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Effect of different cultural conditions on micropropagation of rose (*Rosa sp*. L.) and globe artichoke (*Cynara scolymus* L.)

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To my father

(*in memoriam*)

Zusammenfassung

Zur Optimierung der Mikrovermehrung von Rosen und Artischocken wurde der Einfluss ausgewählter Kulturfaktoren während der in-vitro Phasen untersucht, sowie die Nachwirkung dieser Faktoren im weiteren Kulturverlauf geprüft, vor allem bei der Etablierung *ex vitro*.

Die Entwicklung von Rosen in vitro wurde durch die Nährbodenverfestigung stark beeinflusst, die besten Ergebnisse wurden mit 6,0 g/l Agar erzielt, während die Verwendung von Gelrite bzw. eine Kultur mit Flüssigmedien sich als weniger geeignet erwiesen. Die Entwicklung während der Bewurzelungsphase wurde vor allem durch Auxingaben, Saccharosekonzentration und Gefäßverschluss beeinflusst. Die Kultur von Sprossen auf 0,1 mg L^{-1} NAA im Vergleich mit 0,5 mg L^{-1} NAA führte zu besserer Sprossentwicklung und einer höheren Etablierungsrate *ex vitro*. Eine Reduktion der Saccharosekonzentration in der Bewurzelungsphase ist vorteilhaft. Die Verwendung von Glaskappen, Steristopfen oder Aluminiumfolie als Verschluss ergab bessere Resultate als ein Verschluss mit Plastikfolie. Während der Bewurzelungsphase war ein deutlicher Effekt der Temperatur festzustellen: Die Wurzelbildung war bei 16°C besser als bei 20 bzw. 24°C, während das Sprosswachstum durch die höheren Temperaturen gefördert wurde. Die Dauer der Bewurzelungsphase soll nach den vorliegenden Ergebnissen etwa fünf Wochen betragen. Bei der Übertragung des Pflanzenmaterials in unsterile Bedingungen konnte die Erfolgsrate durch die Verwendung von Substraten mit erhöhter Luftkapazität gesteigert werden, z.B durch die Verwendung einer Substratmischung 1 Teil Torf / 3 Teile Perlite. Insgesamt war bei Rosen in allen Phasen ein ausgeprägter Einfluss des Genotyps auf die Entwicklung festzustellen.

Bei der Mikrovermehrung von Artischocken führt eine Auxin- / Cytokininkombination 2,0 mg L^{-1} NAA + 2,0 mg L^{-1} BA zwar zu einer hohen Vermehrungsrate, jedoch in der anschließenden Bewurzelungsphase wurden bessere Ergebnisse erzielt, wenn in der vorausgegangenen Vermehrungsphase mit 0,2 mg L^{-1} NAA + 0,2 mg L^{-1} BA geringere Konzentrationen oder mit 2,0 mg L^{-1} NAA + 2,0 mg L^{-1} KIN ein schwächer wirksames Cytokinin benutzt wurde. Als Auxingabe zur Bewurzelung war 0,5 mg L^{-1} NAA geeignet, eine Zusatz von Gibberellinsäure erwies sich als nachteilig. Untersuchungen zum Einfluss von Saccharose und Lichtintensität wurden durch Chlorophyllfluoreszenzmessungen begleitet und zeigten bei geringerer Sacharosekonzetration und niedrigerer Lichtintensität bessere Pflanzenentwicklung in der Bewurzelungsphase. Geringere F_v/F_m-Werte bei 210 µmol m^{-2} s^{-1} im Vergleich zu 110 µmol m^{-2} s^{-1} weisen auf eine Photoinhibition hin.

Abstract

The objective of the present study was to investigate the influence of different cultural conditions in the multiplication, rooting and acclimatization phases on the growth of rose and globe artichoke and their effects on the subsequent micropropagation phases.

