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TESI PER IL CONSEGUIMENTO DEL TITOLO DI DOTTORE DI RICERCA:

WATER CONTROL IN CUT STEMS OF ROSE AND CARNATION

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FOREWORD

In cut flowers, water homeostasis involves the simultaneous activation of different physiological mechanisms including responses to physical stress (cutting), the induction of senescence processes, the activation of strategies aimed at maintaining cell hydration and turgor (Dodge et al., 1998), which will overall determine the commercial quality of cut flowers.

The maintenance of post harvest turgor is a critical aspect of cut flowers shelf life, this must be "controlled" in a system in which transpiration water losses, through the leaf surface, must be compensated by a water uptake and transport occurring in the absence the root system, an essential organ for these functions (Singh e Moore, 1992). From a physiological-functional perspective, plant stress adaptation is mediated by pre-existing and stress-induced adaptation mechanisms (Hasegawa et al., 2000). When a whole plant is exposed to water shortage, pre-existing and induced defense mechanisms may be equally important for adaptation. In contrast, when an entire organ or part of it is removed from the plant (i.e. root system), it is conceivable that pre-existing stress control mechanisms may be important as well as the induced one as far as they effectively last over time. Loss and recovery of cellular turgor typically occur as a consequence of water stress and adaptation, upon which plants must control water transport and tissues hydration. Respect to those mechanisms that may improve water uptake in response to a stress, hydraulic conductivity variations and accumulation of compatible solutes are the most documented (Ehlert et al., 2009; Maggio & Joly, 2000; Chen & Murata, 2002; Delauney & Verma; 1993). Compatible solutes are organic molecules generally produced to reduce the cellular water potential and to facilitate water uptake under water shortage and hyperosmotic stress (Rhodes & Hanson, 1993; Iriti et al., 2009). Many plants accumulate organic osmolytes in response to

environmental stresses as a mechanism to overcome the negative consequences of water deficit (Serraj & Sinclair, 2002). This mechanism is called “osmotic adjustment” or “osmoregulation”.

These compounds have been constitutively overproduced via genetic engineering to improve plant stress tolerance or directly fed to control plant stress response in both field and protected cultivation (Zhang et al., 2004). Thus we hypothesized that exogenous applications of compatible solutes as proline and glycinebetaine may improve water absorption and cellular turgor of cut stems.

In addition to physiological mechanisms that can be exploited to improve water uptake, tissue water conservation can also be controlled. The reduction of transpirational flux has traditionally been one of the main objectives for controlling the cellular turgor after vegetables and ornamental harvest. Even though it is not yet clear if an extension of the vase life of cut flowers may be more related to a sustained ability of the cut stem to maintain high water fluxes or to control its hydration state, the control of the stomatal conductance is a fundamental determinant of the overall tissue water balance (Fuchs & Livingston, 1996, Woodward et al., 2002). Generally antitranspirants are made of natural emulsions that, diluted in water and sprayed on the plant tissue can make a film able to reduce leaf transpiration (Nitzsche et al., 1991; Iriti et al., 2009). Alternatively to traditional anti-transpirants that mechanically act on the transpiration (direct effect), the effect of compounds that may act on stomata closure through the induction of functions associated to the synthesis of abscissic acid (indirect effect) could also be considered. Applications of ß-aminobutyric acid (BABA) may increase the tolerance to abiotic stress with positive effects against pathologies, by activation of defence mechanisms mediated by abscissic acid (ABA) and salicilic acid (SA) (Zimmerli et al., 2000).

During the cut flowers shelf life and in general during the ripening of climacteric fruits,

the influence and the effects of endogenous ethylene have been thoroughly studied (Have & Woltering, 1997). Moreover, the presence of exogenous ethylene in the cut flowers storage environment can affect, even at very low concentrations (0.1-1 ppm), the cut stems longevity and quality (Abeles et al., 1992; Reid, 1995; Dodge et al., 1998). The influence of senescence process on cell turgor and water status of cut stems can be evaluated using anti-ethylene compounds. Artificial senescence is induced as a consequence of detachment (wounding), together with the other stresses involved, including water and nutrient deficiency (Ella et al., 2003). Vase life can therefore be potentially regulated through the use of compounds that inhibit ethylene action and delay senescence processes. Objectives of this work were to:

1) Assess cut stems water balance and its variation during post-harvest storage;

2) Evaluate the effect of pre-harvest treatments with osmoprotectants (L-Proline and Glycinebetaine) on cut stems and quantify in terms of water relations (water potentials, leaf gas exchanges and hydraulic conductivity) and vase life (longevity and post-harvest quality);

3) Manage cut stems water use and transpiration rate through the application of antitranspirants with direct effect (Pine resine) and β-aminobutyric acid (BABA), which acts indirectly on stomatal closure improving biochemical signals via abscisic acid (ABA);

4) Evaluate the effect of senescence delay, induced by treatments with anti-ethylene compounds (1-MCP), on water relations and vase life of cut flowers. Furthermore the inclusion of 1-MCP in nanosponges, for a gradual release of the active principle, was investigated.

ABSTRACT

This PhD Thesis address, on cut flowers vase life, physiological mechanisms aimed at maintaining and regulating tissues hydration, cellular turgor, water use and senescence phenomena. Specifically we considered the effect of compatible solutes (osmoregulation) on water status, hydration state and vase life of cut stems of rose and carnation (chapter 1). In the second chapter we evaluated the application of anti-transpirant compounds that may act indirectly or directly on rose cut stems transpiration, in order to control the water use and longevity during the vase life. Finally (Chapter 3) we assessed how anti ethylene compounds may delay senescence phenomena of carnation cut stems, with the purpose to evaluate this effect in terms of on water balance, hydration and longevity during the vase life.

RIASSUNTO

Il presente elaborato analizza, nel corso della *vase life* dei fiori recisi, meccanismi fisiologici finalizzati al mantenimento e alla regolazione dell'idratazione dei tessuti vegetali, del turgore cellulare, dei consumi idrici e dei fenomeni di senescenza.

Nello specifico è stata valutata l'azione di soluti compatibili (osmoregolazione) su stato idrico, condizioni di idratazione e longevità di steli recisi di rosa e garofano (Capitolo 1). Nel secondo capitolo è stato analizzato l'effetto di molecole ad azione diretta o indiretta sul flusso traspirativo di steli recisi di rosa, con l'obiettivo di controllarne i consumi idrici e la longevità nel corso della *vase life*. Infine (Capitolo 3) è stato studiato come formulati ad azione anti etilenica potessero ritardare i fenomeni di senescenza di steli recisi di garofano, in modo da stimarne l'effetto su bilancio idrico, idratazione e longevità degli steli.

CHAPTER 1

OSMOREGULATION AND VASE LIFE OF ROSE AND CARNATION CUT FLOWERS

1.1. Introduction

Accumulation of compatible solutes, usually called 'osmotic adjustment' or 'osmoregulation' is a mechanism that plants have developed to overcoming abiotic stresses that cause inevitably depletion of cellular water (drought, salinity and temperature extremes) (Hare et al., 1998; Serraj and Sinclair, 2002; Maggio et. al., 2002; Kavi Kishor et al., 2005;).

Osmoregulation induces a decrease of the cell osmotic potential in order to improve and preserve water absorption and cellular turgor pressure which may contribute to support physiological mechanisms such as stomatal opening, photosynthesis, and growth (Ludlow & Muchow 1990; Blum, 1996). In response to NaCl stress, cellular accumulation of some inorganic ions such as sodium (Na+) and compatible organic solutes like proline, glycinebetaine, and soluble sugars have been documented (Flowers & Colmer 2008).

Compatible solutes can accumulate to high levels without disturbing intracellular biochemistry (Bohnert & Jensen 1996), protecting membrane functionality and protein structure (Crowe et al., 1992; Zhao et al., 2007), contrasting oxidative damage by scavenging free radicals (Rhodes & Hanson 1993). However their role includes mainly protection against the deleterious effects of the low water activity with appropriate cell volume maintenance (Csonka & Hanson, 1991). Increased levels of compatible solutes (or osmoprotectans) were obtained through genetic engineering (Maggio et al., 2003;

Sakamoto & Murata 2000) or exogenous applications on vegetable and herbaceous plants with the aim of improving their performance in the presence of stress (Heuer, 2003). Proline and glycinebetaine are the most representative compatible solutes involved in osmotic adjustment and their major proportion is located in the chloroplast (Hanson et al., 1985). Most molecules likely accumulate in the cytoplasm and contribute to cellular osmotic adjustment (Yoshiba et al., 1997) and it is largely demonstrated that presence of high concentrations of proline and/or glycinebetaine gives better protection against the biologically unfavorable consequences of dehydration (Hamilton & Heckathorn, 2001). In many cases, exogenous applications of L-Proline or Glycinebetaine enhanced drought/salt stress tolerance in different species (Okuma et. al., 2004; Demiral & Turkan, 2004; Makela et.al., 1999).

While the application of these molecules has been shown to improve yield of salt stressed crops, it is rather unknown its effect on cut stems to preserve water homeostasis and a positive water balance in post harvest. Once the flower is harvested the physiological and biochemical processes are altered by the water and nutrient shortage and the hormonal imbalance induced by the cut (wounding). In this work it has been hypothesized that exogenous applications of L-Proline and glycinebetaine could improve the capacity of water absorption and cellular turgor of cut stems of rose and carnation (two species of commercial importance in the Italian floriculture) during their vase life. It was also evaluated the possible influence of the improved water status on cut stems vase life.

1.2 Materials and methods

Two experiments were performed on Rose plants cv. *Lovely Red* and on Carnation plants cv. *Special white*. Rose plants were hydroponically grown in an heated greenhouse of the "Consorzio Per lo Sviluppo della Floricoltura nel Meridione" (Con.Flo.Mer) located in Ponticelli (NA). Carnation plants were grown in an unheated greenhouse of the farm "Bruno Antonio", Torre Del Greco (NA). Cultivation was carefully monitored during growth. Lab measurements were conducted at the Chemical and Terminological Laboratory of the "Department of Agricultural Engineering and Territorial Agronomy", Faculty of Agriculture, Portici (NA).

1.2.1 Treatments

Rose plants were treated with a solution of 10 mM L^{-1} L-Proline in distilled water (200 ml/plant). Control plants were treated with distilled water only. Otherwise carnation plants were treated with a solution of 7 g L^{-1} *Biohelp* (Biolchim, 97 % Glycinebetaine). On control plants was applied distilled water only.

