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CONTENTS

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Ontogenetic changes of the water status and accumulated soluble compounds in developing and ripening mume (*Prunus mume*) fruit measured by ^{1}H-NMR analysis

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Abbreviations: CPMG= Carr-Percell- Meiboom-Gill; FAA= formalin acetic acid alcohol; NMR= nuclear magnetic resonance; T1= spin-lattice relaxation times; T2= spin-spin relaxation times.

Key words: Histological observation, membrane integrity, mobility of water, NMR relaxation times (T1, T2), water content.

Abstract: The physiological changes of intact mume (*Prunus mume* Sieb. et Zucc. cv. Rinshu) fruit tissues were examined by measuring the physical states of cell-associated water in the fruit tissues with developing and ripening using ^{1}H-NMR spectroscopy. We found that the water molecules in mume fruit tissues existed in several different compartments with different mobilities. Additionally, spectral recovery in the water proton indicated reverse relationships between the pericarps and seeds at the immature and mature stages. In the pericarp tissues, the longest *T*1 and longer *T*2 markedly increased, while those in the seeds decreased. From these results, the change in the water status with growth stage had reverse trajectories in the pericarp and seed of the fruit. In the pericarp tissues, both water uptake and dry weight prominently increased with ripening. The epidermis and inner parenchymal cells of the pericarp tissues remarkably enlarged as a sigmoidal growth curve. Membrane permeability, indicating a loss of membrane integrity, increased in the pericarp tissues. The elongation in the fully vacuolated cells and changes in the membrane permeability in the pericarp tissues with ripening correlated to the longest *T*1. In contrast, the high mobility of water in the seeds began to decrease with maturation, while oil began to accumulate. Thus, the mobility of water, as analyzed in this study, is considered to reflect the results of physiological changes such as cellular heterogeneity and spatial arrangements both in the pericarp and in seed tissues for mume fruit with development and ripening.

1. Introduction

The Japanese apricot, mume (*Prunus mume* Sieb. et Zucc.) fruit, is widely distributed among the different climate regions of East Asia, and is harvested at the yellow-peel stage for pickles called "umeboshi" or at green-peel stage for its juice. Mume is known to be a climacteric fruit that produces large amounts of ethylene as it ripens (Inaba and Nakamura, 1981; Koyakumaru, 1997; Mita *et al*,. 1999), making it difficult to preserve for long periods of time as the characteristic taste and firmness is quickly lost. The reason for the degradation of the fruits and measures to prevent this degradation have never been thoroughly investigated. Investigating harvest timing and improving conditions for fruit quality preservation during commercial processes may make the fruit more common.

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NMR spectroscopy is a promising tool for exploring new aspects of plant science, especially given that there are various types of plant materials in which the physical states of cell-associated water change naturally occur, e.g. in the maturation process of red raspberry (Williamson *et al.,* 1992), gooseberry (Williamson *et al.,* 1993), olives (Gussoni *et al.,* 1993), kiwifruit (Callaghan *et al.,* 1994), coconuts (Jagannathan *et al.,* 1995), cherry tomatoes (Ishida *et al.,* 1994), cherry fruits (Ishida *et al.,* 1997), in floral malformation in mangos (Usha *et al.,* 1994) and maturation in apples (Wang *et al.,* 1988). In addition, the detection of water in different subcellular organelles is often used to understand the changing water distribution among plant components. It has been suggested that multi-exponential ^{1}H-NMR relaxation times (*T*1 and *T*2) in plant tissues reflect water in different plant cell compartments (Burke *et al.,* 1974; Stout *et al.,* 1978; Gusta *et al.,* 1979; Bacic and Ratkovic, 1984; Hills and Duce, 1990; Isobe *et al.,* 1999), and can be ascribed to several different causes: cellular heterogeneity and subcellular compartmentation (Belton

and Ratcliffe, 1985; Snaar and Van, 1992; Kumamoto *et al.,* 1998; Iwaya-Inoue *et al.,* 2004 a, b). The movement of water is controlled by cellular organization, such as compartmentalization by membrane structures (Tanner, 1978, 1983), and water is ordered by macromolecules, such as proteins and polymers of organic compounds (Hazlewood, 1995). Thus, the dynamic states of water in cells closely correlate with the organic properties of macromolecular structures.

The objectives of the present research were (1) to examine the ontogenetic changes in the state of water by ^{1}H-NMR spectroscopy, water content, membrane permeability and histochemical observation during development and ripening of Japanese apricot fruit, and (2) to study the interrelationships among them.

2. Materials and Methods

Plant materials

Japanese apricot (Prunus mume Sieb. et Zucc, cv. Rinshu) fruits cultivated in the orchard at the University Farm of Kyushu University were harvested at each stage (a total of four times) from April to June. The fruits were defined as belonging to four ripening stages as follows (Fig. 1 and Table 1): Stage 1, fruits at approximately 2.0 g in fresh weight and 1.5 cm in transverse diameter, peels green, and seeds immature and 0.1 g in fresh weight; Stage 2, fruits at 15.0 g in fresh weight and 3.0 cm in transverse diameter, and seeds 0.5 g in fresh weight; Stage 3, fruit at their maximum size, seeds 0.7 g in fresh weight; Stage 4, fruit at maximum size and 45.0 g in fresh weight, 4.0 cm in transverse diameter, the firmness in the pericarp softening, the peels yellow, and seeds hard with a fresh weight of 0.8 g. Fruits were separated into pericarp (including epicarps) and seed, and both tissues were used for experiments. Tissue water contents were determined by obtaining the weight loss after drying in an oven at 90°C for 20 h. Eight to ten fruits were used in each stage in the following experiments.

^{1}H-NMR analysis

An NMR spectroscope with a magnet operating at 89.5 MHz for ^{1}H (JEOL EX 90A) was used for the measurement of spin-lattice relaxation times ($T1$), ^{1}H-NMR spin-spin relaxation times ($T2$) and ^{1}H-NMR spectra. A piece of pericarp or seed of an intact Japanese apricot fruit was placed in an NMR tube (8 mm in diameter) which was then placed in an outer glass tube (10 cm in diameter) containing 99.8% D_2O as an internal lock signal; spectroscopic measurement at 25±1°C was then undertaken. The repetition time was 15 s with four accumulation transients for each tissue. The decay between scans was always greater than five times $T1$.

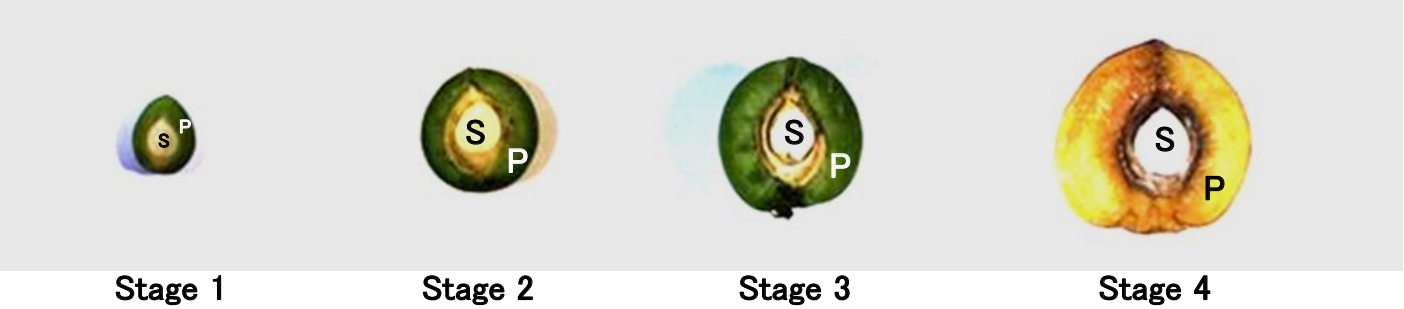

Fig. 1 - Mume (*Prunus mume* Sieb.) fruit during four ripening stages. P= pericarp; S= seed.

Table 1 - Characteristics of mume fruit tested

Characteristics		Stage			
		1	2	3	4
		Harvested day			
		April 9 ~ 24	May 6 ~ 12	May 29 ~ June 4	June 13 ~ 30
Skin color		green	green	green	yellow
Endocarp		hard	hard	soft	soft
Size (cm)	Longitudinal diameter	1.6±0.1	3.2±0.4	3.9±0.1	4.2±0.1
	Transverse diameter	1.3±0.1	2.9±0.1	3.5±0.9	4.2±0.1
Weight (g)	Whole [z]	1.7±0.2	14.8±1.1	32.8±3.0	45.8±2.4
	Pericarp	1.1±0.3	11.2±1.1	27.7±2.4	35.6±2.6
	Seed	0.1±0.0	0.5±0.1	0.8±0.1	0.7±0.1

Values represent the mean of eight to ten fruits±SE.
[z] The weight of the stone is not included.

*T*1 measurements were determined by the inversion recovery (180°- τ -90°pulse sequence) method. τ is the time between the radio-frequency pulses in the sequence, and the indicated angles are between the average direction of the original proton spins and that induced by the radio-frequency pulse (Farrar and Becker, 1971). Twenty-two values of pulse interval (τ) ranging from 0.001 s to 15 s were used to acquire a *T*1 data set. *T*1 was determined from the slope of ln (M0 - M) /2M0 versus τ, where M is the amplitude of the FID of the water proton signal following the 90° pulse at τ, and M0 is the limiting value of M. The existence of water components with different *T*1 values was revealed from spectral recovery and semi-log plots of signal intensity of ^{1}H-NMR according to Ishida *et al.* (1994). A graphical method was used to determine the relaxation time of water and the percentage of the fraction (Hazlewood and Nichlos, 1969; Belton and Packer, 1974). Short *T*1 values including those below 0.1 s were considered from spectral recovery ranging in the pulse intervals of 0.03 s and 0.05 s.

*T*2s measurements were determined by the CPMG (Carr-Percell-Meiboom-Gill) method from the slope of ln M/M0 versus t, where M0 is the magnetization amplitude of the water proton signal occurring at time τ after the initial 90° pulse in the CPMG (90°-τ-180°-2τ-180°-2τ---) pulse sequence. t = 2nτ, where n is the number of refocusing pulses (25 points from 2 to 4000 loops) and τ was 0.001s. *T*2 was also determined from a semi-log plot of signal intensity as in the case of *T*1.

Histological observations

Materials were fixed in FAA (formalin acetic acid alcohol; 80% ethanol: 100% acetic acid: formalin = 90:5:5), and 20-μm sections were cut using a microtome (Cryostat HM500-OM, Microm Co. Ltd) at -20°C. The sections were stained with 0.01% Ruthenium red for pectic substances in the middle lamella (Fig. 2A) and by Sudan III for lipid substances (Fig. 2B), respectively. They were then subjected to microscopic observations (Axiphot, Carl Zeiss Co. Ltd).

Leakage of electrolytes

Pericarp tissues of Japanese apricot fruit (about 3.0 g) were cut into pieces of about 2-mm square. These small pieces were immersed in distilled water (50 ml) and stirred at 180 cycles/min. The extent of leakage of electrolytes was determined with an electrolytes conductivity meter (Toa conductivity meter, Model CM-20E, Toa Electronics Ltd.) and expressed as the percentage of the total electrolytes in each sample measured after samples were killed by a cycle of freezing and thawing (Iwaya-Inoue *et al.,* 2004 a, b).

3. Results

Histochemical characteristics of fruit tissues with ripening

Mume fruit are characterized by four stages (Fig. 1 and Table 1). The histochemical changes of the mume fruit tissues with ripening are shown in figure 2. The individual pericarps and seeds are mainly comprised of the parenchymal tissue, beneath the epidermal tissue and around the vascular tissue. Pectic substances stained by Ruthenium red are abundant in the middle lamella of cell walls in the pericarp parenchymal tissues from the small green fruit to the large green fruit (Fig. 2A, Stages 3 and 4). The components in the cell wall of pericarp tissues began to change with matura-

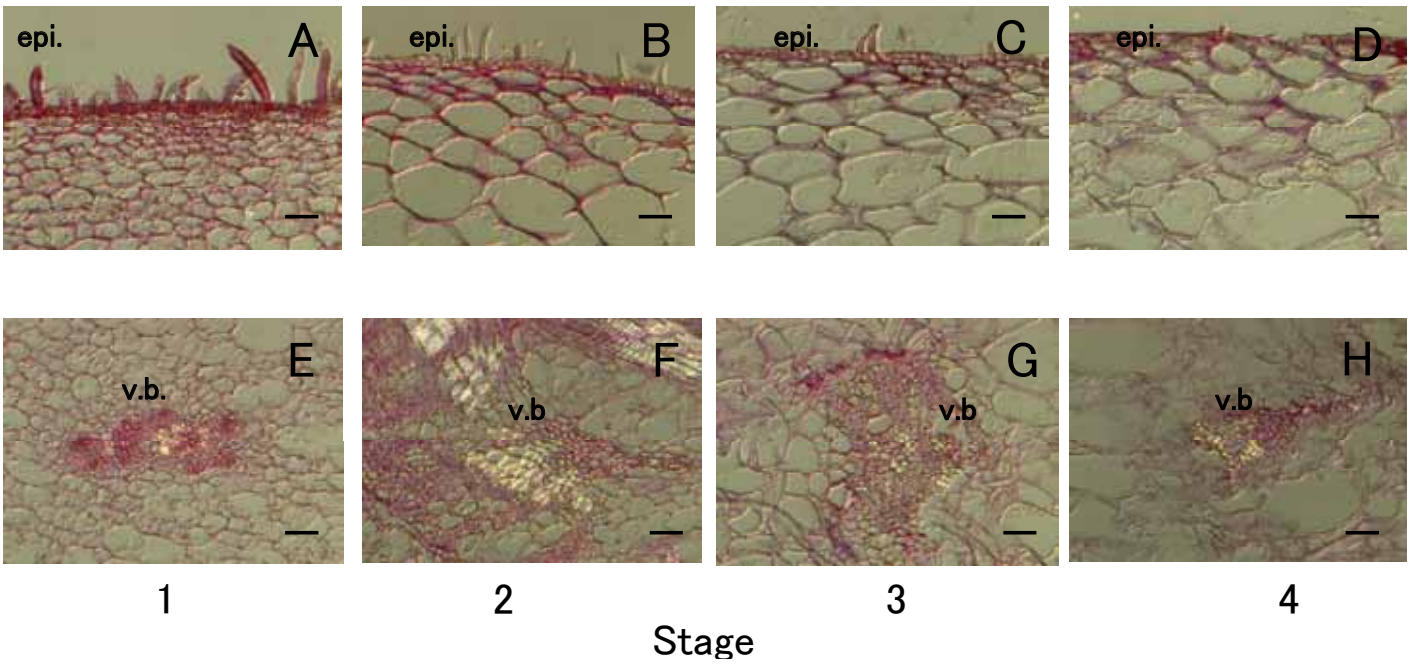

Fig. 2A) Photomicrographs of cross-sections of the pericarp tissues in mume fruit with ripening (A ~ D). Parenchymal tissues beneath epidermis. A= Stage 1; B= Stage 2; C= Stage 3; D= Stage 4. (E ~ H) Vascular bundles. E= Stage 1; F= Stage 2; G= Stage 3; H= Stage 4. epi= epidermis; v.b.= vascular bundle. Tissues were stained with Ruthenium. Photomicrographs of cross-sections of the pericarp tissues in mume. Bars indicate 50 μm.

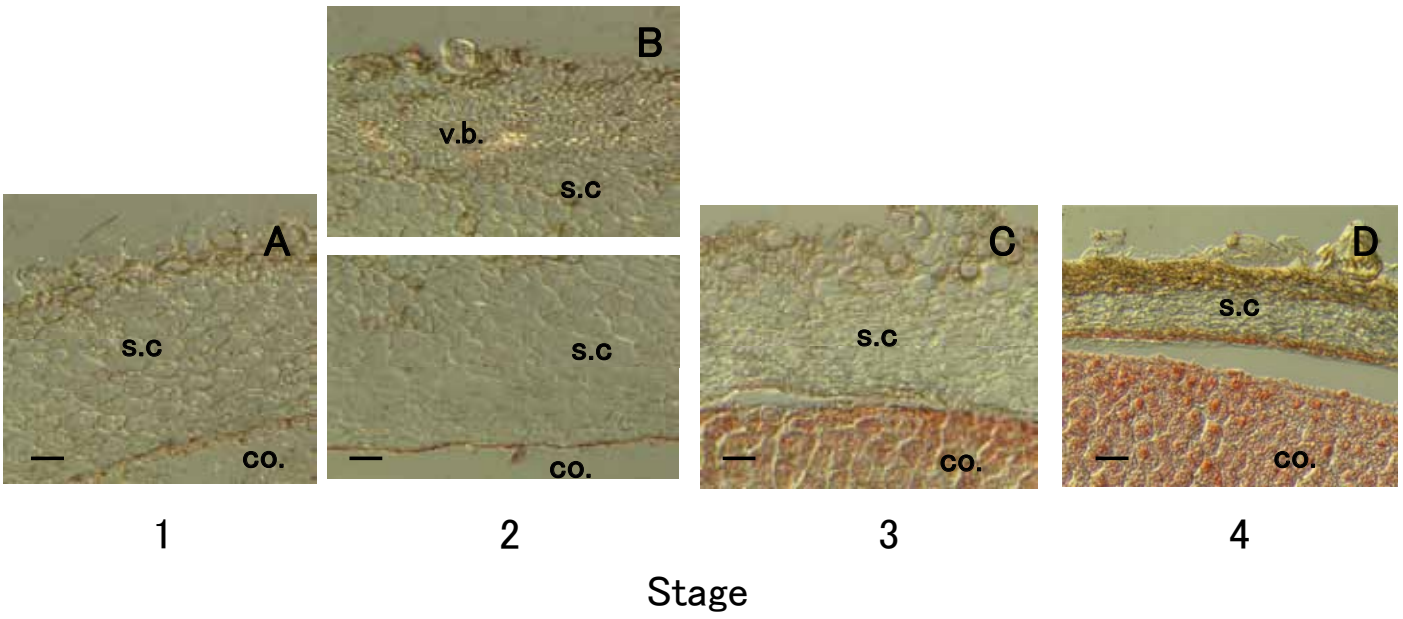

Fig. 2B - Photomicrographs of cross-sections of the seed tissues in mume fruit with ripening (A ~ D). Parenchymal tissues beneath epidermis. A= Stage 1; B= Stage 2; C= Stage 3; D= Stage 4. s.c.= seed coat; co.= cotyledon. v.b= vascular bundle. Tissues were stained with Sudan III. Bars indicate 50 µm.

tion of the fruit (Stage 3). As the peel of the fruit assumes a yellow color, pectic substances in the cell wall structures in the parenchymal tissue collapse, except for the epidermis and vascular bundles (Fig. 2A, Stage 4). On the other hand, lipids in the seeds stained with Sudan III were observed in the epidermis of seed tissues, indicating that the cells of the epidermis are abundant in suberin (Fig. 2B). Moreover, oil bodies were also observed in the parenchyma of the cotyledons in the matured and ripened fruit seeds (Stages 3 and 4).

Water uptake in relation to cell enlargement

In the pericarp tissues, both water uptake and dry weight prominently increased during ripening, especially from the large green-fruit stage to the matured-fruit stage (Fig. 3). Vacuoles occupy a large part of individual cells in the tissues at these stages (data not shown). The epidermis and inner parenchymal cells of the pericarp tissues remarkably enlarge during ripening in a sigmoidal growth curve (Table 2). The enlargement of cells in the pericarp region closely correlated with that for fruit diameter, accompanied by a marked increase in water uptake and dry matter accumulation (Tables 1 and 2). In seed tissues, water uptake did not change, while dry weight remarkably increased in both the matured and ripened fruit (Fig. 2). Therefore, the seed water content remarkably decreased in the mature stages (Fig. 4, Stage 3).

Membrane integrity in the pericarp tissues

Changes in the membrane permeability as well as the cell wall integrity of mume fruit with ripening were considered. Leakage of electrolytes from the pericarp tissues increased during ripening, and 90% of the total electrolytes were found to have leaked at fruit maturation (Fig. 4, Stage 3). Thus, membrane permeability, indicating a loss of membrane integrity, arises in the pericarp tissues.

Tissue specificity of 1H-NMR spectra with various pulse intervals between 180 and 90°

The physiological changes of intact tissues were examined by measuring the physical states of cell-associated

Table 2 - Changes in cell size of pericarp during ripening of mume fruit

		Cell size (µm)			
		Stage			
		1	2	3	4
Epidermis	Long side×short side	25.1× 9.9	39.0×12.3	38.9×22.9	49.5×49.5
Parenchyma	Small cells				
	Long side×short side	44.7×18.6	97.9×54.4	119.7×68.9	142.9×102.9
	Larage cells				
	Diameter	61.4	145.5	181.2	243.9

Values represent the mean of ten cells of the pericarp tissues in three fruits.

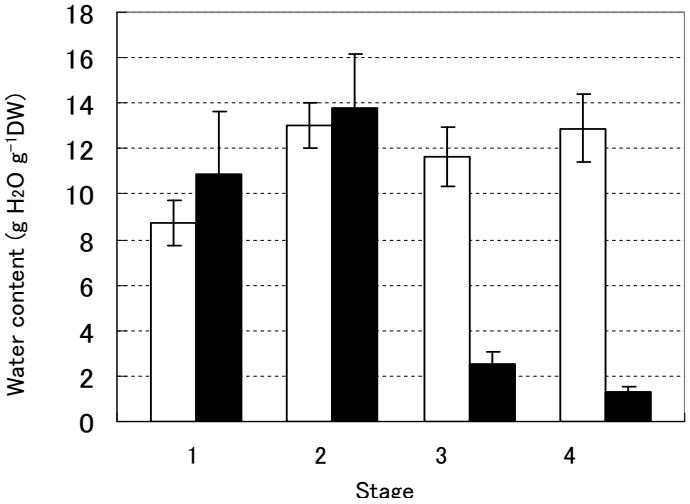

Fig. 3A - Changes in the water content of pericarp (□)and seed (■) for mume fruit with ripening. Values represent the mean of eight to ten fruits ± SE.

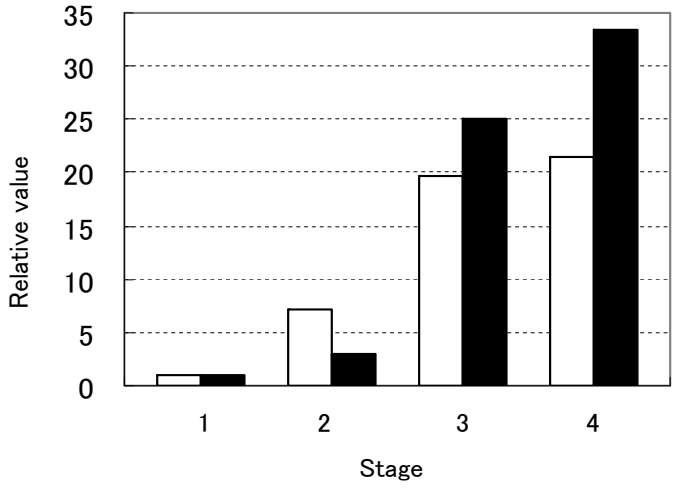

Fig. 3B - Changes in the dry weight of pericarp (□)and seed (■) for mume fruit with ripening. Values represent the mean of eight to ten fruits ± SE.

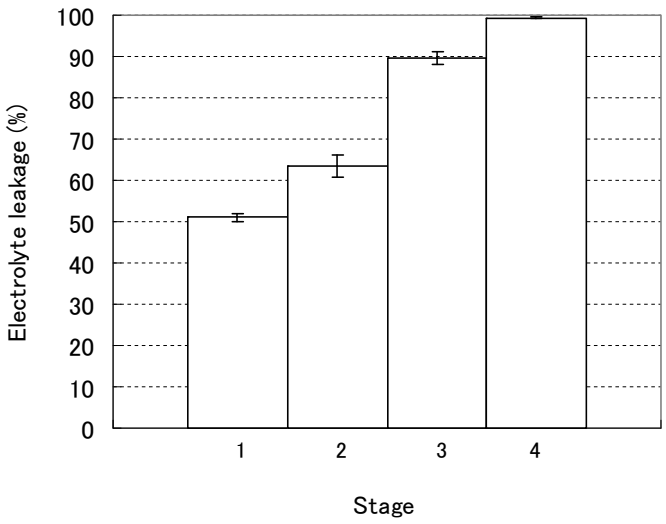

Fig. 4 - Changes in electrolyte leakage of pericarp during ripening of mume fruit. Values represent the means of three fruits ± SE.

water in fruit tissues with ripening using ^{1}H-NMR spectroscopy. ^{1}H-NMR spectra of both intact pericarp and seed in the fruit tissues were examined by spectral recovery of ^{1}H-NMR with the inversion recovery method. The pulse interval was varied between 180° and τ- 90° pulses. An ^{1}H-NMR spectral peak was observed at 4.8 ppm of chemical shift, which corresponds to the ^{1}H nuclei of water. ^{1}H-NMR spectra of both the pericarp and seed tissues were not symmetrical, indicating that the peak consists of components with different chemical shifts and various recovery times (Fig. 5).

In pericarp tissues of the small green fruit, major spectral recoveries were observed in the pulse intervals of 0.3 s and 0.5 s, while in the ripened fruit, these were 0.9 s and 1.5 s (Fig. 5A and B, Stage 1). On the other hand, in seeds of the small green fruit, several peaks of spectral recovery were mainly observed at pulse intervals of 0.8 s and 1.0 s,

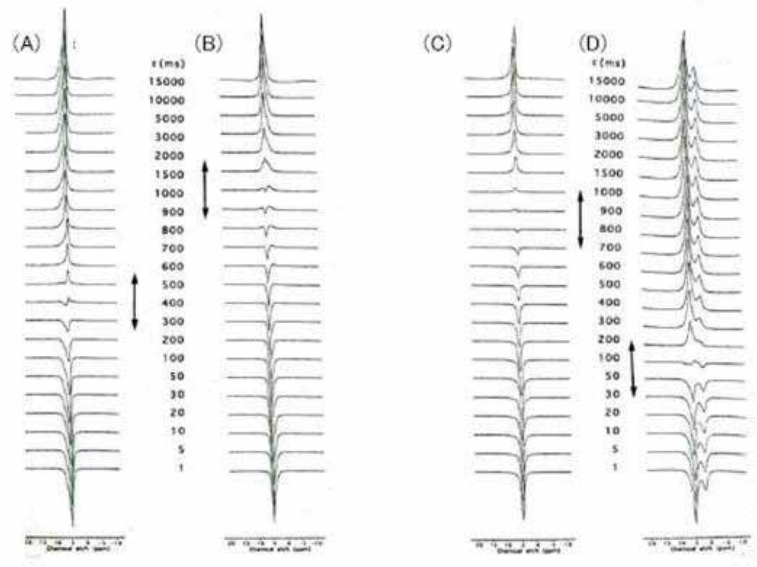

Fig. 5 - Spectral recovery of 1H-NMR of mume fruit with inversion recovery method. T: pulse interval. A= Spectra of pericarp tissues at Stage 1; B= Spectra of pericarp tissues at Stage 4; C= Spectra of seed tissues at Stage 1; D= Spectra of seed tissues at Stage 4. 1; D= Spectra of seed tissues at Stage 4.

while they occurred between 0.05 s and 0.2 s in ripened fruit (Fig. 5C and D, Stage 4). The individual values of the *T*1 components can be obtained by pulse interval (τ) at null point, *T*1 = null / ln2. Thus *T*1 values that are due to major spectral recovery times in pericarp tissues became longer in the ripened fruit, while the tendency was reversed in the seed tissues.

Changes of T1 components in the fruit tissues

The relaxation times and estimated amounts of the individual water fractions calculated from semi-logarithmic plots of ^{1}H-NMR signal intensities in the fruit tissues with ripening processes are listed in Table 3. The spin-lattice NMR relaxation time (*T*1) describes the process of realigning the magnetic moment with the external magnetic field.

