labortechnik), followed by sonication, centrifugation at 4000 rpm for 10 min at 4°C, and filtration. Finally, 1 mL of filtrate was taken by each falcon tube, put in an Eppendorf tube and stored at -20°C till the chromatographic analysis.

3.4.2 HPLC analysis

The HPLC methodology, acronym of High Performance Liquid Chromatography, is the evolution of liquid chromatography on column. This technique shows many advantages than others conventional technique, for example:

- It is faster during the extraction process, it performs with higher efficiencies;
- It has better and clearer resolutions in substances peeks;
- It shows simultaneously the compounds outgoing from the column;
- It records every moment of the chromatography process;
- It determines quantitatively the components of the mixture.

The HPLC system (Figure 11) works with two different phases: mobile phase and stationary phase (i.e. the column). The column is packed with micro-scale beads and these interact with all the components and compounds present in the liquid that flows in the overall system (i.e. mobile phase). Thus, the mobile phase held in solvent reservoirs, is pumped through the system by pumps at constant flow rate. In our case, we utilized HPLC system for the four analysed compounds (Vitamin C, folates, phenolic acids, Anthocyanins), with chromatographic columns of different lenghts, 4.5 mm diameter, 5 µm internal pore diameter.
In detail, for the Vitamin C analysis, the entire system was composed of:

• Pump: PU2089 Plus (Jasco);
• Autosampler: AS-4050 (Jasco);
• Column: Ascentis Express (C18 150 mm x 4.6 mm column - Supelco);

• Pre-column (for the protection of the column from unwanted external particles);
• UV/VIS Detector: UV-2070 Plus (Jasco).

The analysis was carried out in groups of 33 samples, according to the autosampler capacity, and every analysis in HPLC method is performed at higher flow rate and higher pressure than classical column chromatography. This gets easier the interactions among beads in column and particles in mixture. For vitamin C analysis, the elution was performed with 50 mM phosphate buffer at pH 3.2, at a flow rate 0.5 ml/min for 15 minutes, according to Johannes et al. (2002). Ascorbic acid was detected at 260 nm, the column was washed with 50% acetonitrile and 50% 50 mM phosphate pH 3.2, for 5 minutes, and equilibrated in the original solvent for 10 minutes before the next analysis.

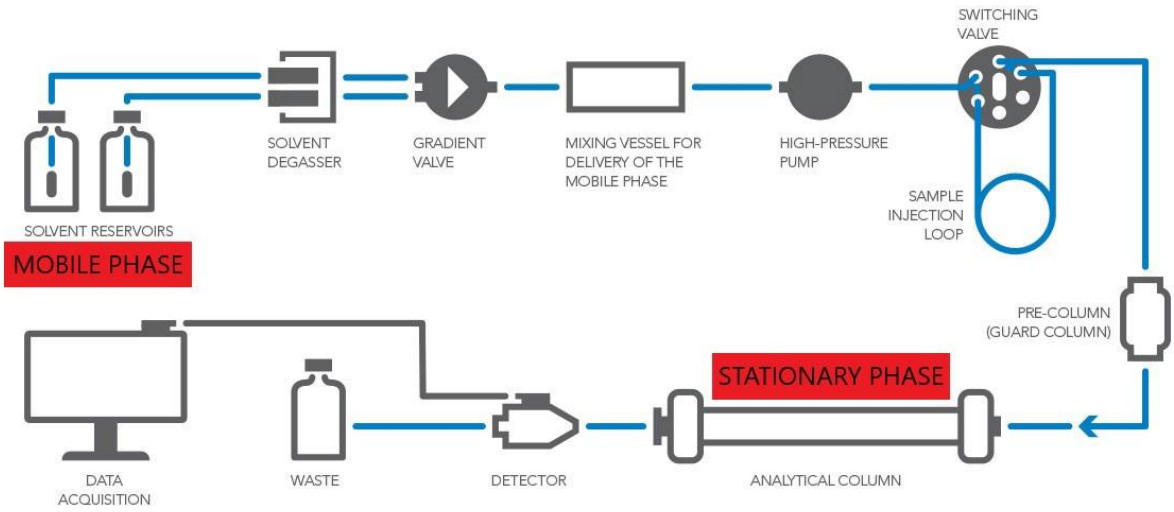

Figure 11: HPLC system.
Source: www.tudelft.nl

3.5 Folates analyses

3.5.1 Folate extraction

The folate extraction followed two different steps: the first extraction of folates by the strawberry samples and the Solid Phase Extraction for the extract purification.

First step: folates from strawberries samples were extracted according to the procedure described by Strålsjö et al. (2002), alongside some modifications. Strawberries samples (2 g) have been defrosted for 12 min in a boiling water bath, in 8 mL phosphate buffer (composed of 17.42 g K_2HPO_4 and 13.62 g of KH_2PO_4 in 100 ml distilled water, 1% (w/v) ascorbic acid, 0.1% 2-mercaptoethanol, pH 6.1). After cooling, the samples were incubated with 1.5 ml hog kidney conjugase at pH 4.9 in a shaking water bath at 37°C for 3 h and the conjugase was then inactivated in a boiling water bath for 5 min. Subsequently, two centrifugations were carried out at (4500rpm, 4°C and 30min) and the supernatant collected and made to 25 mL mark of the falcon tube, with the same phosphate buffer used for extraction. The final extraction was then stored at -20°C until purification.

Second step: in order to separate and purify the folate from unwanted compounds, SPE technique was employed (Figure 12).

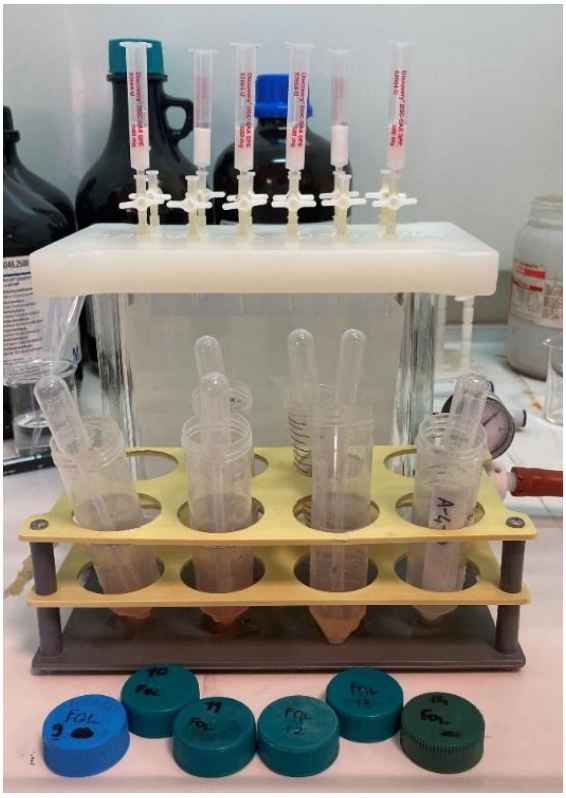

Figure 12: Solid phase extraction manifold.