In both experiments, two applications of these solutions were administered to the cultivation substrate in the 2 days before harvest and at the end of the last daily irrigation to avoid washout of these compounds.

1.2.2 Lab measurements and storage

In both experiments, cut stems were immediately transferred, after the harvest, to the laboratory, re-cut at the base (2-3 cm) and placed in graduated glass cylinders with distilled water and sodium hypochlorite (50 mg L^{-1}). Part of the stems, weighted and sized (length and diameters of the stems), was placed on 10 precision balances connected via USB to a computer for automatic detection of weight through a specific

software (*RS-com*®). These cylinders were sealed with *parafilm* to avoid water losses by evaporation. *RS-com*® software was set to record 3 daily weights in order to determine stems water consumptions over the storage days. Cut stems of rose and carnation were stored for 12 days measuring daily mean temperatures and Relative Humidity using a thermo-hygrometer (Delta OHM DO 9847K). At storage days 2, 4 and 6, water flux measurements were made, by using a pressure chamber (Scholander chamber): 20 cm stem segments (5 cm below the corolla after measuring stems diameter) were immersed into a cylinder containing water, placed in the pressure chamber, while the other extremity was connected to Falcon tubes to collect and weight the water efflux (Fig. 1). The system was then subjected to increasing pressure (P = 0.05, 0.1, 0.2, 0.3 MPa). The pressure inside the chamber was gradually increased and maintained at each value for 5 minutes up to a constant outflow from the stem.

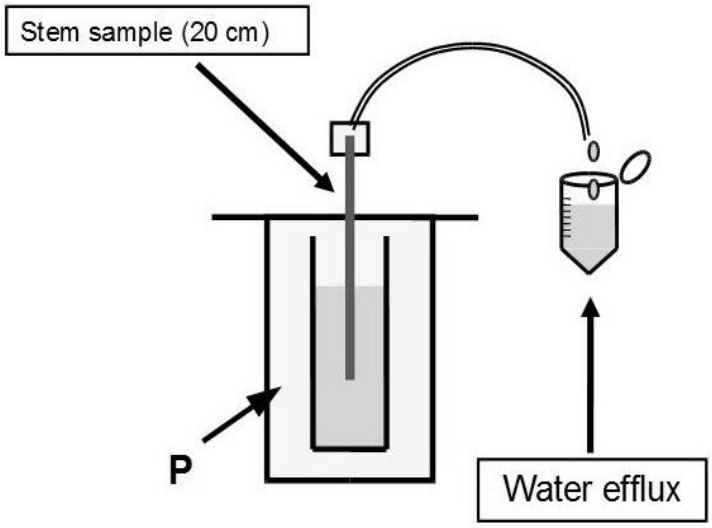


Figure 1. Scholander pressure system

Water flux (Jv) was expressed as Jv = kg H_2O m^{-2} s^{-1} m^{-1}. Water conductivity (Lp) of stems was then expressed by the slope of the regression function of Jv vs. P (figure 2). Volumes of collected efflux per unit of time (Jv) were normalized to the cutting section surface.

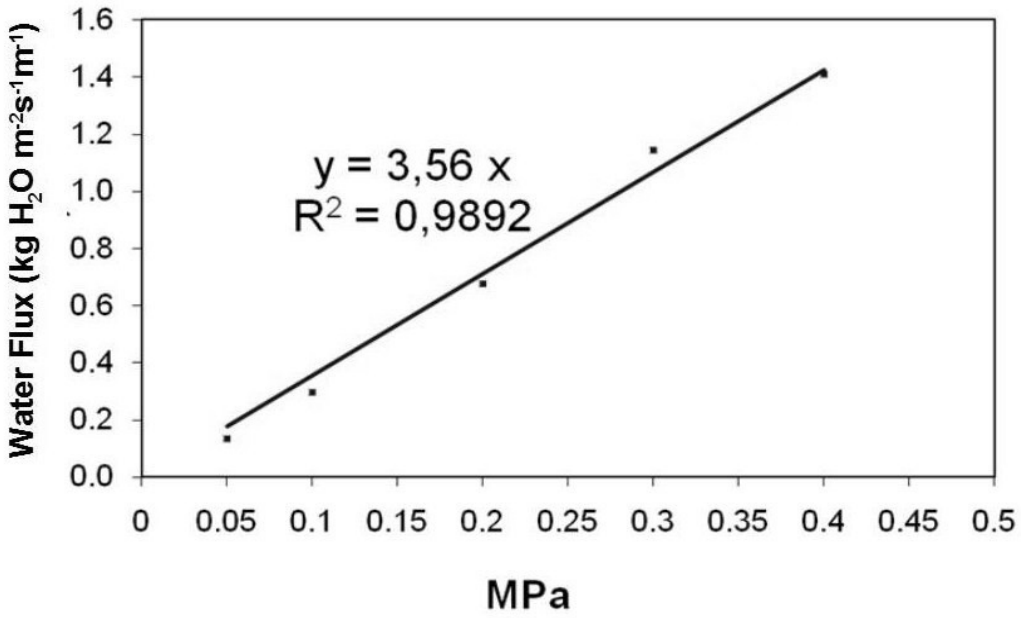


Figure 2. Water Conductivity (Lp) of stems: Relation between Water Flux and applied hydrostatic pressure

Stomatal conductance (*gs*) was determined at storage days 2, 4 6 using a diffusion porometer (delta P-4, Delta-T Devices, Cambridge) in 3 daily measurements (h 9:00 a.m; h 1:00 p.m; h 7:00 p.m.). Xylematic potential was obtained using the Scholander Chamber (days 2, 4 and 6) on young leaves increasing pressure level until vascular leakage from cut petioles was reached (Cochard et. al., 2000). Relative Water Content value was calculated as: RWC = (leaf fresh weight - leaf dry weight)/(leaf saturated weight – leaf dry weight) (Morgan, 1984). Leaf area was estimated by scanning cut stems leaves and using the *Image J*® software (Abramoff et al., 2004) for image processing. The cut stems vase life was assessed visually using a "quality score" from 4 to 0.

1.3 Results and discussion

Exogenous applications of L-Proline enhanced water fluxes of rose cut stems respect to the control, for all the 3 days of measurements (fig.3). During storage, water flux decreased from day 2 to day 6 in treated stems (fig.3). Moreover, stems water conductivity (Lp) was 3.05 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in control and 3.55 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in L-Proline treatment.

Increased stomatal conductance (gs) and RWC were observed in L-Proline treatment compared to control (tab.1). Consistent with this physiological status, water consumptions normalized per leaf area were higher in treated stems (fig.4). As a result of cellular osmotic adjustment due to L-Proline application, water potential was lower for treated stems compared to control (tab.1). The improved water status of L-Proline treated stems influenced positively cut stems longevity extending the vase life by 2 days compared to the untreated control (fig.5).

Even for glycinebetaine treatment on carnation plants, water fluxes were higher compared to the control (fig.6). The water flux trend decreased during storage (fig.6).

In this experiment stems water conductivity (Lp) was 1.01 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in control and 1.42 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] upon glycinebetaine treatment. Glycinebetaine applications increased the stomatal conductance (gs) and RWC of stems compared to control (tab.2) leading to higher water consumptions, in treated flowers (fig.7). Effects on water potentials were not significant (tab.2) even considering that osmoregulation improved the cut stems hydration state (improved gs, Lp, RWC, Water Consumptions) . The shelf life was not extended by glycinebetaine treatment (12,5 days vs. 12 days in control stems) (fig.8).

The osmotic adjustment contributes to maintain water uptake and cellular turgor, which

are essential to sustain physiological processes such as cell expansion, stomatal opening, photosynthesis, and many others plant functions (Zhang et al., 1999, Maggio et al., 2002a). One of the first plant responses to abiotic stress (as for cutting) is the stomatal closure. This mechanism provides protection against tissues dehydration by reducing transpiration from the leaf surface (Hare et al., 1998).

Upon L-Proline treatment, gs increased (tab.1). This was associated with a significant decrease of water potential (tab.1).

It has been suggested that proline, exogenously applied via foliar spraying or through the irrigation water, should localize into the cytoplasm to reduce the cellular osmotic potential and to restore cellular hydration (Gadallah, 1999). L-Proline treatment on rose plants has shown a to substantially improve water homeostasis of the cut stems. This was correlated with increased stomatal conductance, water conductivity and leaf RWC (tab.1). During the vase life, water fluxes were higher for L-Proline treated stems (fig.3), which confirmed the improved water status of the vegetal tissues. Osmotic adjustment is a fundamental adaptation mechanism in response to abiotic stress to guarantee cellular turgor and water uptake, (Zhang et al., 1999, Hasegawa et al., 2000) and sustain stomatal opening (Chaerle et al., 2005;Yancey, 1982, Stoop et al., 1996)., Water consumption per unit of leaf area, daily and cumulated, were higher in samples treated with L-Proline compared to control (fig.4). These results confirmed the measured water relation parameters. During storage, water potential of cut flowers declines with time as well as water uptake, water loss and water conductivity. The decline in stem tissues water conductivity is one of the main reasons for impaired water balance. Consequently, water stress is the most common reason for reduced cut flowers vase life (Halevy, 1976; Joyce & Jones, 1992). In rose cut stems, L-Proline treatment extended flowers vase life by 2 days compared to the control probably as a result of an

improved water status (fig.5).

Glycinebetaine (GB) is considered one of the most effective compatible solutes involved in osmotic adjustment (Siqueira Boniolo et al., 2009; Papageorgiou et al. 1991, Rhodes & Hanson 1993). Many studies indicate that GB might play an important role in enhancing plant tolerance to drought and heat stress (Khan et al., 2009). In plants under drought and salinity, exogenously applied Glycinebetaine has led to enhanced relative water content (RWC), improved activity of some antioxidant enzymes, higher photosynthetic rate and stomatal conductance (Makela et. al., 2000; Demiral & Turkan, 2004). Treatments with Biohelp (97% glycinebetaine) on carnation plants increased gs and RWC (tab.2) compared to untreated stems. Moreover, a significantly higher water consumption per unit of leaf area, in the treated stems (fig.7) was consistent with an increased stomatal opening (tab.2). Also in this experiment, Glycinebetaine treatment has led to improved water fluxes (fig.6) and Lp (1.42 vs. 1.01 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}]) respect to the control. The water potential during storage was not affected by the treatment (tab.2), despite the enhanced water balance of the stems. Substantial improvement in the water homeostasis of cut stems in carnation was not accompanied by a significant prolongation of the vase life (12,5 vs. 12 days in control stems) (fig.8) suggesting that , in this floral species, additional factors such as hormonal control (in particular ethylene) may affect post-harvest senescence (Have & Woltering, 1997).