Cellular water exists in two to three components, which are shown by NMR relaxation times; in plant tissues, these water components consist of three states of water: i.e. free water, loosely bound water, and tightly bound water (Iwaya-Inoue and Nonami, 2003). The three compartmentalizations of water originally identified as the vacuole, cytoplasm, and cell wall/extracellular space (apoplast) are reflected by the different relaxation times in the parenchymal tissues of apples (Snaar and Van As, 1992; Hills and Remigereau, 1997). Thus, differences in the relaxation times (*T*1, *T*2) of biological tissues can be interpreted as differences between the ratio of free water to bound water. Semi-logarithmic plots of ^{1}H-NMR signal intensity with the inversion recovery method of mume fruit were multi-exponential. The long *T*1 fraction could be understood as highly mobile water (free water) derived from the vacuoles, while the short *T*1 fraction with restricted mobility represents loosely bound and bound water from the cytoplasm and the apoplastic region, respectively.

In the pericarp tissues, *T*1 values of the longest water component markedly increased from 0.8 to 2.0 s from the small green fruit to the large green fruit (Stages 1 and 2). The major fraction of the longest *T*1 was about 80% and the ratio was constant during the ripening stage. The fraction of the shortest component of *T*1, at 0.4 s was about 10% and was also constant through all stages. In addition, vacuoles stained by neutral red were observed in a large proportion of individual cells in the pericarp tissues with ripening (data not shown). Thus, water in the pericarps was considered to increase the amount of free water with ripening. On the other hand, in the seeds of the small green and large green fruits, *T*1 values and the fractions of the longest component were about 2.0 s and 75%, respectively (Table 3, Stages 1 and 2). *T*1 values of the longest water component in the seeds markedly decreased from 2.0 to 0.4 s from the large green-fruit to matured-fruit stages (Stage 2 and 3). The *T*1 values did not change further for the ripened fruit. However, the fraction ratio of the longest water component in the seed decreased from 45 to 30 % during that stage (Stage 4).

Table 3 - Changes in components of ^{1}H-NMR spin-lattice relaxation times (*T*1)s during ripening of mume fruit

	Stage							
	1		2		3		4	
	*T*1 values (ms)	Fraction (%)	*T*1 values (ms)	Fraction (%)	*T*1 values (ms)	Fraction (%)	*T*1 values (ms)	Fraction (%)
Pericarp	412±13	12.1±3.2	416± 1	12.1±0.5	413± 5	12.3±0.8	424±13	11.3±1.7
	603±55	7.4±3.4	1171±44	9.2±2.4	1332±63	11.9±1.1	1723±28	10.2±2.1
	758±54	80.5±1.6	1367±34	78.7±1.7	1619±91	75.8±2.1	2057±94	78.5±1.1
Seed	349±11	13.5±4.5	785± 6	12.2±1.1	181± 3	20.2±1.1	138± 2	18.5±1.2
	1414±28	13.4±4.3	1443±25	15.9±1.3	321±49	33.7±3.6	193± 3	50.6±1.2
	2216±15	73.0±0.4	2040±19	75.6±0.6	443±66	46.1±2.6	370± 2	30.9±0.8

Values represent the means of eight to ten samples±SE..

Table 4 - Changes in components of ^{1}H-NMR spin-spin relaxation times (*T*2)s during ripening of Japanese apricot fruit

	Stage							
	1		2		3		4	
	*T*2 values (ms)	Fraction (%)	*T*2 values (ms)	Fraction (%)	*T*2 values (ms)	Fraction (%)	*T*2 values (ms)	Fraction (%)
Pericarp	151±12	28.1±6.0	289±17	26.4±1.2	394±34	25.9±0.9	404±38	26.9±6.7
	211±10	71.9±6.0	442±10	73.6±1.2	731±26	74.1±0.9	713±14	73.1±6.7
Seed	143±11	12.8±2.8	205±19	16.7±6.3	36± 2	16.7±2.8	27± 2	44.8±2.8
	323±24	87.2±2.8	317±14	83.3±6.3	56± 2	83.3±2.8	47± 6	55.2±2.8

Values represent the means of eight to ten fruit±SE.

Changes of the T2 components in the fruit tissues

The spin-spin NMR relaxation time (*T*2) describes the time-dependent decay of NMR signal due to the dephasing process of the individual spins with respect to each other. The water component estimated by *T*2 was divided into two fractions (Table 4). The regions with long *T*2 values in *Glycine max* seed tissues had high concentrations of free water, while the regions with short *T*2 values had high concentrations of loosely bound and bound water (Ishida *et al.,* 1987). In the pericarps of the mume fruit, the fractions in *T*2 comprised about 70% through the four stages. *T*2 values of both long and short components increased until fruit maturation (Stage 3); however, they did not prolong further at the ripened-fruit stage (Stage 4). By contrast, *T*2 values of the longer water component in seeds markedly decreased from about 300 ms to 60 ms from the large green fruit to the matured fruit (Stage 2 to Stage 3).

4. Discussion and Conclusions

Relationship between NMR relaxation time T1 and the characteristics in mume fruits during development and ripening

Nuclear magnetic resonance (NMR) spectroscopy is a useful technique to follow physiological changes with respect to the state of water in developing Japanese apricot fruits.

Water in living tissues is known to consist of several components with regard to the relaxation of the magnetized protons (Hazlewood *et al.*, 1969; Hazlewood, 1995; Isobe *et al.,* 1999; Iwaya-Inoue, 2004 a, b). In developing Japanese apricot fruits, three components with different *T*1 values were distinguished on the semi-logarithmic plots of the recovery of the ^{1}H-NMR signal (Fig. 5 and Table 3). Multicomponent water fractions are similar to those reported for other plant tissues (Stout *et al.,* 1978; Gusta *et al.,* 1979; Isobe *et al.,* 1999). Referring to the line width of ^{31}P-NMR signals (Kano *et al.,* 1990; Takagishi *et al.,* 1991), the longest component is ascribed to that of vacuoles containing small molecules, such as metabolic intermediates, secondary products and inorganic ions, and the middle component to that of cytoplasm in plant tissues. The water with the shortest *T*1 is considered to be exchangeable water around macromolecules, such as starches, proteins and strings of macromolecules in vesicles or between cell walls (Rorschach and Hazlewood, 1986).

Large amounts of highly mobile water were detected in the seeds of small green and large green fruits. The mobility of exchangeable water in the seed tissues became higher as fruit developed. However, the mobility became low at fruit maturation; thereafter, it continued to be low. On the other hand, the mobile water in the pericarp of small green fruit was low. This suggests that concentrations of cell components that bind water were high in these tissues. Thereafter, the mobile water in the pericarp increased with enlargement and even more so upon coloring of the fruit (Figs. 1 and 5, Tables 1 and 3). These trends agree with previous reports (Ishida *et al.,* 1989; 1994; 1997). Changes in the mobility of water are considered to be the result of physiological changes in fruit growth including seed development and maturation, which is the most important natural function of the fruits (Crane, 1964).

Relaxation times are strongly influenced by the availability of the water and the presence of macromolecules to which water molecules can be "bound". It was shown that *T*1 closely correlated with water content in developing and maturing rice grains (Funaba *et al.,* 2006), in azalea buds subjected to low-temperature stress (Kaku *et al.,* 1984), and in the heat-tolerant and heat-sensitive cultivars rice grains subjected to high-temperature stress (Tanaka *et al*., 2009).

In the pericarp tissues, the changes in water contents were not correlated with the values of the longest *T*1 component with ripening (Fig. 3A, Tables 3). However, our results suggest that other factors may contribute to the motional restriction of water. A similar tendency was observed in sweet potato tubers exposed to cold stress (Iwaya-Inoue *et al.,* 2004 b).

We found that membrane permeability, indicating a loss of membrane integrity, increased in mume pericarp tissues during ripening. An increase in membrane permeability also has been reported in ripening wild-type tomato fruit, but not in the ripening-inhibited (rin) mutant (Poovaiah *et al.,* 1975). Moreover, the permeability of the plasma membrane increased and the membrane-lipid composition changed in ripening *Malus domestica* fruit (Lurie *et al.,* 1987) and during the development and senescence of *Cucumis melo* fruit (Lester and Stein, 1993). This coincided with our results from the electrolyte leakage study for mume fruit. In wood plants (i.e. galled leaves invaded by insects), a higher ratio of ion leakage was strongly associated with prolongation of the NMR relaxation times (Kaku and Iwaya-Inoue, 1990). In addition, it was demonstrated that the relaxation times strongly related to the size and geometry of the vacuolated cells in mushroom tissues during postharvest senescence (Donker *et al.,* 1997). In our experiment, elongation in fully vacuolated cells and changes in membrane permeability in the pericarp tissues may have contributed to the correlation of the longest *T*1 components with fruit ripening.

However, in seed tissues, a considerable decrease in the longest *T*1 components in seeds of mume fruit was accompanied with markedly decreased water content, from 14 to 1 g H_2O/g dry weight from Stage 2 to Stage 4 (Fig. 3A and Table 3). Phenomena such as cold acclimation in plant tissues correlated with the decrease both in *T*1 and in water content (Burke *et al.,* 1974; Kaku *et al.,* 1984; Fennell *et al.,* 1996; Yoshida *et al.,* 1997). The decrease in water content of the seed tissues during maturation is considered to be associated with a reduction in free water, perhaps due to an increase of dry matter, that is, an increase in the accumulation of cellular substances (Fig. 2A and B and Table 3).

Relationship between NMR relaxation time T2 and characteristics in mume fruits during development and ripening

The larger fraction of the two, with the long *T*2, can still be assigned to vacuolar water, whereas the small fraction with short *T*2 represents water from the cytoplasm and perhaps the contribution of water inside the cell wall and extracellular water (Scheenen *et al.,* 2002).

The amount of water or *T*2 in the pericarp and seed changed inversely according to the progression of growth stages. Mobile water in the pericarp of small green fruit was low (Table 4). Thereafter, the mobile water in the pericarp increased with enlargement, but did not change further at the coloring fruit stage. Thus, the changes in water contents were not correlated with the values of a longer *T*2 component with ripening (Fig. 3A and Table 4).

During the stage at which the fruit peel became yellow, however, pectic substances in the cell wall structures in the parenchymal tissue collapsed except for in the epidermis and vascular bundles (Figs. 1 and 2A, Stage 4).

Kaneko *et al.* (1989) reported changes in the pectic substance components during mume fruit ripening. The cellular changes, such as the changes of polysaccharide components or pectic substances, specific to the pericarp tissue ripening characteristics, may contribute to a more ordered state of water, thereby resulting in unchanged relaxation time. In previous reports, McCarthy *et al.,* (1995) measured a decrease in *T*2 in bruised regions of apples. In addition, marked shortening of *T*2 values compared with *T*1 values in the fruit tissues may reflect a correlation between the relaxation times and compartment size of cross-linked polymer gels (Murase and Watanabe, 1989).

On the other hand, in seed tissues, a considerable decrease in the longer *T*2 components in seeds of mume fruit is accompanied with markedly decreased water content from Stage 2 to Stage 4 (Fig. 3A and Table 4).

Water status can provide useful information about the characteristics of mume fruit development and ripening

When the seed of a small green mume fruit was gellified, the mobility of water was high. Thereafter, when the color inside the seed of a small green mume fruit turned milky white, the mobility of water, water derived from the vacuole and exchangeable water around macromolecules, became low (Fig. 1 and Tables 3 and 4). The decrease in water content of the seed tissues during maturation is associated with a reduction in free water, perhaps due to an increase of dry matter, that is, an increase in the accumulation of cellular substances such as oil bodies (Fig. 2B and Fig. 3B).

Japanese apricot fruit pericarp tissues may act as a sort of storehouse where photosynthates are temporarily accumulated for further transport into the seed during seed formation. Therefore, seed formation, which is the primary object of fruit growth, may require larger amounts of energy than are available in the pericarp. The parenchymal tissues of the pericarps in matured mume fruit began to collapse in the yellow-peel stage (Figs. 1 and 2A), probably because the pericarp tissues are no longer necessary to physiological functions. These are considered to be the result of a loss of membrane integrity and efflux of pectic substances in the cell wall structures (Figs. 2A and 4). Regardless of the constant high water content, the level of high-mobility water, i.e. water derived from vacuoles and exchangeable water, was high (Fig. 3A, Tables 3 and 4). This increase is considered to result from the fact that the increase of the mobile water by cell wall breakdown is greater than the decrease of the free water by cellular accumulation. In addition, the enlargement of cells in the pericarp region closely correlates with increases in fruit diameter, water uptake, and dry matter accumulation. Changes in the fully-vacuolated-cell volume were closely correlated with changes in the values of the longest *T*1 component during ripening (Tables 2, 3 and 4). Thus, the water compartments and the mobility of water analyzed in this study are considered to reflect the result of physiological changes such as cellular heterogeneity and spatial arrangements both in the pericarp and in seed tissues for mume fruit with development and ripening.

In conclusion, the analysis of water components derived from ^{1}H-NMR spectroscopy provides useful information about the characteristics of fruit ripening.

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A preliminary study on pollen compatibility of some hazelnut cultivars in Iran

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Key words: *Corylus avellana* L., Filbert, nut set, pollen incompatibility, pollination.

Abstract: Pollen incompatibility is a major problem among hazelnut cultivars and can result in considerable crop loss in hazelnut orchards. Thus, identifying the level of self-compatibility of a cultivar and compatibility between cultivars are crucial aspects to selecting the proper pollenizers. Controlled self- and cross-pollinations were carried out in three local and four imported hazelnut cultivars. Based on cluster set, partial self-compatibility was found in cultivars Pashmine and Shastak, and complete self-compatibility in 'Tabestane'. The best pollenizers for the cultivars Pashmine, Tabestane, Shastak, Barcelona, Segorbe, Daviana and Merveille were Segorbe, Barcelona, Segorbe, Shastak, Shastak, Pashmine and, Pashmine, respectively.

1. Introduction

Pollen incompatibility is a recognition mechanism that enables plants to prevent inbreeding. Many flowering plants have these systems and prevent self-fertilization and subsequently prevent inbreeding depression (Hiscock, 2002). Pollen compatibility is an essential factor in breeding programs of fruit trees and for selection of the best pollenizers in orchard establishment for all fruit and nut species, including the European hazelnut (*Corylus avellana* L.) (Mehlenbacher, 1997). Most hazelnut cultivars are self-incompatible; self- and cross-incompatibility in hazelnut cultivars are widespread phenomena which are of the sporophytic type (Germain, 1994). Dominance relations may lead to reciprocal differences in pollen incompatibility between cultivars, a result that makes pollinizer selection a complicated decision (Hampson *et al*., 1993). Cross-pollination is crucial for efficient nut set in hazelnuts. At least two different pollinizers are recommended for commercial yields to ensure sufficient amounts of viable, compatible pollen when needed, since flowers continue to emerge for several weeks (Hampson, *et al*. 1993). Therefore, in commercial orchards there should be several verified compatible cultivars to achieve appropriate nut production. To do so, it is suggested to have 6 to 15% of orchard trees as pollenizers (Mehlenbacher and Miller, 1988).

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Several studies have been carried out to determine the level of self- and cross-compatibility in different hazelnut cultivars from different geographical regions (Mehlenbacher and Smith, 1991; Mehlenbacher, 1997; Erdogan and Mehlenbacher, 2000; Mehlenbacher and Smith, 2006; Vicol, *et al*., 2009; Mehlenbacher, 2014). There is a serious need to examine self- and cross-compatibility of hazelnut cultivars in different climates and regions to determine the most compatible pollenizers for main hazelnut cultivars in the region. Accurate information on this aspect could also enhance breeding efficiency and contribute to knowledge about hazelnut pollen-stigma incompatibility as well as helping to improve further efforts to study interspecific and intraspecific crosses (Molnar, 2011).

Notwithstanding the importance of this requirement, there is little information on pollen compatibility of hazelnut cultivars grown in Iran. The present investigation was undertaken as a preliminary study to determine the level of self- and cross-compatibility of three Iranian and four imported hazelnut cultivars.

2. Materials and Methods

The study was carried out in winter 2010 at the Astara Hazelnut Research Station in Astara, province of Guilan, Iran. Three Iranian cultivars including Shastak, Pashmine, and Tabestane, and four imported cultivars Barcelona, Daviana, Segorbe, and Merville (= Merveille de Bollwiller) were selected. Three trees of each cultivar and some

shoots of the trees were selected: for each tree of Iranian cultivars six shoots were selected (for self-pollination, open-pollination and pollination with four imported cultivars); and also for each tree of imported cultivars four shoots were selected (for open pollination and pollination with three Iranian cultivars). The experimental design was completely randomized blocks with each tree considered as one block.

Based on the method described by Mehlenbacher (1997), after removing the catkins, the selected shoots were isolated with long paper bags. The selected shoots for open-pollination were left unbagged to receive airborne pollen. At the same time pollination and pollen receptivity period of all cultivars were monitored and recorded. Since the life of pollen and the receptivity of stigma are short and there is a gap between the periods of female and male flowers bloom, it is essential to know the duration of their activity for all cultivars in the region.

Pollen was collected in January, before the beginning of pollen shedding, and kept in vials at -18°C. Controlled pollinations were performed by hand when styles appeared and became receptive. The number of pollinated pistillate inflorescences was recorded after pollination. Produced nut clusters were picked and counted in early September. The percentage of nut cluster set was determined as the ratio of the produced nut clusters to the pollinated inflorescences.

To evaluate the level of self- and cross-incompatibility, the index of self-incompatibility (ISI) was applied (Zapata and Arroyo, 1978). ISI is the ratio of nut set after self- or cross-pollination to nut set after open-pollination, as a potential compatible cross. When the ratio is ≤ 0.2, the cross is incompatible, 0.2-1 is partially compatible, and ≥ 1 is completely compatible. Since this ratio was applied to evaluate both self- and cross-incompatibility, the index of pollen-incompatibility (IPI) was used instead of ISI, as suggested by Seifi *et al*. (2011). The obtained data were analyzed by analysis of variance (ANOVA) using SAS software version 9.1 for Windows (SAS Institute, 2001).

3. Results and Discussion

Pollination and pollen receptivity of all cultivars are illustrated in figure 1. Dichogamy was predominant in all cultivars. All seven cultivars were protanderous, which was expected according to the literature (Germain, 1994). The most distinct dichogamy was obvious in the cultivar Tabestane. Although there was complete self-compatibility through hand pollination in this cultivar, its pollen will not touch its stigma when such dichogamy exists. However, it is noticeable that dichogamy is influenced by climatic conditions and may differ from year to year. The shortest and longest period of pollen release were recorded in 'Shastak' (20 days) and 'Merville' (35 days), respectively. Further, the shortest and longest period of pollen receptivity by stigma of pistillate flowers were recorded in 'Shastak' (10 days) and 'Tabestane' (45 days), respectively (Fig. 1). The activity of male inflorescence of 'Merville' had an appropriate overlap with receptivity of the female inflorescence of all studied cultivars.

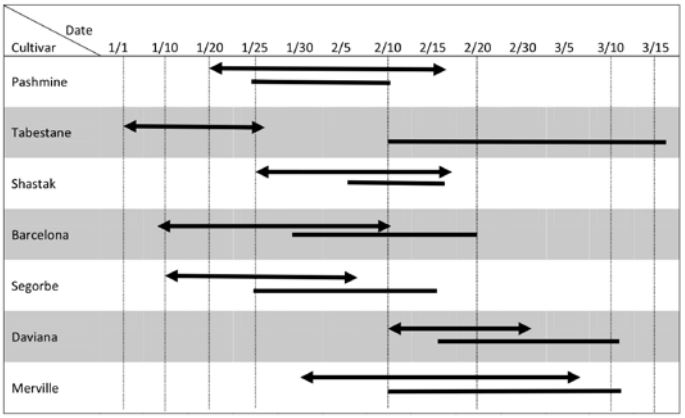

Fig. 1 - Period of bud break and female and male bloom of 10 hazelnut cultivars. ◄——► Male bloom; ▬▬ female bloom.

Pashmine

'Pashmine' is a local Iranian cultivar that is widespread throughout Iran. Little information is available about this cultivar's characteristics. Results showed that there was a significant difference between pollenizers in terms of nut set in 'Pashmine'. As can be seen in Table 1, there was no significant difference between self-pollination and open-pollination in 'Pashmine' which produced 39.4% nut set after self-pollination, indicating its partial self-compatibility. This result is roughly similar to nut set after self-pollination in the cultivar Tombul (44%) (Mehlenbacher and Smith, 1991). This level of self-compatibility is noticeable in this species and is reported here for the first time in Iran. The pollen of 'Barcelona', 'Daviana' and 'Merville' was completely incompatible with 'Pashmine' (IPI < 0.2) and produced no nuts. Pollen of 'Segorbe' was partially compatible with 'Pashmine' (IPI = 0.21) (Table 2).

Tabestane

This cultivar has a small nut and kernel and is widespread throughout traditional orchards in Iran. Statistical analysis revealed a highly significant difference between treatments (pollenizers) ($P < 0.01$) (Table 1). There was no significant difference between self- and open-pollination, and self-pollination resulted in 59.82% nut set, indcating complete compatibility (IPI = 1.22) (Table 2). This is a rare and interesting phenomenon in hazelnut. This level of self-compatibility was higher than the two other local pollenizers 'Pashmine' (39.4%) and 'Shastak' (19.33%). Nevertheless, this nut set percentage was lower than that after self-pollination in some genotypes reported by Mehlenbacher and Smith (2006).

Shastak

The size of nut and kernel of this local cultivar is medium. According to analysis of variance, pollenizers had a significant difference in terms of nut set. The highest nut set was obtained through open pollination (63.14%). 'Shastak' showed partial self-compatibility (IPI = 0.31) (Table 2). 'Barcelona', 'Daviana' and 'Merville' with IPI lower than 0.2 were considered as completely incompatible pollenizers (Table 2).

Barcelona

In the literature, the pollenizers 'Casina', 'Daviana' and 'Merville' have been suggested for 'Barcelona' (Wilkinson, 2005). Pollenizers for this cultivar were significantly different. Open pollination resulted in 50% nut set which was statistically different from other pollenizers ($P < 0.01$) (Table 1). Pollen of 'Pashmine' was completely incompatible with 'Barcelona' (IPI = 0) and produced no nut and consequently is not suggested for 'Barcelona'. More research is needed to ensure this finding.

Segorbe

This cultivar originated from France and has upright growth with medium to large nuts. Its pollen grain is shed in early winter and pistillate flowers are observable in mid-winter (Wilkinson, 2005). Observations demonstrated significant differences between treatments. The highest nut set in 'Segorbe' was obtained after open pollination (82.62%). This could be as a result of the effect of mixing various cultivars' pollen grains from more than 40 genotypes at the station. Based on calculated IPI, 'Tabestane' (0.22) and 'Shastak' (0.24) were partially compatible with 'Segorbe'; but 'Pashmine' was incompatible (Table 2).

Daviana

'Daviana' originated from England and is an upright growing cultivar with few root suckers and medium and oblong nuts. Its pollen grain is shed in mid-winter and pistillate flowers are observed in late winter. This cultivar is considered an appropriate pollenizer for 'Barcelona' and 'Butler' (Wilkinson, 2005). Based on analysis of variance, there was a significant difference between all pollenizers. The highest nut set resulted from open pollination (26.78%) which actually was not that much in such an orchard containing almost 40 different cultivars. 'Pashmine' was partially compatible (IPI = 0.35); however, the amount of nut set was low (Tables 1, 2). The pollenizers 'Tabestane' and 'Shastak' showed complete incompatibility. Therefore, according to the preliminary findings, the three mentioned pollen sources are not suggested for 'Daviana'.

Table 1 - Cluster set in controlled self- and cross-pollinations of some hazelnut cultivars (%)

Pollenizer \ Main cultivar	Pashmine	Tabestane	Shastak	Barcelona	Segorbe	Daviana	Merville
Pashmine	39.4±20.8 ab	-	-	0.00±0.00 c	2.38±2.38 b	9.48±1.97 a	25.16±3.41 b
Tabestane	-	59.82±15.61 a	-	17.26±11.28 b	18.35±6.33 b	1.59±1.59 b	18.38±6.62 b
Shastak	-	-	19.33±8.51 bc	17.76±5.88 b	19.44±15.47 b	1.19±1.19 b	20.73±1.49 b
Barcelona	8.4±4.55 bc	13.63±9.77 b	9.42±8.14 c	-	-	-	-
Segorbe	12.3±6.32 bc	10±3.71 b	30.47±4.38 b	-	-	-	-
Daviana	7.5±1.5 bc	6.72±4.15 b	5.84±2.95 c	-	-	-	-
Merville	5.61±1.98 c	5.75±3.02 b	2.3±1.15 c	-	-	-	-
Open	57.38±6.81 a	49.02±8.22 a	63.14±6.31 a	50±1.92 a	82.62±7.98 a	26.78±9.71 a	76.18±6.17 a

Mean±SE. Means within a column followed by the same letter are not significantly different ($P<0.01$).
Within the table, hyphen (-) means that the cluster set was not determined where it was not aimed.

Table 2 - Index of pollen incompatibility (IPI) in different crosses of hazelnut cultivars

Pollenizer \ Main cultivar	Pashmine	Tabestane	Shastak	Barcelona	Segorbe	Daviana	Merville
Pashmine	0.69	-	-	0.00	0.03	0.35	0.33
Tabestane	-	1.22	-	0.35	0.22	0.059	0.24
Shastak	-	-	0.31	0.36	0.24	0.044	0.27
Barcelona	0.15	0.28	0.15	-	-	-	-
Segorbe	0.21	0.20	0.48	-	-	-	-
Daviana	0.13	0.14	0.09	-	-	-	-
Merville	0.0017	0.12	0.04	-	-	-	-
Open	-	-	-	-	-	-	-

Within the table, hyphen (-) means that the cluster set was not determined where it was not aimed.

Merville

This cultivar is known as Hall's Giant in the United States and Australia and has been created from a seedling selection in Germany in 1788 (USDA, 2010). Its pollen is shed in late winter, after other cultivars, for a short time. Its pistillate flowers also appear in late winter (Wilkinson, 2005). Results showed that open pollination led to the highest nut set (76.18%) which was significantly different from the other pollenizers. 'Pashmine', 'Tabestane' and 'Shastak' showed partial compatibility with 'Merville', without any significant difference between them (Table 1).

4. Conclusions

In conclusion, based on cluster set observations, partial self-compatibility was found in the cultivars Pashmine and Shastak, and interestingly complete self-compatibility in Tabestane. The best pollenizers for the cultivars Pashmine, Tabestane, Shastak, Barcelona, Segorbe, Daviana and Merveille were Segorbe, Barcelona, Segorbe, Shastak, Shastak, Pashmine, and Pashmine, respectively. Since the observed compatibility in hazelnut cultivars may not be consistent over the years, complementary pollination studies should be carried out to verify the previous compatibility results and therefore document more reliable data.

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Origin of *Prunus* × *yedoensis* 'Somei-yoshino' based on sequence analysis of *PolA1* gene

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Key words: Flowering cherry, phylogenetic relationships, *PolA1* gene, RNA polymerase I largest subunit.

Abstract: *Prunus* × *yedoensis* 'Somei-yoshino' is the most popular cultivar of flowering cherry in Japan. Although the origin of this cultivar has been considered hybrid between *P. pendula* f. *ascendens* and *P. lannesiana* var. *speciosa*, the paternity of *P. lannesiana* has not been clearly proven by molecular analysis. To reveal the origin of 'Somei-yoshino,' we analyzed sequences of intron 19 and exon 20 of *PolA1*, a single-copy nuclear gene encoding the largest subunit of RNA polymerase I. One of two exon 20 sequences found in 'Somei-yoshino' was the same as that of *P. pendula*, whereas the other sequence was shared with several taxa in seven wild species, including *P. jamasakura* and *P. lannesiana*. 'Somei-yoshino' contained two different haplotypes of the intron 19 sequences; one was the same as that of *P. lannesiana*, which is endemic to the Izu and Boso Peninsula in Japan. While another haplotype of 'Somei-yoshino' was different from that of *P. pendula* by two SNPs but identical to one of two haplotypes of *P. pendula* 'Komatsu-otome,' which is a cultivar found in the Ueno Park, Tokyo. These results indicated that 'Somei-yoshino' probably originated by the hybridization of cultivars derived from *P. pendula* and *P. lannesiana*.