The SPE cartridges were conditioned (activated and made more effective) using 2.5ml of methanol which was run twice through the cartridge and later equilibrated with same volume of water to ensure the interaction of the SPE material with the sample. Aliquots of samples were then loaded to the preconditioned cartridges and allowed to flow under vacuum through the SPE material. 0.7ml of eluting solution (0.1 mol/L sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid, 0.1% 2-mercaptoethanol), was then used to wash away unwanted materials from the cartridges and was discarded, and finally the second portion (3.5 ml) was collected and stored at -20°C until further analysis.

3.5.2 HPLC analysis

Quantification of folates by HPLC was carried out according to Strålsjö et al., (2003), with some modifications. The HPLC system comprised a pump model PU-2089 (Jasco, Easton, MD, USA), a Fluorescence detector (FLD) FP-2020 Plus (Jasco, Easton, MD, USA) set at wavelengths of 290 nm excitation and 360 nm emission, and an autosampler AS-4050 (Jasco, Easton, MD, USA). The analytical column was a Luna C18, 250×4.6, 5 µm (Phenomenex, Torrance, California, USA). The mobile phase consisted of 30 mmol/l phosphate buffer, pH 2.3, using a gradient with acetonitrile starting at 6%, a lag time of 5 min and rising linearly to 25% within 20 min. The total run time was 33 min. Retention time of 10mins was used for peak identification, and quantification of folates content was determined through a calibration curve prepared by running standard concentrations of 5-methyl-tetrahydrofolic acid (5-CH3-H4folate). Results are expressed as µg 5-CH3-H4folate per 100g of fresh weight of strawberry (µg 5-CH3-H4folate/100g FW). All the samples were analyzed in triplicate.

3.6 Polyphenols analyses

3.6.1 Methanolic extraction

The strawberry samples were subjected to a methanolic extraction to obtain the extract for the analysis of polyphenols and specifically anthocyanins, phenolic acids and antioxidant capacity. 10 g of fruits pieces, previously obtained, were weighed and collected into a falcon tube, taking attention to take pieces of strawberries from different fruits, to have a representative sample. The extraction was done in methanol, because it allowed the stability of antioxidant compounds during extraction and during sample storage. Methanol was added to 10 g of strawberries in 1:4 ratio (1 part of fruits and 4 parts of methanol) in two steps (a double extraction was performed). During the first extraction, the procedure was quite simple: the

tube, where previously were placed 10 g of strawberries, was filled with 20 ml of methanol. The whole material, strawberries and methanol, was homogenized with the ultraturrax-T 25 (Janke & Kunkel, IKA-Labortechnik) for 20/30 seconds. Then, the tube with the methanolic suspension was closed and placed in a rotary shaker for 30 minutes, protected from light. Subsequently, at the end of shaking time, the suspension was centrifuged at 4000 rpm for 10 minutes, at 4°C. At the end of the centrifugation, 1 ml of the supernatant homogenate was taken and placed in amber vials. These vials were stored in freezing conditions (-20° C). The residual pellet, obtained after the centrifugation, was re-filled with other 20 ml of methanol, and placed in a rotary shaker for 30 minutes again, protected from light. Then, the tube was centrifugated at 4000 rpm for 10 minutes, at 4°C. At the end of this second centrifugation, 1 ml of supernatant was withdrawn and added to the prior vials. That extract was then conserved at freezing temperature (-20° C). The whole procedure was repeated for each strawberry sample. At the end, for each replica of the remontant strawberry (324 replicas), there have been 8 vials, with a final amount of 2592 vials.

3.6.2 Spectrophotometry (Folin Ciocalteu)

The determination of total phenolics has been carried out through the Folin-Ciocaleteu assay. Folin–Ciocalteu (F–C) is an assay regularly used to predict total phenolics in strawberry as well as in a variety of other fruits and vegetables (Prior et al., 2005). The original F–C spectrophotometric method created to detect total phenolics in fruits and vegetables was developed by Folin and Ciocalteu (1927) and was later modified by Singleton and Rossi (1965). The modified F–C method uses molybdotungstophosphoric heteropolyanion reducing reagent which indirectly detects phenolics (Medina, 2011a), but lacks specificity (Prior et al., 2005). The total phenolics assay does not only determine phenolics but also reducing agents like ascorbic acid because the basic mechanism is

an oxidation/reduction reaction. The exact chemical nature is not known but is believed to contain heteropolphosphotunstates molybdates. Molybdenum seems to be easier reduced in the complex. An electron-transfer reaction occurs between reductants and Mo (VI) under alkaline conditions which results as blue colour with an absorbance maximum at about 760 nm (Figure 13). The total phenolic contents (TP) were calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per kg of the sample. The calibration is calculated by linear regression (ΔA = ac + b, c = concentration of Gallic Acid mg/L, ΔA=absorbance, a = slope, b= intercept):

$$TP(Mg\ Gallic\ Acid\frac{eq}{kg}Fruit = \frac{(\Delta A - b)\ \times\ F}{A\ \times e}$$

ΔA = A sample/standard (sample)

a = slope (calibration line)

b = intercept (calibration line)

F = Diluition factor (20)

E = sample weight [kg/L extracting agent]