1.4 Tables and Figures

Table 1.

Results of the ANOVA. Effect of exogenous L-Proline on Stomatal conductance (gs), Relative Water Content (RWC) and Water Potential (Ψ) of rose cut stems.

Treatment	Stomatal conductance ($cm\ s^{-1}$)	RWC (%)	Ψ (MPa)
L-Proline	0,42	89	-0,34
H_2O	0,38	84	-0,17
Significance	*	*	*

** significant at $P \leq 0.05$*

Table 2.

Results of the ANOVA. Effect of exogenous glycinebataine on Stomatal conductance (gs), Relative Water Content (RWC) and Water Potential (Ψ) of carnation cut stems.

Treatment	Stomatal Conductance ($cm\ s^{-1}$)	RWC (%)	Ψ (MPa)
Glycinebetaine	0,20	99	-0,48
H_2O	0,17	84	-0,45
Significance	*	*	ns

*ns, not significant; * significant at $P \leq 0.05$*

Figure 3.

Effect of exogenous L-Proline on water flux of rose cut stems during storage.

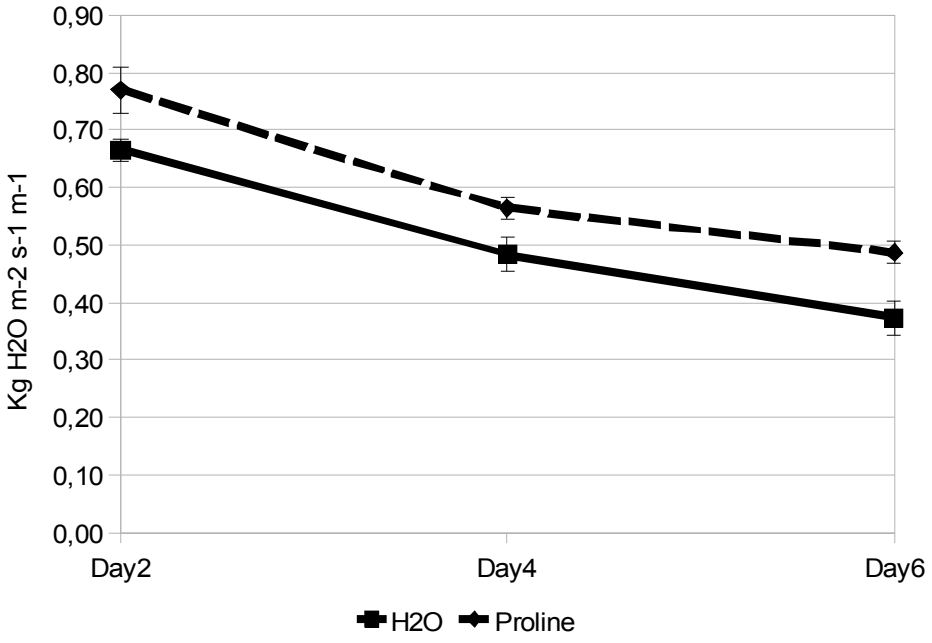


Figure 4.

Effect of exogenous L-Proline on water consumptions of rose cut stems during storage.

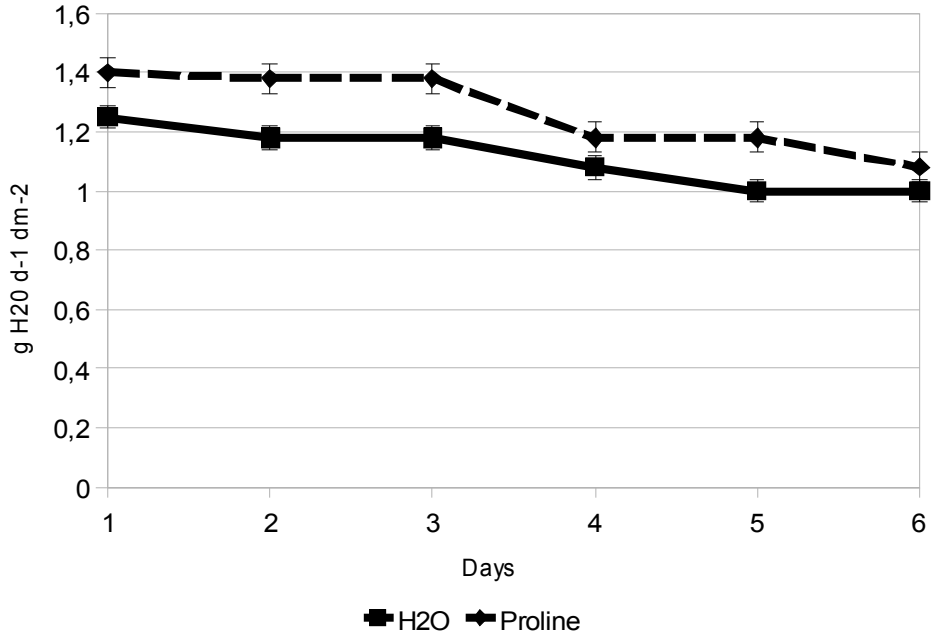


Figure 5.

Effect of exogenous L-Proline on the vase life of rose cut stems during storage.

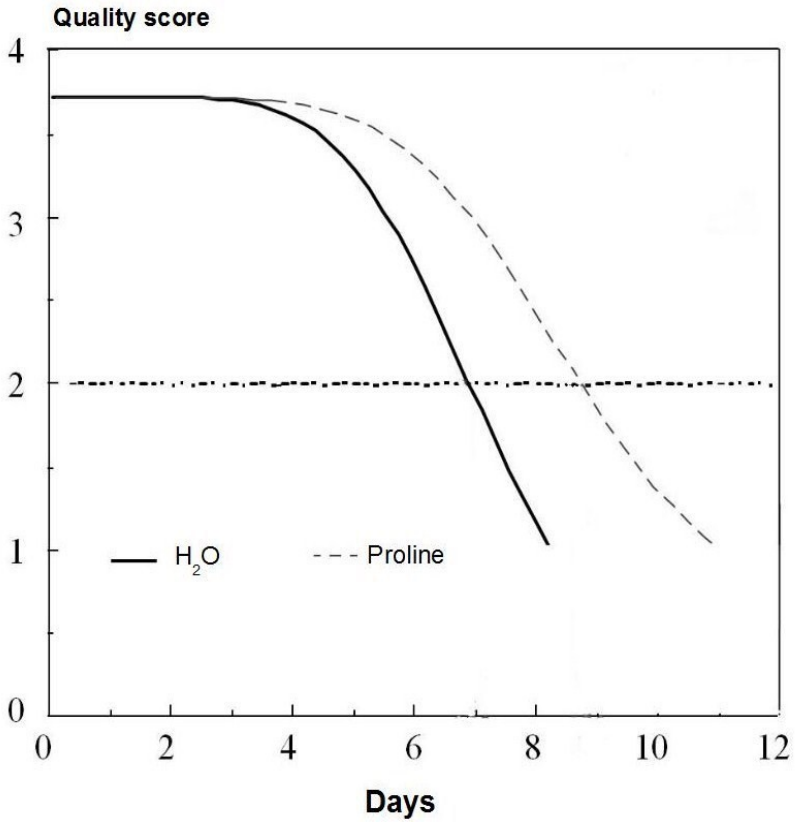


Figure 6.

Effect of exogenous glycinebetaine on water flux of carnation cut stems during storage.

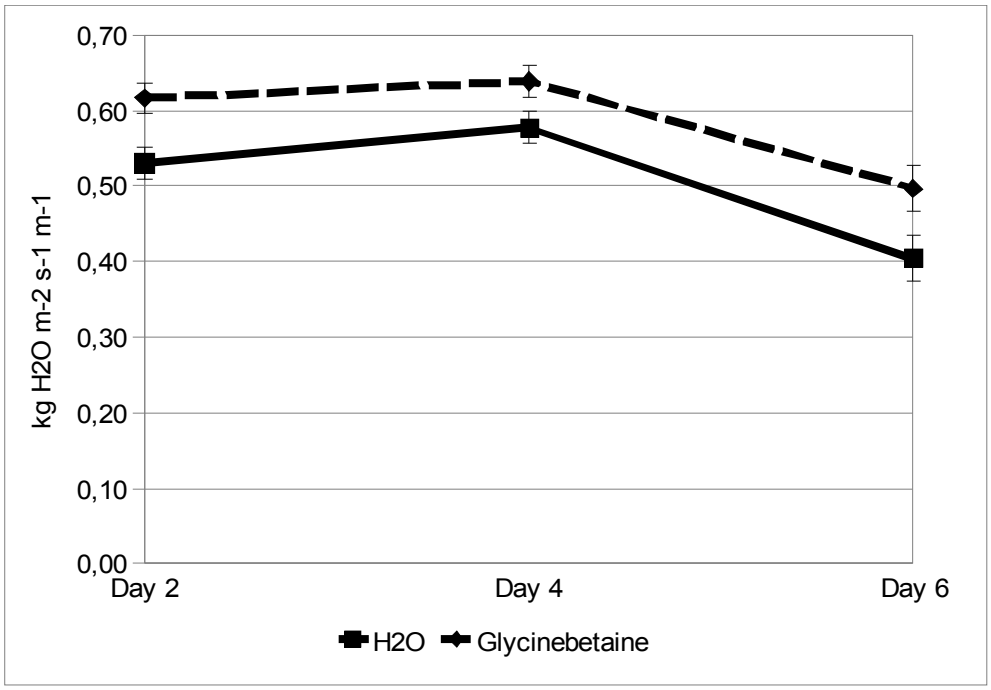


Figure 7.

Effect of exogenous glycinebetaine on water consumptions of carnation cut stems during storage.