The growth of rose plants *in vitro* was affected by the type of gelling agent added to the culture medium. The best results concerning plant growth were found by using agar as gelling agent at the concentration of 6 g L^{-1}. On the other hand, the use of gelrite as gelling agent in the culture medium cannot be recommended for the micropropagation of rose cvs. 'Frisco' and 'Lambada', as well as the liquid medium in rose cv. 'Kardinal'. Moreover, explants grown at 0.1 mg L^{-1} of NAA (auxin) in the rooting phase presented better growth and higher survival rate in the acclimatization phase when compared to explants grown at 0.5 mg L^{-1}, as well as at 10 g L^{-1} sucrose in comparison to 20 or 40 L^{-1}. The type of closure used also played an important role in the rooting phase. The use of glass, steristop or aluminium as closure showed better results than plastic film for growth of shoots for all tested cultivars ('Frisco', 'Kardinal' and 'Lambada'). In addition, the growth of 'Kardinal' was not influenced by the use of Magenta B-CAP covers with or without filter in baby food culture jars. Temperature in the rooting phase clearly influenced rooting formation. The formation of roots was improved at 16°C, whereas shoot growth showed better results at higher temperatures (20°C or 24°C), being different temperatures required for the optimum growth of roots and shoots. Additionally, a minimum duration of the rooting phase of five weeks should be used in the micropropagation of rose. Substrates with higher drainage capacity in the initial phase of acclimatization (as e.g. a mixture of peat and perlite in the proportion 1peat:3perlite v/v) increased the survival rate of plants. The use of perlite mixed with peat should be recommended in order to increase the looseness, permeability and aeration of the substrate.

The growth of globe artichoke cv. 'Green globe' plants *in vitro* was affected by the type and concentration of growth regulators supplemented to the culture medium. The use of 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA in the culture medium positively influenced the growth of shoots in the multiplication phase. However, in the subsequent phases (rooting and acclimatization) this effect was not maintained. In these phases, higher shoot development, rooting percentage, and lower mortality rate of plants were found with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA or with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin in the multiplication phase. Concerning the rooting phase, the addition of auxin (NAA) to the rooting medium at a concentration of 0.5 mg L^{-1} is suitable for the micropropagation of globe artichoke. Moreover, the supplementation of the rooting medium with gibberellic acid (GA_3) is not beneficial. Light intensity of 110 μmol m^{-2} s^{-1} can be used for the micropropagation of globe artichoke, since plants were not photoinhibited under this condition. Lower values of the F_v/F_m ratio may indicate the occurrence of photoinhibition due to damage to photosystem II reaction centers in response to light intensity of 210 μmol m^{-2} s^{-1}. The results indicate that a reduction of sucrose concentration is advantageous for the micropropagation of globe artichoke.

Contents

List of Figures

List of Tables

1 Introduction

Roses (*Rosa* sp. L.) are one of the most important commercially produced cut flowers in the world. In Germany, the genus *Rosa* took first place of the top 10-list in the commerce of cut flowers in 2002 (Source: www.florakom.de), with a market volume estimated at 990 million Euro (approximately 33% of the total amount commercialized of cut flowers) (Source: www.flowerpr.nett24.de).

The genus *Rosa*, member of the family Rosaceae, comprises more than 100 species (Horn, 1992). Most of the cultivated roses occur indigenously in the northern temperate zone and in tropical mountain areas (Horn, 1992). There are more than 20,000 commercial cultivars, which collectively are based on only 8 wild species (Kim et al., 2003). In Germany, rose is the third most micropropagated species within the woody plants, with approximately 350,000 plantlets per year (N.N., 2004).

The production of globe artichoke (*Cynara scolymus* L.), in turn, has a smaller economical significance in Germany in comparison to rose. The world cultivation is mainly concentrated in the Mediterranean region, where approximately 85% of the world production is grown (ca. 119,000 ha). Italy is the biggest world producer with more than 50% of the total production (Halter, 2003). In Germany, artichoke is much appreciated and, thus, there is a potential for increasing its cultivation. At the Technical University Munich, a research project is being carried out with the objective to optimize the micropropagation and to generate cultivation methods for globe artichoke under the conditions of the Bavarian state.

The genus *Cynara* belongs to the family Asteraceae (formerly Compositae) and is probably native to the southern Europe around the Mediterranean and Canary Islands (Source: www.explore-plants.com).

Rose and globe artichoke can be propagated under *in vitro* conditions. Micropropagation is a technique used for producing plantlets and implies the culture of aseptic small sections of tissues and organs in vessels with defined culture medium and under controlled environmental conditions and has become an increasingly important tool for both science and commercial applications in recent years. It is the foundation on which all biotechnological research rests, because almost all uses of plant biotechnology ultimately require the successful culture of plants cells, tissues or organs.

This technique has many advantages over conventional vegetative propagation, as e.g. the propagation of a great number of pathogen-free plants in a short time with high uniformity.

The success of micropropagation involves several factors, as the composition of the culture medium, culture environment, and genotype. The development of procedures for rapid *in vitro* clonal micropropagation of rose and globe artichoke may be a great commercial value to the industry. Tissue culture techniques should minimize the time necessary for the introduction of new cultivars into the commercial market and thus increase the availability of plants with improved horticultural characteristics (Bressan et al., 1982).