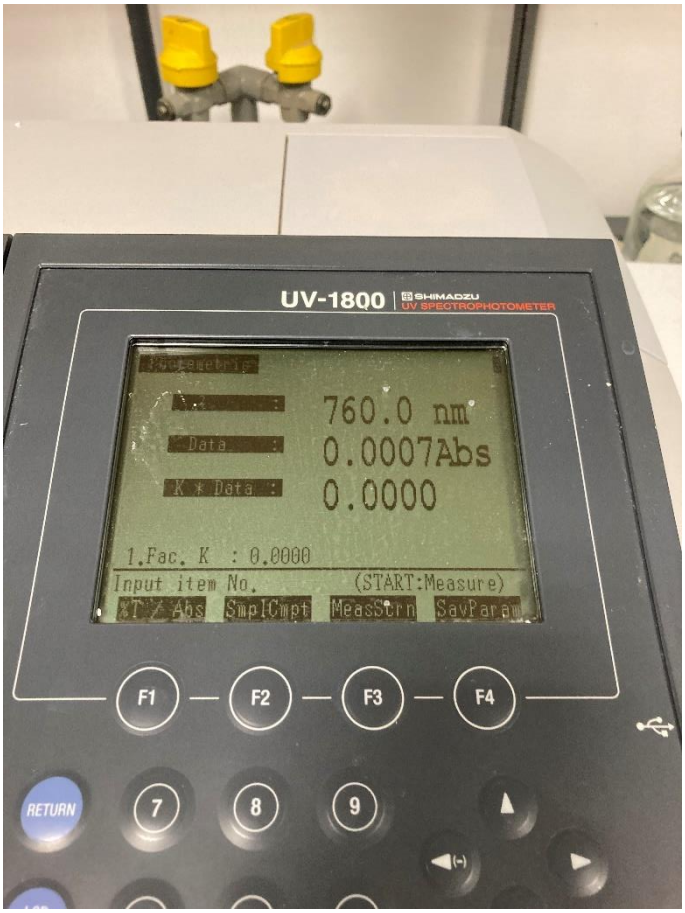

Figure 13: Spectrophotometer with the absorbance set at 760 nm.

3.7 Anthocyanin analyses

3.7.1 Methanolic extraction

Reference to protocol described in 3.6.1 Methanolic extraction.

3.7.2 Spectrophotometry (pH shift method)

Anthocyanin pigments change hue and intensity at different pH values: at pH 1.0, anthocyanins exist in the colored oxonium or flavylium form, and at pH 4.5 they are predominantly in the colorless carbinol form. An aliquot of an aqueous anthocyanin solution is adjusted to pH 1.0 and another

aliquot to pH 4.5. The difference in absorbance is proportional to the anthocyanin content. Determination of anthocyanin content is based on Lambert-Beer's Law. Molar absorbance values for purified pigments taken from literature are used, making it unnecessary to determine them. Pelargonidin-3-glucoside is the major anthocyanin in strawberry, so the total anthocyanin content is calculated as pelargonidin-3-glucoside equivalents. Amount of anthocyanins is given as Pel-3-gl [mg/kg FW] fruit, and it is expressed by the formula:

$$mgPel-3-\frac{glu}{kg}FW=\frac{[(A\lambda\max-\mathrm{A700})\mathrm{pH4,5}]\ \mathrm{x\ MW\ x\ F\ x\ 1000}}{\varepsilon\ x\ \delta\ x\ E}$$

A= absorbance [-]

MW= molecular weight of pelargonidin-3-glucoside=433.2 [g/mol]

F= dilution factor [-]= 10

d= cell pathlenghts [cm]

ε= molar absorbance of Pel-3-glu= 15600 $[\frac{L}{mol\ x\ cm}]$

E= sample weight [kg/L extracting agent]

1000= Factor for mg.

3.8 Phenolic acids analyses

3.8.1 Methanolic extraction

Reference to protocol described in 3.6.1 Methanolic extraction.

3.8.2 HPLC analysis

Even for phenolic acids analysis, the double methanolic extraction was utilized. For HPLC analysis, the method of Frederick et al. (2012) was performed. Two mobile phases were prepared for HPLC analysis, and they flowed in the system, at different ratio, according to the following plan:
The mobile phase "A" was a water solution with 2% of acetic acid (CH_3COOH), while phase "B" was composed by 50% acetonitrile (C_2H_3N), 49% water and 1% acetic acid. The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (1 min), and 10% B for 5 min before injecting the next sample.
As well as anthocyanins extraction, also for phenolic acids three standard solutions were prepared from the following pure phenolic acids:

- Chlorogenic acid (Figure 14);
- Caffeic acid;
- Ellagic acid.

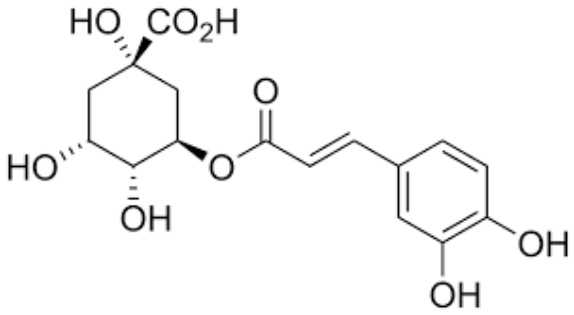

Figure 14: Chlorogenic acid.
Source: https://en.wikipedia.org/wiki/Chlorogenic_acid

Figure 15: HPLC system.

These are the phenolic acids commonly present in strawberry fruit. Similarly to what happened for anthocyanins, also for the three phenolic acids were prepared three mother solutions at a concentration of 1 mg/ml. For caffeic and chlorogenic acids, ethanol (C_2H_6O) was used as solvent. For ellagic acid, sodium hydroxide (NaOH, 1M) was used as solvent.

As for the anthocyanins, serial dilutions were performed from each mother solution, obtaining six dilutions, for each phenolic acid. These solutions were injected to the HPLC (Figure 15) to build the standard calibration curves. The UV/VIS detector was set to 320 nm to quantify and recognize only phenolic acids. The final results were expressed as mg of phenolic acids per 100 g of fresh fruit.

3.9 Antioxidant capacity analyses

3.9.1 Methanolic extraction

Reference to protocol described in 3.6.1 Methanolic extraction.

3.9.2 Spectrophotometry (TEAC)

The determination of antioxidant capacity has been carried out through a TEAC-Decolorization. TEAC assay is evaluated by the oxidation of ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) with potassium persulfate. The blue/green radical of $ABTS^{\bullet+}$ is generated. Its absorption maximum is at 734 nm. In the presence of hydrogen-donating antioxidants it is reduced resulting in the decolorization of the $ABTS^{\bullet+}$ radical. The decolorization is determined as a function of concentration. Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), a water-soluble vitamin E analogue, serves as a standard. The Total Antioxidant Capacity of the sample is expressed as Trolox equivalents per g of fresh weight (Re et al. 1999). The day before the extraction, the stock solution of the $ABTS^{\bullet+}$ was generated by oxidation of ABTS with potassium persulfate ($K_2S_2O_8$) overnight. Additional 1 ml of the $ABTS^{\bullet+}$ radical solution was added to 10 µl of reagent, that can be ethanol for the blank of the standard curve, Trolox solution for the standard curve, methanol: water 80:20 for the blank of the strawberry extracts, or strawberry extract for the analysis. After this step, the analysis solution must be vortexed for 20 seconds, and after 6 minutes must be red in the spectrophotometer at 734 nm, measuring the color inhibition of the $ABTS^{\bullet+}$ radical (Figure 16).