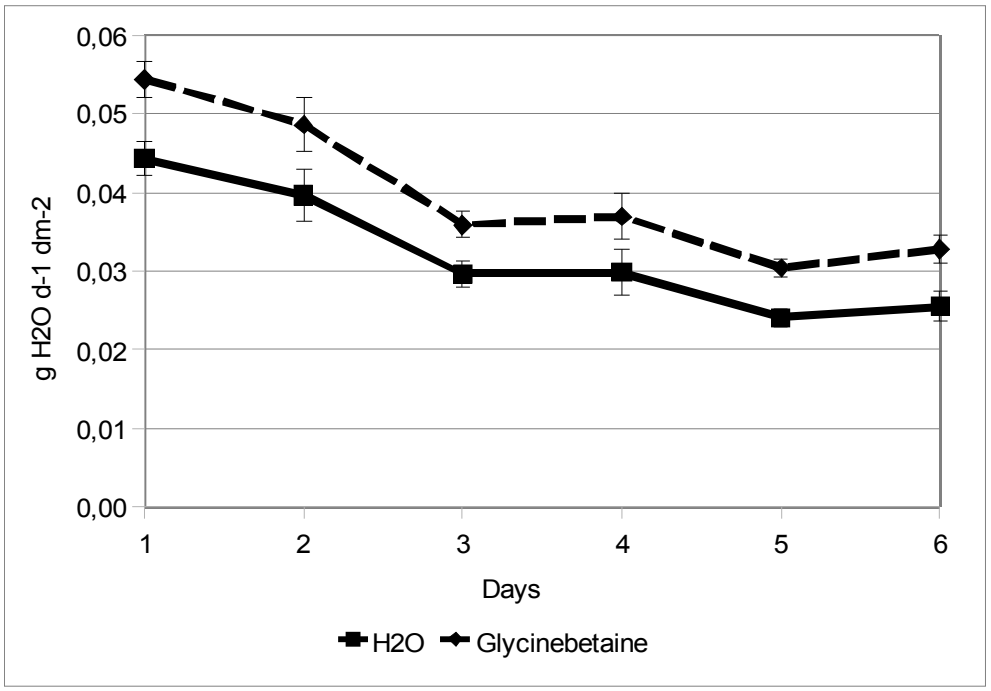
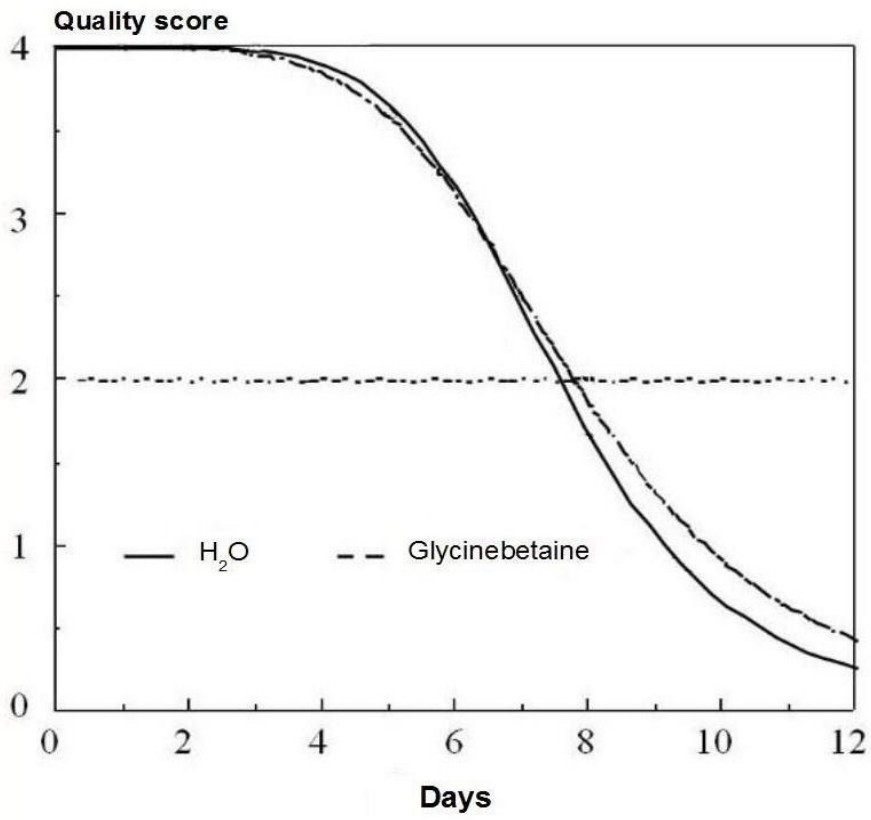


Figure 8.

Effect of exogenous glycinebetaine on the vase life of carnation cut stems during storage.



CHAPTER 2

TRANSPIRATION RATE CONTROL AND VASE LIFE OF ROSE CUT FLOWERS

2.1 Introduction

In nature it is well known that plants control water losses by regulating transpiration in response to environmental factors. In some respect cut flowers respond to the same stimuli and the difference between the rate of water uptake and the transpiration rate is one of the parameters that will define their hydration state The control of water use is strictly related to variation of flux resistance mainly controlled by the stomatal resistance (De Pascale & Viggiani, 1998). Indeed stomata aperture is one of the main factors influencing water loss by transpiration (Chaerle et al., 2005). An improved abscissic acid (ABA) level may determine an increase in the level of cytoplasmic Ca++ and a subsequent activation of the membrane ion channels, depolarization of the guard cells and efflux of K+ ions (Hamilton et al., 2000; Zhang et al., 2001 Schroeder et al., 2001). This causes a cell volume decrease, turgor loss and finally leads to stomatal closure (Pospísilová et al., 2005). Applications of ß-aminobutyric acid (BABA) may increase the tolerance to drought and salt stress through the induction of functions associated to the synthesis of ABA, such as stomatal closure. A pre-treatment with BABA (300 μM) in Arabidopsis exposed to stress, has been shown to have beneficial effects on plants (Jakab et al, 2005). ß-aminobutyric acid (BABA) is a non-protein amino acid and is known as a potent inducer of resistance against infection by many pathogens (Jakab et al., 2001; Pajot et. al., 2001; Baider & Cohen, 2003). In different cases BABA acts through potentiation of ABA-dependent signaling pathways (Ton &

Mauch Mani, 2004). Conceivably, BABA may have indirect effects on transpiration trough an induced stomatal closure mediated by ABA.

Alternatively, a direct effect on the transpiration rate can be obtained with antitranspirant compounds that physically affect transpiration. Generally, antitranspirants are made of natural emulsions (eg. pine resine: poly-1-p-menthene) that, diluted in water and sprayed on the plant tissue can make a film able to reduce leaf transpiration (Nitzsche et al., 1991). These polymers, also called "Film Forming antitranspirants", sprayed on crops have the main function to reduce weathering and extend pesticide efficacy, improving distribution and adherence of agrochemicals, and decrease water loss and wilting of young transplants (Gale & Hagan, 1996; Percival & Boyle, 2009). In this experiment, it was evaluated the effect of exogenous applications of ß-aminobutyric acid (BABA) and Pine resine (Pinolene) on rose cut flowers transpiration rates. It was hypothesized that the two compounds may act indirectly and directly on cut stems transpiration in order to control the water use. Their effects were evaluated on the water balance and the vase/shelf life of Rose cut flowers.

2.2 Materials and Methods

Two experiments were carried out on Rose plants cv. Lovely Red hydroponically grown in an heated greenhouse at the "Consorzio Per lo Sviluppo della Floricoltura nel Meridione" (Con.Flo.Mer) located in Ponticelli (NA). All the lab measurements were performed at the Chemical and Terminological Laboratory of the "Department of Agricultural Engineering and Territorial Agronomy", Faculty of Agriculture, Portici (NA).

2.2.1 Treatments

In pre-harvest, rose plants were treated with a solution of 0,5 mM L^{-1} of ß-aminobutyric acid (BABA) in distilled water (200 ml/plant). On control plants it was applied distilled water only. The solution was given twice to the cultivation substrate: 2 days before harvest and at the end of the last daily irrigation.

In the second experiment, in post harvest, rose cut stems were sprayed with a solution of 50 g L^{-1} of Pinolene (NU-film- Intrachem bio, 96% poly-1-p-menthene) in distilled water. Control stems were sprayed with distilled water only. The application was done after transferring the stems to the laboratory.

2.2.2 Lab measurements and vase life

Upon harvest, cut stems were immediately transferred to the laboratory, re-cut at the base and placed in glass cylinders containing distilled water and sodium hypochlorite (50 mg L^{-1}). Rose cut stems, weighted and sized, were placed in glass cylinders on 10 precision balances for the daily weights detections through the *RS-com*® software;

Concerning the BABA treatment, at storage days 2, 4 and 6, were made water flux measurements, using the Scholander pressure chamber. Stems water conductivity (Lp)

was then expressed by the slope of the regression function of *Jv* vs. the applied pressure. Moreover in the same storage days were measured: 1) the stomatal conductance (gs) with a diffusion porometer (delta P-4, Delta-T Devices, Cambridge) in 3 daily measurements (h 9:00 a.m; h 1:00 p.m; h 7:00 p.m.); 2) the xylematic potential with the Scholander Chamber; 3) Relative Water Content.

At the end of the cut stems vase life was estimated the leaf area using th Image J® software.

The cut stems treated with pinolene were stored in 2 groups: 1) one group was kept in distilled water and weighted with the *RS-com*® software to determine the cut stems water use; 1) the remaining stems were stored "dry" to register the daily weight loss.

In both the experiments cut stems were stored for 12 days assessing the vase life visually (quality score from 4 to 0).

2.3 Results and discussion

Stomatal conductance was reduced by BABA treatment respect to the control (tab.3). Consequently, daily and cumulated water consumptions, normalized per leaf area, were lower for treated stems (fig.9). The application of 0.5 mM of BABA significantly reduced water fluxes during storage (fig.10) and the water conductivity (*Lp*) of rose cut stems (2.6 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in control vs. 1.54 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in BABA treatment). RWC was significantly higher in the control (tab.3) and it decreased during storage. The water potential decreased over time and it was lower for rose stems treated with BABA (tab3). The vase life, however, was not influenced by treatment because in BABA treated stems the improvement of the water balance was accompanied by premature yellowing of the leaves.

The water use of pinolene treated cut stems was always lower compared to control stems (fig.11). This was associated with a medium increase of the vase life by 1.5 days in the former (fig.12). In dry-stored stems , no significant differences in weight loss were found.