1. Introduction

The subgenus *Cerasus* of the genus *Prunus* includes more than 50 species, most of which are distributed in temperate areas in the Northern Hemisphere, especially in China, where 33 wild species occur (Yu and Li, 1986). In Japan, nine native species are recorded: *P. jamasakura* Sieb. ex Koidz., *P. sargentii* Rehder, *P. verecunda* (Koidz,) Koehne, *P. incisa* Thumb. ex Murray, *P. nipponica* Matsum., *P. apetala* (Sieb. et Zucc.) Fr. et Sav., *P. lannesiana* (Carr.) Wilson var. *speciosa* (Koidz.) Makino, and *P. pendula* f. *ascendens* (Makino) Ohwi. In addition, three wild species, *P. pseudo-cerasus* Lindl., *P. cerasoides* D. Don and *P. campanulata* Maxim., have been popularly cultivated since their introduction from China, Taiwan, and Nepal, respectively (Kawasaki, 1991).

Several classifications based on morphological observations have been proposed for Japanese flowering cherries (Kawasaki, 1991; Kobayashi, 1992; Ohba, 1992), and they have been classified into five sections: *Apetalae* (*P. apetala*), *Incisae* (*P. incisa* and *P. nipponica*), *Sargentiella* (*P. jamasakura*, *P. sargentii*, *P. verecunda*, and *P. lannesiana*), *Phyllomahaleb* (*P. maximowiczii*), and *Microcalymma* (*P. pendula*). The phylogenetic relationships among these taxa have been investigated using restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA (Kaneko *et al*., 1986), randomly amplified polymorphic DNA (RAPD) analysis (Shimada *et al*., 2001), and analyses of rDNA ITS sequences (Lee and Wen, 2001), SSR markers for nuclear DNA (Ohta *et al*., 2005), and plastid subtype identity (PSID) sequences (Ohta *et al*., 2006).

More than 250 cultivars of flowering cherries, including *Prunus* × *yedoensis* Matsum. 'Somei-yoshino' (Iketani *et al*., 2006), have been created through repeated natural and artificial hybridizations among wild *Cerasus* species (Kawasaki, 1993). 'Somei-yoshino' was first proposed to have arisen as a hybrid between *P. lannesiana* var. *speciosa* and *P. pendula* f. *ascendens* (Wilson, 1916). Takenaka (1962, 1965) produced hybrids between the two species and noted that they had similar morphological characters to 'Somei-yoshino.' However, these hybrid plants, such as 'Amagi-yoshino' and 'Izu-yoshino,' were taller and produced many more flowers with white petals than 'Somei-yoshino.' Based on SSR marker analysis, Iketani *et al*. (2007) pointed out clonal status of 'Somei-yoshino,' which has been propagated by grafting.

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Previously, Kaneko *et al*. (1986) showed that *P. pendula* might be the maternal parent of 'Somei-yoshino' based on RFLP patterns of chloroplast DNA. A recent analysis of PSID sequences provided additional support that 'Somei-yoshino' had the 10A-T-4A haplotype specific to *P. pendula*, but not the 14A haplotype for *P. lannesiana* and *P. jamasakura* (Ohta *et al*., 2006). Recently, Roh *et al*. (2007) proposed that variations for nuclear ISSR markers and two plastid DNA sequences of the *P. yedoensis* population on Korean Jeju Island overlapped those of 'Somei-yoshino.' Their data, however, did not prove the paternal origin of 'Somei-yoshino.' Utilizing the associations of nuclear DNA markers dispersed over the genome, it is especially difficult to identify the paternal parent in *Cerasus* species. Because the *Cerasus* species have complete self-incompatibility, those DNA markers recombine every generation.

To resolve the paternity of 'Somei-yoshino,' we decided to compare a relatively short sequence within a single-copy gene, because a short DNA sequence is thought to be a block consisting of many closely linked DNA markers, for which recombination is difficult. Sang (2002) stated that the sequences of single- or low-copy nuclear genes are particularly helpful for understanding the inter- and intraspecific relationships of various plant groups. Recently, we were interested in *PolA1* as a candidate single-copy gene, which encoded the largest subunit of the RNA polymerase I complex. The DNA sequences of intron 19 of the *PolA1* gene were highly polymorphic whereas the exon 20 sequences showed species-specific variations in the genera *Petunia* (Zhang *et al*., 2008), *Oryza* (Takahashi *et al*., 2009), and *Triticum* (Takahashi *et al*., 2010).

The present study was initiated to reveal the origin of 'Somei-yoshino' through the analysis of intron 19 and exon 20 sequences in *PolA1* gene.

2. Materials and Methods

Plant material

Most of the DNA samples used in this study were provided from the Faculty of Agriculture, Shizuoka University, and some DNA samples were extracted from leaves of the clonally propagated plants that were maintained in the Tama Forest Science Garden, Tokyo, Japan. A total of 42 individuals of nine wild species native to Japan (Table 1) were analyzed; *P. apetala* (three individuals), *P. incisa* (four), *P. nipponica* (four), *P. jamasakura* (eight), *P. sargentii* (two), *P. verecunda* (four), *P. lannesiana* (three), *P. maximowiczii* (three), and *P. pendula* (six), and three alien wild species, *P. campanulata* (two), *P. cerasoides* (two), and *P. pseudo-cerasus* (one) (Table 1). Two cultivars, 'Somei-yoshino,' *P. pendula* f. *ascendens* 'Komastuotome' Hayashi & Nshida (Hayashi, 1989), and five Edohigan trees were collected in Ueno Park, Tokyo, Japan. One individual of apricot (*Prunus armeniaca* L.) was also analyzed as out-group material.

Genomic DNA isolation and PCR amplification

Genomic DNA was extracted from *ca*. 50 mg of young leaves using a modified CTAB method (Doyle and Doyle, 1987). The forward primer designated as 19ex5P (5'- CTCGCTGGACGGGGTGAGATGAATG-3') and the reverse primer designated as 21ex3P (5'-ATTTACTGGCAATCCAAGACAGAT-3') were designed based on *PolA1* gene (GenBank accession No. NM_125397) of *Arabidopsis thaliana* and EST (GenBank accession No. BQ641151) of almond (*Prunus dulcis* Mill.), respectively. DNA fragments containing intron 19 and exon 20 sequences of *PolA1* gene were amplified by PCR using a pair of 19ex5P and 21ex3P primers (Fig. 1). The reaction mixture of 25 µl contained 10-50 ng of genomic DNA, 1 unit of *Ex Taq* DNA polymerase (TaKaRa Co., Japan), 2.5 µl of 10× buffer (100 mM Tris-Cl, 500 mM KCl, and 15 mM $MgCl_2$, pH8.0), 2 µl of 2.5mM dNTPs, 1 µl of 2.0 µM each primer (19ex5P and 21ex3P), and 17.5 µl of distilled water. PCR was performed with a condition of 35 cycles of 94°C for 1 min denaturation, 58°C for 1 min annealing, and 72°C for 2 min elongation in PTC200 Thermocycler (MJ Research Co., USA).

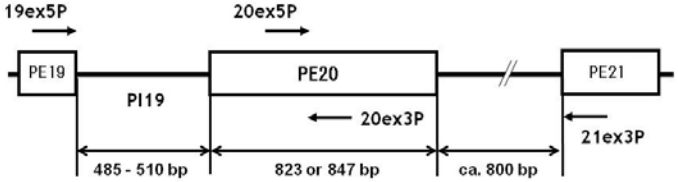


Fig. 1 - DNA fragments containing intron 19 (PI19) and exon 20 (PE20) of *PolA1* gene were amplified using a pair of 19ex5P (PE19) and 21ex3P (PE21) primers. The sequences were determined by direct sequencing using primers for the initial amplification and internal sequence primers, 20ex5P and 20ex3P.

Direct sequencing of PCR products containing the intron 19 and exon 20

The amplified PCR products were subjected to 1.2% agarose gel electrophoresis, purified using QIAquick PCR Purification Kit (Qiagen Co., USA), and directly sequenced with 19ex5P or 21ex3P primer used for the PCR-amplification by ABI3100 Automated DNA Sequencer with a BigDye Terminator Cycle Sequencing Kit (Life Technologies Co., USA). Either 20ex3P (5'-TTGAAGATGTTCAGGTATGGGGAG-3') or 20ex5P (5'-ATAAGTTGAAGAAAATCAC TGTGG-3') primer were also used as an internal sequencing primer. The two internal primers were designed based on the partially determined sequences of *PolA1* exon 20 of *Cerasus* in this study.

The determined sequences of the intron 19 and exon 20 of *PolA1* gene were analyzed using a NCBI web-based Blast server (Altschul *et al*., 1990), and aligned using web server of Mafft ver 6.0 (Kato and Toh, 2008), and then the aligned sequences were subjected to phylogenetic analysis using UPGMA software, with bootstrap analysis using 1,000 replicates, in the Mega 4.0 (Tamura *et al*., 2007).

Table 1 - Samples used in this study

Species	Name [Z]	Locality	PI19 [y]	PE20 [y]
Prunus apetala (Sieb. et Zucc.) Fr. et Sav.	TJ063	Kawaguchiko, Yamanashi	507	nd.
	TJ093	Chino, Nagano	507	847
	TJ164	Hachioji, Tokyo (TFSG)	507	847
P. incisa Thumb.	MM048	Gotenba, Shizoka	507	847
	MM131	Fujimi, Nagano	507	847
	MM160	Amatsukominato, Chiba (TFSG)	507	847
	MM165	Fujiyoshida, Yamanashi (TFSG)	507	nd.
P. nipponica Matsum.	TK077	Shizuoka, Shizuoka	507	nd.
	TK113	Ashiyasu, Yamanashi	507	nd.
	TK140	Fujimi, Nagano	507	847
	TK188	Kusatsu, Gunma	507	847
P. jamazakura Sieb. ex Koidz.	YM001	Morimachi, Shizuoka	507	nd.
	YM011	Ishikawa Forest Exper. Station	485,507	nd.
	YM038	Amagiugashima, Shizuoka	485	823
	YM154	Hachioji, Tokyo (TFSG)	507	nd.
	YM245	Kushikino, Kagoshima	507	847
	YM256	Izumi, Kumamoto	507	847
	YM272	Kinkai, Nagazaki	507	nd.
	YM277	Yayoi, Notsu, Oita	485,507	nd.
P. sargentii Rehder	OY024	Ishikawa Forest Exper. Station	507	nd.
	OY162	Mamurogawa, Yamagata (TFSG)	507	847
P. verecunda (Koidz.) Koehne	KS016	Ishikawa Forest Exper. Station	507	847
	KS136	Fujimi, Nagano	507	nd.
	KS183	Yahiko, Niigata	507	nd.
	KS211	Nishiki, Yamaguchi	507	847
P. lannesiana (Carr.) Wilson var. speciosa (Koidz.) Makino	OS017	Ishikawa Forest Exper. Station	507	847
	OS166	Miyake, Tokyo (TFSG)	507	847
	OSMTD	Matsudo, Chiba	507	nd.
P. maximowiczii Rupr.	MY076	Shizuoka, Shizuoka	507	823
	MY139	Fujimi, Nagano	498	nd.
	MY158	Chichibu, Saitama (TFRG)	507	823
P. pendula Maxim. f. *ascendens* (Makino) Ohwi.	EH015	Ishikawa Forest Exper. Station	506	nd.
	EH149	Ochiai, Okayama (TFRG)	506	nd.
	EH150	Takekawa, Yamanashi (TFRG)	506	823
	EH155	Oya, Hyogo (TFRG)	506	nd.
	EH163	Oguchi, Kagoshima (TFRG)	506	823
	EHFSG	a mountain behind TFRG	506	nd.
P. pseudo-cerasus Lindl. Shinami	SN009	Ishikawa Forest Exper. Station	507	823
P. campanulata Maxim.	KN014	Ishikawa Forest Exper. Station	507	823
	KN101	Tsukubo Botanical Garden	507	nd.
P. cerasoides D. Don.	HM001	Katomandu, Nepal (Shizuoka U.)	507	823
	HM002	Katomandu, Nepal (Shizuoka U.)	507	nd.
P. armeniaca L.	ANZ	Chiba University	510	823
P. × *yedoensis* Matsum. 'Somei-yoshino'		Chiba University	505, 507	823, 847
P. pendula Maxim. 'Komatsu-otome'		Ueno Park, Tokyo	505, 506	823

[Z] Nos. are according to Ohta *et al*. (2006).
[y] length (bp).
Nd= not determined.

3. Results

Polymorphisms of the PolA1 intron 19 sequences

Using total DNA extracted from 43 individuals in ten species as template, *ca*. 2.2-kb-long DNA fragments containing intron 19 and exon 20 of the *PolA1* gene were clearly amplified by PCR (Fig. 2). The *PolA1* intron 19 sequences of most *Cerasus* species were 507 bp in length (Table 1). All six individuals of *P. pendula* contained 506 bp because of a one-base insertion at position 49 and a two-base deletion at position 349-350. One individual (YM038) of *P. jamasakura* and one individual (MY139) of *P. maximowiczii* had shorter intron 19 lengths of 485 and 498 bp, respectively. Two individuals (YM011 and YM277) of *P. jamasakura* had two intron 19 sequences of different lengths (485 and 507 bp), although these sequences could not be confirmed.

The DNA sequences described in this paper have been deposited in DDBJ DNA database (accession nos. LC010372- LC010416).

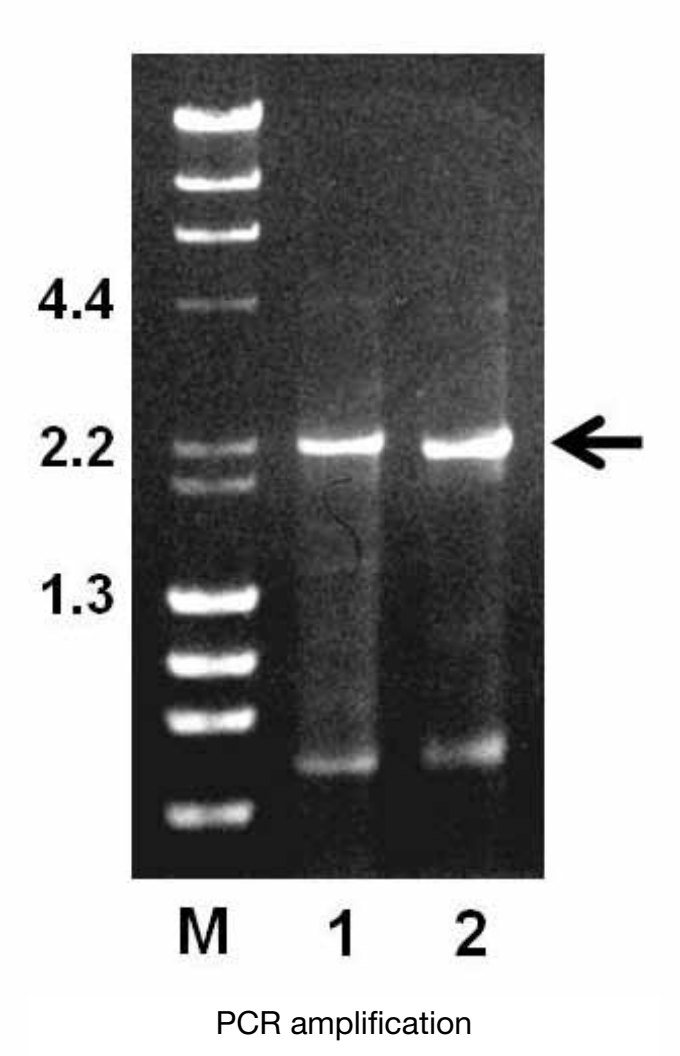


Fig. 2 - PCR products (arrow) of DNA fragments containing intron 19 and exon 20 of *PolA1* gene in the *Cerasus* species, 1: *Prunus lannesiana* var. *speciosa*, 2: *P. pendula* f. *ascedens*, M: marker (λDNA/*Hin*dIII plus øx144 DNA/*Hae*III).

Polymorphisms of the PolA1 exon 20 sequences

Prunus pendula, P. maximowiczii, P. pseudo-cerasus, P. campanulata, P. cerasoides, and *P. armeniaca* (outgroup) contained an 823-bp exon 20 (Table 1), whereas, seven species (*P. apetala*, *P. incisa*, *P. nipponica*, *P. jamasakura*, *P. sargentii, P. verecunda*, and *P. lannesiana*) showed a long 847-bp-long exon 20 with a 24-bp insertion. One individual (YM038) of *P. jamasakura* had the short exon 20 (823 bp), and two individuals (YM011 and YM277) possessed both long and short exons 20 (Table 1).

The DNA sequences described in this paper have been deposited in DDBJ DNA database (accession nos. LC010540 - LC010565).

Phylogenetic tree of the PolA1 intron 19 and exon 20 sequences

The 41 sequences determined for intron 19 and the 23 sequences for exon 20 were aligned using Mafft and subjected to phylogenetic analysis using the UPGMA method in MEGA 4.0 with 1,000 bootstap replicates. In the phylogenetic tree for exon 20, the nine Japanese wild species were classified into two groups, Jamasakura and Pendula (Fig. 3). *Prunus campanula* belonged to a distantly-related clade. In the Jamasakura group, *P. jamasakura*, *P. nipponica*, *P. incisa*, *P. lannesiana*, and *P. apetala* shared a long exon 20 and formed a closely-related clade, and ten individuals of these five species contained the same sequence for the exon 20. One individual (KS016) of *P. verecunda* and one individual (OY162) of *P. sargentii* also shared an identical exon 20. One individual (YM038) possessed the short exon 20 and was distantly related to the other individuals in the Jamasakura group. The remaining two species, *P. pendula* and *P. maximowiczii*, formed the Pendula

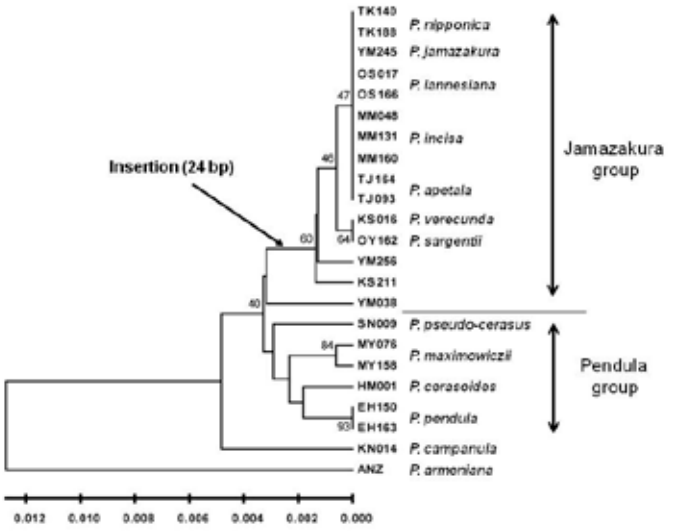


Fig. 3 - Phylogenetic UPGMA tree of the exon 20 sequences in the *PolA1* genes from 22 individuals in nine *Cerasus* species. Individuals used are listed in Table 1.

group together with *P. pseudo-cerasus* and *P. cerasoides*. Unlike the species in the Jamasakura group, the four species in the Pendula group were clearly differentiated from one another (Fig. 3).

In the phylogenetic tree for intron 19, *P. pendula* was positioned as the most distantly-related clade (Fig. 4) because this species contained a unique insertion (position 49) and a unique deletion (positions 349-350) (Fig. 5). Except for *P. pendula*, the individuals in the Jamasakura and Pendula groups formed two independent clades. Although the species in the Pendula group were clearly differentiated from one another, concurring with the results for exon 20, most individuals of the six species in the Jamasakura group, except for YM038, shared similar sequences at intron 19, and 14 individuals of the six species possessed the same intron 19 sequence. Three individuals of *P. lannesiana* had the same sequence and belonged to an independent sub-clade (Fig. 4).

Analysis of the origin of P. × yedoensis 'Somei-yoshino'

When the two allelic sequences of exon 20 of 'Somei-yoshino' were determined, one was identical to that of *P. pendula*, and the other was the same as that shared by five species in the Jamasakura group. For the intron 19 sequence, the (O) haplotype of *P. lannesiana* was distinguished from the haplotypes of *P. jamasakura* by three unique single-nucleotide polymorphisms (SNPs) (positions 25, 101, and 171), which were also found in one of two 'Somei-yoshino' haplotypes (Fig. 5). By contrast, the other (K) haplotype was found to differ from the (E) haplotype of *P. pendula* by one base deletion at position 49 and one base substitution of C to A at position 392 (Fig. 5). Consequently, we found that *P. pendula* f. *ascendens* 'Komatsu-otome' possessed two haplotypes (K and E): one was the same haplo-

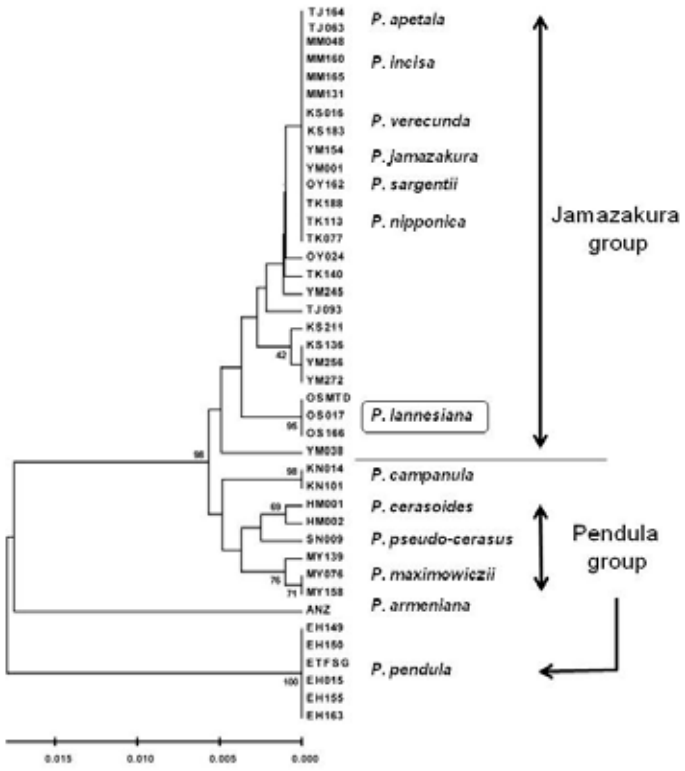
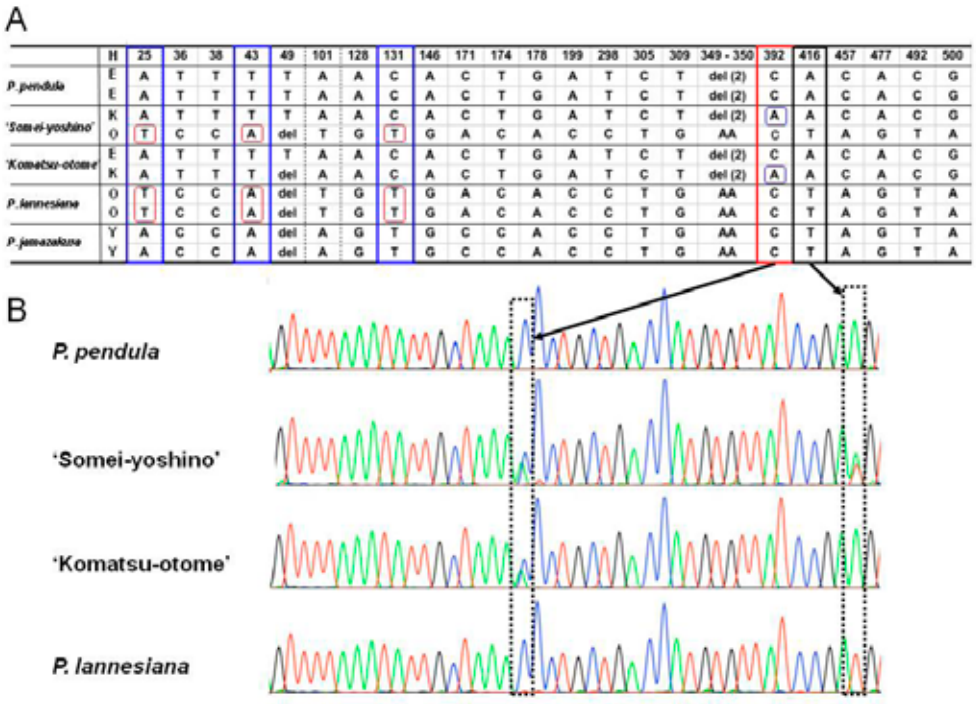


Fig. 4 - Phylogenetic UPGMA tree of the intron 19 sequences in the *PolA1* genes from 40 individuals in nine *Cerasus* species. Individuals used are listed in Table 1.



	H	25	36	38	43	49	101	128	131	146	171	174	178	199	298	305	309	349 - 350	392	416	457	477	492	500
P. pendula	E	A	T	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	C	A	C	A	C	G
	E	A	T	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	C	A	C	A	C	G
'Somei-yoshino'	K	A	T	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	A	A	C	A	C	G
	O	T	C	C	A	del	T	G	T	G	A	C	A	C	C	T	G	AA	C	T	A	G	T	A
'Komatsu-otome'	E	A	T	T	T	T	A	A	C	A	C	T	G	A	T	C	T	del (2)	C	A	C	A	C	G
	K	A	T	T	T	del	A	A	C	A	C	T	G	A	T	C	T	del (2)	A	A	C	A	C	G
P. lannesiana	O	T	C	C	A	del	T	G	T	G	A	C	A	C	C	T	G	AA	C	T	A	G	T	A
	O	T	C	C	A	del	T	G	T	G	A	C	A	C	C	T	G	AA	C	T	A	G	T	A
P. jamazakura	Y	A	C	C	A	del	A	G	T	G	C	C	A	C	C	T	G	AA	C	T	A	G	T	A
	Y	A	C	C	A	del	A	G	T	G	C	C	A	C	C	T	G	AA	C	T	A	G	T	A

Fig. 5 - Haplotypes (H) of the intron 19 sequences in the *PolA1* genes among 'Somei-yoshino' (KO), *P. pendula* (EE), 'Komatsu-otome' (KE), *P. lannesiana* (OO), and *P. Jamasakura* (YY), A: polymorphic bases and their positions in two haplotypes are shown, B: Sequence charts were produced using 21ex3P primer and converted to the complementary charts using the 4Peak software.

type of 'Somei-yoshino' and the other was identical to that of a wild *P. pendula* individual. Except for cultivars derived from 'Somei-yoshino,' we found that 'Komatsu-otome' and two other trees (Nos. 142, 145) in Ueno Park had the same haplotype as 'Somei-yoshino' (Fig. 6).

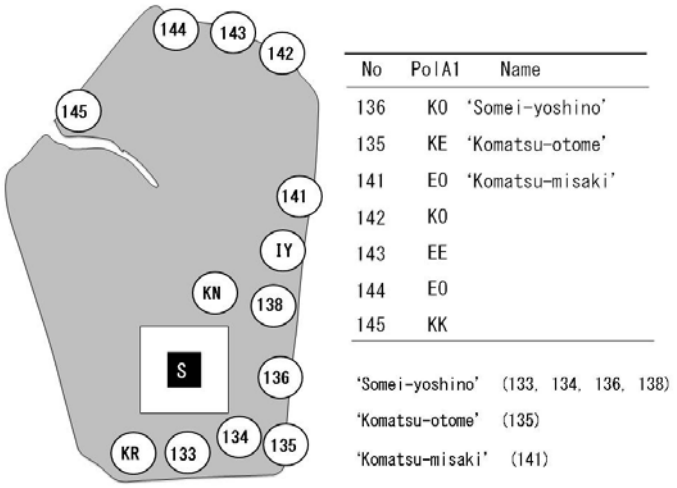


Fig. 6 - Haplotype of the *PolA1* intron 19 of trees (Nos. 135-145) found around "Komatsu no miya" statue (S) in the Ueno Park. 'Somei-yoshino' (133, 134, 136, 138) and 'Komatsu-otome' (135) share a haplotype K, *P. pendula* (E), *P. lannensiana* (O). IY, KN and KZ are flowering cherry cultivars, 'Ichi-yo,' 'Kanzan,' and 'Kanzakura,' respectively.