The objective of the present study was to investigate the influence of different cultural conditions in the multiplication, rooting and acclimatization phases on the growth of rose and globe artichoke and their effects on the subsequent micropropagation phases.

In rose, there are numerous examples in the literature in which the results obtained during tissue culture have varied from one cultivar to another (Sauer et al., 1985; Alderson et al., 1988; Leyhe & Horn, 1994). Therefore, the effect of the following conditions was studied in different rose cultivars (‘Frisco’, ‘Lambada’ and ‘Kardinal’):

1) type and concentration of gelling agents in the culture medium;
2) type of vessel closure and concentration of sucrose in the culture medium;
3) room temperature;
4) duration of the rooting phase;
5) consistency of the culture medium and concentration of growth regulators;
6) substrates in the acclimatization phase.

For globe artichoke, in turn, fewer studies on micropropagation have been published in comparison to rose. Thus, only a little amount of information concerning the *in vitro* cultivation is available. For this species (cv. ‘Green globe’), the effect of the following cultural conditions was investigated:

1) concentration and type of growth regulators in the culture medium;
2) sucrose concentration, type of vessel closure and light intensity.

The purpose of the present study was to establish appropriate procedures for the micropropagation of both species, in order to increase the quality and survival *ex vitro* of plantlets.

2 Literature Overview

2.1 Micropropagation

2.1.1 Principles of tissue culture

Plant micropropagation is an integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized, and incubated in a growth-promoting aseptic environment to produce many clonal plantlets (Altman, 2000).

The technique of cloning isolated single cells *in vitro* demonstrated the fact that somatic cells, under appropriated conditions, can differentiate to a whole plant. This potential of a cell to grow and develop a multicelular organism is termed cellular totipotency (Razdan, 1993; Torres et al., 1999; Wetzstein & He, 2000). This potential of cells or tissues to form all cell types and regenerate a plant is the basic principle of tissue culture.

The growth of *in vitro* cultured tissues and morphogenesis are very influenced by the genotype (Pierik, 1997), even more than by any other factor (George, 1993). The expression of the genetic make-up also depends on physical and chemical conditions, which have to be created *in vitro* (Pierik, 1997).

Great differences in morphogenic responses *in vitro* among genera and species within a plant family have been observed. Even within a species, there are notable differences among subspecies and cultivars, so that a single plant growth medium formulation is often inadequate for stimulating morphogenesis for a wide range of cultivars (Litz, 1993). For instance, different responses to the growing conditions *in vitro* have been found between rose cultivars (Sauer et al., 1985; Alderson et al., 1988; Horn et al., 1988; Leyhe & Horn, 1994).

According to Debergh & Read (1991) and Altman (2000), the micropropagation process can be divided in five different stages:

Phase 0: growing mother plants under hygienic conditions. It involves the production of stock plants in greenhouse.

Phase I: initiation of culture. The purpose of this stage is to initiate axenic cultures. It involves the selection of explants, desinfestation and the cultivation under aseptic conditions.
Phase II: rapid regeneration and multiplication of numerous propagules (multiplication phase). Masses of tissues are repeatedly subcultured under aseptic conditions onto new culturing media that encourage propagule proliferation. The culture can supply shoots for the subsequent propagation phases as well as material that is required to maintain the stock.
Phase III: elongation and root induction or development (rooting phase). This phase is designed to induce the establishment of fully developed plantlets. It is the last period *in vitro* before transferring the plantlets to *ex vitro* conditions.
Phase IV: transfer to *ex vitro* condition (acclimatization). Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant that has been moved to a new environment (Kozai & Zobayed, 2000).

2.1.2 Composition of nutrient media

The culture media must provide all the essential elements and nutrients necessary for the growth of plants *in vitro*. The selection or development of a culture medium is an essential step in any tissue culture project. Components of the media used for the growth of explants are as follows:
- water (distillated and de-ionized water)
- mineral elements (macro- and micro-salts)
- organic substances (vitamins and carbon source)
- growth regulators (cytokinins, auxins, gibberellins and other regulators)
- gelling agent (as e.g. agar)

2.1.2.1 Mineral nutrition

Minerals are important components of the culture medium. There is a large choice of combinations of macro- and micro-salt mixtures. The most widely used culture medium is described in Murashige and Skoog (1962) (MS medium), because most plants react to it favorably. It contains all the elements that have been shown to be essential for plant growth *in vitro*. It is classified as a high salt medium in comparison

to many other formulations, with high levels of nitrogen, potassium and some of the micronutrients, particularly boron and manganese (Cohen, 1995). Due to the high salt content, however, this nutrient solution is not necessarily always optimal for growth and development of plants *in vitro* (Pierik, 1997).