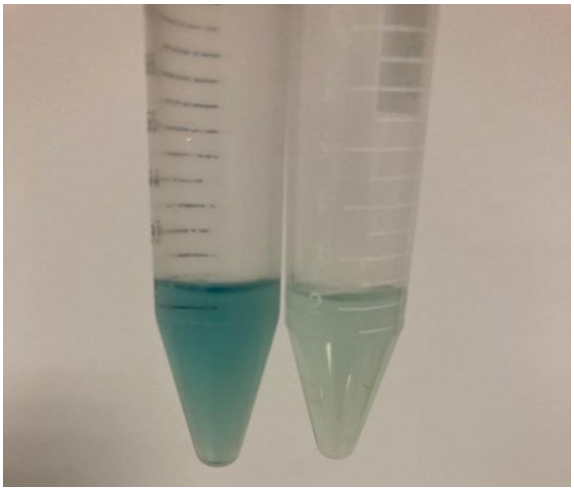

Figure 16: Difference in color inhibition of the ABTS•⁺ radical.

The % of inhibition must be calculated following this formula:

$$\Delta A = \frac{blank\ Abs - sample\ or\ standard\ Abs}{blank\ Abs}$$

ΔA =% inhibition
Abs = absorbance read at 734 nm

TEAC value was determined comparing the % inhibition with the standard calibration curve of Trolox, the mean of absorbance is set due to the 3 measurements. The antioxidant capacity values is expressed as

$$\mu mol\ Trolox\ equivalents/g\ FW = \frac{(\Delta A - b) \times F}{a \times E} \times 100$$

ΔA =% inhibition (sample)
a = slope (calibration line)
b = intercept (calibration line)
F = dilution factor
E = sample weight.

4 Statistical analysis

The fruits' phytochemical parameters data were analyzed using two-way analysis of variance to test the effect of cultivar and treatment, and corresponding interactions. Statistically significant differences in means were determined with Fisher (Least Significant Difference, LSD) test ($p \leq 0.05$). Principal component analysis (PCA) was also used to evaluate the levels of association among the phytochemical parameters, and among the evaluated genotypes. The two most significant factors were used to identify the most important variables and observations in each dimension. The factor loading values are the correlations of each parameter with the principal component (PC). They are given as vectors (positions) in the space represented by the axes of the PCA bi-plot. In the graph, the parameters and the genotypes that are closest to each other in the same geometric plane of the bi-plot are considered to be interrelated, and consequently the parameters and the genotypes that are distant from each other are not related or are negatively related. The greater the distance of a vector from the origin of the axis, the higher the correlation of the variable with the PC represented in that dimension (axis). All the analyses were performed with Statistica 7 software (StatSoft, TIBCO Software, Palo Alto, CA, USA).

5 Results

The 3 cultivars (Albion, Monterey and S. Andreas) showed a different response in terms of fruit nutritional quality to the different water and nitrogen regimes applied during the cultivation. The ANOVA analysis applied on data collected from the different treatments allowed to understand which parameters, and which among their interactions, have influenced the nutritional quality of strawberry fruits object of the study (Table 3). The fruit content of vitamins(Vitamin C and folates, which

correspond to Vitamin B9) has been mostly influenced by the factors of the study, namely genotype and treatments (different amount of water and nitrogen). Vitamin C content is significantly influenced by water and nitrogen regimes, and by the interaction of the cultivar with both treatments. The same is for Folates, even if in this case seems that the genotype is not a critical factor in the determination of Vitamin B9 content.

Colonna1	Vit C	Phenolic acids	Folates	TPH	ACY	TAC
Cultivar	**	**	n.s.	n.s.	**	n.s.
Fertilization	*	n.s.	**	n.s.	n.s.	n.s.
Irrigation	**	n.s.	**	n.s.	n.s.	*
Cultivar x Fertilization	**	n.s.	**	n.s.	n.s.	n.s
Cultivar x Irrigation	**	n.s.	**	n.s.	n.s.	n.s.

Table 3: ANOVA Analysis. *: Significant interaction with $p<0.05$; **: Significant interaction with $p<0.01$; n.s.: not significant interaction (ANOVA analysis).

5.1 Ascorbic acid

The Vitamin C amount evaluated on 100g of fruit weight, seems to be influenced by the different nitrogen supply, depending to the genotype (Table 4). For each analyzed cultivar, in fact, the application of the highest nitrogen amount negatively affected the total concentration of this compound. For Monterey and San Andreas, N100 resulted with fruits having significant lower values of Vitamin C than N60, while in Albion the trend was the same but without significant difference. Albion and Monterey presented significantly higher amount of Vitamin C in their fruits in respect to S. Andreas, for each nitrogen supply.

Sample	Vit C (mg/100g FW)	Standard error (mg/100g FW)	Statistical analysis
Albion N60	25.79	0.03	ab
Albion N80	28.09	2.69	a
Albion N100	25.79	0.27	ab
Monterey N60	26.91	1.22	a
Monterey N80	28.37	3.53	a
Monterey N100	23.91	0.04	b
S. Andreas N60	21.11	0.04	c
S. Andreas N80	15.42	0.01	e
S. Andreas N100	18.14	0.06	d

Table 4: Vitamin C concentration (mg/100g FW) depending on nitrogen supply. Different letters mean significant difference (Fisher test, $p<0.05$).

The fruit vitamin C content seems to be influenced also by the different water supply and by the genotype (Table 5). Fruits of Albion and Monterey differed for the higher content Vit. C value at W100 and S. Andreas at W60. Monterey fruits show the significantly highest contents in vitamin C (at W100 with 26.63 mg/100g FW, and at W80 with 25.23 mg/100g), while S. Andreas fruits show the significantly lowest vitamin C contents at all water trials.