ß-aminobutyric acid (BABA) is found rarely in plants and is known as a potent inducer of a broad-spectrum disease and abiotic stresses resistance in different species (Cohen et. al., 2001; Jakab et. al., 2001). Recently, Jakab et al. (2005) have found that BABA is also effective against osmotic stress. This BABA-induced protection against drought and salinity was found in Arabidopsis genotypes impaired in Salicilic acid, Ethylene, and Jasmonic acid signalling but was not functional in Arabidopsis mutants impaired in the production or sensitivity to the abscisic acid (ABA) (Ton & Mauch Mani, 2004). Ton et al. (2004; 2005) have indicated that BABA applications on wild-type plants, that had been moderately salt stressed, have induced protection from osmotic stress through

priming of ABA dependent defense mechanisms. The reduction of the transpiration flux subsequent to a stress is regulated by the abscissic acid (ABA) (Zhu, 2002). ABA, moreover, controls other mechanisms involved in water homeostasis. The accumulation of proline, for instance, documented for several species as a response to many stresses, is induced by ABA, that promotes proline biosynthesis and inhibit its degradation (Hasegawa et al., 2000). It is acknowledged that ABA acts as cellular signal for the expression of a number of salt and water deficit responsive genes (Jia et al., 2002). Increased synthesis and redistribution of ABA, in response to water deficit stress, activates different signalling pathways in guard cells leading to stomatal closure (Desikan et al., 2004). In rose cut stems, BABA treatment has induced a decrease in stomatal conductance compared to control (tab.3), with the consequent reduction of water consumptions. Along with this decline of the transpirational flux, water fluxes and RWC (fig.10, tab.3) decreased over time and they were generally lower in BABA treated stems. Also Lp was lower in BABA-treated stems (2.6 [(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in control vs. 1.54[(kg H2O m^{-2} s^{-1} m^{-1}) MPa^{-1}] in BABA treatment). The decreased RWC and water potential (tab.3), together with a ruduction of the cut stems hydration state, can probably be related to senescence phenomena that occurred with the premature yellowing of the leaves, which is also mediated by ABA (Hunter et al., 2004; Ferrante et. al., 2006). It has been demonstrated that application of ABA to rose cut flowers accelerate the senescence phenomena (Mayak & Halevy 1972).

In the second experiment, we tested the effects of an antitranspirant polymer (pinolene) on water loss and water use of rose cut flowers. Film forming polymers act as a physical protective barrier (Han, 1990) able to reduce water losses by transpiration (Iriti et al., 2009). Water consumptions normalized per leaf area, daily and cumulated, of rose cut stems was significantly reduced by pinolene application during the vase life (fig.11).

The reduced water use was correlated to an extended vase life of 1.5 days compared to the water-treated control (fig.12). In cut flowers, transpiration water losses must be compensated by a water uptake and transport occurring in the absence the root system, an essential organ for these functions (Singh & Moore, 1992). Concerning the stems kept in absence of water, no differences in weight loss were registered between the 2 treatments. It is possible that the action of pinolene is manifested in a system in which water losses, resulting from a reduced transpiration, must be offset by absorption of water (even if limited by the absence of roots).

2.4 Tables and Figures

Table 3.

Results of the ANOVA. Effect of exogenous ß-aminobutyric acid (BABA) on Stomatal conductance (gs), Relative Water Content (RWC) and Water Potential (Ψ) of rose cut stems.

Treatment	Stomatal conductance ($cm\ s^{-1}$)	RWC (%)	Ψ (MPa)
BABA	0,25	74	-0,27
H_2O	0,41	79	-0,17
Significance	*	*	*

** significant at $P \leq 0.05$*

Figure 9.

Effect of exogenous ß-aminobutyric acid (BABA) on water consumptions of rose cut stems during storage.

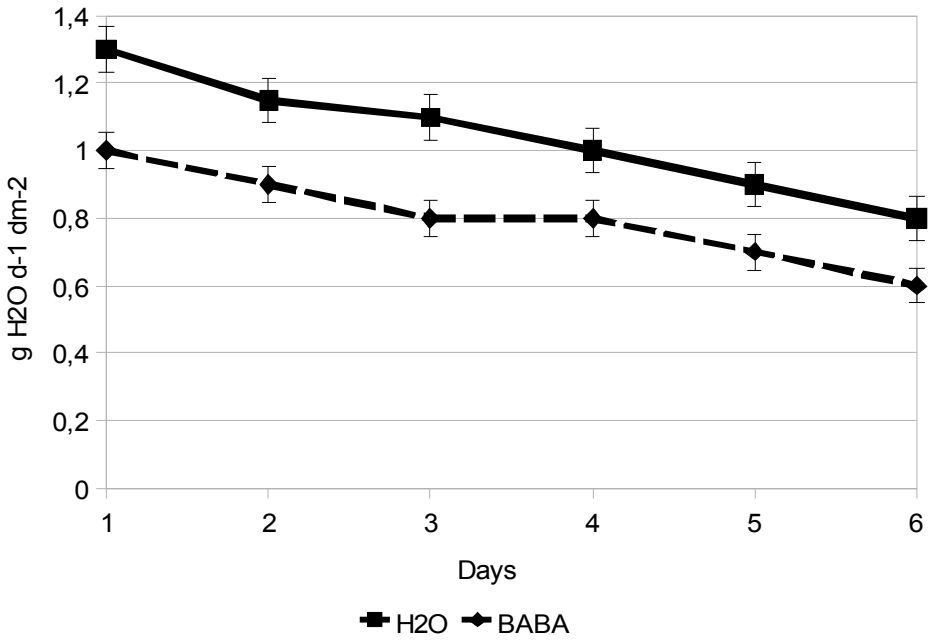


Figure 10.

Effect of exogenous ß-aminobutyric acid (BABA) on water flux of rose cut stems during storage.

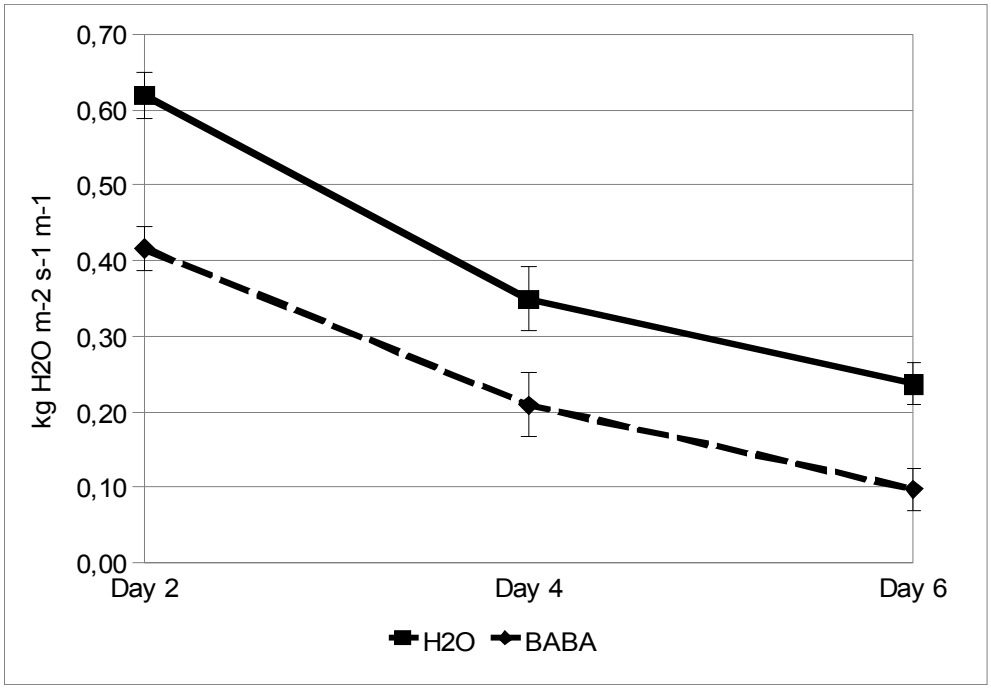


Figure 11.

Effect of pinolene application on water use of rose cut stems during storage.

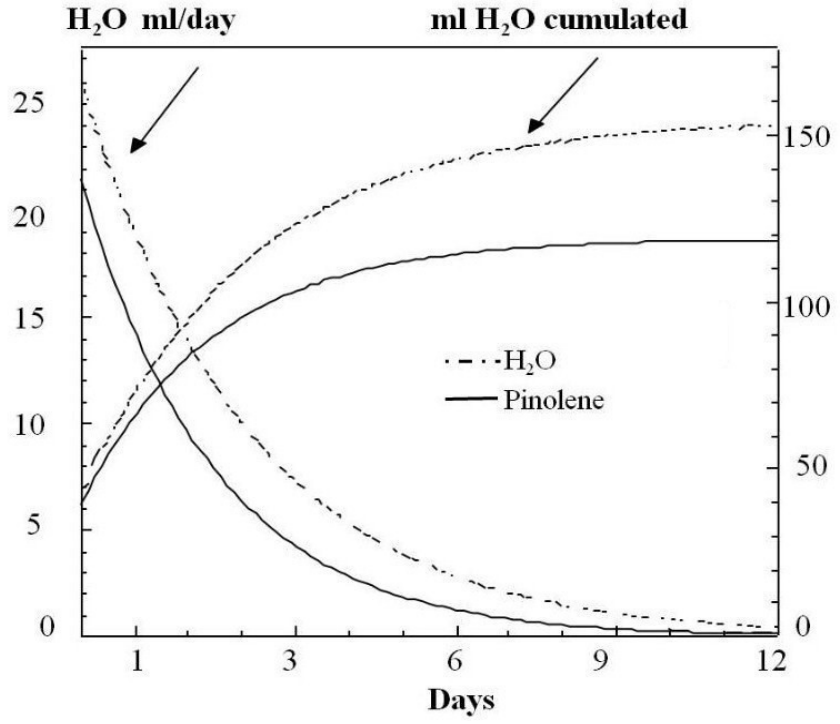
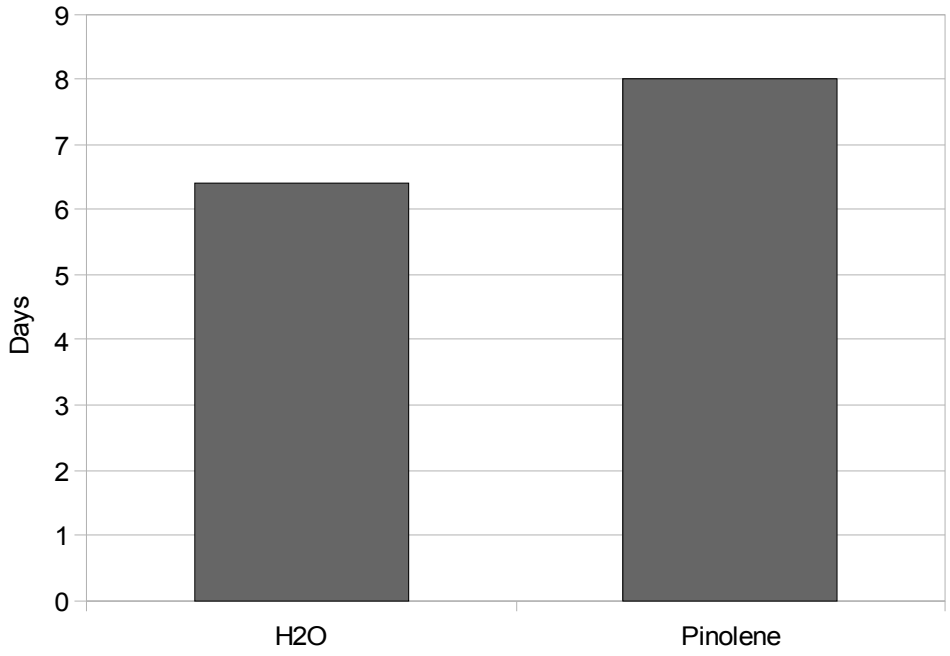


Figure 12.