For that reason, the use of dilute media formulations has generally promoted better formation of roots, since high concentration of salts may inhibit root growth, even in presence of auxins in the culture medium (Grattapaglia & Machado, 1998).

The ability of rose explants to produce shoots and initiate roots was studied by Kim et al. (2003). They concluded that optimum shoot proliferation was obtained in full-strength MS salts, while rooting improved with 1/4 strength. Sauer et al. (1985) reported that 1/3 strength MS salts proved to be suitable for rooting of rose. For globe artichoke, 1/2 strength MS salts have been used in the rooting medium (Ancora, 1986; Lauzer & Vieth, 1990; Iapichino, 1996).

2.1.2.2 Carbon source

Sucrose is by far the most used carbon source, for several reasons. It is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants. Other carbohydrates can be also used, such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol (Fowler, 2000).

The carbohydrates added to the culture medium supply energy for the metabolism (Caldas et al., 1998). The addition of a carbon source in any nutrient medium is essential for *in vitro* growth and development of many species, because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (in darkness). Normally, green tissues are not sufficiently autotrophic under *in vitro* conditions (Pierik, 1997) and depend on the availability of carbohydrates in the growing medium. In fact, only a limited number of plant cell lines have been isolated which are autotrophic when cultured *in vitro* (George, 1993).

On the other hand, the presence of sucrose in the culture medium may inhibit the carbon metabolism, the potential photosynthesis *in vitro* and the formation of chlorophyll as well as promote a decrease in the Rubisco activity (Campostrini & Otoni, 1996; Huylenbroeck & Debergh, 1996).

The reduction of the sugar concentration in the growing medium, associated with an increase in the CO_2 concentration and light intensity, may induce plants to become mixotrophic, using then CO_2 as carbon source (Langford & Wainwright, 1987; Gourichon et al., 1996). Kozai et al. (1997) pointed out some advantages of sugar-free growing media over conventional ones (heterotrophic or mixotrophic), as e.g. faster and more uniform growth and development of plants *in vitro* and lower occurrence of physiological and morphological disorders as well as a reduction of *in vitro* contaminations and increased survival rate under *ex vitro* conditions. Hence, productions costs could be reduced and plant quality could be improved.

Podwyszyńska et al. (2003) studied the effect of sucrose concentration, CO_2 enrichment and light intensity in rose and gerbera. In rose, the CO_2 enrichment had a positive effect on *ex vitro* rooting and establishment only when combined with the lowest level of sucrose in the medium (10 g L^{-1}) and high light intensity. Rose shoots were characterized by significantly higher photosynthetic activity compared to those of gerbera. Furthermore, the highest percentage of gerbera microcuttings rooted *ex vitro* was found when they were multiplied *in vitro* at the higher sucrose concentrations (30-40 g L^{-1}) under low light intensities. Thus, presumably, the higher level of CO_2 may affect positively only *in vitro* cultures of plant species that are able to respond to increased light intensities and CO_2, and have higher photosynthetic activity *in vitro* (Podwyszyńska et al., 2003).

2.1.2.3 Growth regulators

Growth regulators are organic compounds naturally synthesized in higher plants, which influence growth and development. Apart from the natural compounds, synthetic chemicals with similar physiological activities have been developed which correspond to the natural ones (Pierik, 1997).

There are several classes of plant growth regulators, as e.g. cytokinins, auxins, gibberellins, ethylene and abscisic acid.

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (George, 1993). A balance between auxin and cytokinin is most often required for the formation of adventitious shoots and roots. In tobacco cultured *in vitro*, it was found that the formation of roots and shoots depended on the

ratio of auxin to cytokinin in the culture medium. High levels of auxin relative to cytokinin stimulated the formation of roots, whereas high levels of cytokinin relative to auxin led to the formation of shoots (Taiz & Zeiger, 1991).

The balance of growth regulators depends on the objective of the cultivation *in vitro* (as e.g. shoot, root, callus or suspension culture) and on the micropropagation phase considered (initiation, multiplication or rooting). In the multiplication phase, the level of citokinins should be normally higher than of auxins. In the rooting phase, in turn, the use of cytokinin is, in some cases, not necessary and higher levels of auxins can be supplemented to the culture medium (Torres et al., 2001).