Sample	Vit C (mg/100g FW)	Standard error (mg/100g FW)	Statistical analysis
Albion W60	23.72	0.01	cd
Albion W80	23.38	0.06	d
Albion W100	23.89	0.47	c
Monterey W60	22.18	0.03	e
Monterey W80	25.23	0.00	b
Monterey W100	26.63	0.03	a
S.Andreas W60	18.08	0.07	f
S.Andreas W80	16.90	0.14	g
S.Andreas W100	17.76	0.25	f

Table 5: Vitamin C concentration (mg/100g FW) depending on water supply. Different letters mean significant difference (Fisher test, $p<0.05$).

5.2 Folates

As previously anticipated in Table 3, Folates content is influenced by both nitrogen and water supply, and is not significantly influenced by genotype (Tables 6 and 7). In Albion, the N100 treatment resulted with the significantly lowest folates content in fruits, It's evident the slight difference between the highest value, while in Monterey the significantly lowest folates content was detected in fruits deriving from N60 trial. S. Andreas showed similar values of folates in fruits obtained at the three fertilization levels.

Sample	Folates (µg/100g FW)	Standard error (µg/100g FW)	Statistical analysis
Albion N60	30.35	0.48	ab
Albion N80	31.32	0.05	a
Albion N100	27.39	0.10	d
Monterey N60	27.18	0.52	d
Monterey N80	29.71	0.71	bc
Monterey N100	29.52	0.16	bc
S. Andreas N60	29.85	0.15	bc
S. Andreas N80	28.87	0.08	c
S. Andreas N100	29.09	0.16	c

Table 6: Folates concentration (µg /100g FW) depending on nitrogen supply. Different letters mean significant difference (Fisher test, $p<0.05$).

The folates content, in modified water supply strawberries, did not show a significant variation among genotypes, while the amount of water significantly influenced folates accumulation in fruits. In particular, the general trend shows that a reduction of irrigation water stimulates the production of fruits with increased folates content in all the tested genotypes. Monterey plants at W60 thesis produced fruits with significantly higher content of folates than fruit from plants at W100, while plants of S. Andreas at W60 thesis produced fruits with significantly higher content of folates than W80 (Table 7).

Sample	Folates (µg/100g FW)	Standard error (µg/100g FW)	Statistical analysis
Albion W60	28.47	0.38	cde
Albion W80	28.51	0.67	cde
Albion W100	27.21	0.43	de
Monterey W60	33.12	0.11	a
Monterey W80	31.50	0.39	ab
Monterey W100	28.87	0.19	cd
S. Andreas W60	30.78	0.38	b
S. Andreas W80	27.02	1.37	e
S. Andreas W100	29.94	0.42	bc

Table 7: Folates concentration (µg /100g FW) depending on water supply. Different letters mean significant difference (Fisher test, $p<0.05$)

5.3 Polyphenols

The total phenolics amount in the strawberry fruits was not significantly influenced by nitrogen supply, cultivar and their interaction (Table 8). The highest fruit content was detected for Monterey plants grown at N100, with 3495.89 mgGA/100g FW. The lowest content was detected in fruit of S. Andreas plants grown at N60, with 3027.84 mgGA/100g FW. In Monterey and S. Andreas the highest nitrogen fertilization have induced an higher accumulation of TPH in fruits, while in Albion the best result was obtained in fruits from N80 trial, but differences are not significant.

Sample	TPH (mgGA/100g FW)	Standard error (mgGA/100g FW)	Statistical analysis
Albion N60	3293.48	151.35	ab
Albion N80	3355.95	62.14	ab
Albion N100	3194.76	30.30	ab
Monterey N60	3274.14	123.25	ab
Monterey N80	3291.20	72.71	ab
Monterey N100	3495.89	44.45	a
S. Andreas N60	3027.84	207.79	b
S. Andreas N80	3128.35	94.38	b
S. Andreas N100	3234.07	115.27	ab

Table 8: Total phenolics (mgGA/100g FW) depending on nitrogen supply. Different letters mean significant difference (Fisher test, $p<0.05$)

The fruit accumulation of total phenolic compounds was not significantly influenced nor by water regimes nor by its interaction with genotype (Table 9). The highest accumulation was detected in fruit from S. Andreas plants at W60, with 4225.03 mgGA/100g FW; on the contrary the lowest accumulation was detected in fruits harvested from Albion plants at W80, 3513.80 mgGA/100g. Differently from these cultivars, S. Andreas plants produced fruit with the lower accumulation of TPH at W100 in respect to W80 and W60 (the highest).

Sample	TPH (mgGA/100g FW)	Standard error (mgGA/100g FW)	Statistical analysis
Albion W60	3593.86	76.46	b
Albion W80	3513.80	145.72	b
Albion W100	3678.09	270.05	ab
Monterey W60	3911.53	99.41	a
Monterey W80	3587.29	14.68	b
Monterey W100	3671.02	35.82	ab
S. Andreas W60	4225.03	338.13	a
S. Andreas W80	3931.19	317.70	a
S. Andreas W100	3498.12	226.95	b

Table 9: Total phenolics (mgGA/100 g FW) depending on water supply. Different letters mean significant difference (Fisher test, $p<0.05$)

5.4 Anthocyanins

The anthocyanins content in strawberries fruit of the different cultivars did not presented significant differences in response to the different nitrogen treatments, but among cultivars S. Andreas differed for a significantly higher amount of fruit anthocyanins compared to the other 2 cultivars (Table 10).

Sample	ACY (mg PEL-3-GLU/100g FW)	Standard error (mg PEL-3-GLU/100g FW)	Statistical analysis
Albion N60	35.54	2.43	bcd
Albion N80	35.38	0.37	bcd
Albion N100	33.91	2.20	d
Monterey N60	36.01	1.76	bcd
Monterey N80	32.12	1.63	d
Monterey N100	35.81	3.13	bcd
S. Andreas N60	40.63	2.92	abc
S. Andreas N80	42.15	1.06	ab
S. Andreas N100	44.11	1.72	a

Table 10: Anthocyanins (mg PEL-3-GLU/100 g FW) depending on nitrogen supply. Different letters mean significant difference (Fisher test, $p<0.05$)

The same response in terms of fruit anthocyanins content was detected also in plants treated with water regimes, where no significant differences were detected among treatments, exception for the slight increase of ACY by W60 to W100 in Albion and Monterey. Again S. Andreas resulted with the significantly highest amount of fruit anthocyanins, compared to other 2 cultivars (Table 11).