4. Discussion

Speciation of wild Cerasus species *in Japan*

Comparing the sequences of intron 19 and exon 20 (Figs. 3 and 4), the nine wild species of Japanese *Cerasus* were clearly classified into two groups; the Jamasakura group of seven species (*P. apetala*, *P. incisa*, *P. nipponica*, *P. jamasakura*, *P. sargentii*, *P. verecunda*, and *P. lannesiana*) and the Pendula group of two species (*P. pendula* and *P. maximowiczii*). Although the former group had been grouped into sections, *Apetalae*, *Incisae*, and *Sargentiella* based on morphological differences (Kawasaki, 1966, 1991; Kobayashi, 1992; Ohba, 1992), all seven species shared the same 24-bp insertion within the long exon 20 (847 bp), suggesting that they originated from the same ancestor. The remaining two wild species, *P. pendula* and *P. maximowiczii*, contained the short exon 20 (823 bp), in common with three alien wild species (*P. pseudo-serasus*, *P. campanulata*, and *P. cerasoides*), and with *P. armeniaca* (out-group). The results from the sequence analysis of exon 20 were thought to be more reliable than those for intron 19 for classifying the subgenus *Cerasus*, and polymorphisms found in intron 19 will be useful for discriminating among closely-related taxa and cultivars.

Although the seven species in the Jamasakura group have clearly different phenotypes, such as the apetala flower of *P. apetala* and dwarf stature of *P. incisa* and *P. nipponica*, these species have formed a large hybridizing population because they share the same sequences for intron 19 and exon 20. The short intron 19 found in three individuals (YM011, YM038, and YM277) of *P. jamasakura* might have been derived from an ancestral cryptic species. These results suggest that the classification of seven species in the Jamasakura group remains to be revised based on further molecular information.

Origin of 'Somei-yoshino'

'Somei-yoshino' is the most popular flowering cherry cultivar in Japan and the rest of the world. Ever since Wilson (1916) proposed a hypothesis for the hybrid origin of 'Somei-yoshino,' the biological and geographical origin of this cultivar has been disputed in Japan. In this study, we found that the O haplotype for intron 19 of *P. lannesiana* contained three unique SNPs, and these SNPs were also found in one of the two haplotypes (K and O) in 'Somei-yoshino' (Fig. 5). This indicates that the paternal parent of 'Somei-yoshino' was *P. lannesiana* or its cultivars. As *P. lannesiana* is endemic to the Izu Peninsula and the Izu Oshima Islands, 'Somei-yoshino' may have originated on the the Izu Peninsula (Takenaka, 1962) or in Edo and Tokyo (Iwasaki, 1989), and not on Jeju Island, Korea (Park *et al*., 1984; Roh *et al*., 2007).

The other (K) haplotype of intron 19 in 'Somei-yoshino' was identical to that (E) of *P. pendula*, except for two SNPs (Fig. 5). We also found that one of the two haplotypes (K and E) for intron 19 of 'Komatsu-otome' was the same as that of 'Somei-yoshino.' The original individual of 'Komatsu-otome' grows inside the Ueno Park, Tokyo (Fig. 6) and has a dwarf stature with pinkish flower petals. This implies that the maternal origin of 'Somei-yoshino' is a cultivar related to 'Komatsu-otome.' Out of five trees grown in the same position with 'Somei-yoshino' and 'Komatsu-otome' shown in Figure 6, two trees (Nos. 142, 145) contained K haplotype and three (Nos. 141, 142, 144) were hybrids between *P. pendula* f. *ascendens* and *P. lannesiana* var. *speciosa*.

These results suggest that there were sufficient genetic resources to develop 'Somei-yoshino.' Because *P. pendula* and 'Komatsu-otome' bloom two weeks earlier than *P. lannesiana*, 'Somei-yoshino' and 'Komatsu-misaki' were probably produced in Tokyo through artificial hybridizations between 'Komatsu-otome' or a related cultivar, and *P. lannesiana* or a related cultivar, before the end of the Edo Period (Iwasaki, 1989).

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Designing a modified atmosphere packaging (MAP) for fresh-cut artichokes

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Key words: *Cynara scolymus* L., enzimatic browning, gas transmission rate, high CO_2, low O_2, polylactic acid, visual quality,

Abstract: 'Catanese' artichoke quarters were packaged in active modified atmosphere (5% O_2+10% CO_2) in four different materials or in air in macro-perforated bags used as control (CTRL), and stored at 4°C. Materials used for modified atmosphere packaging (MAP) were: polylactic acid (PLA), polylactid acid with one line of micro-perforation (PLA MF1), polypropilene with two lines of micro-perforations (PP MF2), and polypropylene + polyamide with two lines of micro-perforations (PP+PA MF2). Initially and after 2, 4, and 9 days, overall artichoke appearance, color and weight loss were monitored. O_2 and CO_2 concentrations within the packages were detected initially and after 1, 2, 17, 24.5, 41.5, 49.5, 120 and 216 h (end of the experiment). Also, at the last sampling date ethanol and acetaldehyde accumulations in artichoke tissue were measured. All the micro-perforated films maintained gas levels within the range of O_2>3% and CO_2<15%, defined as "safe", with a positive effect on quality: all samples remained above the limit of marketability until the end of the experiment, without significant differences among them, but showed a slight better overall appearance and, accordingly, a better retention of color parameters when compared with CTRL samples. Complete anaerobic condition (16% CO_2 and 0% O_2) developed in PLA bags where blackening of cut bracts and receptacle was observed, while black spots appeared on outer bracts, causing drastic quality reduction; samples fell below the limit of marketability after just 2 days. Also a significant accumulation of ethanol and acetaldehyde was found in these samples. Optimizing MAP made it possible to maintain the desired gas condition, with positive effects on quality of the produce in absence of any stabilizing treatment, for 9 days.

1. Introduction

Fresh-cut artichokes suffer several degradative reactions which limit their marketability. Enzymatic browning is caused by the oxidation of phenols, catalysed by polyphenol oxidase enzymes (PPO), with the subsequent formation of dark compounds (Lattanzio *et al*., 1994; Tomás-Barberán and Espín, 2001). Non-enzymatic browning reactions are caused by iron-polyphenol complexes: chlorogenic acid, the most representative phenolic compound of artichoke heads, in presence of O_2 forms dark-coloured complexes with Fe^{3+}, while the same substrate in anoxic conditions forms colourless complexes with Fe^{2+}, but after exposure to air the Fe^{2+} complex is quickly oxidized to Fe^{3+} to give coloured compounds (Lattanzio, 2003). Also, mechanical wounding enhances a different array of enzymatic pathways, many of which are associated with volatile accumulation, such as ammonia, ethanol and acetaldehyde, which leads to the darkening of tissues and onset of off-flavors (Salunkhe and Do, 1976; Rolle and Chism, 1987).

The use of modified atmospheres can promote or, otherwise, inhibit these degradative reactions, and differences between beneficial and harmful effects of gas mixtures may be small. Increased levels of CO_2 are used in combination with low O_2 concentrations to maintain the visual quality of several types of fresh-cut produce (Beaudry, 1999). Carbon dioxide is considered a competitive inhibitor of PPO but increases in ammonia were observed in leafy tissues stored in high CO_2 (Cantwell *et al*., 2010); similarly, if the O_2 level in the package decreases below the fermentation threshold, anaerobic respiration is triggered leading to the accumulation of anaerobic metabolites (i.e. ethanol and acetaldehyde) and stimulating the growth of some anaerobic pathogens (Oms-Oliu *et al*., 2009). The presence of a very high CO_2 concentration (25%) in the storage atmosphere has been proved to be deleterious for fresh-cut artichokes (la Zazzera *et al*., 2012), while only slight beneficial effects were observed at lower concentrations (5 and 15%). Other authors, combining a soy protein isolate enriched with cysteine (Cys) and different modified atmospheres (Active with 5 kPa O_2+15 kPa CO_2; active with 80 kPa O_2 and passive), found that the MA did not increase shelf-life compared to coated samples obtaining only 4 days of shelf-life, but the same authors refer an accumulation up to 30% of CO_2, which can be the cause of

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low shelf-life (Ghidelli *et al*., 2015). Therefore, the avoidance of extreme conditions in terms of CO_2 and O_2 concentrations within the package should be the main objective when designing a modified atmosphere packaging (MAP) system for fresh-cut artichokes.

In this work an active MAP was designed to maintain inside the package, at a steady state, a gas concentration of 5% O_2+10% CO_2 (target atmosphere) or at least within a "safety range" ($O_2 \geq 3\% + CO_2 \leq 15\%$) as defined in previous experiments. The use of micro-perforated films, allowing a greater permeability to O_2 and CO_2 than non micro-perforated films, should be recommended for fresh-cut produce with a very high respiration rate, like artichoke (Kader, 2002), since an accumulation of CO_2 can still be reached within the package but avoids an extreme concentration and total O_2 depletion. A simple recyclable film (polypropylene), a two-layer non recyclable film (polypropylene and polyamide), and a bio-based compostable film (polylactic acid), with different levels of micro-perforations, were used in this work as MAP materials.

2. Materials and Methods

Freshly harvested artichokes (*Cynara scolymus* L. 'Catanese') were collected in the area of Brindisi (Italy). In previous studies (Cabezas-Serrano *et al*., 2009), 'Catanese' showed the best attitude for processing as a fresh-cut produce compared to other cultivars, as it is less susceptible to post-cutting degradation phenomena. Raw heads were immersed for 2 min in a 100 mg L^{-1} sodium hypochloride solution in order to reduce the microbiological contamination and eliminate residual soil particles. Artichokes were then hand-trimmed using sharp stainless steel knives in order to remove external bracts, leaves and stalks; heads were washed in a NaOCl solution (100 mg L^{-1} of free chlorine). After washing, head trimming was completed by further removal of external greener and tougher bracts (inedible fraction) so as to keep just the innermost tender bracts, and by cutting about 2 cm from the top. Finally, artichokes were cut into quarters and closed in active modified atmosphere packaging (MAP) with the initial gas composition of 5% O_2+10% CO_2 using a Tecnovac packaging machine (Mod. T520, Grassobbio, BG; Italy). Four different packaging materials were used (Table 1): polylactic acid (PLA), polylactid acid with one line of micro-perforation (PLA MF1), polypropilene with two lines of micro-perforations (PP MF2), and polypropylene + polyamide with two lines of micro-perforations (PP+PA MF2). Control samples were passively packaged in macro-perforated PP MF2 bags in which additional macro holes (four per side) were manually made with a needle, insuring the complete gas exchange through the film and no atmosphere modification inside the packaging, while protecting the product from excessive weight loss (CTRL).

Evolution of gas composition inside the package, once it has been sealed, is due to the gas permeability of the plastic material used, to the respiration rate of the packaged commodity and, consequently, to the ratio between the package dimension and product weight. In order to reach and maintain the target atmosphere within the bags, packaging dimensions for a produce weight of 150 g were optimized knowing the film properties (Table 1), and the desired Gas Transmission Rate for O_2 (OTR) and CO_2 (CO_2TR) being respectively 221 ml m^{-2} day^{-1} for O_2 and 354 ml m^{-2} day^{-1} for CO_2, using the following formula:

$$A = \frac{W * RR}{GTR * (\%G_{atm} - \%G_{pkg})}$$

where A = packaging surface (m^2); W = product weight (kg); RR = respiration rate (ml kg^{-1} day^{-1}); GTR= gas transmission rate (ml m^{-2} day^{-1}) % G_{atm} = percentage of the gas in the atmosphere (Bar) ; % G_{pkg} = percentage of the gas in the packaging (Bar).

Only for PLA MF1 was a produce weight of 120 g used, and the optimal dimension could not be optimized since, based on its low GTR, too-large and not feasible bag dimensions would have been needed.

All samples were stored at 4°C. Initially and after 2, 4, and 9 days, artichoke colour, overall appearance and weight loss, were monitored. O_2 and CO_2 concentrations

Table 1 - Film material and packaging characteristics

Film material	Packaging dimension (cm)	Thickness (µm)	Diameter of holes (µm)	Number of holes/m2	OTR (ml m^{-2} day^{-1} bar^{-1})	ß (CO_2TR/OTR)	WVTR (gm^{-2} day^{-1})
PLA	20x25	30	-	-	570	4.2	317
PLA MF1	10.6X17	30	60	138	570	4.2	317
PP MF2	24.6X13	30	60	160	1100*	2.4*	5*
PP+PA MF2	22.5X25	67	60	222	29*	2.75*	2.01*

* refers to the film without micro perforation.

PLA= polylactic acid without micro-perforation; PLA MF1= polylactid acid with one line of micro-perforation; PP MF2= polypropilene with two lines of micro-perforations; PP+PA MF2= polypropylene + polyamide with two lines of micro-perforations; OTR= Oxygen Transmission Rate; β = CO_2TR/OTR; WVTR = Water Vapor Transmission Rate.

within the packages were detected initially and after 1, 2, 17, 24.5, 41.5, 49.5, 120 and 216 h (end of experiment). On the last sampling date, also ethanol and acetaldehyde accumulations in artichoke tissue were measured. O_2 and CO_2 concentrations within the packages were measured using a WITT gascontrol 100-model MAPY 4.0 (WITT-GASETECHNIK GmbH & Co KG, Germany).

Overall appearance was evaluated by assigning a score to each artichoke quarter, from 5 (excellent) to 1 (very bad) using as a reference a photographic scale associated to brief descriptions (Amodio *et al*., 2007). Samples in the bags were weighed at each storage period and the weight loss was calculated as percent of the initial fresh weight.

Colour in CIE L*a* b* scale was measured on the external surface of the outer bract and on the cutting surface, elaborating the images acquired with a Spectral scanner (DV SRL, Italia); Hue angle (h°) and Chroma were calculated from the primary L*, a*, and b* values.

Ethanol and acetaldehyde accumulation in artichoke tissues were measured only on the last sampling date with the method of Mateos *et al*. (1993) using a gas chromatograph Shimadzu GC-14A equipped with a FID detector (temperature 150°C); separation was carried out isothermally at 80°C on a capillary column 5% CBWX 20M on Carbograph 1AW20 80/120, 6'x 1/8'' x 0.0085 (Alltech). Ethanol and acetaldehyde were identified and quantified by comparison with standard curves, and expressed respectively as umol ethanol g^{-1} and nmol acetaldehyde g^{-1}.

The experiment was organized in a Split-plot design with the packaging condition as the main factor and the time of storage as the secondary factor. The most conservative degrees of freedom were used to determine the effect of time, and packaging x time interaction. At each storage time the effect of packaging treatments was tested performing a one-way ANOVA. Means were separated using the Tukey test. For the O_2 and CO_2 concentrations within the packages and ethanol and acetaldehyde contents, standard deviations (STD) were calculated.

3. Results and Discussion

Packaging treatment and storage time significantly influenced all the quality parameters with few exceptions (a* value of bract cut surface was not influenced by packaging and b* value of the receptacle was not affected by time of storage). Also interactions were found to be significant for all attributes except for a* value in bract cut surface (Table 2).

The evolution of gas composition within the packages is shown in figure 1. All the micro-perforated films maintained gas levels within the safety range (O_2 higher than 3% and CO_2 lower than 15%). For samples stored in PP+PA MF2 and PP MF2 gas evolution showed the same trend: the O_2 level decreased in the first hours, due to the high respiration rate of cut artichokes, reaching a steady state at 12% and 10% respectively for PP+PA MF2 and PP MF2; CO_2 concentration increased slightly for both films, reaching a steady state at 11.5% for PP+PA MF2 and 12.5 for PP MF2. In PLA MF1 bags, O_2 concentration increased progressively, reaching a maximum of 14.5% after 216 h, while CO_2 concentration decreased sharply just after sealing, falling from 10 to 6%, and then rapidly increased, reaching at equilibrium a concentration of about 7.5%. In non-micro-perforated PLA packages, anaerobic conditions (0% O_2 and 16% CO_2) developed after 17 h; then the CO_2 level increased further, reaching a maximum of 29% after 120 h of storage.

All the samples stored in micro-perforated bags showed a better or comparable visual quality than CTRL samples, allowing the produce to keep marketability until the end of the experiment without significant differences among them (Fig. 2). On the contrary, artichokes stored in non-perforated PLA resulted heavily damaged: black spots appeared on outer bracts, a blackening of cut bracts and receptacle was observed and off-odours were perceived. After 2 and 4 days of storage, the injuries did not show up until removal from low O_2 atmosphere and exposure to

Table 2 - Results of ANOVA test for treatment and time of storage on quality attributes of fresh-cut artichoke quarters during storage in different atmospheres

Parameter	Packaging	Time	Packaging x Time
Appearance score			
Bract cut surface and receptacle cut surface	****	****	****
External bracts	****	****	****
Color			
External surface			
L*	****	****	****
a*	****	****	****
b*	***	****	****
Chroma	***	****	****
Hue Angle	**	***	**
Bract cut surface			
L*	****	****	****
a*	ns	****	ns
b*	****	****	****
Chroma	****	****	****
Hue Angle	*	****	**
Receptacle cut surface			
L*	***	****	*
a*	****	****	****
b*	***	ns	****
Chroma	****	*	****
Hue Angle	****	****	***
Weight loss	****	****	****

Within each row, each factor (Treatment and Time) and their interaction are significantly different for P≤0,05 (*); P≤0,01 (**); P≤0,001 (***); P≤0,0001 (****), or not significant (ns).

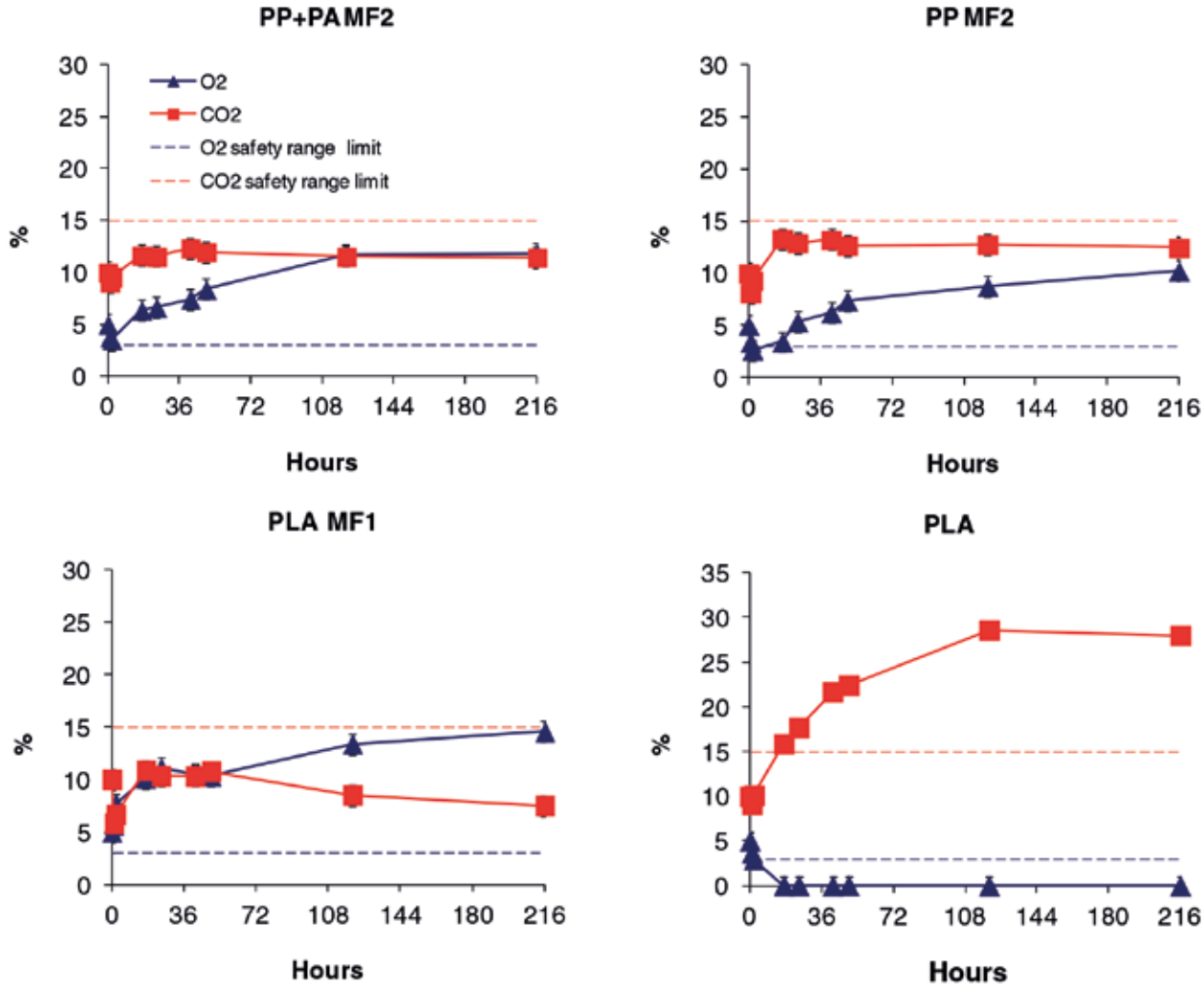

Fig. 1 - O_2 and CO_2 concentrations within different packages used for artichoke quarters stored at 4°C: PP+PAMF2 = polypropylene + polyamide with two lines of micro-perforations; PP MF2 = polypropilene with two lines of micro-perforations; PLA MF1= polylactid acid with one line of micro-perforation; PLA= polylactic acid without micro-perforation. Mean values of three replicates ± STD.

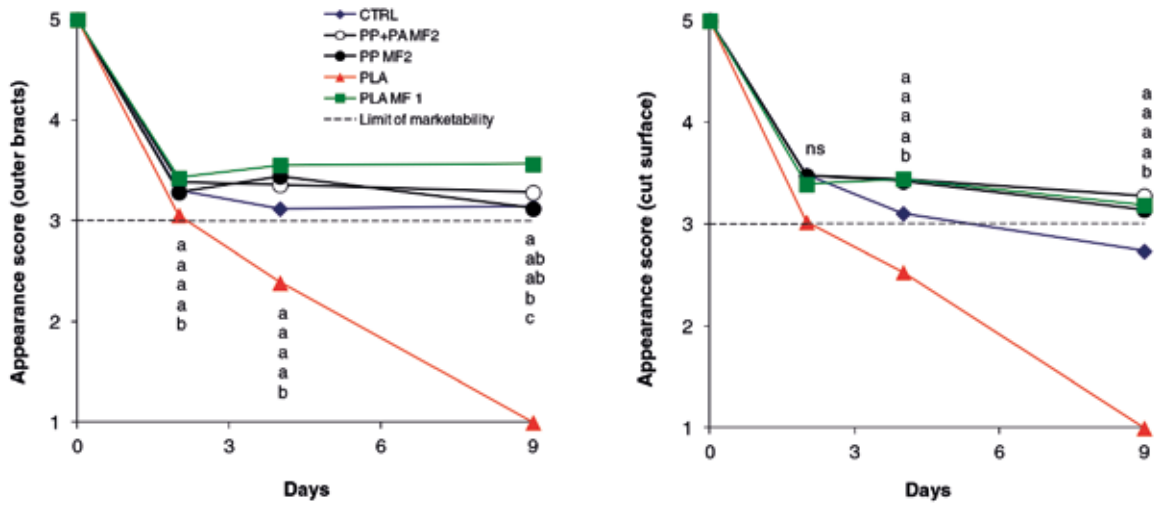

Fig. 2 - Changes over time of overall appearance in fresh-cut artichoke quarters packaged in active modified atmosphere (5% O_2+10% CO_2) and stored at 4°C: PP+PAMF2= polypropylene + polyamide with two lines of micro-perforations; PP MF2= polypropilene with two lines of micro-perforations; PLA MF1= polylactid acid with one line of micro-perforation; PLA= polylactic acid without micro-perforation. CTRL represents samples stored in air in macro-perforated bags. At each storage sampling different letters indicate significant differences ($P<0.05$).

air, when in few minutes they became completely inedible, with the onset of off-odours. Black spots and blackening could be due to non-enzymatic browning, as suggested by Lattanzio (2003) on artichokes: chlorogenic acid, the most representative phenolic compound of artichoke heads, in the absence of O_2 forms colourless complexes with Fe^{2+}, but after exposure to air the complexed Fe^{2+} is quickly oxidized to Fe^{3+} to give coloured compounds. In the same way, also enzymatic reactions could have taken place after exposure to O_2. At the last sampling, after 9 days of storage in extreme atmosphere condition, black spots and blackening began to appear in the produce when still inside the PLA package, suggesting a possible necrosis of the tissues.

Colour data (data not shown) confirmed the deleterious effect of the extreme gas conditions reached within the PLA bags, indicating a severe browning (drop on L and b*, increase of a* and higher variation of Hue Angle) and a better retention on colour parameters for samples stored in micro-perforated films, also compared to CTRL samples.

Weight loss (Fig. 3) was greater in CTRL samples and in samples stored in PLA bags (7.5, 5.7 and 3.7% for CTRL, PLA and PLA MF1 respectively), than in samples stored in PP MF2 and PP+PA MF2 (below 1%). Weight loss in non micro-perforated PLA bags was significantly higher than in bags of the same material but with micro-perforations. Most probably the deleterious atmosphere condition within these bags played a more important role on weight loss than the possible higher transpiration which the presence of micro-perforations might have generated.

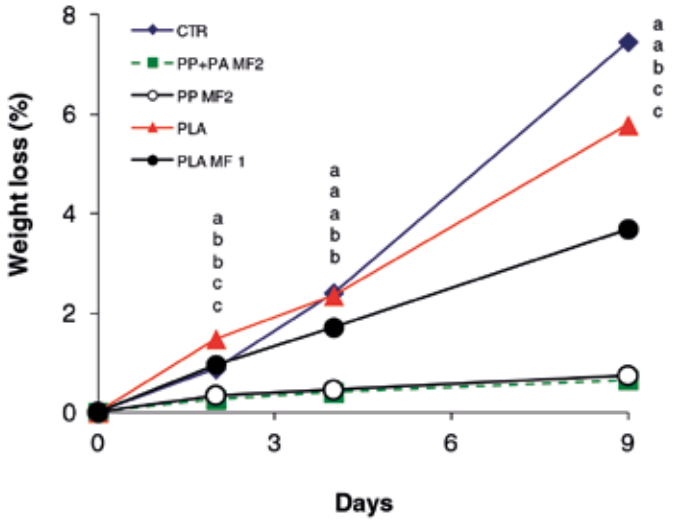

Fig. 3 - Weight loss over time of cut bract and external surfaces of fresh-cut artichoke quarters packaged in active modified atmosphere (5% O_2 + 10% CO_2) and stored at 4°C: PP+PAMF2 = polypropylene + polyamide with two lines of micro-perforations; PP MF2= polypropilene with two lines of micro-perforations; PLA MF1= polylactid acid with one line of micro-perforation; PLA= polylactic acid without micro-perforation. CTRL represents samples stored in air in macro-perforated bags. At each storage sampling different letters indicate significant differences (P<0.05).

A clear accumulation of ethanol and acetaldehyde was found in artichokes stored for 9 days in PLA (Fig. 4), where complete anaerobic conditions developed. The synthesis of these volatile compounds caused by anaerobic respiration was demonstrated by several authors (Shaw, 1970; Woodward and Topping, 1972; Prasad and Stadelbacher, 1974). Ke *et al.* (1991) found strong correlations between off-flavour development and ethanol content, and to a lesser extent, acetaldehyde. Severe off-odours were produced by broccoli under anaerobic conditions developed after few days in MAP (Forney *et al.*, 1991). Nichols and Patterson (1987) observed that in apples the injury due to ethanol and acetaldehyde accumulation in anaerobic conditions or high CO_2 atmospheres is post-anoxic: it occurs after removal from low O_2 exposures, when ethanol is oxidized to acetaldehyde.