Effect of pinolene application on the quality (days for the decay of 50% of quality) of rose cut stems during storage.



CHAPTER 3

EFFECT OF 1-MCP AND ITS INCLUSION IN B-NANOSPONGES ON VASE LIFE AND WATER BALANCE OF CARNATION CUT FLOWERS

3.1 Introduction

Ethylene (C_2H_4) is a plant hormone involved in fruit ripening and flower senescence (Beaudry, 2003; van Loon & Glick, 2004). It is in gaseous form and its biosynthetic pathway, starting from methionine, is well known and all the enzymes involved were characterized and cloned (Kende, 1993; Have & Woltering, 1997). Carnation is very sensitive to ethylene. In carnation flowers, ethylene is the primary plant hormone involved in plant senescence (Abeles et al., 1992; Reid & Wu, 1992) and a large amount of it is synthesized after the full flower opening (Woodson et al., 1992; Shibuya et al., 2000). Following pollination, a signal passes from the style to the petals through the ovary and initiates a burst of ethylene production (Jones & Woodson, 1997; Wagstaff et al., 2005).

Cut flowers, during their post-harvest life, can be affected by exogenous and endogenous ethylene. The presence of exogenous ethylene in a storage environment can strongly influence the longevity and quality of cut flowers (Reid, 1995; Dodge et. al., 1998). The exogenous source of ethylene can be biological or non-biological. In the first case, biosynthesis by bacteria, fungi and other plant material (fruit and vegetables) in the storage environment can be the main sources. In the second case, the production of ethylene is due to chemical reactions as oxidative co-product of the flue gas of engines used for heating and transport, transport vehicles with internal combustion and cigarette

smoke. In addition to all the rational operations aimed at preserving cut flowers shelf life from harvest to detail, the shelf life can therefore be potentially regulated either by reducing the causes of exogenous ethylene in storage environments or through the use of compounds that are able to inhibit ethylene action. The more efficient ethylene action inhibitor is the 1-methylcyclopropene (1-MCP) that competitively blocks the hormone action by its irreversible binding to the ethylene receptor (Sisler & Serek, 1997). 1-MCP treatment has been shown to extend the shelf life of various detached leaves (Jiang et al., 2002; Hassan & Mahfouz, 2010).

In cut flowers, water homeostasis is a complex phenomenon since it involves the simultaneous activation of different physiological mechanisms including responses to physical stress (cutting), the induction of the senescence process and the activation of strategies aimed at maintaining cell hydration and turgor (Mayak, 1987; Dodge et al., 1998).

In carnation cut stems, the activation of senescence processes may be directly (stem bending) associated to turgor loss and consequently to an impaired water transport through the stem (Botondi et al., 1998; Ferrante & Serra, 2009). The aim of this work was to evaluate the effect of 1-MCP on water balance and hydration of carnation cut stems during the vase life. 1-MCP was applied as gaseous or soluble forms in post-harvest to carnation stems. Soluble 1-MCP was included in β-nanosponges for a gradual release in the storage water. B-nanosponges are nano-sized colloidal carriers recently proposed for drug delivery and prolonged release of the compounds (Cavalli et al., 2006). Nanosponges have been synthesized from natural derivatives of starch (cyclodextrins) (Cavalli et al., 2006). The cyclodextrin-based nanosponge are structures with both cyclodextrins lypophilic cavities and carbonate bridges, leading to a reticulum of hydrophilic channels (Trotta & Cavalli, 2009). Nanosponges are solid compounds

insoluble in water and in common organic solvents, non-toxic, porous, stable above 300 ◦C, and may be used to encapsulate, carry, and/or release a great variety of substances (Seglie et. al., 2010). They facilitate the gradual release of different compounds over time, increasing their bioavailability (Vyas et al., 2008)

3.2 Materials and Methods

3.2.1 Cultivation

Carnation plants cv. Special white were grown in an unheated greenhouse, on soil, at the farm 'Bruno Antonio', Torre Del Greco (NA). Cultivation was carefully monitored during growth. Lab measurements were conducted at the Chemical and Terminological Laboratory of the 'Department of Agricultural Engineering and Territorial Agronomy', Faculty of Agriculture, Portici (NA).

3.2.2 Treatments

Two forms of 1-methylcyclopropene (1-MCP) were utilized in this experiment: 1-MCP gaseous (*SmartFresh*^{TM} – AgroFresh; 3.3 % 1-MCP): granular, to be activated by adding distilled water (Beaudry, 2003);

A formulate of granular 1-MCP included in β-nanosponges (1-MCP + B-nanosponges 1:8) (synthesized by "Department of Agronomy, Forest and Land Management", Faculty of Agriculture University of Torino - Italy; 6% 1-MCP): to be solubilized by the storage water (Seglie et al., 2010).

At harvest carnation cut stems were transferred to the chemical laboratory and divided in 4 groups for the following treatments:

N. 15 cut stems were placed in sealed box and exposed to the gaseous treatment with 1-MCP (*SmartFresh*^{TM} 2.5 μL L^{-1}) for 6 h in the dark (Sisler et al., 1996). N. 15 stems were placed in glass cylinders and treated with 0.128 g L^{-1} of the 1-MCP+ β-nanosponge formulate in the storage water. The experiment included 2 controls: stems stored in distilled water and stems stored in distilled water+ β-nanosponge 1:8.

3.2.3 Lab measurements and storage

At harvest, stems were transferred in the laboratory and prepared for the treatments. All the treated stems were placed and stored as described in Chapters I and II. Measurements started on the third day from harvest to allow the 1-MCP to begin its activity.

At storage days 3, 5 and 7, on carnation flowers treated with gaseous 1-MCP (control in distilled water) were measured stomatal conductance, water fluxes, Relative Water Content, and water potential (same methodology described in Chapter I and II).

During storage days 4, 6 and 8, the same measurements were done on carnation stems treated with soluble 1-MCP+ β-nanosponge (control in distilled water+ β-nanosponge).

Stems were stored for 13 days measuring daily water consumptions and assessing the vase life using a quality score from 0 to 5 (according to a ranking scale determined at the “Department of Agronomy, Forest and Land Management”, Faculty of Agriculture University of Torino – Italy). The leaf area was measured at the end of the stems vase life.

3.3 Results and discussion

The cut stems water fluxes were significantly enhanced by the 1-MCP gaseous treatment (fig.13) until the 5th day of storage. This was probably confirming a gradual decay of 1-MCP effectiveness over time. The application of 1-MCP+β-nanosponge also improved water fluxes of carnation cut stems (fig.14) although lower values respect to the 1-MCP gaseous treatment were measured (fig.13, fig.14). A prolonged effect beyond the 7th day of storage was observed when plants were treated with 1-MCP+β-nanosponge (fig.14).

Stems water conductivity (*Lp*) was higher respect to the control in both 1-MCP applications (soluble or gaseous forms) (tab.4, tab.5) This contributed to an improved water use of the 1-MCP -treated stems (soluble and gaseous) (fig.15, fig.16).

1-MCP-treated stems had higher quality score during storage (fig.17, fig.18). However, 1-MCP treated flowers did not have an extended vase life compared to the non-treated controls. No significant differences were found in terms of RWC, stomatal conductance and water potential (tab.6, tab.7).

Carnation cut flowers are highly perishable products for which extending the vase life has an important commercial value.

In order to ensure this, it is possible to delay the senescence of these flowers using various compounds such as acetaldehyde (Podd & van Staden, 1999) or 1,1-dimethyl-4-(phenylsulfonyl)semicarbazide (Onoue et al, 2000). It is well documented that ethylene is the primary plant hormone involved in carnation senescence (Borochov & Woodson 1989; Abeles et al., 1992). 1-methylcyclopropene (1-MCP, C_4H_6) is a strong ethylene inhibitor and its affinity for the receptor is estimated approximately 10 times greater than that of ethylene (Blankenship & Dole, 2003). 1-MCP has been frequently

used to study ripening and senescence phenomena of fresh vegetables (Watkins, 2006) and is efficient in protecting plants from both endogenous and exogenous sources of ethylene (Blankenship & Dole, 2003). Studies on carnation have demonstrated that applications of 1-MCP may reduce up to 2/3 the effects of exogenous ethylene during the shelf life, but they do not effect endogenous biosynthesis (Blankenship & Dole, 2003). In general, in many flower species 1-MCP has shown its effect mainly in presence of exogenous ethylene (Celikel & Reid, 2002; Serek and Sisler, 2001; Serek et al., 1994b)

During cut flowers vase life, the absence of roots along with the senescence phenomena, inevitably lead to a loss of cellular turgor and cell wall rigidity (Botondi et al., 1998). In our experiment, water conductivity and water fluxes decreased over time although they were enhanced by 1-MCP treatment compared to the control. The effect of gaseous 1-MCP treatments declined after the 5th storage day while the stems treated with the 1-MCP+β-nanosponge formulate maintained high water fluxes until the 7th storage day (fig.13, fig.14). The effectiveness of the 1-MCP treatment may depend on many factors such as temperatures and O_2 concentration of the storage environment that influence the receptor functionality (Mir et al., 2001; DeEll et al., 2002). Also the concentration of the compound can influence the the application effect. The effective concentration of 1-MCP may differ with the type of product (es. granular to solubilize by the storage water, or gaseous) and, within the same formulation, respect to time, temperature and method of application (Watkins, 2006). The minimum 1-MCP concentration required was found on carnation (2.5 nl l^{-1}), while in apples, studies have shown that 1 ml l^{-1} was required to block ethylene action (Jiang & Joyce, 2002; Sisler et al., 1996a). Serek et al. (1995a;1995b) tested different 1-MCP concentrations from 0.6 to 20 nl l^{-1} on cut carnation 'Sandra' flowers and their results have shown that 90% of possible carnation

longevity was obtained with a 10-20 nl l^{-1} concentration range. Evermore on carnation, Sisler et al. (1996a) tested concentrations of 1.25, 2.5, 5, and 10 nl l^{-1} for 24 h on young flowers (Stage II) and reported that at least 2.5 nl l^{-1} was required for prevent effects of exogenous ethylene. Even plant developmental stage and maturity may influence the 1-MCP action (Blankenship & Dole, 2003). Single flowers (i.e. carnations stems) may be completely protected from ethylene, but *spray* flowers like delphinium or gypsophila may be only partially protected, presumably due to different tissue ages (Sisler et al., 1996a; Newman et al., 1998).