The cytokinins are derived from adenine (aminopurine) and play an important role in the *in vitro* manipulation of plant cells and tissues (Torres et al., 2001). Cytokinins stimulate plant cells to divide, and they were shown to affect many other physiological and developmental process. These effects include the delay of senescence in detached organs, the mobilization of nutrients, chloroplast maturation, and the control of morphogenesis (Taiz & Zeiger, 1991). Added to the culture medium, these compounds overcome apical dominance and release lateral buds from dormancy (George, 1993). The most common cytokinins used are kinetin, BA and 2iP (Pierik, 1997).

Also auxins (IAA, IBA, NAA or 2,4-D) are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinin (George, 1993). Auxins are involved in the regulation of several physiological processes, as e.g. apical dominance and formation of lateral and adventitious roots. This growth regulator generally causes cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). Normally, the concentration of auxin used in the culture medium varies between 0.01 and 10 mg L^{-1} (Torres et. al, 2001). The IAA is a natural auxin, whereas 2,4-D and NAA are synthetically produced and have similar effect in comparison to natural-occurring auxins.

According to most of the studies that have been published concerning the effect of auxin type and concentration in rose, low concentrations of this growth regulator should be used in the culture medium. The rooting of rose shoots was improved with

IAA (considered a weak auxin) supplementation at 1.0 mg L^{-1} (Kim et al., 2003), 0.1 mg L^{-1} NAA (Rahman et al., 1992; Leyhe & Horn, 1994) or even in absence of auxin (Ibrahim & Debergh, 2001). The combination of two types of auxin can be also used to increase root formation in rose. Kosh-Khui & Sink (1982) found that the best combination for the production of rooted plants was 0.1 mg L^{-1} NAA with 0.05 mg L^{-1} of either IAA or IBA. The combination of two auxins was more effective for root formation than either auxin alone.

In globe artichoke, the most effective auxin for rooting was NAA (0.1-2.0 mg L^{-1}) also combined with IAA (2.0 mg L^{-1}) (Rossi & Paoli, 1992). Ancora et al. (1981) and Kanakis & Demetriou (1993) found optimal results concerning the production of roots in globe artichoke plants grown at high concentrations of NAA (2.0 mg L^{-1}).

Gibberellins are a group of compounds that is not necessarily used in the *in vitro* culture of higher plants. In some species, these growth regulators are required to enhance and in others to inhibit growth (Razdan, 1993). Gibberellic acid (GA_3) is the most common gibberellin used. It induces the elongation of internodes and the growth of meristems or buds *in vitro* (Pierik, 1997). Furthermore, the use of gibberellins in the rooting medium may reduce or prevent the formation of adventitious roots and shoots, although it can stimulate root formation when present in low concentrations. Morzadec & Hourmant (1997) showed the beneficial effect for globe artichoke of using gibberellin at a concentration of 1.0 or 5.0 mg L^{-1} GA_3 in the rooting medium, resulting in a rapid root expression and in the formation of high quality explants.

2.1.2.4 Gelling agents

Culture media can be classified as liquid or solid. The liquid media have the advantage of faster (and cheaper) preparation than the solid ones. Furthermore, liquid media are more homogeneous, since gradients of nutrients may appear during tissue growing in solid media. This phenomenon is not observed in liquid media (Caldas et al., 1998). Furthermore, it has been shown that the propagation ratio of some species is higher in liquid than in solid media (Debergh et al., 1981; Pateli et al., 2003).

One serious disadvantage of using liquid media for shoot growth and multiplication is that shoots, which are perpetually submerged in liquid cultures, may become

hyperhydric and will then be useless for micropropagation (George, 1993; Debergh, 2000). Ebrahim & Ibrahim (2000) reported that the solid medium should be used to overcome the production of vitrified shoots of *Maranta leuconeura* and to insure obtaining vigorous plants with higher chlorophyll content.

Agar has traditionally been used as the preferred gelling agent for tissue culture, and is very widely employed for the preparation of semi-solid culture media (Torres et al., 2001). It is a polysaccharide extracted from species of red algae which are collected from the sea (Torres, 1999).

Concerning the optimal agar concentration in the culture medium, large differences between two rose cultivars were observed by Acker & Scholten (1995). The cv. 'Motrea' preferred higher concentrations of agar (7 g L^{-1}). At this concentration, completely developed shoots were formed. The cv. 'Sweet Promise', in turn, showed the best results with extremely low concentrations (4 g L^{-1}).