Sample	ACY (mg PEL-3-GLU/100g FW)	Standard error (mg PEL-3-GLU/100g FW)	Statistical analysis
Albion W60	32.25	1.48	b
Albion W80	33.58	1.51	b
Albion W100	35.49	0.19	b
Monterey W60	34.06	1.87	b
Monterey W80	33.82	1.60	b
Monterey W100	36.24	1.11	b
S. Andreas W60	44.63	0.73	a
S. Andreas W80	42.50	1.59	a
S. Andreas W100	44.27	1.92	a

Table 11: Anthocyanins (mg PEL-3-GLU/100g FW) depending on water supply. Different letters mean significant difference (Fisher test, $p<0.05$)

5.5 Phenolic acids

The analyses on fruit content of phenolic acids revealed that for all cultivars the ellagic acid is predominant, followed by chlorogenic acid, and finally caffeic acid (Figures 17 and 18). Fruits of the 3 cultivars showed a

different content of phenolic acids but it was not influenced by intensity of the nitrogen treatment. The fruit of Monterey showed the highest content of phenolic acids, regardless of the nitrogen supply considered. The highest content of phenolic acids determined for Monterey was mostly due to the highest accumulation of Chlorogenic acid (Figure 17). In fact, the amount of Caffeic and Ellagic acid seemed to be similar in all the analyzed genotypes.

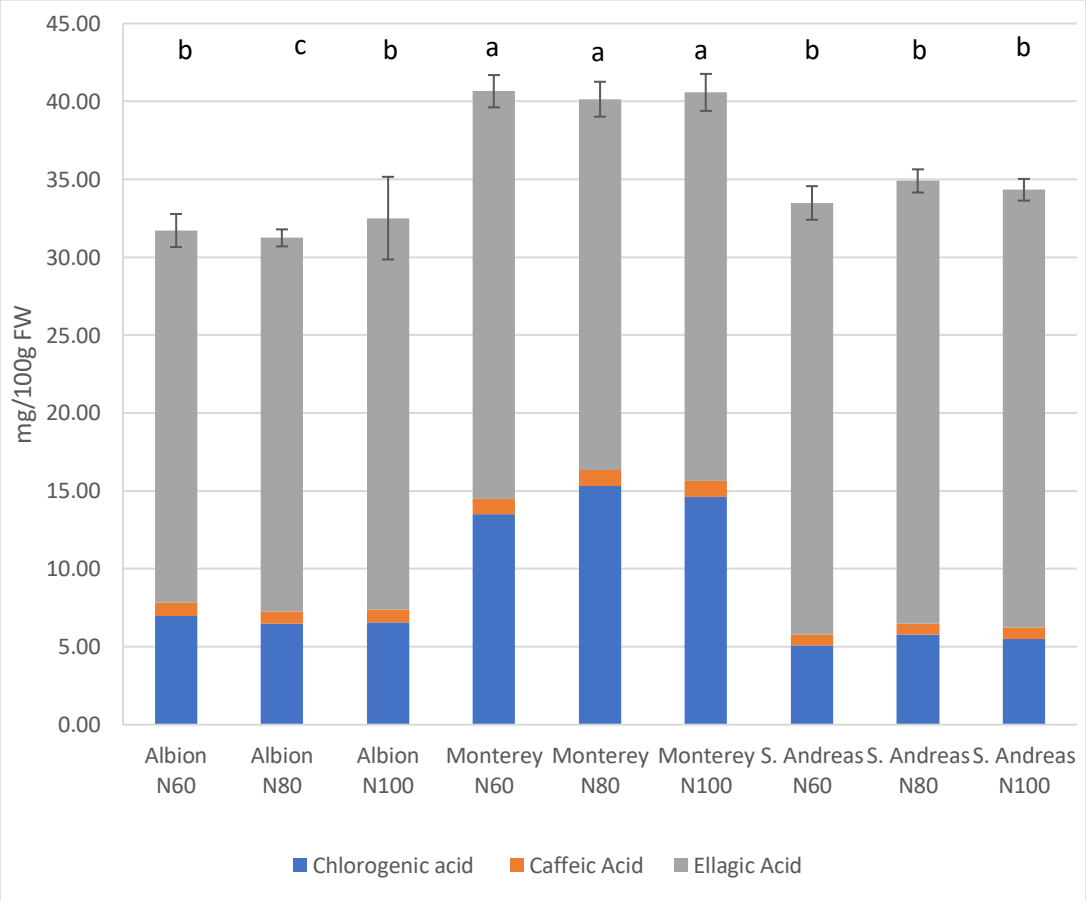

Figure 17: Phenolic acids content (mg/100g FW) based on different nitrogen supply. Different letters mean significant difference (Fisher test, $p<0.05$)

Similar results were observed in fruits analyzed from the irrigation trial. In fact, the different water regimes did not affect in a significant manner the amount of these compounds in strawberry fruits, while again the

genotype resulted a determinant factor. In fact, fruits of Monterey confirmed the highest content of phenolic acids, presenting the significantly highest amount in fruits obtained at W60 trial. In this case, both Monterey and S. Andreas showed a similar trend, with phenolic acids increasing at decreasing water supply. Furthermore, fruits of S. Andreas showed a significant difference between the phenolic acids content detected at W60 (30.52 mg/100g FW) and at W100 (27.37 mg/100g FW) (Figure 18).