The interaction of 1-MCP with other substances with regulatory action on senescence can improve the maintenance of vegetables quality over time (Macnish et al., 2004). Cavalli et al. (2006) evaluated the capacity of the nanosponges to incorporate molecules within their structure using drugs with different structures and solubilities (i.e. Dexamethasone, Doxorubicin).

Seglie et al. (2010) evaluated the effects of 1-MCP included in cyclodextrin-based nanosponges on the vase life of carnation cut flowers; the inclusion of 1-MCP in nanosponges promoted cut flower longevity better than the commercial 1-MCP gaseous treatment. In this work, the inclusion of 1-MCP in β-nanosponges may have extended the activity of this molecule (fig.13, fig.14). Moreover applications of 1-MCP on carnation has contributed to improve the water conductivity of stems during the vase life (tab.4, tab.5). Consequently, cut stems daily water consumption, was higher for 1-MCP treated stems (fig.15, fig.16).

Despite the enhanced *Lp*, water flux and water use of the 1-MCP treated stems, non significant effects were recorded in RWC, water potential and *gs* (tab.6, tab.7). During storage, gaseous 1-MCP treated plants had higher quality score compared to those treated with soluble 1-MCP (fig.17, fig.18). 1-MCP applications did not extend the

stems longevity probably because of loss of efficacy of the two treatments over time.

3.4 Tables and Figures

Table 4.

Results of the ANOVA. Effect of gaseous 1-MCP on water flux, Water Conductivity (Lp) of carnation cut stems.

Treatment	Water Flux kg H_2O m^{-2} s^{-1} m^{-1}	Lp (kg H_2O m^{-2} s^{-1} m^{-1}) MPa^{-1}
1MCP gas	0,17	0,80
H_2O	0,17	0,74
Significance	ns	*

*ns, not significant; * significant at $P \leq 0.05$*

Table 5.

Results of the ANOVA. Effect of 1-MCP+β-nanosponges on water flux, Water Conductivity (Lp) of carnation cut stems.

Treatment	Water Flux kg H_2O m^{-2} s^{-1} m^{-1}	Lp (kg H_2O m^{-2} s^{-1} m^{-1}) MPa^{-1}
1MCP+β nanosponges	0,17	0,85
H_2O	0,15	0,63
Significance	*	*

significant at $P \leq 0.05$

Table 6.

Results of the ANOVA. Effect of gaseous 1-MCP on Stomatal Conductance (gs), Relative Water Content (RWC) and Water Potential (Ψ) of carnation cut stems.

Treatment	Stomatal conductance ($cm\ s^{-1}$)	RWC (%)	Ψ (MPa)
1MCP gas	0,03	94	-
H_2O	0,02	97	-
Significance	ns	ns	-

ns, not significant at $P \leq 0.05$

Table 7.

Results of the ANOVA. Effect of 1-MCP+ β-nanosponges on Stomatal Conductance (gs), Relative Water Content (RWC) and Water Potential (Ψ) of carnation cut stems.

Treatment	Stomatal Conductance ($cm\ s^{-1}$)	RWC (%)	Ψ (MPa)
1MCP+β nanosponges	0,03	92	-
H_2O	0,03	92	-
Significance	ns	ns	-

[c]*ns, not significant; * significant at $P \leq 0.05$*

Figure 13.

Effect of gaseous 1-MCP on water flux of carnation cut stems during storage.

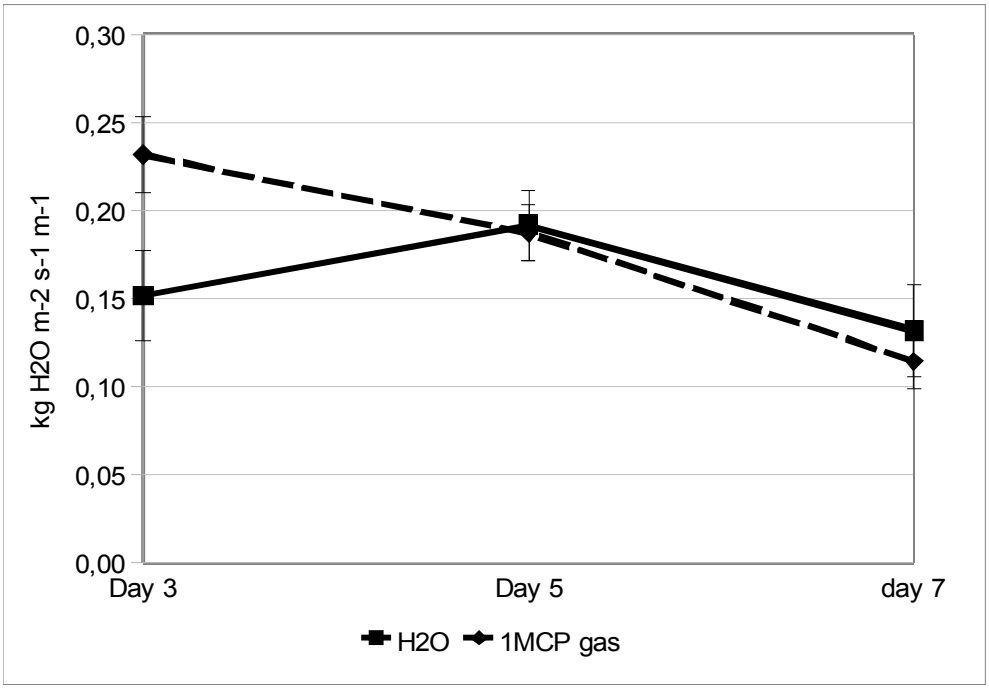


Figure 14.

Effect of 1-MCP + β-nanosponges on water flux of carnation cut stems during storage.

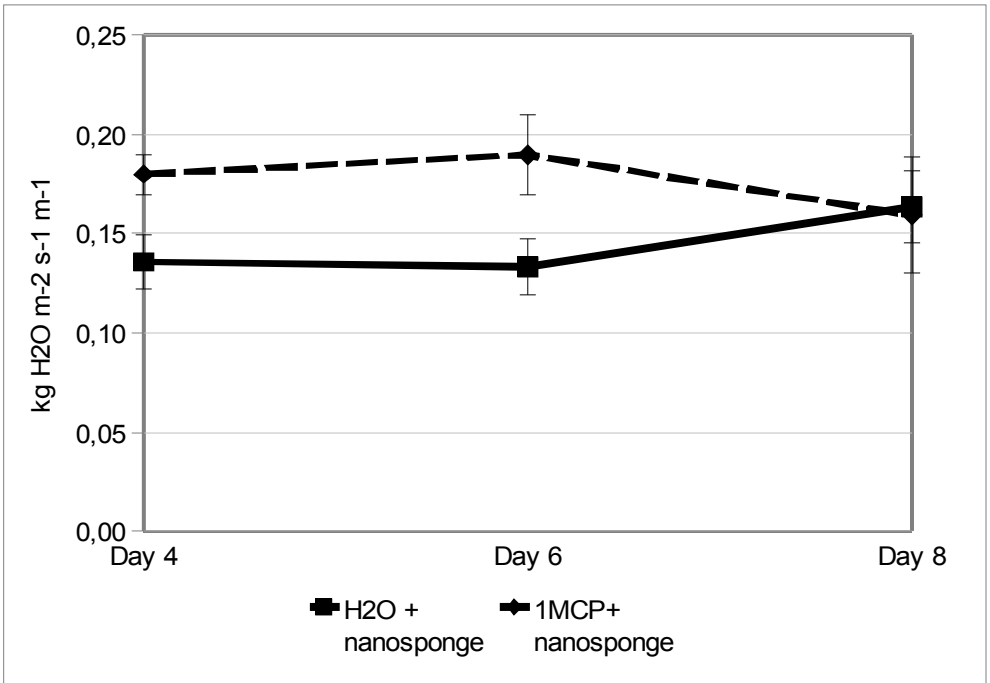


Figure 15.

Effect of gaseous 1-MCP on water consumption of carnation cut stems during storage.

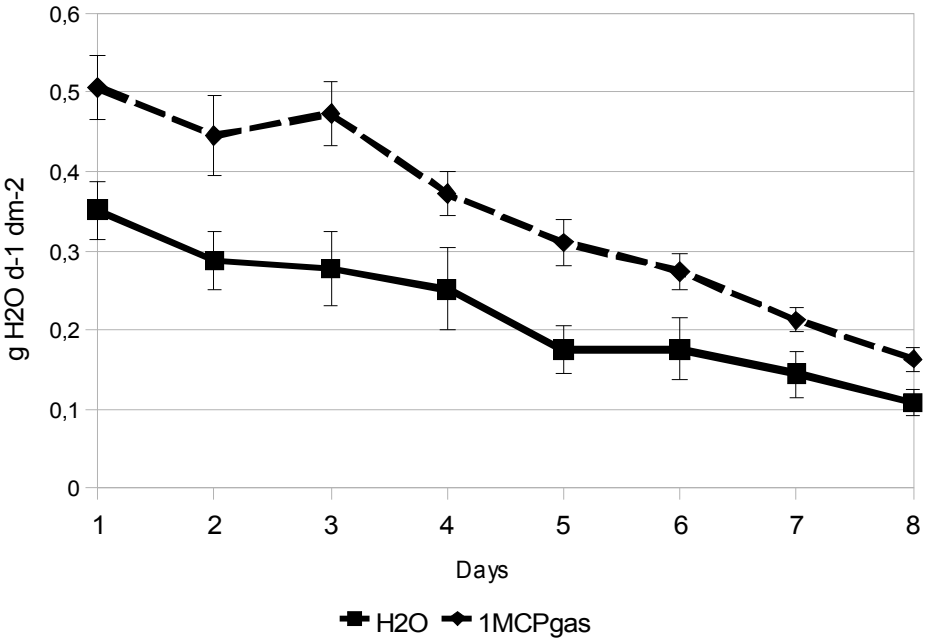


Figure 16.