Pâques (1991) pointed out that there is a strong connection between culture medium hardiness, proliferation ratio and hyperhydration. Normally, an increase in the agar concentration promotes a reduction in the occurrence of hyperhydration symptoms in plants. However, the propagation rate can be drastically reduced and, consequently, the efficiency of micropropagation (Debergh, 2000).

The concentration of agar in the medium may also affect the formation of roots. Rahman et al. (1992) reported that rooting performance of rose decreased with increasing agar concentration (from 6 up to 15 g L^{-1}). At 6 g L^{-1}, optimal rooting induction was achieved.

An alternative to agar is the use of a gelling agent named gelrite. Gelrite is a gellan gum – a hetero-polysaccharide produced by the bacterium *Pseudomonas elodea* (Kang et al., 1982). Gelrite is an attractive alternative to agar for plant tissue culture because its cost per liter of medium is lower, and it produces a clear gel which facilitates the proper observation of cultures and their possible contamination (George, 1993). Williams and Taji (1987) found that several Australian woody plants survived best on a medium gelled with gelrite rather than agar.

2.1.3 Physical environment

2.1.3.1 Gas exchange and relative air humidity inside the vessel

The response of plant tissue culture *in vitro* can be significantly affected by the gaseous constituents in and adjacent to the culture vessel. Carbon dioxide, oxygen and ethylene are the most frequently studied constituents of the culture atmosphere (Read & Preece, 2003). The culture vessel is usually a closed system, but some gas exchange may occur depending upon the type of vessel, the closure and how tightly they are sealed together.

The sealing of the vessels must allow sufficient ventilation to prevent significant accumulation of ethylene and depletion of CO_2 (Buddendorf-Joosten & Woltering, 1994). Carbon dioxide concentrations inside the vessels alter due to respiration and photosynthesis of the plant. In the dark, CO_2 concentrations increase due to respiration, whereas during the light period the concentration decreases (Buddendorf-Joosten & Woltering, 1994).

The utilization of tightly closed vessels that reduce the gas exchange may affect negatively the normal growth and development of plants during cultivation *in vitro* (Campostrini & Otoni, 1996). Several studies have shown the advantages of using closures with filters or vented vessels, which allow gas exchange, increasing the photosynthetic capacity, the multiplication rate, and the survival of plants after transfer to *ex vitro* conditions (Chuo-Chun et al., 1998; Murphy et al., 1998; Zobayed et al., 2000; Benzioni et al., 2003; Gribaudo et al., 2003; Lucchesini & Mensuali-Sodi, 2004; Park et al., 2004).

The increased availability of CO_2 by using vessels with filter may also influence the amount of photosynthetic pigments. *Nicotiana tabacum* plants grown in vessels with closures with microporous vents (better supplied with CO_2) had higher contents of chlorophyll *a*, *b* and ß-carotene, higher photochemical activity of photosystem II and electron transport chain. Furthermore, plants grown under this condition had higher net photosynthetic rate, lower transpiration rate and stomatal conductance under *ex vitro* conditions than plants grown in glass vessels tightly closed (Haisel et al., 1999).

Water status of cultures is influenced by the growing medium used, the culture vessel itself, and the physical environment. The medium affects water status in various ways, including the gelling agent used (or its absence), the osmotic pressure

(influenced by e.g. salt concentration, amount and type of carbohydrate and quantity and type of other constituents), and changes in the medium with time (Zimmerman, 1995).

It is generally accepted that the relative humidity in the vessel is approximately 98-100% (Altman, 2000). The plants that develop under higher relative humidity *in vitro* have more transpiration and more anatomical abnormalities under *ex vitro* conditions, which may result in high mortality rate during acclimatization.

Therefore, different methods to reduce the relative air humidity inside the vessel have been tested, including the opening of culture containers for some days before acclimatization (Brainerd & Fuchigami, 1981; Kirdmanee et al., 1996), the use of special closures that facilitates water loss (Gribaudo et al., 2003) or the cooling of container bottoms, which increases the condensation of water vapour on the gel surface (Ghashghaie et al., 1992). However, methods to improve gas exchange and reduce relative humidity inside the vessel should be carefully used, in order to prevent excessive water loss during cultivation.

A study carried out with grapevine shoots testing different hole diameters in the vessel closure showed that shoots cultivated in vented vessels were taller than shoots grown in unvented ones, and had higher chlorophyll content. On the other hand, the largest holes (40 mm) caused an excessive water stress. Shoots became more resistant to wilting, but their growth was seriously retarded (Gribaudo et al., 2003). In chrysanthemum, an increase in the mortality of plants as well as a reduction in root formation were found by using silica gel and lanolin oil to reduce air humidity inside the vessel (Wardle et al., 1983).