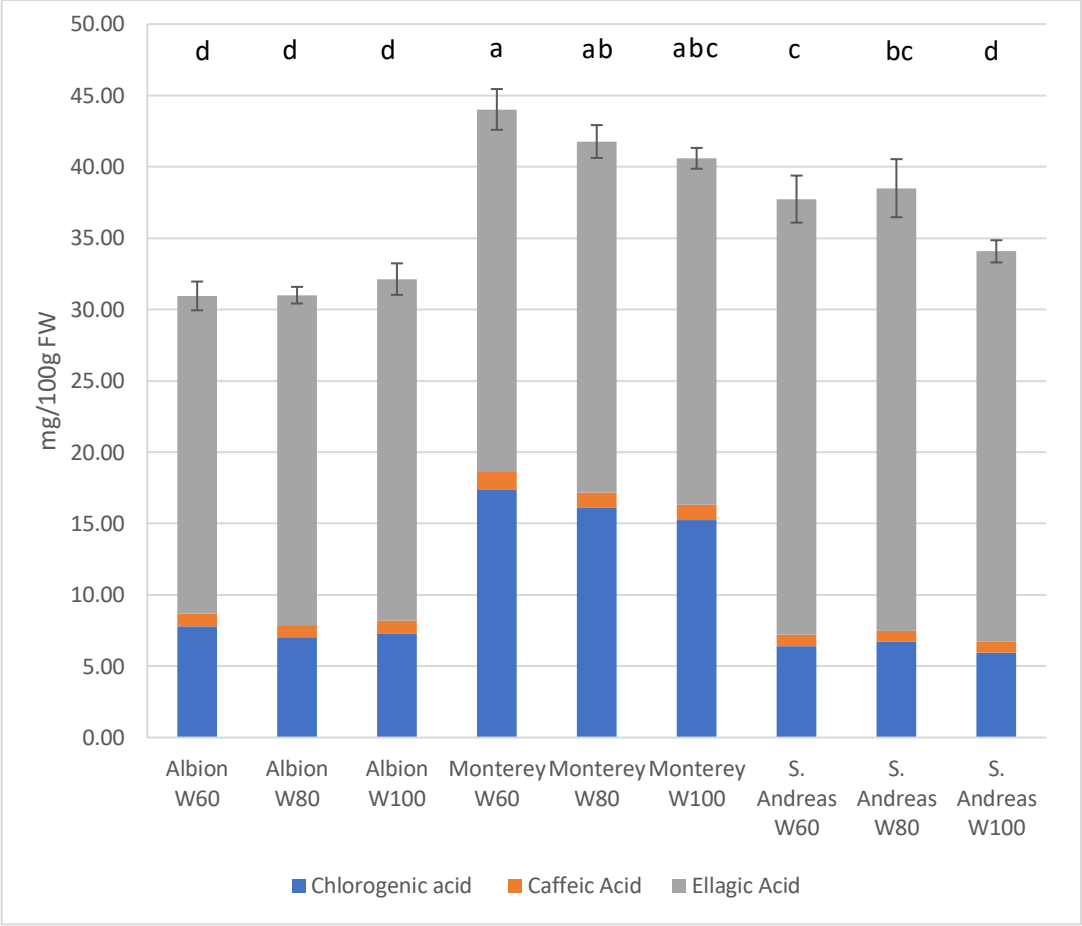

Figure 18: Phenolic acids content (mg/100g FW) based on different water supply. Different letters mean significant difference (Fisher test, $p<0.05$)

5.6 Total Antioxidant Capacity

The total antioxidant capacity (TAC) seems to be not dependent by the different amounts of nitrogen fertilizer, nor by the genotype (Table 12). In fact, the data did not show any significant difference among treatments and genotypes in terms of fruit TAC. The highest value is reported for Monterey N100 fruits, that show a TAC of 509.16 mg Trolox eq/100g FW. The lowest is the S. Andreas N80, with the value of 324.44 mg Trolox eq/100g FW.

Sample	TAC (mg Trolox eq/100g FW)	Standard error (mg Trolox eq/100g FW)	Statistical analysis
Albion N60	485.50	18.77	a
Albion N80	496.94	48.28	a
Albion N100	447.04	12.88	a
Monterey N60	484.46	19.52	a
Monterey N80	473.07	6.47	a
Monterey N100	509.16	38.31	a
S. Andreas N60	454.46	36.57	a
S. Andreas N80	423.44	30.31	a
S. Andreas N100	483.79	38.36	a

Table 12: Antioxidant capacity (mg Trolox eq/100g FW) based on different nitrogen supply. Different letters mean significant difference (Fisher test, $p<0.05$)

The antioxidant capacity of strawberries treated with different water regimes, demonstrates lower values for all the fruits from plants of the 3 cultivars grown with the 100% of water restitution, compared to the same cultivars treated with lower % of water restitution (Table 13). Even if this trend was confirmed in each cultivar, only S. Andreas presented significant differences, with the fruits obtained at W60 trial presenting significantly higher value of TAC (599.23 mg Trolox eq/100g FW) than W100 (506.66 mg Trolox eq/100g FW). The cultivar effect did not affect in a significant manner the TAC of strawberry fruits in this trial.

Sample	TAC (mg Trolox eq/100g FW)	Standard error (mg Trolox eq/100g FW)	Statistical analysis
Albion W60	535.92	21.15	bc
Albion W80	539.86	28.44	bc
Albion W100	509.13	11.50	bc
Monterey W60	561.27	24.44	ab
Monterey W80	559.79	10.65	abc
Monterey W100	552.06	5.19	abc
S. Andreas W60	599.23	7.57	a
S. Andreas W80	557.57	21.86	abc
S. Andreas W100	506.66	18.43	c

Table 13: Antioxidant capacity (mg Trolox eq/100g FW) based on different water supply. Different letters mean significant difference (Fisher test, $p<0.05$)

6 Discussion

This study is putting new and useful information about the relation between the strawberry fruit quality and the supply of different inputs (water and nitrogen in the specific case) depending to the cultivars. At our knowledge, in literature the great number of studies dealing with nitrogen fertilization took into account the vegetative and productive responses of strawberry plants (Trejo-Tellez and Gomez-Merino, 2014), without considering in a deeper manner the relation between the application of different amount of this macronutrient and the fruit nutritional quality. In our study, it was demonstrated that the reduction of nitrogen amount did not caused a significant change in the polifenolic pattern (in terms of total phenolics, total anthocyanins and phenolic acids), as well as in the Total Antioxidant Capacity, in none of the studied cultivars. Conversely, the nitrogen reduction act significantly in the increase of the vitamins pattern in the studied cultivars, registering the highest content of both Vitamin C and Folates at the N80 thesis for fruits of Albion and Monterey, and N60 thesis for fruits of San Andreas.

Regarding the water trial, much more studies have been previously performed to analyze the influence of different water irrigation regimes on strawberry fruit nutritional analysis. The fruit content of phenolic compounds was not influenced by the water treatments, differently from what stated by Adak et al. (2017), but antioxidant capacity and folates were stimulated by water stress treatments, with Monterey and San Adreas presenting highest values for these parameters in fruits harvested at W60, and Albion at fruits harvested at W80. These results are in agreement with Adak et al. (2017), which stated that water stress positively influence the Total Antioxidant Capacity, and with Terry et al. (2007) and Bordonaba et al. (2010), both stating that water stress in strawberries influences the fruit biochemical properties. In addition, vitamin C was found to be influenced by water treatment, but in different manner depending to the cultivar: fruits of Albion and Monterey showed the highest content at W100, while San Andreas at W60 (even if statistically similar to W100). This data seems to indicate that the water reduction has a negative effect on the fruit accumulation of Vitamin C, differently from what stated by Ghaderi et al. (2011), which affirmed that water stress did not have any influence on the vitamin C.