4. Conclusions

All the tested micro-perforated materials made it possible to reach and maintain the atmosphere composition within a safety range (O_2>3% and CO_2<15%), with a posi-

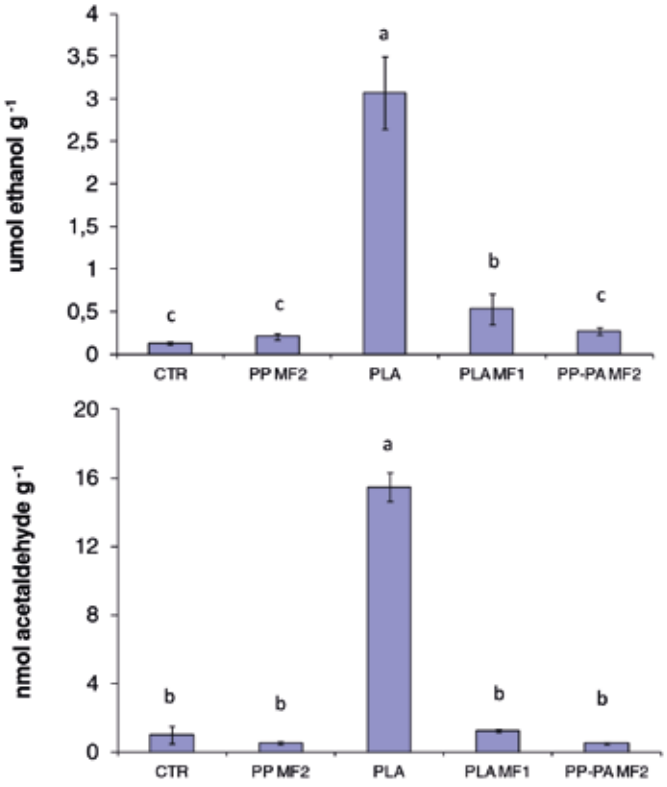

Fig. 4 - Ethanol and acetaldehyde accumulation, after 9 days of storage, in fresh-cut artichoke quarters packaged at 4°C in active modified atmosphere (5% O_2+10% CO_2), in different packaging conditions: PP+PAMF2 = polypropylene + polyamide with two lines of micro-perforations; PP MF2 = polypropilene with two lines of micro-perforations; PLA MF1 = polylactid acid with one line of micro-perforation; PLA = polylactic acid without micro-perforation. CTRL represents samples stored in air in macro-perforated bags. Mean values of three replicates ± STD.

tive effect on quality of stored fresh-cut artichokes. Artichokes packaged in PLA MF1 suffered a higher weight loss than produce stored with other packaging materials, which however did not seem to influence its overall appearance. On the contrary, the use of non micro-perforated films was not suitable for cut artichoke, probably due to its high transpiration rate: serious damage occurred to the produce in PLA bags, causing a drastic reduction in its quality. Extreme conditions in terms of CO_2 and O_2 concentrations may be very deleterious for packaged vegetables and the avoidance of these conditions within the packages must be the main objective when designing a MAP system for fresh-cut artichokes. MAP optimization for fresh-cut artichokes was possible with the auxilium of micro-perforation, which permitted establishment and maintenance of the desired gas conditions, regardless of the plastic material. The designed MAP allowed storage of cut artichokes in the absence of any stabilizing treatment for 9 days.

Acknowledgements

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Innovative application of NIR-AOTF and MRI to study water behaviour in cut flowers

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Key words: Dry matter, flower, image software, MRI, NIR-AOTF, plugging, water content.

Abstract: In order to study the water status of cut flowers, a comparision study was made between flowers stored in water and flowers stored «dry pack», by using a portable NIR (near infrared)-AOTF(acousto-optical tunable filter) instrument and MRI (magnetic resonance imaging). As model flower, *Zantedeschia aethiopica* (commercially known as Calla lily) was used. To predict the weight loss and water content by NIR-AOTF, cut flowers were dried in a cold room at 10°C (±1°C) and 85% (±5%) relative humidity (RH), and measured for weight loss. For MRI application, 4 and 20°C storage temperatures were used for flowers kept in water or dry; two stem sections, basal and middle, were measured. Significant correlation results for weight loss and water content (R^2 in calibration = 0.98 for estimated % of water loss and 0.96 for % of water content; R^2cv = 0.95 and 0.90) were obtained by NIR-AOTF spectra acquisitions. MRI detected vessel degradation in the stem of the water-stored flowers at 4°C but at 20°C in dry storage no vessel degradation appeared and images were correlated with dry matter values. The use of image software allowed the transformation of images in normalized population and pixel intensity, which gave hints about the potential use of these data to combine with NIR-AOTF data. NIR-AOTF, an easy-to-use and non destructive instrument, can be used to predict the vase life of cut flowers by measuring water content or weight loss. MRI is a powerful tool to identify the plugging of vessels but it is destructive; the image software used represents a useful tool to correlate MRI with NIR-AOTF to predict vessel plugging.

1. Introduction

It is well known that the vase life of cut flowers depends on water quality in terms of sanitation, nutrients and acidity regulators. Water lost during the postharvest period can normally be replaced through the vase solution. Nevertheless, desiccation is one of the most important postharvest problems for cut flowers due to the plugging of xylematic vessels (i.e. bent neck of cut roses or stem break of cut gerbera flowers), disorders caused by plugging of the cut surface of the stem due to bacteria, exudations or colloidal materials. Thus, non destructive identification of the water status in the stem of cut flowers can be useful to predict postharvest vase life. *Zantedeschia aethiopica* (commercially known as Calla lily) has long been an important cut flower, and new green-tinged and different-shaped variants are increasingly important. The flowers are normally pulled from the rhizome, and re-cut to ensure adequate water uptake (Reid, 2004). This practice is very important to guarantee a long shelf life if flowers are maintained at the correct, low temperature. The fresh weight of spathe of *Z. aethiopica* decreases with time while that of scape and spadice increases (Tjia and Funnel, 1986). A standard preservative solution used to prolong the longevity of cut flowers (8-HQC + sucrose) was deleterious to *Zantedeschia* foliage, reducing display life several fold (Skutnik *et al*., 2001). In a recent paper (Ahmad *et al*., 2013 a) it was shown that floral preservative was ineffective to prolong vase life of Calla while it was tolerant of high water pH (8.1) and vase life varied from 9.2 d for acidic solutions (pH 3.2) to 10.1 d for solutions with intermediate pH (6.3). All of these results are conditioned by the water quality and behaviour. MRI (magnetic resonance imaging) has been used to monitor the developmental change of *Zantedeschia* Spreng. tuber (Robinson *et al*., 2000) but no paper has reported, to our knowledge, its application on flower stems. NIR (near infrared) spectroscopy is an excellent technique to detect the water content inside the tissue in a non destructive way because water has a very strong signal (Cozzolino *et al*., 2006). It has been applied to measure the water potential in vine leaves (De Bei *et al*., 2011) as well as the water loss of grape berry during dehydration (Bellincontro *et al*., 2011). In the research reported here, a portable NIR-AOTF spectrometer was used to non destructively measure the water content and weight loss of Calla lily, as model cut flower, to predict its vase life;

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subsequently, MRI was applied to study the water traslocation in Calla lily stems kept at two storage temperatures (4 and 20°C). Image software was used to transform the MR images in pixel intensities for graphical representation and comparative evaluation. In subsequent investigations, the correlation between MRI results and NIR-AOTF spectra will be tested with the aim of performing a calibration model able to predict vessel plugging of flowers.

2. Materials and Methods

Material and experimental procedure

Calla lily (*Zantedeschia aethiopica*) cv. Childsiana flowers, at commercial stage of development, were picked in the morning and immediately placed in water, the stem surface was placed in sterilized water after recutting. The average weight of flowers was 80 g; color of spathe L= 75.7±2.9 a= -9.7±1.2 b= 44.5±4.5; color of stem L= 50.1±6.6 a= -8.8±0.5 b= 28.6±1.3 measured by a CM-2600d colorimeter (Konica Minolta Inc., Ramsey, NY) set at SCE (specular component excluded) measuring CIELAB coordinates L, a, and b. In order to build a calibration curve for weight loss, in the first experiment, Calla flowers (50) were kept dry, horizontally placed in cardboard boxes covered with plastic film to avoid excess air flow, in a cold room at 10°C (±1°C) and 85% (±5%) RH, with coil fans blowing during the cooling intervals. Each single flower was weighed on a scale (Cubis mod., Sartorius-Stedim Italia spa, Florence, Italy) every 8 h. Weight loss and water content were calculated and expressed as %.

NIR spectra collection

A Luminar 5030 miniature, hand-held NIR-AOTF analyzer (Brimrose Corporation, Baltimore, Maryland, USA) was used for the NIR spectra acquisitions, obtained by putting the optical sensor in contact with four sections of the flower: basal, middle, upper, and spathe. Ten spectral acquisitions were run for each section (along and all around), recorded in transmittance mode (Bellincontro *et al.*, 2011) and then averaged. A single measurement, at the speed of 16000 wavelength sec^{-1}, was conducted in the 1100-300 nm range, with 2 nm wavelength increments and 50 spectra per average, which represents a good compromise between acquisition speed and signal quality of the spectrum.

Near infrared spectroscopy analisys and chemometrics

Raw spectra were statistically pre-treated for absorbance (log 1/T) transformation using SNAP 2.03 software (Brimrose Corporation, Baltimore, Maryland, USA). The absorbance spectra, obtained as spectral average, were used as X-variables and opposite to the Y-variables (water content and water loss) in model calculation, performed by chemometric procedures of filtering and partial least square (PLS) calculation. Model validation in parameter prediction was obtained by full cross-validation (leave-one-out) method, and no outlier identification and elimination was applied. The statistical R^2 indexes (coefficient of multiple determination) in calibration (R^2_c), and in cross-validation (R^2_{cv}) were defined to establish the correlation between NIR spectra and destructive measurement. Root Mean Standard Error of Cross Validation (RMSECV) and the number of latent variables (LVs), minimizing the error in modeling, were used to determine the significance of the calculations. Finally, the RPD values, defined as the ratio between SD and SECV, were also calculated in order to define the robustness of predicting responses. This experiment was run until complete wilting of the flowers (10 days). A parallel destructive experiment was carried out: flowers (50) were kept under the same conditions and NIR acquisitions were performed in a similar way as described above; after each NIR acquisition, sections were cut and used to measure water content by drying them in an electric ventilated oven (Tecno-lab srl, Brescia, Italy) at 70°C for 72 h. Ten flowers were used for each sampling time.

MRI experiment

In the second experiment, flowers were collected and in the laboratory the stems were recut in sterilized water and then placed in the same. Flowers were divided into two lots: 60 flowers were kept in water (10 flowers per vase, 6 vases); other 60 flowers were placed horizontally, wrapped, not tightly, in plastic film as used commercially. Both lots were split and stored in two storage rooms at 4 or 20°C (±1°C) at 70% (±5%) RH. The experiment lasted 12 days. Dry matter was measured by using a ventilated oven as described above, at each sampling time. MRI measurements were performed on flower sections at time 0, after 3 and 12 days. At each sampling time, MRI analyses were performed on two sections (15 cm long) of the stem, cut from 5 cm above the stem cut surface and from 15 cm below the spathe, named basal and middle, respectively. A Bruker AVANCE 300 MHz spectrometer (Bruker Biospin Corp, Bilerica, MA, USA) equipped with cylindrical birdcage single-tuned nucleus (^{1}H) coil probehead with an inner diameter of 20.0 mm was used (Taglienti *et al.*, 2009). The water signal was monitored and used for the image reconstruction. Gradient-Echo (GEFI) and Multi-Slice-Multi-Echo (MSME) experiments, m_gefi_ortho and m_msme_ortho, respectively (Bruker library), were performed according to standard procedures. In GEFI measurements, which generate echoes by applying gradient pulses, the field of view was 20.0 mm x 20.0 mm, the matrix size 128 x 128 pixels and spectral width 100.0 kHz. The echo and repetition times were set equal to 2.445 ms and 60.0 ms, respectively. The number of scans was 1; slice thickness was 1.01 mm; and excitation pulse was a sinc3. The data were processed to obtain images 128 x 128 in size and a field of view of 20.0 mm x 20.0 mm. The processing mode was FT_MODE, the latter was complex_FFT and spikes elimination was allowed. In MSME experiments, which produce echoes via a spin-echo-based sequence, the field of view was 20.0 mm x 20.0 mm; the

matrix size 128 x 128; spectral width 100.0 kHz; the echo and repetition times were set equal to 17.5 ms and 6000.0 ms, respectively; the number of echoes and images were 196; number of scans and dummy scans was 1; slice thickness was 1.0 mm; and the excitation pulse was a sinc3. The data were processed to obtain images 128 x 128 in size, a field view of 20.0 mm x 20.0 mm, the processing mode FT_MODE, the latter complex_ FFT and with spikes elimination allowed. The intensity decay of the NMR signal *vs*. the echo time was calculated by the Bruker software ParaVision 3.0.2 in order to calculate the local transverse relaxation time, i.e. T_2. GEFI and MSME images were also analysed by ImageJ 1.41 (Rasband, 2007) which allows for greyscale analysis. For GEFI experiments, the axial image was analysed by ImageJ software, while for MSME the intensities of the 24th acquired image were reported to a scale from 0 to 255, the first value corresponding to full black colour and the second to complete white. The whole sample was selected, and parameterised images were used to create curves according to the pixel intensities.

3. Results and Discussion

The absorbance mean spectra of the acquisitions in the floral sections showed characteristic molecular references of the absorbance peaks (data not shown). Spectra were characterized by two principal water absorption bands around 1450 nm and 1920-1950 nm (Nicolai *et al*., 2007). These bands are assigned to the first overtone of the symmetric and asymmetric OH stretching and/or combination bands (1450 nm), and to the combination of the OH stretching band and to the OH bending band (1920-1950 nm), respectively (Shenk and Westerhaus, 1996). This spectral response is strictly correlated with the floral water content; thus the weight loss estimation, mainly due to water loss, is predictable. In Table 1 analytical measurements of weight loss (%) and water content (%) are statistically defined by descriptive indexes. Data range (as min and max values), mean, and SD (standard deviation) are reported to describe the variability of the data sets destined to the multivariate calibration models. For regression models, different pretreatments were tested on the spectra sets (data not shown) previously transformed in absorbance (log 1/T). First derivation by Savitzky-Golay filter (11 points of smoothing, 2nd order) proved to be the best performing and were used in subsequent chemometric applications and regressive PLS1 calculation. Calibration and cross-validation results for the models obtained from the two tested parameters, are reported, as characteristic scatter plots of multivariate regressions, in Figure 1. Significant correlation results were achieved for both models: the determination coefficients (R^2) in calibration were 0.98 and 0.96 for the estimated percentage of the weight loss and the estimated percentage of the water content, respectively; the coefficients of determination in cross-validation (R^2cv) were, respectively, 0.95 and 0.90. For the estimation of the predictive accuracy of the models, an R^2cv greater than 0.9 represents a valid quantitative information (Maeda *et al*., 1995). Cross validation is a practical method to demonstrate how NIRS can predict a qualitative attribute, even if it would be better to estimate the accuracy of the application by using an appropriate, preferably external, test or validation set (Dardenne, 2010). In leave-one-out cross validation, one sample is removed from the dataset and a calibration model is built on the basis of the remaining subset, using that samples to calculate the residual prediction (Cozzolino *et al*., 2011). The significant results obtained in terms of correlation on the predictive models can also be attributed to the high degree of accuracy and precision of the reference data (Bellincontro *et al*., 2012).

Table 1 - Statistical analyses of sample sets relative to the two analyzed parameters (weight loss and water content) during the first experiment

Parameter	Samples	Mean	SD	Min	Max
Weight loss (%)	95	7.95	4.45	1.51	19.60
Water content (%)	75	93.11	3.31	93.11	97.11

Mean, standard deviation (SD), range (min. and max.) are reported expressed as %. Number of sample s (i.e. the number of destructive measurements carried out) is reported

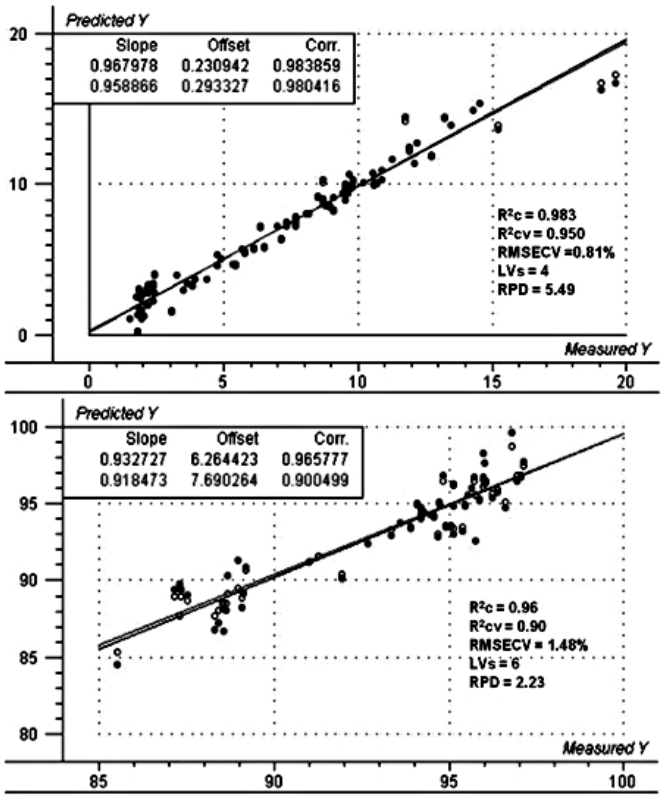

Fig. 1 - Scatter plots relative to the prediction models for the percentage of weight loss of flowers (top). Scatter plots relative to the prediction models for the percentage of water content of flowers (bottom). Measured values are plotted versus predicted values and significant indexes of calibration and validation are reported.

However, the real and applicative performance of the predictive models is better defined if combined with the estimation indexes referring to potential errors in calibration and prediction or cross-validation (RMSEC, RMSEP and RMSECV). Here we report RMSECV (root mean standard error in cross-validation) which was 0.81% and 1.48% in predicting the flowers' weight loss and water content, respectively. Quite low LVs values were obtained for both PLS models (Fig. 1), and it is possible to observe that a small number of latent variables could reduce the possible errors in predictive responses of the models. RPD (Ratio of Performance to Deviation) indexes are also reported in figure 1. The RPD ratio is another statistical index useful for evaluating the predictive ability of the NIRS and is calculated as the ratio between SD of destructive measurements and the standard errors of prediction. A RPD below 2.5-3 means that the model has low ability of discrimination from high values of the response variable; values above 5 indicate good discrimination, especially if destined for quality control (Williams and Sobering, 1996).

As regards the second experiment on the use of MRI, the dry matter in the basal (cut surface) and middle sections were similar at the start of the experiment (Table 2). After 3 days, at 4°C, no differences were observed among samples, whatever the location of the section or the method of storage (dry or water). At 20°C, after 3 days, the dry matter was similar (4.7 and 4.9%) in the middle sections regardless of the storage method and similar to the one at 4°C while, the basal sections had 3.4 and 4.1% of dry matter in flowers kept in water or dry, respectively; after 12 days the values significantly decreased, 3.7 and 2.8%, in the middle and basal sections, respectively, of flowers kept in water. The results for dry matter indicate that storage temperature plays an important role in preserving the integrity of the stem as has been reported recently for *Lilium* (Prisa *et al*., 2013) and also to maintain fresh and dry weight (Ahmad *et al*., 2013 b). Dry matter was also affected by storage method: storage in water preserved the integrity of the internal structure of the Calla stem. Dry matter concentration is mainly due to sugars concentration which are known for their importance in vase life of cut flowers. The significant reduction of dry matter in middle and basal sections of the flowers kept in water at 20°C might be due to the presence of bacteria. Bacteria can enter maintenance solutions from the external surface of the flowers (Teixeira da Silva, 2003). In tap water, *Acinetobacter* sp., *Bacillus pumilus* and *Pantoea agglomerans* cells moved from the outside to inside the flower parts, becoming endophytic bacteria with a role in stem break of gerbera flowers (Balestra *et al*., 2005). Xylematic vessel blockage could be due to some amorphous or physiological deposition and rod-shaped bacteria located within the 5 cm stem end of the cut flower as shown by scanning electron microscope (Wang *et al*., 2014). A partial relationship between the lower content of dry matter and the tissue degradation seems to be confirmed by MRI. In figure 2, degradation of the cut stem surface (white area indicates that water moves freely) is clear, while the middle section still maintains intact tissue and its vessels are

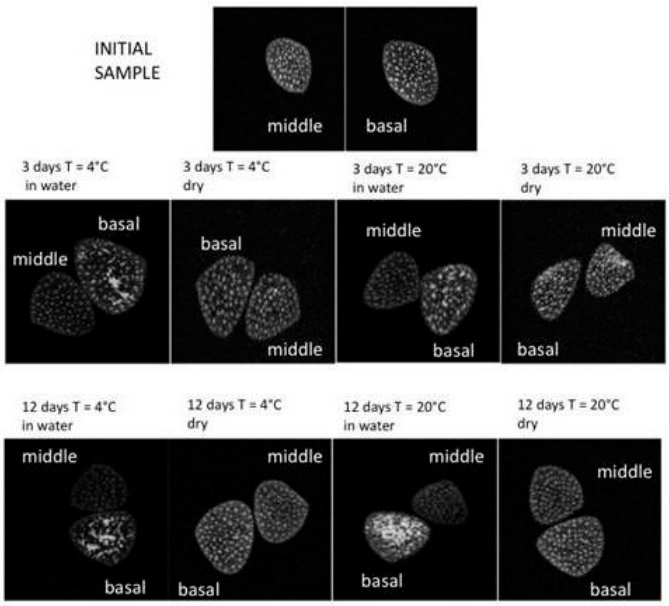

Fig. 2 - MSME MRI images of flowers, middle and basal sections. On top, initial samples. Ten images were taken for each sample at each sampling time. The reported images are representative of the pool of 10 for each sample, which showed similarity of behaviour.

Table 2 - Dry matter content (%) of sections (basal and middle) of Calla lily flowers kept at 4 or 20°C, dry or in water

	Water M.S.	Dry M.S.	Water B.S.	Dry B.S.	Middle section	Basal section
Initial time					4.8±0.3 bcd	4.4±0.4 cd
4°C						
After 3 days	5.2±0.3 ab	5.2±0.2 ab	4.7±0.4 bcd	4.7±0.4 bcd		
After 12 days	4.9±0.4 abc	4.9±0.5 abc	4.9±0.4 abc	4.9±0.5 abc		
20°C						
After 3 days	4.7±0.4 bcd	4.9±0.4 abc	3.4±0.4 ef	4.1±0.2 de		
After 12 days	3.7±0.2 e	4.5±0.4 cd	2.8±0.2 f	4.3±0.2 cd		

Data are the mean of 10 flowers at each sampling time ± SD. Values with different letters are significantly different ($p<0.05$) by LSD. (M.S.= medium section; B.S.= basal section).

visible (white dot); this image is very clear in the initial samples. At 4°C in water, the degradation is less diffuse than at 20°C, both in the basal and middle sections. These images do not reflect the data of dry matter probably because the lower temperature reduces the consumption of sugars by respiration. In contrast, similarity of the images between the initial samples and the samples kept dry at 4 or 20°C is evident, in agreement with dry matter data. In water at 20°C, the images show a great degradation which is in line with the data of dry matter loss.

Elaboration of the images in terms of normalized population *vs* pixel intensity, in order to have numbers to combine with the NIR data, made it possible to emphasize that the basal sections of the flowers kept in water, not only at 20°C but also at 4°C, are contaminated already in the first 3 days of vase life. The GEFI image (MR signal prosectional to water content) of the basal section of flowers kept at 20°C, at a pixel intensity of 100, shows how great the difference is (0.2 vs 0.05-0.1 normalized population) between the initial sample (black line) and the lines (red and bleu) of the flowers kept in water, at 3 and 12 days (Fig. 3 a). This result pinpoints a greater water content in the basal tissue than in the middle section where the difference is not evident (data not shown). The same images elaborated as MSME signal (MR signal prosectional to water mobility) (Fig. 3 b) show a shift of the red and blue lines (flowers in water at 3 and 12 days, respectively) toward the left (Y axis), compared to the other samples, and a particular peak at 200 pixels (which suggests a change in water mobility) similar to the one observed in the GEFI image (water content) but at 100 pixels (Fig. 3a). Both these results point out that a change in water content and mobility occurs; this event could be attributed to the presence of bacteria. The curve pattern of the middle section is similar but with a greater distance between the two pairs of lines (red and bluu vs green and light blue) and no peak is observed at 200 pixel (Fig. 3 c). The peak of the normalized population for red and blue lines is at 25 pixels while for green and light blue lines it is between 50 and 75 pixels, meaning a clearer image, as can be observed in Figure 2. The patterns of curves of basal and middle sections are very different, especially for red and blue lines: at 50 pixels the value of

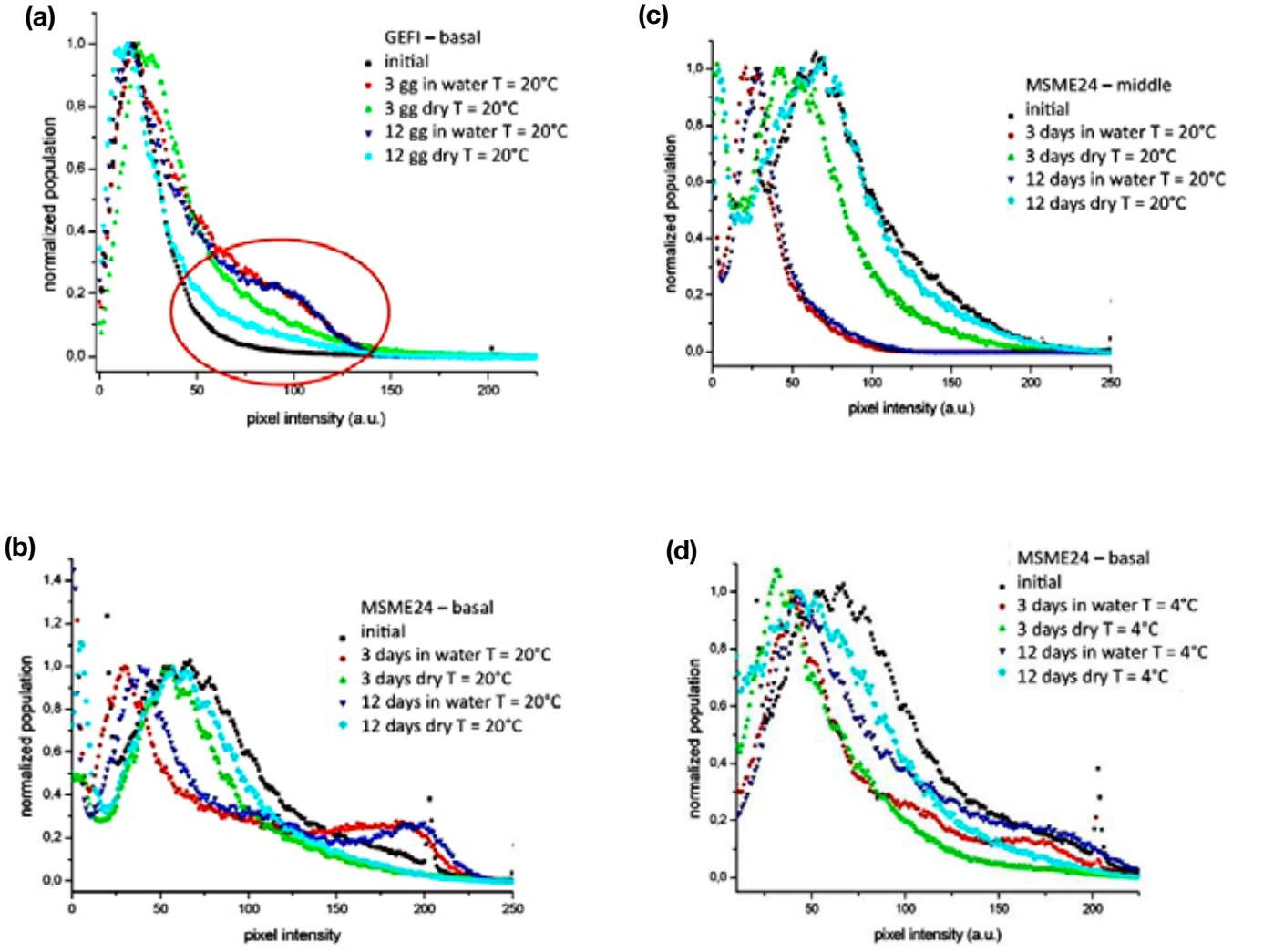

Fig. 3 - Normalized population vs pixels (a.u. = auxiliary units) elaboration of GEFI (3a) and MSME (3b,c,d) images.

the basal section is 0.4 normalized population while the one of the middle section is 0.2; for the green and light blue lines (dry flowers at 3 and 12 days) the 100-pixel values are similar for the basal and middle sections. Thus, for flowers that were kept dry, clearer images and less difference in the images of the sections can be noted.