2.1.3.2 Light

Light is an important environmental factor that controls plant growth and development, since it is related to photosynthesis, phototropism and morphogenesis (Salisbury & Ross, 1994; Read & Preece, 2003). The three features of light, which influence *in vitro* growth, are wavelength, flux density and the duration of light exposure or photoperiod (George, 1993).

Several studies showed that light enhanced root formation and shoot growth (Cabaleiro & Economou, 1992; Cui et al., 2000; Lian et al. 2002; Kumar et al., 2003), whereas in others darkness favored root formation (Hammerschlag, 1982). The

reduced rooting in presence of light is due to the degradation of the endogenous IAA (George, 1993).

Some species may react positively to an increase in the photosynthetic photon flux, especially under photoautotrophic/mixotrophic growing conditions (low sugar levels and CO_2 enrichment).

Limonium grown under photoautotrophic conditions *in vitro* associated with a higher light intensity (200 µmol m^{-2} s^{-1}) had more leaves, higher chlorophyll and sugar contents, higher net photosynthetic rate and percent survival of plants *ex vitro* than plants grown at lower light intensities (50 and 100 µmol m^{-2} s^{-1}) (Lian et al., 2002). Under heterotrophic growing conditions, no significant changes were observed with increasing light intensity. Matysiak & Nowak (1998) investigated the influence of CO_2 concentrations (350 and 1200 µmol mol^{-1}) on the growth of *Ficus benjamina* microcuttings at two light levels (50 and 150 µmol m^{-2} s^{-1}) under *ex vitro* conditions. The increase in CO_2 concentration and irradiance had positive effects on shoot and root fresh weights and leaf area. The elevation of the CO_2 concentration at the lowest light intensity did not increase growth.

2.1.3.3 Temperature

Temperature influence on various physiological processes, such as respiration and photosynthesis, is well known and it is not surprising that it profoundly influences plant tissue culture and micropropagation. The most common culture temperature range has been between 20°C and 27°C, but optimal temperatures vary widely, depending on genotype (Altman, 2000; Read & Preece, 2003).

Horn et al. (1988) studied the effect of different temperatures in the multiplication phase of 14 rose cultivars. The best overall results were obtained at 18°C, but certain ‘thermonegative’ cultivars gave best results at 12°C, while other ‘thermopositive’ cultivars had their optimum at 18°C or 24°C. Alderson et al. (1988) observed that the temperature during the rooting phase affected the timing of root emergency, the final rooting percentage and shoot health. For the cv. ‘Dicjana’, roots emerged first at 25°C and approximately 2 and 4 days later at 20°C and 15°C, respectively, but at 25°C the final rooting percentage was lower. The shoots remained green and looked healthiest at 20°C.

2.1.4 Characteristics of micropropagated plants

2.1.4.1 Anatomy

Culture *in vitro* may result in anatomical abnormalities in leaves, stems and roots of micropropagated plants. It may also affect the major physiological processes, reducing the survival rate of micropropagated plants in the acclimatization phase.

Leaves of plants grown *in vitro* are thinner than leaves formed *ex vitro* and the mesophyll is generally not differentiated into palisade and spongy regions. *In vitro* leaves, in comparison to *ex vitro* ones, have a poorly developed palisade layer with a significant amount of mesophyll air space (Waldenmaier & Schmidt, 1990; Preece & Sutter, 1991; Wetzstein & He, 2000). Moreover, no cuticule is formed and chloroplasts are flattened (Waldenmaier & Schmidt, 1990). In some species, *in vitro* leaves presented open stomata and collapsed guard cells (Romano & Loução, 2003). Furthermore, the internal membrane system of chloroplasts was irregularly arranged and lacked organization into grana and stroma lamellae (Wetzstein & He, 2000).

Shade-leaf structure was observed in transverse sections of cork oak *in vitro* leaves, with large intercellular air spaces and low mesophyll cell density. Leaves from acclimatized plants, in turn, showed a sun-leaf structure with small intercellular air spaces, high cell density and two or three palisade cell layers (Romano & Loução, 2003).

Leaves of certain species, in turn, have a considerably capacity of adaptation to different growing conditions. These changes occur at the subcellular level via modifications in cell ultra-structure and at the cell and tissue levels, where differences in cell number and histological organizations may arise (Wetzstein & He, 2000).