Effect of soluble 1-MCP + β-nanosponges on water consumption of carnation cut stems during storage.

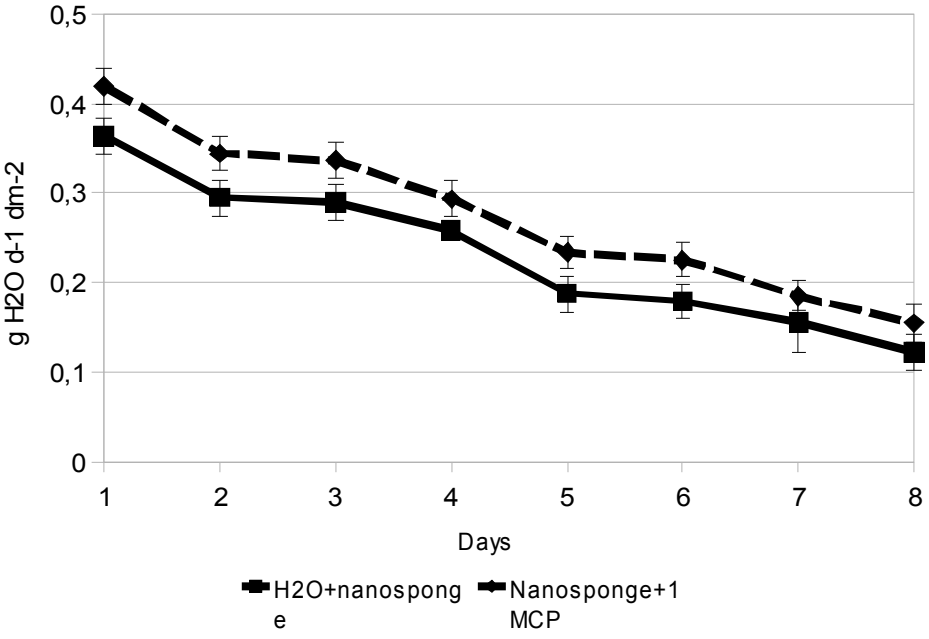


Figure 17.

Effect of gaseous 1-MCP on the vase life of carnation cut stems.

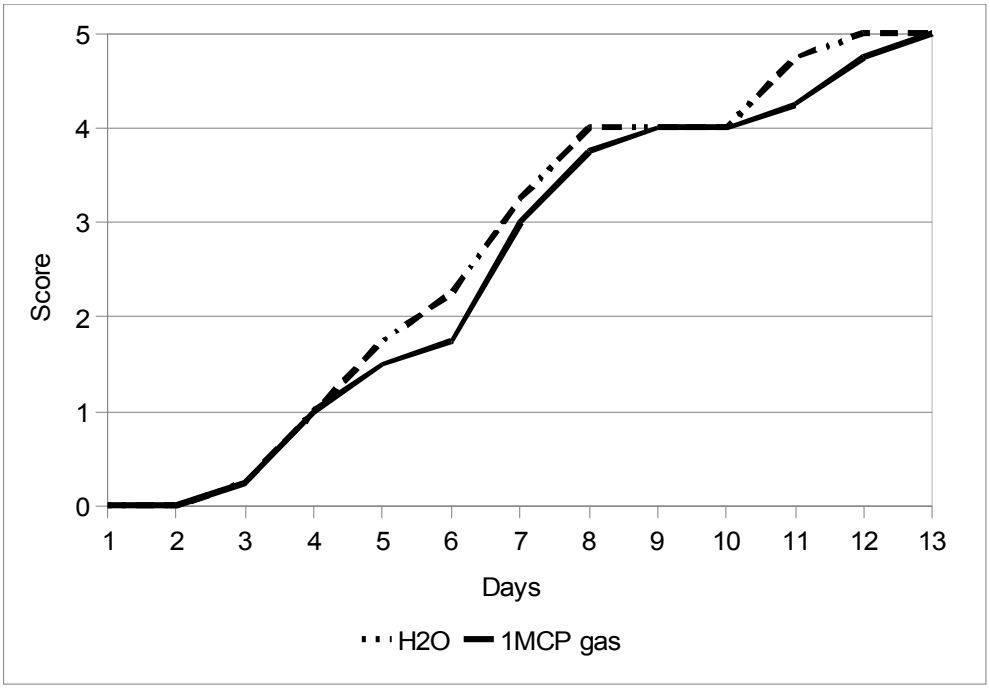
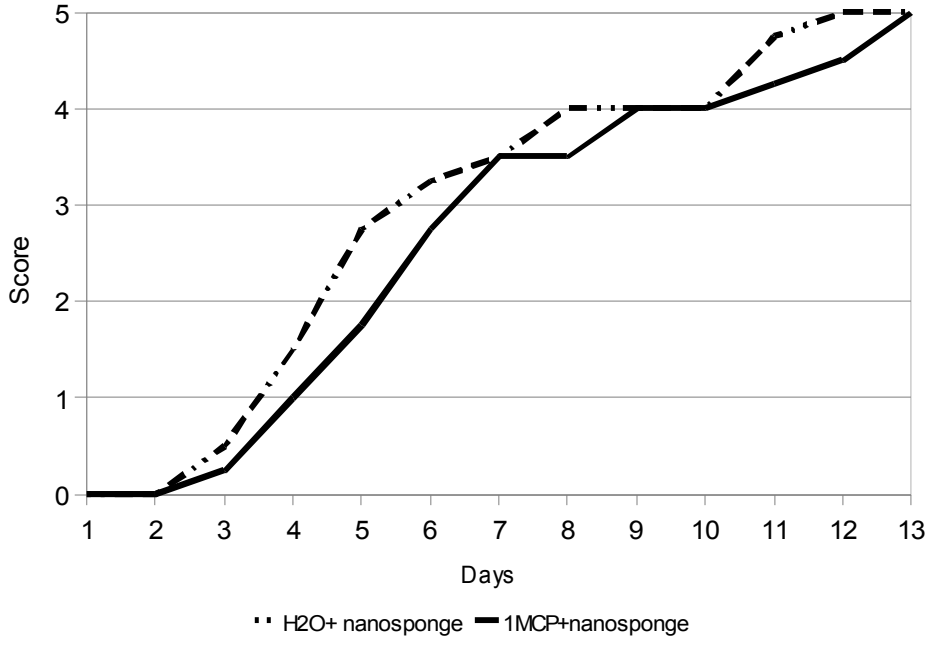


Figure 18.

Effect of soluble 1-MCP + β-nanosponges on the vase life of carnation cut stems.



CHAPTER 4

CONCLUSIONS

In cut flowers, wounding inevitably leads to stomatal closure and activation of several physiological mechanisms that may reduce water loss and prevent wilting.

On rose plants, treatment with 10 mM L-Proline has allowed the maintenance of higher stomatal aperture and improved cut stems RWC and Lp during storage compared to untreated control. The same result was obtained by applying a solution of 97% glicynebetaine on carnation plants. The positive effects on cut stems were measured as increased leaf water potential and increased stomatal aperture consequent to osmoregulation. These physiological conditions are crucial for prolonging the vase life of cut flowers because, despite the absence of the root system, allow the plant to partially continue its metabolic functions. The cut stems vase life was especially extended by the L-Proline treatment while in carnation most likely the senescence processes were influenced more by other physiological determinants (hormonal control). In both rose and carnation treated with osmoprotectants, higher values of gs, water fluxes, *Lp* and RWC reflected an improved hydration state. Overall, treatment with these compatible solutes improved the cut stems water status, influencing positively their water balance and vase life.

In cut flowers, the reduction of transpiration is considered a functional target for controlling cellular turgor after harvest. Since ABA is involved in the induction of physiological mechanisms that facilitate adaptation to abiotic stress, it has been hypothesized that the administration of BABA, a mediator of ABA functions, may confer a stress protection that could result in enhanced turgor and vase life of cut stems. In rose, applications of 0.5 mM BABA, has reduced water consumption through the activation of functions associated with the synthesis of ABA including stomatal closure.

However, this was associated with a more rapid decay of the cut stems quality probably for earlier oncoming of senescence phenomena. Pinolene treatment, significantly prolonged the post-harvest life of cut stems, by reducing water losses through transpiration. This was likely due to the formation of a "film" at the leaf surface that acts as a physical barrier to gas exchanges. Based on these results we may conclude that the extension of vase life of rose cut stems is more closely related to the reduction of transpiration rates incurred after the cut rather than mechanisms associate to water absorption.

Anti-ethylene compounds not only delayed senescence phenomena but, by improving the general physiological state of the stem, contributed to ameliorate the water balance of carnation cut stems. 1-MCP is an efficient ethylene antagonist and its application on carnation cut flowers significantly improved the hydration state of the stems. 1-MCP, gaseous or solubilized into the storage water, enhanced water flux, water conductivity and water use of the stems during storage. In comparison with the commercial gaseous 1-MCP, the inclusion of 1-MCP in β-nanosponges has prolonged the effect of this compound. Stems treated with gaseous 1-MCP reported higher water fluxes until the 5^{th} day of storage. In contrast, the formulate of 1-MCP+β-nanosponges enhanced water fluxes until the 7^{th} storage day. The vase life was non extended by the treatments despite the higher *quality score* of 1-MCP treated stems during storage. It is conceivable that the loss of efficacy of the 2 formulates has improved the shelf life only until the 5^{th} day for gaseous 1-MCP treated stems and to the 7^{th} day for the 1-MCP+ β-nanosponges treatment.

Overall the extended vase life of cut rose stems appears to be more related to the maintenance of transpiration fluxes supported after cutting, rather than mechanisms

aimed at conserving cut flowers hydration water.

The control of stomata opening and cellular turgor was critical for the water balance regulation during cut flowers vase life. However, on carnation plants, the substantial improvement in water homeostasis of the cut stems was not accompanied by a significant prolongation of the vase life, confirming the crucial role of hormonal control (in particular of ethylene) on carnation post-harvest senescence.

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