At 4°C, the basal section showed coupled lines on the basis of sampling times, with a shift towards the Y axis, compared to the black line (initial sample): the peak of the normalized population of the initial sample is around 75 pixels, while for the rest of the lines it is between 25 and a little bit more than 50 pixels (Fig. 3 d). In the range 50 and 100 pixels, the green and red lines, which refer, respectively, to dry and water samples at 3 days, had lower values than the bleu and the light bleu lines, referring to water and dry samples but at 12 days. Even in this case, at 150 pixels an increase of the values of normalized population of the red and blue lines was observed as was shown at 20°C, in line with the presence of degraded tissue. In the middle section, the cited increase at 150 pixels is not visible and the pattern of lines is more confused (data not shown).

4. Conclusions

Application of the portable NIR-AOTF instrument to the stem of cut flowers (Calla lily in the present study) to non-destructively measure weight loss and water content, especially for dry shipped flowers, can be very useful to predict vase life. MRI is a very powerful tool to study the water status in the xylematic vessels and the use of of ImageJ software permits transformation of the image into numbers (pixel intensity or normalized population). Unfortunately the cost of the instrument, which depends on magnet size, makes commercial use of the instrument impossible. The use of software will permit correlation of the pixel intensity or normalized population data to spectra values of other non destructive instruments, such as the NIR-AOTF as will be examined in our future research.

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A Simple approach to assess common root rot severity incidence data in wheat

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Key words: *Cochliobolus sativus*, common root rot, incidence, severity, wheat.

Abstract: Common root rot (CRR) of wheat, caused by *Cochliobolus sativus*, produces discoloration of the subcrown internodes (SCIs) and is directly related to yield losses. It is critical to clearly define and standardize the CRR assessment methods to avoid subjectivity and variability between assessors. Therefore, in this study, a comparison between the incidence (I; proportion of diseased SCIs) and the severity (S; proportion of SCI showing CRR symptoms) was investigated to explore the possibility of simplifying disease rating. Assessments were made visually at multiple sample sites in artificially- and naturally-inoculated research and production fields for three growing seasons. Significant differences (*P* = 0.001) in mean I and S values were found among cultivars, with values being consistently higher in the susceptible ones. However, CRR severity increased linearly as incidence increased in both *Triticum durum* and *T. aestivum* wheat. Their slopes and intercepts of the I–S relationship were consistent over the three growing seasons. This result may be considered a significant contribution for CRR assessment in wheat breeding programs.

1. Introduction

Common root rot (CRR) caused by *Cochliobolus sativus* (Ito & Kurib.) Drechsler ex Dastur (anamorph *Bipolaris sorokiniana* (Sacc.) Shoemaker), is consistently one of the most damaging diseases of wheat and barley worldwide (Gurung *et al.,* 2013; Fernandez *et al*., 2014). CRR is considered economically important because it can cause marked reduction in yield and quality of the crop (Kumar *et al.,* 2002). This disease produces a brown to black discoloration of the subcrown internode (SCI), therefore presence and severity can be determined by pulling up plants and examining SCI for disease (Kokko *et al.,* 1995; Mathre *et al.,* 2003).

Efforts to minimize the impact of CRR have been centered around the use of management strategies such as host resistance, crop rotation, tillage, and fungicide application (Fernandez and Conner, 2011; Burlakoti *et al.,* 2013). From a management perspective, the comparison of CRR epidemics across years and locations is necessary to determine the effects of the environment on the efficacy of a given management approach, under similar environmental conditions, and to develop or recommend management strategies or decision thresholds (Fernandez *et al*., 2014).

The first step to quantify the effect of CRR is to develop a key that clearly defines and standardizes the assessment methods to avoid subjectivity and variability between assessors. Therefore, CRR evaluation methods need to easily provide objective measurements so that data from different sources are comparable, and provide an adequate sample of the crop for assessment (Mathre *et al.,* 2003). Reaction of wheat to CRR is commonly measured either by incidence (I, proportion of SCI units diseased) or severity (S, proportion of SCI showing CRR symptoms). However, incidence is a binary measurement (Madden and Hughes, 1999), meaning it is a measure of only one of two possible states, diseased or not diseased. Moreover, in spite of the drawback, however, severity is often considered a more important and useful measure of disease intensity than incidence to evaluate yield loss and to deterimine the effectiveness of disease management strategies (Fernandez *et al.,* 2009).

Since measurements of incidence are more easily acquired and more reliable than measurements of severity, and severity is more useful than incidence for certain objectives, a quantitative relationship between incidence and severity would greatly facilitate the evaluation of disease intensity when accurate assessments of severity are not available or possible (Seem, 1984; Fernandez *et al.,* 2014). Therefore, in this study, the I-S relationship of CRR was investigated to explore the possibility of simplifying disease assessment.

2. Materials and Methods

Disease assessment sites

In order to acquire data from CRR epidemics of different intensities and to represent a range of environmental, cropping, and management conditions likely to influence

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the development of CRR, three different locations with several research plots and production fields were selected for CRR assessment in three growing seasons (Table 1).

Inoculum preparation

The *C. sativus* isolate (Pt4) has been proved to be one of the most virulent isolates to all barley and wheat genotypes available so far (Arabi and Jawhar., 2002). In the present study, the fungal mycelia were transferred from a stock culture into Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/I kanamycin sulphate and incubated for 10 days at 21±1°C in the dark.

Host genotypes

The ten wheat cultivars (six *Triticum durum* and four *T. aestivum)* used in this study were chosen for their wide genetic variability for *C. sativus* reaction from highly susceptible to highly resistant (Table 2). The local susceptible landrace Salamoni was included in each set as check.

Experimental design

Seeds were artificially inoculated with Pt4 isolate following the procedure set out by Arabi and Jawhar (2013). The experimental design was a randomized complete block design with three replicates. The seeding depth was 6 cm (Kokko *et al.,* 1995). Plot area was 1 x 1 m with a 1 m buffer. Each plot consisted of five rows 25 cm apart with 50 seeds sown per row. Experimental design, cultural practices, and inoculation methods were performed as described by Arabi and Jawhar (2002). Weeds were controlled by pre- and post emergence herbicides as appropriate. Soil fertilizers were drilled before sowing at a rate of 50 kg/ha urea (46% N) and 27 kg/ha superphosphate (33% P).

Disease assessment

In each field/plot, I and S were estimated visually at several systematically selected sampling sites, 20-25 subsampling from each row were taken at random from each replication.

Incidence (I) was recorded as the proportion of diseased SCIs (number of SCIs with nonzero severity divided by the total number of plants sampled). Severity (S) was recorded as infected SCIs expressed as a proportion of the total area.

Statistical analysis

Data for I and S were analyzed by analysis of variance (Newman-Keuls test), using the STAT-ITCF program (ITCF, 1988). The assumption of coincidence for each year was tested using the ANOVA procedure implemented in the software package Statistica 6.1. Years were set as the categorical variable and coincidence was tested by simultaneously checking the year's effect combined with its interaction with the incidence. For all experimental data, each pair of I and S values from each sampling site was considered an observation for data analysis. The experimental data were edited to remove observations with no diseased plants (i.e., I = 0 and S = 0), since the I-S relationship is only defined when disease is present.

3. Results

Significant differences ($P = 0.001$) in mean I and S values were detected, with values being consistently higher in the susceptible cultivars for the three growing seasons (Table 2). The data show that the highest mean I and S were recorded in the *T. aestivum* landrace Salamoni (I and S =100), whereas the lowest was found in the *T. durum* landrace Horani (I and S ≈ 7). In general, the *Triticum durum* genotypes were more resistant than *T. aestivum* (Table 2), in agreement with data presented by Bhandari and Shrestha (2004).

Additionally, the data demonstrate that S increased linearly as I increased (Fig. 1). There was no difference in the slopes and intercepts of the I-S relationship among the three years, as was shown by the coincidence test ($F_{3, 32} = 0.309$, $P = 0.585$). In some cases I = S for one or more observations such as in the susceptible landrace Salamoni (Table 2). This can be explained by the fact that when all plants in the sample are diseased, there is no longer any information on the magnitude of (mean) S in relation to I, other than being larger than the (mean) S when some plants are disease-free. In this extreme situation, I was equal to S for wheat CRR reaction. These findings are in agreement with the results of Paul *et al*. (2005) for fusarium head blight on winter wheat.

The overall response to CRR for the three growing seasons considered in this study differed with the differences

Table 1 - Range of magnitude of environmental conditions encountered during three growing seasons (2011, 2012 and 2013)

Location	No. fields	Temperature (°C) [z]	Relative humidity (%)[z]	Average rainfall (mm) [y]	Altitude (m)	Directions
Draa (south)	4	33-39	40-51	256	716.5	36°06'23.86'' E 33°06'55.71'' N
Allepo (north)	4	25-36	41-79	360	297.4	33°55'56.99'' E 36°01'31.14'' N
Hassaka (north east)	5	35-48	35-42	228	313.9	40°40'02.31'' E 36°31'53.73'' N

[z] Average during April, May and June.
[y] Average from November to April.

Table 2 - Mean common root rot disease incidence (I) and severity (S) of the most frequently grown wheat cultivars in Syria under field conditions for 3 years, combining data for three locations

Cultivar						
	S	I	S	I	S	I
Triticum aestivum						
Sham 2	15.20 e	20.00 e	22.50 de	20.50 e	18.30f	17.60 e
Bouhouth 4	33.16 d	40.00 d	25.40 d	33.00 d	22.50 e	30.00 d
Bouhouth 6	42.60 c	50.50 cd	48.20 c	47.60 c	40.30 c	43.00 c
Salamoni (Landrace)	95.50 a	100.00 a	90.07 a	100.00 a	89.30 a	91.00 a
Doma 4	60.16 bc	73.00 c	52.90 c	50.60 c	40.50 c	39.00 c
Mexipak	66.50 b	80.00 b	70.30 b	78.30 b	63.20 b	86.20 b
T. durum						
Doma 1	31.13 d	33.00 de	27.00 d	30.00 d	35.70 d	39.50 c
Sham 3	10.30 e	18.00 e	9.10 e	15.10 f	12.20 g	18.30 e
Horani	7.50e	10.60f	7.80e	8.50fg	5.50h	9.67f
Bouhouth 7	30.06 d	38.00 d	22.33 de	25.60 de	31.70 d	33.50 d
LSD	8.83	6.11	7.42	5.3	4.09	4.01

Values followed by different letters columns are significantly different at *P*= 0.001 according to Newman-keuls test. LSD: Least Significant Difference at P<0.05.

in susceptibility levels of the cultivars. However, cultivars that are resistant to CRR may in fact have different resistance response to the spread of the fungus within the infected plants. Hence, for any given I value, a wide range of S values may be observed across cultivars. McRoberts *et al.* (2003) reported that incidence severity analysis was directly useful in evaluating resistance response.

In particular, the I–S relationship could be used to draw conclusions about the relative rate of disease increase among cultivars with different levels of resistance.

4. Discussion and Conclusions

Our results show that neither differences in weather conditions for the three growing seasons, nor geographical locations resulted in any different patterns in the I-S relationship. Although the locations were up to 50 km apart, it appeared that within a climatologically similar region, I-S relationships did not show distinct differences among sites. Moreover, in this study, the number of plants sampled and the small distance among locations did not affect the I-S relationship either.

We undertook this study to determine an I-S relationship for CRR and then to establish whether that relationship would remain the same for different years, locations and cultivars. The results reveal a positive correlation between CRR parameters I and S in wheat which was consistent among seasons and locations. However, characterizing the functional relationship between I and S is still critically important, because through this relationship researchers can identify the cultivars with unusually large or small S for a given I (McRoberts *et al.,* 2003), or through covariance analysis (when there are several pairs of I-S points for each cultivar), identify cultivars with an unusual I-S relationship compared with others. Moreover, the es-

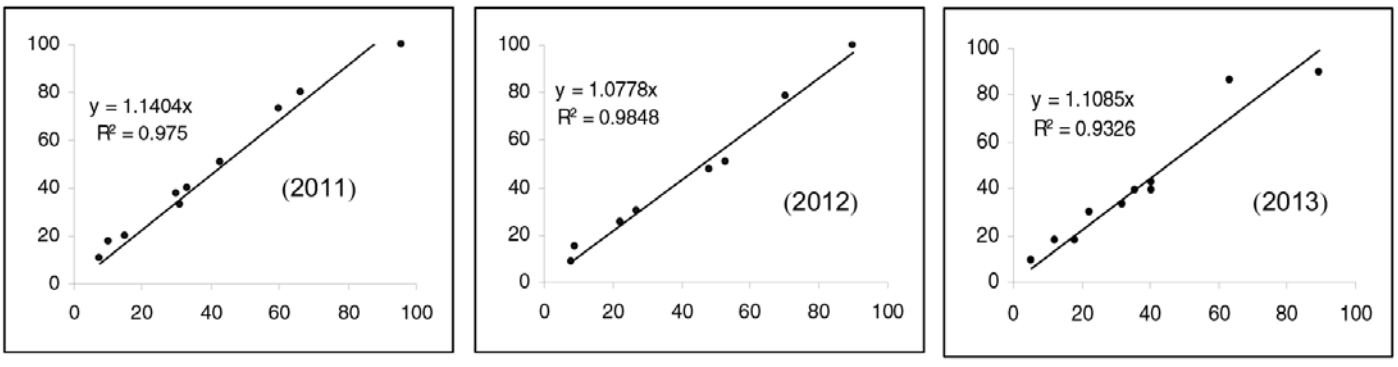

Fig. 1 - Relationship between incidence (I; proportion of diseased SCIs) and severity (S; proportion of SCI showing CRR symptoms) of wheat common root rot for three growing seasons.

timation of mean I from S would substantially reduce the work load in CRR quantification in field surveys and treatment comparisons.

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Mini-review

Pteridophytes as active components in gardening, agricultural and horticultural ecosystems in Japan

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Key words: Environment, fern, gardening, insects, Japan, mycorrhizae.

Abstract: Many members of Pteridophytes have been traditionally used in the designs of Japanese gardens and a large variety of ferns attract gardeners as greening pieces in the garden designs. However, details or examples of practical uses of ferns in traditional gardening in Japan have only rarely been introduced in non-Japanese literatures to date, despite the importance of ferns in Japanese gardening traditions. In addition to the discussion of ferns in gardening, the use and association of these plants in Japanese agricultural and horticultural sceneries are addressed. The presence and importance of 40 familiar fern species in local gardening, agricultural and horticultural ecosystems are also discussed, as well as the roles of introduced ferns as key elements of ecosystems and their interaction with neighboring biota. Finally, some examples of uses of fern species in environmental science and engineering are also reviewed.

1. Introduction

It is widely known that traditional Japanese gardening requires moss species as key botanical components for covering natural rocks, wooden and stone walls, tree trunks and sidewalk surfaces. The use of mosses is frequently described in the world of Japanese literatures, chiefly in Haiku, as symbolized by a mythical conversation between legendary Haiku poet Basho and Zen master Buccho in which they discuss the Buddhist philosophy on growing green mosses (Suzuki, 1975). In typical Japanese gardens, natural rocks partially covered by a layer of thick moss and ponds surrounded by mossy stones represent miniature-sized rocky mountains with forestry slopes and lakes located deep in the forest. These approaches for miniaturizing natural landscapes are well in line with the Buddhist-affected and Shintoist-mixed nature-respecting philosophical preferences in Japan which encourage people to be surrounded by pieces of nature.

In Japan, second to mosses, many Pteridophytes (ferns and fern allies) have also been traditionally used in Japanese garden designing. Interestingly, some ground-covering ferns such as members of Selaginellaceae have been considered as mosses by gardening practitioners lacking taxonomical background, largely due to their moss-like appearance (Fig. 1). In place of mossy mats, two Selagi-

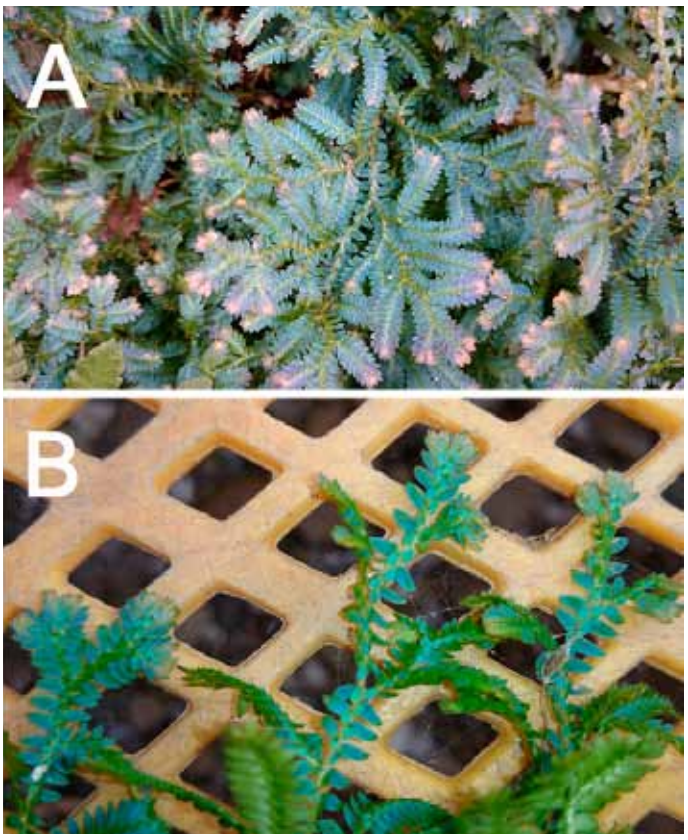

Fig. 1 - Living *Selaginella remotifolia* found in a private garden (Kiyotake-cho, Miyazaki, Japan). (A) Sheet of growing *Selaginella remotifolia* covering the ground. (B) Blue-green colored leaflets.

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nellaceae members, such as *Selaginella remotifolia* Spring (Japanese name: Kuramagoke) and *Selaginella unicinata* (Desv.) Spring (Japanese name: Konterikuramagoke), can be installed in Japanese gardens. Japanese names ending with -goke or -koke are conventionally given to moss species, despite the fact that these species are taxonomical members of ferns.

Furthermore, a large variety of ferns (including standing types) also attract the gardeners as greening pieces in gardening designs. Not only in Japan but also in other East Asian regions such as Taiwan and Korea, some fern species such as *Lycopodium fordii* Bak. (Lycopodiaceae) are grown as gardening plants and reflect the local flora (Huang *et al*., 2000).

In addition to the Lycopsida members mentioned above (eg. *Lycopodium* among subclass Aglossopsidae and *Selaginella* among subclass Glossopsidae, within the class Lycopsida), *Psilotum nudum* (L.) P. Beauv. (Psilotaceae; Japanese name: Matsubaran) belonging to the class Psilotopsida has long been utilized in Japanese gardening landscapes. Recently it was suggested that this plant communicates with neighboring plants through a arbuscular mycorrhizal network (Winther and Friedman, 2009). Accordingly, *Psilotum nudum*, with its non-photosynthetic life cycle, can thrive underground mostly by depending on the transport of photosynthetically fixed carbon from the green plants growing nearby. In the gardens where this plant has been introduced, the micro-ecosystem which allows such ecophysiological interspecies communication should be developed.

The above fern species have conventionally been considered as members of the phylum Microphyllophyta. As might be expected, other pteridophytes belonging to two other conventional phyla, namely Spherophyta and Pterophyta, are also commonly found in Japanese gardening and agricultural landscapes. Despite the importance of Pteridophytes in Japanese gardening traditions, details or examples of practical uses of ferns have only rarely been introduced in non-Japanese literatures to date. As most ferns favor a humid subtropical climate, this article aims to briefly describe and list the Pteridophytes found in the local gardening and agricultural landscapes in the southwestern half of Japan.

2. Gardening and Ferns

Historically, the inclusion of ferns in gardens has been popular among people of various classes such as farmers, warriors, monks and aristocrats, thus ferns were often found in the gardens and backyards of imperial and aristocratic palaces, Shintoist shrines, Buddhist temples, and ordinary houses. Today, it has become much harder to find fern culturing in ordinary houses in the large cities in Japan, yet in rural areas surrounded by semi-wild forests and far from urban areas, the use of ferns can still be found readily.

In traditional Japanese gardens, the following fern species can be found. *Matteuccia struthiopteris* (L.) Todaro (Athyriaceae; Japanese name: Kusasotetsu also known as Kogomi in the northern Japan) often welcomes the visitor to the gardens. *Cyrtomium falcatum* (L. fil.) Presl. (Dryopteridaceae; Japanese name: Oniyabusotetsu) and *Equisetum hyemale* L. (Equisetaceae; Japanese name: Tokusa) are likely planted surrounding ponds and/or waterfalls. Beneath the bushes, *Polystichum polyblepharum* (Roem. Ex Kunze) Presl. (Dryopteridaceae; Japanese name: Inode) can be frequently found. *Equisetum hyemale* and *Dryopteris erythrosora* (Eaton) O. Ktze (Dryopteridaceae; Japanese name: Benishida) can be found in the shade of structures such as a Chashitsu (a tea-ceremony house). Gaps among the rocks or stones can be naturally filled by *Cyrtomium falcatum* and *Pteris multifida* Poir (Pteridaceae; Japanese name: Inomotosō). *Pteris multifida* is a specific host plant that supports the life of caterpillars of a domestic moth of the Noctuidae family, *Callopistria japonibia* (Fig. 2). Inoue and Sugi (1958) suggested that geographical distribution (size of habitat) of *Callopistria japonibia* largely depends on the distribution of this fern. This could be a model for studying the contribution of garden environments to harbor specific ferns for insect biodiversity.

Moreover, some fern species have been passively but very frequently installed in Japanese gardens, thus their presence strongly reflects the nature of original wild flora. Such species include *Pteris cretica* L. (Pteridaceae; Japanese name: Ōbanoinomotosō), *Osumunda japonica* Thunb., *Botrychium japonicum* (Prantl) Underw. (Ophioglossaceae; Japanese name: Fuyunohanawarabi), *Cyrtomium falcatum*, *Athyrium niponicum* (Mett.) Hance (Athyriaceae; Japanese name: Inuwarabi), *Deparia japonica* (Thunberg) M. Kato (Woodsiaceae; Japanese name: Shikeshida) (Fig. 3A, D) and *Pteridium aqualinum* (L.) Kuhn var. *latiusculum* (Desv.) Underw. Ex Hell (Dennstaedtiaceae; Japanese name: Warabi) (Fig. 3E, F).

Fig. 2 - *Pteris multifida* growing on the stone wall in a private garden (Kiyotake-cho, Miyazaki, Japan). (A) Top view of growing plants. (B) Spore-bearing adaxial side of the leaves. (C, D) Plants being fed on by caterpillars of *Callopistria japonibia*. Arrows indicate the presence of caterpillars.

Among passively introduced ferns, members of Polypodiaceae such as *Lemmaphyllum microphyllum* Pres (Japanese name: Mamezuta), *Lepisorus thunbergianus* (Kaulf.) Ching (Japanese name: Nokishinobu), and *Pyrrosia lingua* (Thunb.) Farw (Japanese name: Hitotsuba) are epiphytic species which are often attached to trees and rocks (Fig. 4). On the surface of rocks and walls, *Lemmaphyllum microphyllum* is often exposed to competition with higher epiphytic plants such as *Ficus pumila* L. (Moraceae; Japanese name: Ōitabi).

In addition to epiphytic plants, a climbing species, *Lygodium japonicum* (Thunb.) Sw. (Lygodiaceae; Japanese name: Kanikusa) is of great interest. Historically, sun-tracking movements associated with a the climbing growth habit were described by Charles Darwin (1875) in two members of *Lygodium* (*L. articulatum* and *L. scandens*). Darwin concluded that "*As ferns differ so much in structure from phanerogamic plants, it may be worthwhile here to show that twining ferns do not differ in their habits from other twining plants.*"

The climbing fern native to Japan, *Lygodium japonicum*, is commonly known as "Japanese climbing fern". This fern grows very fast and thus often covers nearby living trees, rocks and walls (Fig. 5). *Lygodium japonicum* has been exported for ornamental purposes. For instance, this plant was introduced in Florida, USA, in 1932 (Gordon and Thomas, 1997).

Fig. 3 - *Deparia japonica* and *Pteridium aqualinum* sampled in a private garden (Kiyotake-cho, Miyazaki, Japan). (A) Adaxial side (top side) and (B) Abaxial side (bottom side) of mature and young bladed of *Deparia japonica*. (C) Mature and (D) immature sporangia on leaflets of *Deparia japonica*. (E) Living *Pteridium aqualinum*. (F) Roots and young emerging frond of *Pteridium aqualinum.*

Fig. 4 - Epiphytic ferns found in a countryside private garden (Kiyotake-cho, Miyazaki, Japan). (A) *Lepisorus thunbergianus* grown on the trunk of a loquat tree (*Eriobotrya japonica* (Thunb.) Lindl.). (B-D) *Lemmaphyllum microphyllum* growing on the tree and rocks.

3. Ferns and Japanese people

Even before ferns were introduced into man-made gardens, people in Japan traditionally enjoyed going out

Fig. 5 - A climbing fern, *Lygodium japonicum*, found in a countryside private garden (Kiyotake-cho, Miyazaki, Japan). (A) Climbing growth of *Lygodium japonicum* on the wooden wall by competing with other climbing plants such as *Ficus pumila*. (B, C) Aggressive growth of *Lygodium japonicum* winning the competition with other standing plants. (D) Vegetative leaflets. (E, F) Development of sporangia on dorsal side of the leaflets.

to the fields and forests to gather edible young fronds of wild ferns. The relationships between Japanese people and ferns, especially Bracken ferns, are historically, socially and culturally so tight that recreational fern gathering was hardly weakened even among Americans of Japanese origin, as is revealed by the various recipes for cooking ferns found among traditional Japanese communities in the United States (Anderson *et al*., 2000). Edible wild ferns in Japan include *Equisetum arvense* L. (Equisetaceae; Japanese name: Sugina), *Pteridium aqualinum* (popularly known as Warabi), *Osumunda japonica* Thunb. (Osmundaceae; Japanese name: Zenmai), and *Matteuccia struthiopteris*.

Historically, among the Japanese, *Equisetum arvense* has been used as an herbal medicine with diuretic, antitussive, antipyretic, hemostatic, and roundworm-eliminating actions. Therefore, this herbal medicine has been given to patients with gonorrhea, cystitis, edema, bronchitis, and bleeding hemorrhoids (Ikegami, 2013). Furthermore, search for lymphangiogenesis-inhibiting agents in the crude extracts from *Equisetum arvense* was recently attempted (Jeong *et al*., 2013).

Figure 6 shows classical illustrations of rhizome anatomy of *Equisetum arvense* L. These illustrations remind us that, like higher plants, ferns, including *Equisetum arvense*, finely develop rhizomes under the soil, suggesting that ferns are one of the key players in the soil ecosystem.

Apart from edible and medicinal species, Japanese people are also familiar with some fern species for ornamental purposes and crafts. The mature fronds of *Gleichenia japonica* Spr. (Gleicheniaceae; Japanese name: Urajiro) are important for traditional New Year's decorations. In summer, outside doors or windows of houses, one can find hanging fern-bearing, peat moss-based green balls called "*Shinobudama*", which are usually about 10 cm in diameter, as one of the favored customs in Japan. A *Shinobudama* literally means a ball of Shinobu which is the Japanese common name for *Davallia mariesii* Moore ex Baker (Davalliaceae). This ornamental arrangement of *Shinobu* fern is often combined with a wind chime hung outside.