Data correlation

To investigate if one or more nutritional compounds are linked together in their determination, data obtained for the three cultivars were analyzed with the Principal Component Analysis (PCA) (Figure 19). The two main factors reported on the graph justify the 60% of the variability registered in this study. It is interesting to note that the TAC and TPH vectors fall very close each other, indicating a strong relation between the amount of total phenolics and the antioxidant capacity of fruits. This result confirms the finding that the phenolic compounds are the main responsible for the TAC of strawberries. Furthermore, phenolic acids vector is placed in the third quadrant, and this was also expected given the antioxidant capacity exerted also by this class of compounds. It is more surprising that also the

folates vector is located in this quadrant, even if these compounds are not strong antioxidants. ACY and Vit C vectors are placed in opposite quadrant (second and fourth, respectively), indicating that high amounts of one of them in the strawberry fruits of this study corresponded to low amounts of the other, and vice versa. However, they are both good antioxidant compounds, and they are equally distant from the TAC vector.

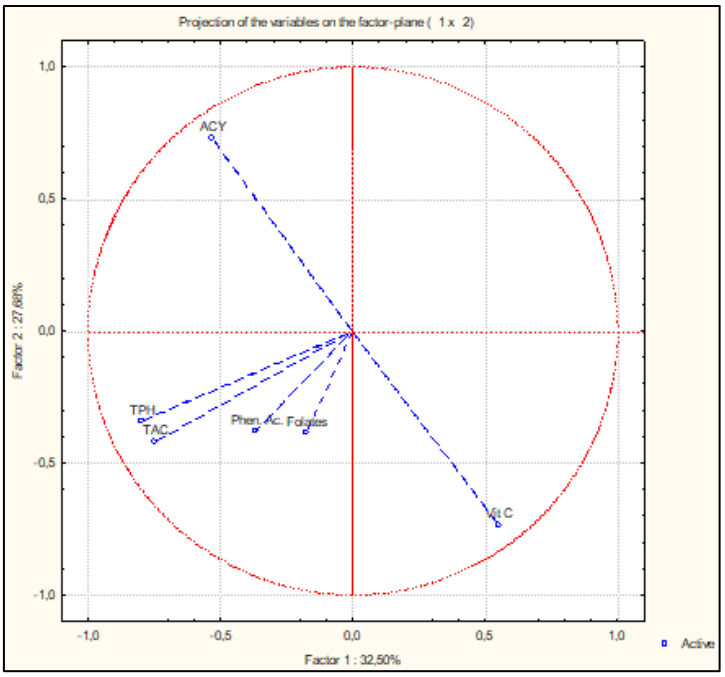

Figure 19: Principal Components Analysis (PCA).

6 Conclusions

Water and nitrogen are two of the main factors that influence the plant performance and adaptability. Be aware about the need to save natural resources, and to reduce agronomical inputs in order to preserve the natural environment, and to face the climate change, is leading to the realization of studies, like the present research, aimed to reduce and optimize the use of inputs in agriculture.

It is clear that each plant can express the best productive and qualitative responses only under certain cultivation conditions, and it is for this reason that for each cultivar grown in different environments and cultivation

systems it is important to identify the most effective practices to improve the productive and qualitative (sensory and nutritional) response in fruit production, with the least use of the 2 main resources necessary for cultivation, namely nitrogen and water.

Based on all analyzed compounds, it is possible to confirm that for fruit content of ascorbic acid, Monterey cultivar obtained the best results, with 80% of nitrogen supply (28.37 mg/100g FW) and 100% of water supply (26.63 mg/100g FW). Folates analyses showed highest contents in fruits of Albion and Monterey, but depending to the conditions: Albion with 80% of nitrogen supply (31.32 µg/100g FW) and Monterey with 60% of water supply (33.12 µg/100g FW). In the evaluation of polyphenols, it is evident the efficiency in fruit content of Monterey, at 80% nitrogen supply (3495.89 mgGA/100g FW), and San Andreas, at 60% of water restitution (4225.03 mgGA/100g FW). San Andreas have had the best results for fruit anthocyanins content, with 100% of nitrogen intake (44.11 mg PEL-3-GLU/100g FW) and with 60% (44.63 mg PEL-3-GLU/100g FW) of water supply. Monterey performed in an excellent way for fruit phenolic acids contents at 60% of nitrogen supply (40.65 mg/100g FW), and 60% of water supply (44.01 mg/100g FW). Finally, the antioxidant capacity confirmed the highest quality of Monterey fruits, with 100% of nitrogen input (509.16 mg Trolox eq/100g FW) and 60% of water input (599.23 mg Trolox eq/100g FW). Considering this research, Monterey variety showed a consistent quality for the main part of the compounds analyzed, compared to the other varieties, in all treatment considered.

An important aspect to underline, is the fact that, as showed in Table 3, the Vitamin C content is significantly influenced, more than other compounds, by the treatments and the type of cultivar. It is also important to underline the influence of water supply on the antioxidant capacity for all the strawberry varieties: reducing the % of irrigation by 100% to 60% involves a consistent increase of the antioxidant compounds in fruits (e.g. San Andreas from 599.23 mg Trolox eq/100g FW in W60 fruits to 506.66 mg Trolox eq/100g FW in W100 fruits).

In conclusion, it is possible to affirm that treatments at reducing irrigation and nitrogen fertilization did not compromise the final quality of Albion, San Andreas and Monterey cultivars, increasing sometimes the nutritional quality in particular in terms of folates and TAC. According to these results, it is possible to obtain the same or improved fruit nutritional quality with these strawberry cultivars, reducing precious resources like water for irrigation, and limiting environmentally harmful substances like nitrogen fertilizer. However, we are aware that more years of study involving different environments and cultivars are necessary to confirm these findings, and the simultaneous evaluation of vegetative, productive and sensorial parameters are fundamental to evaluate the general performance of the cultivars subjected to different irrigation and fertilization regimes.

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