Use of *Equisetum hyemale* L. in traditional craft works should be also noted. The silicate-rich stems of *Equisetum hyemale* L. have long been used for grinding and honing the surface of wooden table wares and furniture.

4. Agriculturally associated flora of ferns in Japan

Paddy fields for culturing rice plants (*Oryza sativa* L.) are man-made wetlands which cover about 50% of the agricultural land in Japan. Some fern allies bloom out in the paddy field after the harvest of rice. Typical examples of dominating aquatic and semi-aquatic ferns in the post-rice paddy include *Ceratopteris thalictroides* (L.) Brongn. (Adiantaceae; Japanese name: Mizuwarabi), *Salvinia natans* (L.) All (Salviniaceae; Japanese name: Sanshōmo), *Azolla japonica* Fr. et Sav. (Azollaceae; Japanese name: Ōakaukikusa), *Azolla imbricata* (Roxb.) Nakai (Azollaceae; Japanese name: Akaukikusa), and *Marsilea quadrifolia* L. (Marsileaceae; Japanese name: Denjisō). *Marsilea quadrifolia* is most likely to be very sensitive to rice field herbicides and thus this plant species is rarely found nowadays (Luo and Ikeda, 2007).

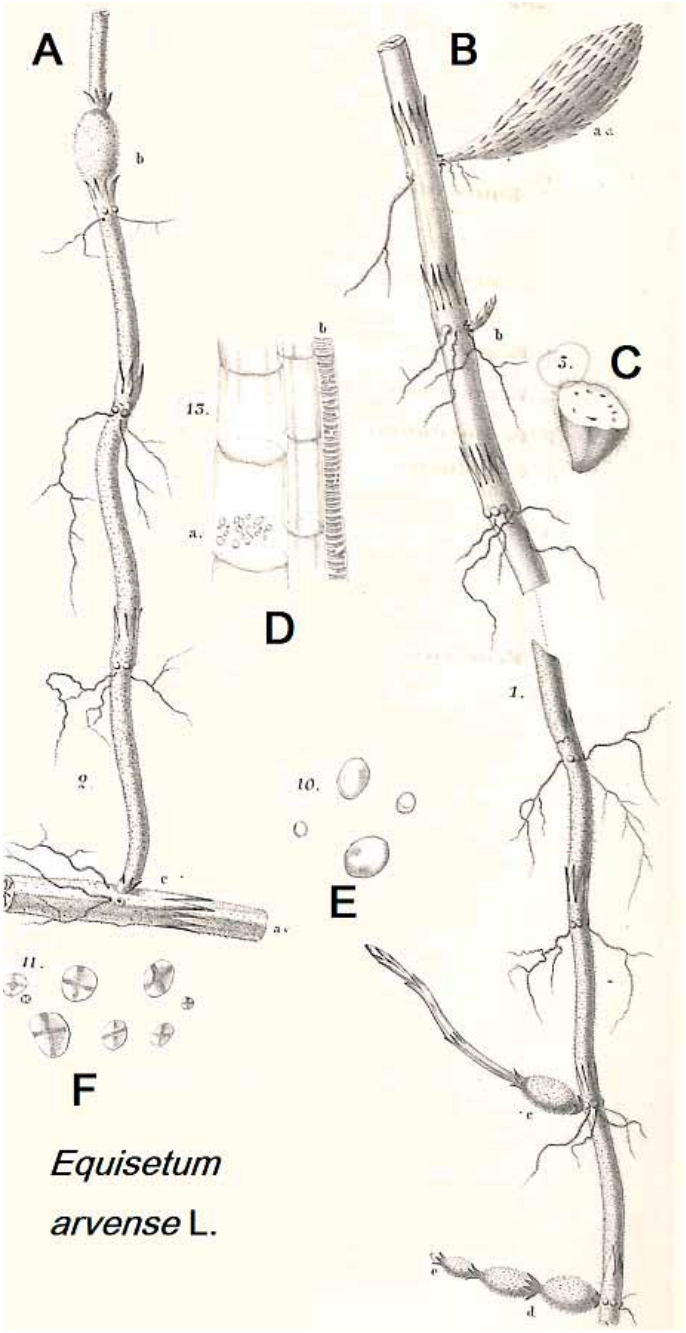

Fig. 6 - Anatomical illustrations of underground structures of *Equisetum arvense* L. found in a classical book by J. Duval-Jouve (1864). (A) Horizontal rhizomes. (B) Vertical rhizomes. (C) Section of large tuber attached to rhizome. (D) Cells of a tuber with starch granules (left) and vessel (right). (E, F) Starch granules. This book was originally collected and preserved through collaborative research between Université Paris Diderot and The University of Kitakyushu (Kawano and Bouteau, 2007; Kawano *et al*., 2008). Original images were digitally scanned and slightly modified (illustrations of other plant species were masked).

5. Breeding for horticultural purposes

Some Pteridophytes have been positively subjected to breeding efforts, especially during the era of Tokugawa Japan known as the Edo period (1603-1868). Among the ferns listed in this section, the breeding history for *Psilotum nudum* (known as Matsubaran) is estimated to have a long history and thus a number of varieties are available (Murata and Yashiro, 2006). Also some ornamental species with attractive leaf orientation, such as *Onychium japonicum* (L.) (Thunb.) Kunze (Parkeriaceae/Adiantaceae; Japanese name: Tachishinobu) and *Nephrolepis cordifolia* (L.) Presl (Lomariopsidaceae; Japanese name: Tamashida) are popularly bred and cultivated for both garden use and for ornamental pots. Note that, nowadays, *Nephrolepis cordifolia* often requires protection under a glass house especially during the winter season.

Some fern species are also favored by gardeners and plant growers and are nowadays bred and available on the market. Such ferns considered as gardening plants include *Selaginella unicinata* (Fig. 1), *Selaginella tamariscina* (Beauv.) Spring (Selaginellaceae; Japanese name: Iwahiba), *Selaginella involvens* (Sw.) Spring (Selaginellaceae; Japanese name: Katahiba), *Adiantum capillus-veneris* L. (Adiantaceae; Japanese name: Hōraishida), *Sphenomeris chinensis* (L.) Maxon. (Lindsaeaceae; Japanese name: Horashinobu), *Pteris nipponica* W. C. Shieh (Pteridaceae; Japanese name: Matsuzakashida) and *Asplenium antiquum* Makino (Aspleniaceae; Japanese name: Ōtaniwatari; this plant often requires green house or similar facilities). *Selaginella unicinata* is believed to have been brought from southern regions in China several centuries ago. *Adiantum capillus-veneris* is naturally found in the western half of Japan, but there are still some discussions on the origins of this fern (Murata and Yashiro, 2006). Among *Adiantum* species, *Adiantum monochlamys* Eaton (Adiantaceae; Japanese name: Hakoneshida) is hardly cultivated in gardens (Murata and Yashiro, 2006). Among the varieties of *Pteris nipponica,* lines or individuals with white spots on the leaves are likely favored on the market (Murata and Yashiro, 2006). It should be noted that even today, many efforts to develop and/or introduce new horticultural fern varieties of East Asian origin are being made (Xu *et al*., 2006).

6. Uses of ferns in environmental science and engineering

Unlike mosses, Pteridophytes possess a highly developed root system (rhisomes) by which minerals and metals are effectively taken up by growing plants. From an environmental point of view, some ferns are receiving more attention than ever, after pioneering works by Japanese fern specialists, since some ferns might be useful indicators and/or tools to assess and remediate contaminated soils.

Most metallic ions present at high concentrations are toxic to living plants by targeting and damaging the root cells exposed to the soil (Kawano *et al*., 2005). *Athyrium yokoscense* (Fr. Rt Sav.) Christ (Athyriaceae; Japanese name: Hebinonegoza), indigenous to East and North East Asia including Japan and Korea, is known to thrive in and around the sites of mining by rooting in soils contaminated with high concentrations of heavy metals (such as Zn, Cd, Pb, and Cu) and As (Morishita and Boratynski, 1992; Van *et al*., 2006). Therefore, the presence or distribution of this plant strongly indicates the history of the soil use or contaminations (Morishita and Boratynski, 1992). It has been well documented that *Athyrium yokoscense* not only tolerates but also effectively takes up the contaminating elements from the surrounding environments, as examined both at cellular level (Yoshihara *et al*., 2005) and field-grown plant level (Morishita and Boratynski, 1992; Kamachi *et al*., 2005).

Similar approaches are now being attempted with regard to other fern species. Use of a hyper-accumulator fern for arsenic and metals, *Pteris vittata* L. (Pteridaceae; Japanese name: Moejimashida) is one successful example of fern-based phytoremediation of metal-contaminated and arsenic-poisoned soils (An *et al*., 2006; Xie *et al*., 2009). Interestingly, a recent proteomic approach by Bona *et al*. (2011) suggested that the hyper-accumulating nature of *Pteris vittata* requires the presence of symbiotically formed arbuscular mycorrhizae. Furthermore, unpublished results presented by Chien *et al*. (2012) during an oral presentation at the 64th annual meeting of the Society for Bioscience and Bioengineering, Japan, suggested a possible relationship between the mechanism of arsenic accumulation by *Pteris vittata* L. and bacterial flora in the rhizosphere. These studies consistently support the view that composition of the micro-flora in the soil inevitably affects the environmental responses performed by rhizome-developed fern species.

7. Preservation of ferns and related flora and fauna in the gardens

Based on statistical techniques, namely two-way indicator species analysis (TWINSPAN) and Detrended Correspondence Analysis (DCA), Murakami *et al.* (2003) reported that there is a relationship between micro-landforms and the fern species composition in forest islands. This is the case of surveys on the ecological status of fragmented forests within the urbanized area of Kyoto city since fern members are largely considered to form a group of environmental indicators in the urban matrix. The authors concluded that the difference in species composition of ferns was shown to reflect differences in micro-landforms. For example, *Polystichum polyblepharum* and many other species are favored in valley bottoms in the small forest islands while some species such as *Dryopteris erythrosora* and *Gleichenia japonica* are favored on the slopes.

Japanese gardens can be considered as a local ecosystem that allows semi-wild propagation of fern species. This is largely due to the diversified micro-landforms in

Japanese garden designs which allocate rocks, mounds, valleys, falls and ponds within small areas. Indeed, Japanese gardens can be considered models for botanical display and/or preservation of semi-wild fern species and interacting flora, fauna and microbiota.

8. Conclusions

In this article, the use and associations of ferns in Japanese agricultural and horticultural sceneries are reviewed and discussed. Through listing 40 key Japanese fern species in gardening, horticulture and environmental studies in Japan, the author has shown that these ferns behave as key building blocks for the semi-wild and half man-made local ecosystems in gardens and agricultural fields. The author has also noted that these ferns are highly and actively communicating and competing with other organisms such as weeds, trees and insects, and also cope with environmental stress through rhizomes. Human-fern interaction may be one such interspecies communication.

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Use of liquefied cold temperature dimethyl ether for extraction of pigments from fresh vegetable tissues

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Key words: Carotenoids, carrot, chlorophyll, DME, Japanese squash, spinach.

Abstract: Dimethyl ether (DME) is known as a useful precursor to other organic compounds and is a promising alternative fuel without issues of toxicity, production, infrastructure, and transportation as is the case with various other fuels. Recently, DME has attracted the attention of scientists and engineers since it behaves as a subcritical solvent or a low-temperature solvent applicable for the extraction of organic molecules from bio-materials. This paper presents the extraction of chlorophylls and carotenoids from green peel and yellow cortex of Japanese squash, spinach leaves and carrot roots using low-temperature liquefied DME. Spectroscopic and fluorescence analyses of the extracted pigments revealed that chlorophylls were successfully extracted by liquefied DME from green materials (squash peel and spinach leaves). HPLC analysis further confirmed that chlorophylls extracted include both chlorophylls *a* and *b*. By using liquefied DME, carotenoids were extracted from all vegetable samples examined. The performance of DME as a novel pigment extracting agent is confirmed in this work and its use as a "green" solvent, as opposed to conventional solvents, for the preparation and extraction of various plant pigments is highly encouraged from an environmental point of view.

1. Introduction

Dimethyl ether (DME), the simplest ether with the formula CH_3OCH_3, is known to be a useful precursor to other organic compounds such as liquefied petroleum gas (LPG) (Zhu *et al*., 2007) and small molecular hydrocarbons (Zhu *et al*., 2008). DME is recognized as a promising alternative fuel for diesel engines, petrol engines, and gas turbines (Gupta *et al*., 2010), enabling clean and high-efficiency combustion with reduced emission of NO_x, SO_x, and particulate matter (Semelsberger *et al*., 2006). Furthermore, DME can be efficiently reformed to H_2 at low temperatures, and most importantly, DME does not have large issues with toxicity, production, infrastructure, or transportation as is the case with various other fuels (Semelsberger *et al*., 2006).

Apart from its use as a fuel or a precursor to other organic chemicals, DME has attracted the attention of scientists and engineers since it behaves as a subcritical solvent. For instance, performance of subcritical DME as an effective media for the extraction of medicinal, flavoring, and pungent agents from some spices (Ginger, black pepper, and chili powder) has been demonstrated (Catchpole *et al*., 2003). In addition to the subcritical approach, DME can be used as a low-temperature solvent or extraction agent, and thus, it is applicable to laboratory procedures for the extraction of organic molecules from bio-materials (Kanda and Makino, 2010; Kanda and Li, 2011). Recently, an industrial use of liquefied DME was reported for rapid removal of water from sub-bituminous coal without any heating process (Kanda and Makino, 2010). This approach encourages us to testify the performance of liquefied DME in de-watering of biomaterials including watery horticultural crops.

One may believe that the usefulness of DME as a solvent is limited by its low boiling point (-23°C). However, this property could be highly beneficial as it may facilitate the removal of the solvent from the extracted samples. In biochemical exercises at ambient temperature, a variety of conventional solvents such as alcohols, acetone, hexane, etc. are used for extraction of organic substances of interest including flavoring agents and pigments from various biological samples. However, extraction of some reactive or highly degradable chemicals requires handling at

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low temperature. Due to the nature of DME in liquefied form at ambient pressure (or relatively low pressure) at low temperature, it may allow us to perform extraction of pigments at low temperature, preferably below 0ºC. In the present study, we examine the application of liquefied DME for the extraction of model plant pigments such as chlorophylls and carotenoids from fresh vegetable tissues by passing the low-temperature solvent through the layer of homogenates of the tissues.

2. Materials and Methods

Plant materials

The pericarp (fruits) of Japanese squash (*Cucurbita moschata*, cv. Ebisu, cultivated in Hokkaido, Japan), leaves of spinach (*Spinacia oleracea* L., cv. Hunter, cultivated in Fukuoka prefecture, Japan), and roots of carrot (*Daucus carota* L., cv. Koyo-2, cultivated in Hokkaido, Japan) were obtained from a local market and used for the extraction of pigments. Squash pericarp was dissected into green peel (1 mm from the surface) and yellow tissue while spinach leaves and carrot roots were used without such separation. Following homogenization of the vegetable tissues in a mixer, the obtained pastes (1-2 g fresh weight, g fw) were layered in the sample holder in the extraction chamber.

Chemicals

Reagents for separation and analysis of pigments such as HPLC grade acetone and methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and ammonium acetate (Sigma-Aldrich Japan, Tokyo, Japan) were obtained through local vendors.

Apparatus for cryo-liquefaction of DME and extraction of pigments

The DME gas cylinder was obtained from a local gas vendor (Air-Gases Kitakyushu Inc.) and supply of gaseous DME was made through 0.5 MPa gauge. The newly-designed apparatus consisted of (1) DME gas cylinder connected to a flow meter, (2) cooling unit, (3) extraction chamber, and (4) liquid trapping port (Fig. 1A). Each of the units was connected with aluminum capillaries. Within the extraction chamber, there was a disk-shaped sample holder for vegetable paste layering. Since the extraction chamber of acryl resin was transparent, the amount of cryo-liquefied DME could be monitored and manually controlled (Fig. 1B, C).

Treatment of plant materials with liquefied DME

For extraction of model pigments from the homogenized vegetable tissues, the apparatus shown in Figure 1 was used. DME gas, supplied directly from the gas cylinder at 0.4 MPa, was rapidly cryo-liquefied in the cooling unit. By handling (opening and closing) of a stopcock between the cooling unit and extraction chamber, collection and release of liquid DME precipitated in the cooling chamber was manually controlled. After loading *ca*. 15 ml liquid DME into the extraction chamber, the vegetable sample was allowed to have contact with liquefied DME only for a few seconds. The mixture of passed DME and accompanying liquid was collected in a test tube by opening the stopcock between the extraction chamber and the collection unit (Fig. 1D, E). In turn, dry matter was left in the extraction chamber. Following passive DME removal under ambient pressure, mixtures of vegetable sap (water) and vegetable-derived oily materials containing pigments were recovered.

Spectroscopic analysis

Pigments recovered after extraction with DME were diluted with acetone and used for spectroscopic scanning and fluorescence analyses using a spectrophotometer (U-3310, Hitachi High-technologies, Tokyo, Japan) and a fluorescence spectrophotometer (F-4500, Hitachi High-technologies, Tokyo, Japan), respectively. For fluorometric analysis of pigments, a three-dimensional (3D) contour plot was performed as described elsewhere (Kawano *et al*., 1999). The fluorescence contour profile was obtained with an excitation range between 250 nm and 700 nm (slit size, 5 nm; sampling interval, 5 nm) and an emission range between 300 nm and 750 nm (slit size, 5 nm; sampling interval, 5 nm), at scanning speed of 30000 nm/min. Recovery of chlorophyll *a* in the extracts was spectroscopically quantified according to the formula proposed by Wellburn (1994) as follows: [chlorophyll *a*] (μg/ml) = 12.25 x $A_{663.2\ nm}$ - 2.79 x $A_{646.8\ nm}$. Similarly, concentration of total carotenoids in the extracts was spectroscopically quantified using the following formula proposed by the National Agriculture and Food Research Organization of Japan: [total carotenoids] (mg/L) = 4.143 x $A_{475\ nm}$ - 0.561.

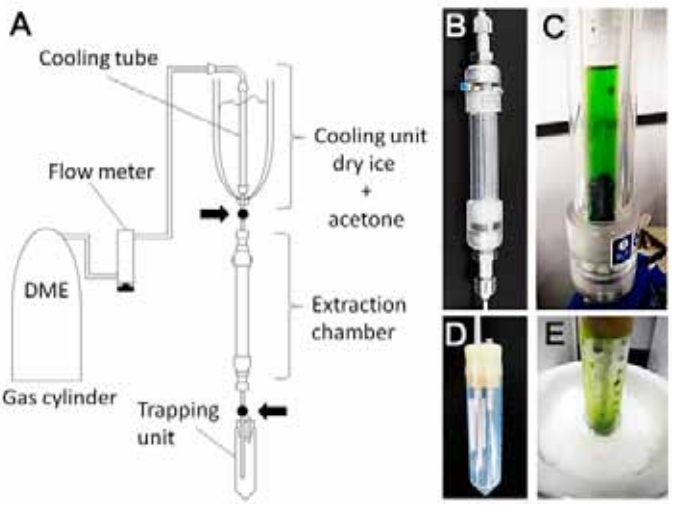

Fig. 1 - Apparatus for pigment extraction with liquefied DME. (A) Composition of the system. Arrows indicate the positions of two stopcocks designed for fine controls of gaseous and liquefied DME flows. (B, C) Extraction chamber. (D, E) Trapping port with a plastic tube.

High-performance liquid chromatography (HPLC)

Aliquots of the extracts containing pigments were analyzed by the reverse-phase HPLC system according to Maeda *et al*. (1998). HPLC (Waters 2690 Separations Module, Waters, USA) equipped with Inertsil ODS-80A column (GL Science, Tokyo, Japan) and two types of detectors, namely, a detector with photodiode arrays (water Alliance PDA system, Waters, USA) and a fluorescence detector (Waters 474 Scanning Fluorescence Detector, Waters, USA), for the separation and detection of pigments.

Analysis of chlorophylls was carried out using the solvent system which consisted of A (1:4 mixture of 1 M ammonium acetate and 80% methanol) and B (1:4 mixture of acetone and methanol). The program started with a linear gradient from A to B (15 min), continued with an isocratic run with B (5 min), and returned to A (2 min), and continued isocratically for 8 min at a flow rate of 1 ml/min. Peaks corresponding to pigments were detected with a fluorescence detector (excitation at 405 nm, emission at 660 nm).

Analysis of carotenoids was carried out using the solvent system consisting of 9:1 mixture of methanol and ethanol at flow rate of 1 ml/min. Peaks corresponding to pigments, chiefly carotenoids, were detected by monitoring absorbance at 455 nm using a detector with PDA.

3. Results and Discussion

Preparation of samples and pigment extraction

Fresh tissues of Japanese squash (green peel and yellow cortex), spinach (green leaves) and carrot (roots) were homogenized and the resultant pastes were prepared for extraction with liquid DME (Fig. 2A-K). After passing liquid DME through the plant sample layer (homogenates) packed in the column of the extraction chamber, a dry powder resembling acetone powder was left in the apparatus (Fig 2L-O). In turn, liquid samples mostly the mixtures consisted of extracted water (due to de-watering action of DME), oil (due to solvent action of DME), and the carrier liquid DME, were collected in the tubes placed beneath the extraction apparatus. As DME can be readily evaporated out under conditions of ambient pressure and temperature, solvent removal from the collected liquid was passively allowed by leaving the tubes with liquid samples at ambient temperature for at least 30 min. As a consequence, dense colored aqueous samples remained in the tubes (Fig. 2P-S) which were a mixture of oil and water. This indicated that both water and oils were extracted from the plant samples by liquid DME. Note that pigments were separately extracted from the green peel (Fig. 2A, D, H, L, P) and yellow cortex (pericarp; Fig. 2A, E, I, M, Q) of Japanese squash. The above extraction processes were repeated three times.

The total yield of oil/water mixture extracted with DME flow largely depends on the water content in the vegetable samples; the highest yield was found with

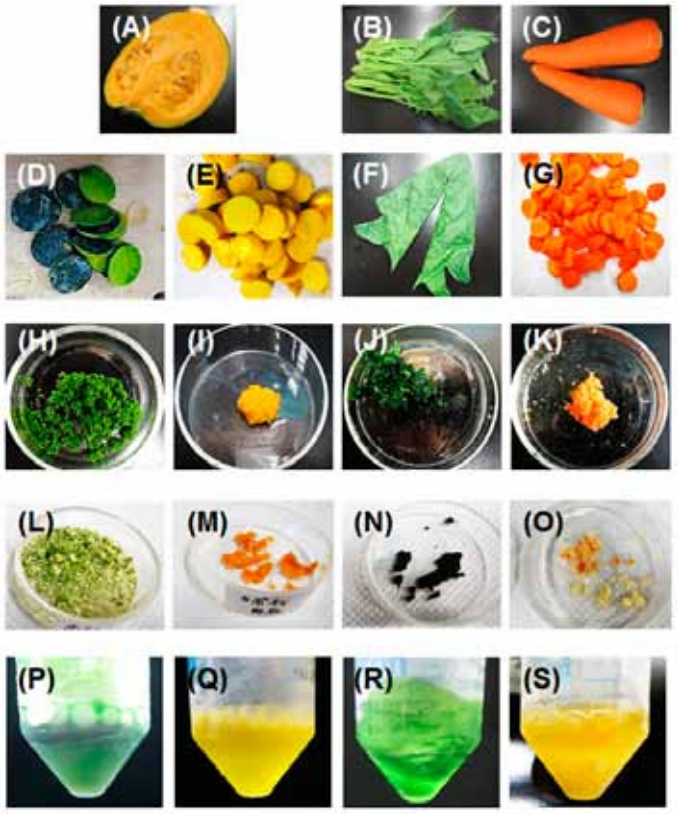

Fig. 2 - Fresh tissues, homogenized paste, dry powder, and pigment mixture of vegetables. Peel and cortex of Japanese squash (A, D, E, H, I, L, M, P, Q), leaves of spinach (B, F, J, N, R) and carrot roots (C, G, K, O, S) were used for extraction of pigments using DME. Whole vegetables (A-C), fresh tissues prior to homogenization (D-E), paste of homogenates (H-K), dry powder remaining after extraction with DME (L-O), and pigment mixtures obtained after evaporation of DME (P-S) are shown.

carrot roots, the most watery sample among those tested (Fig. 3). Since a drastic change in color after DME-based extraction was observed in squash peel (Fig. 2H, L) and carrot root tissues (Fig. 2K, O), we assumed that extraction of pigments was successfully carried out. In

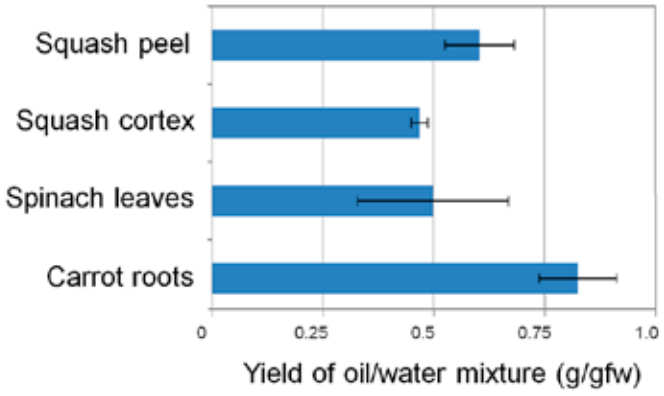

Fig. 3 - Yield of oil/water mixture extracted from vegetables using DME as solvent. Mixture of oil and water containing pigments from vegetables are compared.

contrast, pigment extraction from sticky pastes of yellow squash pericarp and spinach leaves required further modifications (Fig. 2I, J, M, N).

Quantitative and qualitative analysis of extracted pigments

Spectroscopic analyses of the pigments extracted from vegetable samples were performed (Fig. 4 left) and the resultant data was used for quantification (Fig. 5). Carotenoids were observed in all samples. As expected, the presence of chlorophylls was detected only in the samples derived from green tissues. The chlorophyll content in squash peel was found to be higher than that in spinach leaves (Fig. 5 top), possibly due to the loss of chlorophyll during extraction from the leafy sample as previously suggested (Fig. 2N). The total carotenoid contents in vegetable extracts were also compared and revealed that the green peel of squash and carrot roots had the highest values (Fig. 5 bottom).

The presence of chlorophylls (chiefly chlorophyll *a*) in the extracts from squash peel and spinach leaves was further confirmed by fluorescence spectroscopy (Fig. 4 right) and HPLC (Fig. 6). While it was difficult to dissect the spectroscopic and fluorescent signals for chlorophyll *a* from those of concomitantly present chlorophyll *b*, HPLC chromatograms showed clearly separated peaks of both chlorophylls *a* and *b* in the extracts from squash peel and spinach leaves (Fig. 6). With the aid of HPLC, the presence of both α- and β-carotenes were also confirmed in carrot roots while the other three samples showed the presence of β-carotene only (Fig. 7).

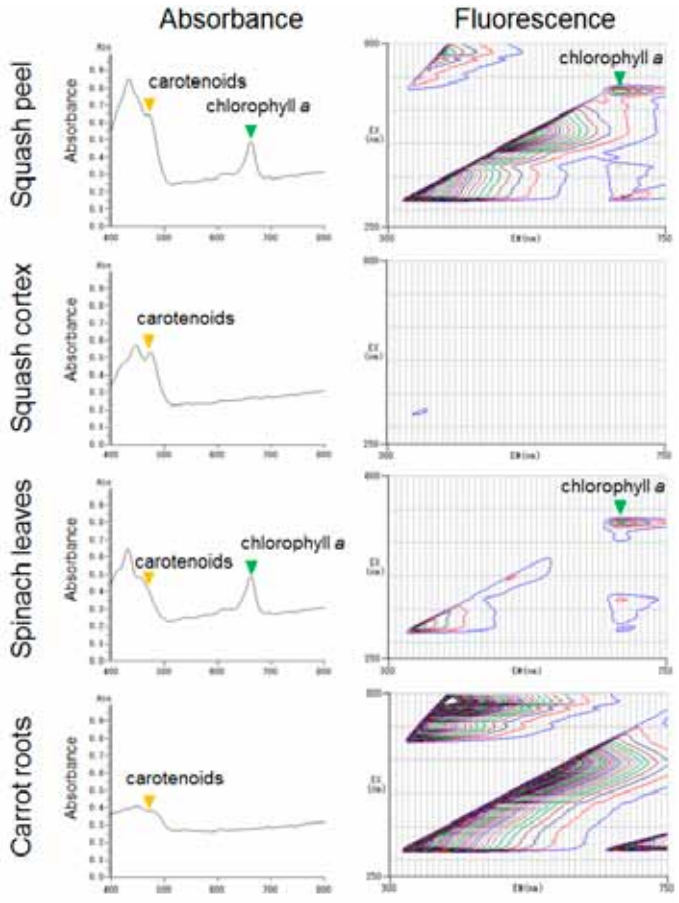

Fig. 4 - Spectroscopic and fluorescent profiles of DME-extracted pigments from vegetable samples. Comparison of absorption spectra (left) and fluorescence spectral contour (right) recorded in extracts from squash peel (top), yellow squash cortex (second line from the top), spinach leaves (third line from the top), and carrot roots (bottom). Prior to assays, extracts (oil/water mixtures) obtained after evaporation of DME were dissolved in acetone.

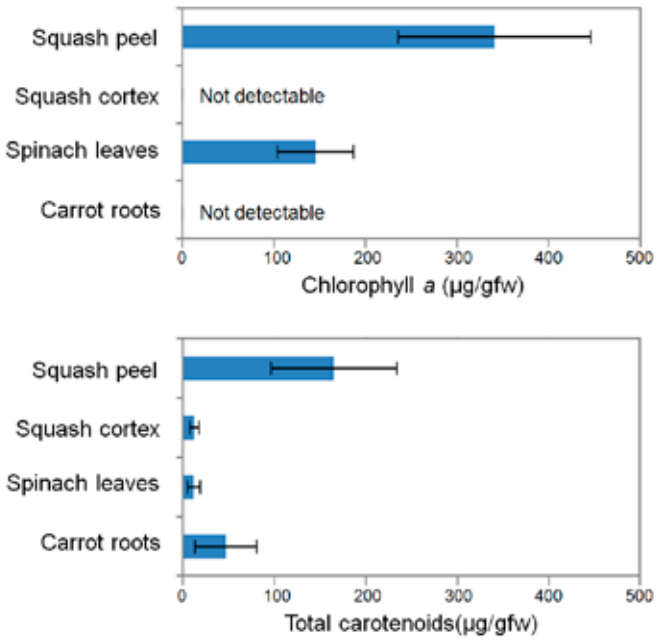

Fig. 5 - Quantification of pigments extracted from vegetable samples. Chlorophyll contents (top) and total carotenoid contents (bottom) in the extracts from green squash peel, yellow squash cortex, spinach leaves, and carrot roots are compared. Bars, S. D. (n = 3).

4. Conclusions

In the present study, the extraction of chlorophylls and carotenoids from green peel and yellow cortex of Japanese squash, spinach leaves and carrot roots using low-temperature liquefied DME has been demonstrated. Spectroscopic and fluorescence analysis of the extracted pigments revealed that chlorophylls were successfully extracted by DME from green materials (squash peel and spinach leaves). HPLC analysis further confirmed that the extracted chlorophylls included both chlorophyll *a* and *b*. Carotenoids were shown to be extracted by DME from all vegetable samples examined as confirmed by spectroscopic and HPLC analyses. The performance of DME as a novel pigment extracting agent is confirmed in this work

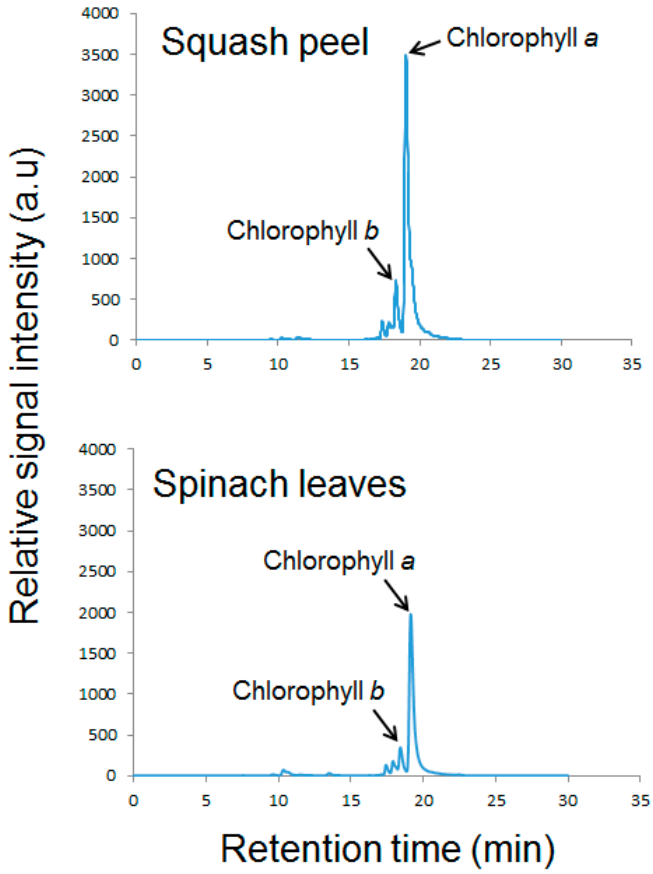

Fig. 6 - Detection of chlorophylls with HPLC. HPLC chromatograms showing the peaks of chlorophylls *a* and *b* extracted from squash peel and spinach leaves using DME. Pigments eluted from Inertsil ODS-80A column were detected by intrinsic fluorescence intensity (excitation, 405 nm; emission, 660 nm).

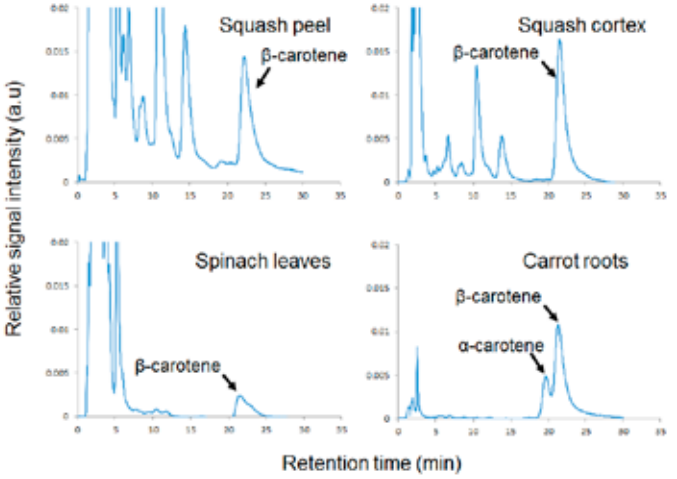

Fig. 7 - Detection of α- and β-carotenes with HPLC. HPLC chromatograms show the peaks of carotenoids extracted with DME from squash peel, squash pericarp, spinach leaves and carrot roots. Pigments eluted from Inertsil ODS-80A column were detected by monitoring the changes in absorption at 455 nm.

and its use as a "green" solvent, as opposed to conventional solvents, for the preparation and extraction of various plant pigments is highly encouraged from an environmental point of view.

Acknowledgements

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Performance of warm-season turfgrasses in an area of Central Italy

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Key words: Bermudagrass, ground cover, growing season, turf quality, weeds.

Abstract: Traditionally, in Italy, the C3 cool-season grasses have been the dominant species used for turfs, even though they do not appear to be the most suitable for the Mediterranean climate. However, recent limited water availability and the need to reduce energy inputs have placed drought tolerant warm-season turfgrasses under the spotlight. These species combine aesthetics with performance advantages in terms of water consumption, and with regard to the reduction of fertilizer and pesticides use. The present research was aimed to test the performance of warm-season turfgrass species (three cultivars of *Cynodon dactylon*, two cultivars of *Paspalum vaginatum* and two of *Zoysia japonica*) in a climatic transition zone in Tuscany, to evaluate their potential for use in this environment. The assessment of several parameters, which were estimated periodically, permitted performance evaluation of each species/cultivar, thereby enhancing the existing knowledge of these species and their potentiality in this environment. Results showed that the species with the best adaptation to the environment was *Cynodon dactylon*, which had higher performances compared to the other species. *Paspalum vaginatum* reported good quality in terms of color and density, but was damaged by low temperatures during winter. *Zoysia japonica* displayed a poor performance during the first year, but quality increased during the second year, yielding satisfactory results.

1. Introduction

Turfs and lawns are an important part of the landscape: they enhance its beauty (Geren *et al.*, 2009) and provide important ecological benefits (Beard, 1973; Linse *et al.*, 2001; Busey, 2003; Argenti and Ferrari, 2009) especially in areas modified by human intervention.

Recently, the need for larger spaces for individual, family environments and common areas for socialization has increased the concept that green spaces represent a higher standard of living in urban areas (Hull, 1990; Roberts, 1990). This awareness has increased interest in turf, as a surface capable of fulfilling technical, aesthetic and recreational goals, ensuring high quality standards, practicability and durability of the turf (Volterrani and Magni, 2007). In turn, this has led to the development of precise management practices (Gaetani *et al.*, 2013) through which to minimize the negative impact on the environment. The main problem connected to turfgrass is the amount of input necessary to obtain a high quality surface (Easton and Petrovic, 2005). The predominant challenge is the utilization of water for irrigation (Youngner *et al.*, 1981; Sevostianova and Leinauer, 2014). This aspect may reach critical levels especially in Mediterranean regions, characterized by low rainfall and hot summer temperatures. These climatic characteristics suggest the use of a low water rate and drought tolerant species such as warm-season turfgrasses (Marchione, 2008) that can provide high quality turf with suitable water consumption and low fertilizer and pesticide input (Turgeon, 2002; Schiavon *et al.*, 2013). The main objection to the use of these grasses is their lack of green color during the winter period, when they enter in dormancy and loose chlorophyll (Bernardini, 2007). This occurs especially in central Italy, an area characterized by high summer and cold winter temperatures, where cold tolerance and rapidity of recovery from winter dormancy are also important qualities (Magni *et al.*, 2014).

The objective of this investigation was to assess performance and adaptation, especially in relation to cold temperature, of three species (seven cultivars) of C4 warm-season turfgrass species and a cool-season grass as a comparison, in an internal area of the Tuscany Region.

2. Materials and Methods

The experimental site was located in a borderline area of Mediterranean climate in the centre of Tuscany, characterized by hot summer and low winter temperatures. The experimental trial started in the late spring of 2011 in Antria (Tuscany, central Italy) on natural soil, contain-

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ing 16.4% sand, 54.1% silt and 29.5% clay. The study was conducted over two growing seasons (2011 and 2012), starting after full establishment of the turf, up to the period of dormancy. Due to the presence of a meteorological station nearby, weather parameters were recorded in detail for the entire experimental period.

The species under comparison included *Cynodon dactylon* ('Black Jack', 'Casino Royale' and 'La Paloma'), *Paspalum vaginatum* ('Marina' and 'Sea Spray'), and *Zoysia japonica* ('Compadre' and 'Zenith') and a cool-season grass as control (*Lolium perenne* 'Kokomo'). The eight different accessions tested were replicated three times for a total of 24 plots (2x3 m with a surface area of 6 m^2) according to a complete randomized block design.

Sowing took place on the 10 June 2011. Seed rate was 25 g m^{-2}, as an average of the suggested dose for warm-season species (Panella *et al.*, 2000). Seeding was performed manually and followed by rolling. Cultural practices for the growing season included four or five applications of standard fertilizers (yearly total amount 318-85-81 kg ha^{-1} NPK). Irrigation was performed to restore 100% of crop evapotranspiration (ETc) and it was applied with a sprinkler system. Periodically, mowing was carried out with a mowing height of 30 mm performed by a rotary mower. A glyphosate treatment was performed during the dormancy period to reduce the presence of weeds (1 l ha^{-1}).

The following parameters were monitored during the trial and evaluated every two weeks (Pardini *et al.*, 2002; Reyneri and Bruno, 2008):

- *Aesthetic quality of the turf*: scores were visually assigned on a scale ranging from 1 to 9 (1= poorest quality, 9= highest quality) (Piano, 2005; Bigelow and Walker, 2008). Quality was also compared to additional parameters measured during the trial (color, ground cover and weeds) by multiple regression analysis to establish which aspects are the most related to quality;
- *Turf color*: scores were visually assigned on a scale ranging from 1 to 9 (1= light green, 9= dark green) (Bullitta *et al.*, 2005; Kir *et al.*, 2010);
- *Ground cover*: estimated visually as a percentage of soil cover;
- *Weed infestation*: estimated visually as percentage of soil cover.

All the data were grouped to obtain seasonal aggregation, where S1 was the first season of trials corresponding to summer 2011, S2 was autumn 2011, S3 spring 2012, S4 summer 2012 and S5 was the end of the study in autumn 2012.

The mean length of winter dormancy was estimated for each species, and plants were considered dormant when color scores means reached a value of 2.

Statistical analysis was carried out by means of ANOVA and Tukey test to discriminate differences among averages species values. Moreover, through multiple regression analysis, it was possible to evaluate which of the tested parameters (color, ground cover and presence of weeds) was strongly related to the quality on a global scale. All analysis were performed utilizing IBM SPSS Statistics software (release 20).

3. Results

Meteorological trends during experimental period

Both years were characterized by very hot and dry summer periods. In particular, 2011 was exceptionally dry until late autumn and had a total rainfall of 500 mm compared to the average of 800 mm (2002-2010). The highest recorded temperature occurred in August (38°C). During winter the coldest month was February 2012 with severe temperatures below 0°C (-9°C minimum recorded) and, even in full dormancy, warm-season grasses can be damage by the cold. Damage is also proportional to the duration of low temperatures. In 2012 there was a total rainfall of approximately 900 mm but it was concentrated at the end of the year. For this reason, during the summer, there was a severe water deficit replaced by irrigation. Spring was exceptionally warm, whereas the summer was one of the driest in recent decades.

Turf quality

Figure 1 shows the interaction between quality and season (S1= summer 2011, S5= autumn 2012) for the eight species or cultivars. The species that showed the best performance over time was *Cynodon dactylon*, with good values and a constant quality trend. All three cultivars belonging to this species performed well: in particular, 'Black Jack', obtained higher scores at the beginning of the trial. The highest value (7.1) was attained during summer 2012 (S4), even though the presence of thatch probably influenced the estimation. *Paspalum vaginatum*, after a good start and an improvement in autumn, proved to be the most sensitive species to low winter temperatures, having slower vegetative growth during the spring 2012 (S3) and the worst score, due to damage that occurred during winter. 'Sea Spray' showed better results in comparison to 'Marina'. *Zoysia japonica* performed badly during the year of establishment until the second growing season. Thereafter,

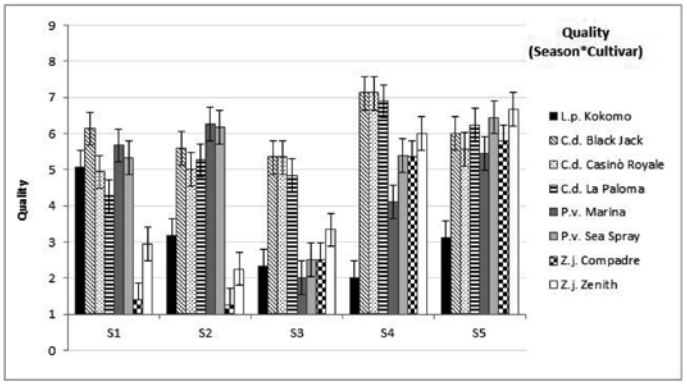

Fig. 1 - Global quality of the species/cultivars under investigation during the trial period (assessment with 1-9 scale). Bars = standard error. L.p. = Lolium perenne, C.d. = *Cynodon dactylon*, P.v. = Paspalum vaginatum, Z.j. = *Zoysia japonica*.

it gradually improved and gave very good scores at the end of the second year. 'Zenith' performed better than 'Compadre' on an overall scale. *Lolium perenne* was the most unsuitable species, showing a rapid initial establishment, after which the quality began to decrease from S2, attaining the worst score (2) in S4.

Color

The best performance regarding color was obtained with *Lolium perenne*: it was clearly superior with a darker and brighter color in each period (Fig. 2). Only *P. vaginatum*, in S3, showed equivalent scores and it resulted to be the species with the best color among the tested warm-season grasses. *Cynodon dactylon* gave unsatisfactory results, showing a decrease in quality in both years (in late summer and in the beginning of autumn), due to a rapid entrance into dormancy. Moreover, the light green color, typical of these seeded cultivars of *Cynodon* and the high production of thatch, influenced the scores. The trend of the two cultivars of *Zoysia japonica* appears to be very similar to that of *Cynodon*.

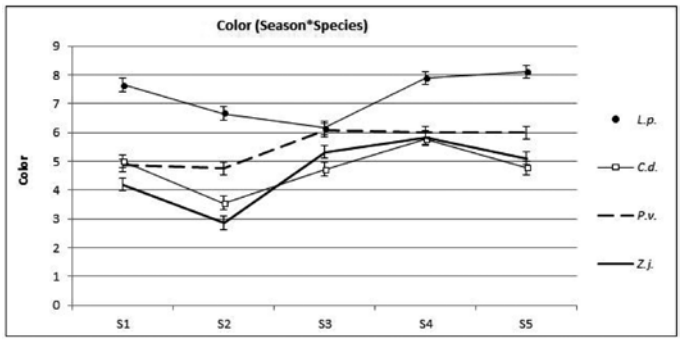

Fig. 2 - Average color of the species under investigation during the trial period (assessment with 1-9 scale). Bars = standard error. L.p. = Lolium perenne, C.d. = *Cynodon dactylon*, P.v. = Paspalum vaginatum, Z.j. = *Zoysia japonica*.

Ground cover and weed infestation

Table 1 shows the average ground cover values for each species during the different seasons of the trial. It is clear that the warm-season grasses increased the soil cover over time, while the percentage of *L. perenne* remained almost unchanged compared to the initial one (approximately 55-60%). *P. vaginatum*, due to the winter cold, had a poor cover percentage in the spring of the second year but it was able to regenerate an efficient soil cover thanks to its strong stolons, and this trend is evident for both cultivars. *C. dactylon* demonstrated good and constant soil coverage and it was more satisfactory than other species, whereas *Z. japonica* displayed the typical behavior of this species, attaining optimal values at the end of the growing season of the second year (S5).

As regards the presence of weeds (Table 1), infestation spread rapidly during the first year on *L. perenne* and *Z. japonica*. The high presence of weeds on *Lolium* is also due to the fact that this species is characterized by a bunch type

Table 1 - Average value (±SE) of ground cover and infestation of the tested cultivars over the 5 seasons

Species/ cultivar	Ground cover (%)				
	S1	S2	S3	S4	S5
Lp	59.0±7.5 a	58.3±2.1 bcd	58.3±4.6 ab	51.7±4.8 d	61.7±2.5 c
Cd1	73.7±5.2 ab	86.3±5.1 a	76.7±8.8 a	92.8±1.5 a	95.0±0.1 a
Cd2	63.7±5.8 ab	81.3±4.4 a	78.3±2.2 a	92.2±1.1 a	94.4±0.6 a
Cd3	57.0±5.6 ab	79.6±4.2 ab	73.3±3.6 a	92.2±1.1 a	93.9±1.1 a
Pv1	67.0±3.2 ab	78.3±2.1 ab	40.0±6.3 b	58.1±9.3 cd	83.3±5.9 b
Pv2	61.7±2.6 ab	76.7±6.9 ab	42.5±14.4 b	71.9±12.3 bc	88.9±5.3 ab
Zj1	24.7±2.4 c	40.8±3.4 d	52.5±6.3 b	78.9±1.5 ab	87.2±2.0 ab
Zj2	39.7±7.6 bc	56.3±6.3 cd	60.0±3.8 ab	83.3±2.5 ab	89.4±0.6 ab
Species/ cultivar	Infestation (%)				
	S1	S2	S3	S4	S5
Lp	4.0±2.3 ab	16.7±4.0 bc	30.0±5.8 c	23.3±4.4 b	30.6±4.0 b
Cd1	0.3±0.3 a	5.8±0.8 ab	8.3±2.2 a	6.7±1.0 a	16.1±2.9 ab
Cd2	1.7±0.9 a	10.8±1.1 abc	8.3±2.2 a	9.4±2.8 a	18.9±5.3 ab
Cd3	1.7±1.2 a	5.4±1.1 a	7.5±2.5 a	9.4±4.4 a	13.9±5.6 a
Pv1	1.3±0.9 a	3.3±1.1 a	19.2±5.8 bc	14.7±6.8 b	20.0±3.3 ab
Pv2	1.0±1.0 a	4.2±0.4 a	15.0±2.5 ab	12.5±2.7 ab	19.4±7.2 ab
Zj1	4.3±1.3 ab	17.9±4.2 c	19.2±4.4 bc	11.7±3.5 ab	18.9±4.5 ab
Zj2	8.7±1.3 b	19.6±1.8 c	23.3±2.2 bc	13.9±2.4 ab	9.4±0.6 a

Lp= *Lolium perenne* Kokomo, Cd1= *Cynodon dactylon* Black Jack, Cd2= *C. dactylon* Casinò Royale, Cd3= *C. dactylon* La Paloma, Pv1= *Paspalum vaginatum* Marina, Pv2= *P. vaginatum* Sea Spray, Zj1= *Zoysia japonica* Compadre, Zj2= *Z. japonica* Zenith.
Values in a column with the same letter are not significantly different (P<0.05) according to Tukey test.

habitus that makes it less competitive with opportunistic weed species in comparison to warm-season grasses. In addition, *Lolium*, after being stressed by the high summer temperatures, was also the only species not treated with glyphosate, taking into account its vegetative activity during the winter. For *Zoysia*, the infestation was easily attributable to a slow establishment that left plenty of space for the development of invasive species at the beginning of the trial. Nevertheless, for all the species, despite the winter control with glyphosate, a manual control against weeds in the second year was necessary. After these operations, the presence of weeds was shown to be reduced, thanks to the ability of these highly-competitive species to suppress weed growth.

Multiple regression analysis

This model permits the calculation of turf quality value (considered the dependent variable) through the other observed parameters. The model applied (a stepwise model) involves a mechanism of removal of the independent variables provided that they do not cause a decline in the power of the model. The elimination of the variables from the model is performed automatically by comparison with a threshold value of significance, which in this case was set to be equal to 0.05.

From the analysis the following formula was obtained:

$$\text{Global quality} = -1.447 + 0.067 * \text{cover} - 0,050 * \text{weeds} + 0.443 * \text{color} \quad (R^2 = 0.884)$$

The parameters were ordered starting from the one most related to overall quality to the one with the lowest influence on the formula. No variable was removed from the model, demonstrating the importance of all investigated parameters in describing the aesthetic value of a turf. The presence of weeds, as expected, produced a negative value, as it was inversely related to the aesthetic appearance of the turf. The very high determination coefficient indicated the good correlation between the independent variables and the dependant variable (turf quality). This type of analysis could be used in future investigations to predict the overall performance of a turf.

Winter dormancy

Table 2 shows the dates at the beginning and the end of the growing season, and the length of vegetation and dormancy periods for the species during the trial years.

At the end of the first year of experimentation, dormancy took place in November for all the species, with differences of about two weeks between the earliest (*Zoysia*) and latest (*Paspalum*) entrance into dormancy.

The period of dormancy during winter 2011-2012 was particularly long for *Paspalum*, also because the growing season in the spring 2012 started at the end of April. *Cynodon* and *Zoysia* showed early vegetation activity at the end of March. The growing season for 2012 was longer for *Z. japonica* (236 days), due to its early vegetative growth and the entrance into dormancy in mid-November, which was exceeded only by *Paspalum vaginatum*. Nearly the same duration was found for *C. dactylon* (233 days), with greater precocity in vegetative growth, but with an earlier loss of green color than with other species. The shortest growing season, as expected, was found in *P. vaginatum* (208 days), despite its late entrance into dormancy.

The period of dormancy during winter 2012-2013 showed a substantial analogy to the previous year for *Zoysia* (137 days in 2011/2012 and 142 in 2012/2013) and *Paspalum* (151 days compared to 159) but a considerable lengthening for *Cynodon* (124 days compare to 160 in the second year). This behavior could be connected to the fact that mean and maximum spring temperatures in 2013 were lower compared with the previous year. This trend definitely affected the vegetative growth of the earliest species (*Cynodon*), highlighting the important role temperatures play in the vegetative activity of warm-season grasses.

4. Discussion and Conclusions

In this study, *Cynodon dactylon* emerged as the species with the best overall behavior and the best potential adaptation to the environment. It displayed good quality standards, rapid establishment, good overall appearance, high ground cover and a competitive behavior against weed

Table 2 - Dates of the beginning and the end of the growing season and length of vegetation and dormancy periods for the species during the years of trial

Species	Beginning	End	Vegetation (days)	Dormancy (days)
Cynodon dactylon		16/11/2011		124
Paspalum vaginatum		26/11/2011		151
Zoysia japonica		08/11/2011		137
Cynodon dactylon	20/03/2012	08/11/2012	233	
Paspalum vaginatum	26/04/2012	20/11/2012	208	
Zoysia japonica	25/03/2012	16/11/2012	236	
Cynodon dactylon	17/04/2013			160
Paspalum vaginatum	28/04/2013			159
Zoysia japonica	07/04/2013			142

infestation. The constancy of studied parameters along experimental trial are consistent with Volterrani *et al.* (1997). The worst performance concerned color of this species that probably resulted remarkably affected by presence of thatch, confirming the observations of Holm *et al.* (1991).

Paspalum vaginatum presented an overall satisfactory appearance. However, the species was highly susceptible to cold winters, in agreement with De Luca *et al.* (2008), and Tesi (2012) reported cold damages on *Paspalum* with temperature around -7°C. Anyway this species performed in a very good manner concerning other variables, as it displayed a dark green-blue color, as well as a good color retention during autumn, as reported previously by Volterrani *et al.* (1996) and Geren *et al.* (2009). Given that the stability of the turf was compromised, this behavior establishes a limit to the use of this species in areas that border the Mediterranean climate or with severe risks of low temperatures during winter.

Zoysia japonica exhibited a typical growing pattern, confirming that observed by Patton and Reicher (2007), and it showed very poor performance in the first year, slow development and not competitive attitude against weeds. Similar results regarding this species were found by Geren *et al.* (2009) in a comparative study among warm-season grasses in Turkey. On the other hand, starting from the beginning of the second year, a gradual increase in quality, color and coverage was observed, and the species achieved remarkable results at the end of second growing season, in agreement with Sladek *et al.*, (2011).

Through multiple regression analysis it was possible to evaluate which of the tested parameters influenced turf quality the most. The results showed that the ground cover and the presence of weeds are strongly related with the overall aesthetic quality.

The winter season revealed important differences in relation to the length of the period of dormancy among species. *P. vaginatum* had the longest period of dormancy (about 155 days as the average of the two years of experimentation). A delay in the vegetative activity in *Paspalum*, in comparison to other warm-season grasses, was also reported by other authors in different climatic areas (Duncan, 1996; Miele *et al.*, 2000; Volterrani *et al.*, 2001), as well as in both coastal and internal areas of Tuscany (Volterrani *et al.*, 2000; Pardini *et al.*, 2002) and in the province of Rome (Croce *et al.*, 2001). *C. dactylon* showed a rapid recovery from winter dormancy in warm springs, while a longer length of dormancy was observed during the cold spring 2013. *Z. japonica* remained dormant for approximately 140 days during both years of the experiment.

In general, in the interior zones of this study, the duration of dormancy was found to be longer than those verified by other experiments along the coastal areas of central Italy (Croce *et al.*, 2004). For this reason, in this environment, it is necessary to plan and analyze costs and benefits associated with the use of warm-season grasses. Where it is not possible to accept the absence of green color during winter, it is evident that the use of warm-season species can be extended to these areas only by adopting appropriate overseeding programs, but these interventions seem possible only in high standard turf such as those related to sport pitches.

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