Brutti et al. (2002) found substantial changes in leaf morphology and anatomy of globe artichoke cv. ‘Early French’ during *in vitro* phases and after transferring plantlets to soil (*ex vitro*). *In vitro* leaf surfaces failed to show any epicuticular wax, whereas *ex vitro* leaf surfaces had scarce granular waxes.

During acclimatization *ex vitro* of tobacco plants, stomatal density was considerably decreased on both leaf sides. This decrease was compensated by an increase in stomatal size and length, and in stomatal area. Elongation of stomata was increased, indicating that the originally circular stomata were changed into elliptical ones in

ex vitro acclimatized plants (Tichá et al., 1999). Elliptical stomata were also observed in *ex vitro* leaves of rose (Johansson et al., 1992). Furthermore, stomatal density was higher in leaves formed *in vitro*.

Plants propagated under *in vitro* conditions may develop anatomical abnormalities known as hyperhydration, which can negative influence the micropropagation of several species. Since affected tissues often exhibit a transluced, vitrescent, hyperhydric, and water-soaked appearence, the condition has also been referred as 'vitrification' and 'glassiness' (Pâques, 1991; Wetzstein & He, 2000). Shoots often have shorter internodes and thickened stems. Microscopy investigations showed a reduction in the number of palisade layers, reduced cuticular wax, thin cell walls, defective epidermal tissues and more intercellular spaces in leaves with hyperhydration symptoms (Debergh, 2000) as well as abnormal guard cells of the stomatal apparatus (Ziv & Ariel, 1994).

The functionality of roots formed under *in vitro* conditions is a controversial point in the micropropagation. Several authors stated that roots are not functional when the plant is transferred from the *in vitro* to the *ex vitro* environment. Therefore, the *in vitro* rooting step may be not necessary, since the roots formed *in vitro* die and a new rooting system is formed in the *ex vitro* phase. In fact, some commercial laboratories do not root microcuttings *in vitro*, because it is labor-intensive and expensive (Hazarika, 2003).

On the other hand, several studies have shown the benefits of the formation of roots *in vitro* for the establishment of plants *ex vitro* (Waldenmaier & Bünemann, 1992; Díaz-Pérez et al., 1995; Bosa et al., 2003a). The presence of *in vitro* roots increased water status and improved physiological activity of tissue-cultured apple shoots in the acclimatization, suggesting that *in vitro* roots are functional in water uptake (Díaz-Pérez et al., 1995). These authors suggested that tissue-cultured plants behave as hydraulically integrated units, in which there must be a coordination between control of water loss by the shoot and uptake of water by the root to maintain a favorable plant water balance.

2.1.4.2 Photosynthesis: heterotrophic vs. autotrophic metabolism

Growth, which depends upon sugar in the culture medium as the sole carbon source, is called heterotrophic growth, and growth, dependent upon CO_2 in the air as the sole carbon source for photosynthesis, associated with a higher light intensity, is called photoautotrophic growth. Growth, which depends upon sugar and CO_2, is called photomixotrophic growth (Kozai & Zobayed, 2000).

Plantlets *in vitro* have been considered to have a little photosynthetic ability and to require sugar as carbon and energy source for their heterotrophic or mixotrophic growth (Kozai, 1991). Recent research, however, has revealed that plantlets can have a relatively high photosynthetic ability *in vitro*, being able to grow under photoautotrophic conditions.

Huylenbroeck et al. (1998) and Grout (1988) described two groups of plants according to their photosynthetic response. In the first group, *in vitro* leaves function as storage organs, from which the accumulated reserves are consumed during the first days after transfer to *ex vitro* conditions, until new leaves appear; these *in vitro* leaves never become fully autotrophic. In the second group, *in vitro* leaves are photosynthetically competent.

2.1.5 Substrates *ex vitro*

The substrate, in which the plants develop secondary roots, and the container used are of fundamental importance in the acclimatization phase. The substrate must allow the formation of roots in this phase, but also depends on the type of container used. For acclimatization, trays are used and the reduced height of the cells determines special patterns of drainage, with greater retention of water. These containers are known as plugs and can be defined as the smallest possible volume for the production of an individualized seedling.

The smaller the plug, the higher is the vulnerability to humidity changes, nutrient deficiency, oxygenation, pH and soluble salts (Styer & Koranski, 1997). These authors emphasized that the cell height in the trays influences the drainage due to the effect of the gravity force, with larger amount of water accumulated in the bottom of the plug. This problem can be solved with the use of substrates with porosity above 85%. Styer (1997) mentioned two important characteristics that should be

taken into account when choosing a substrate, namely water holding capacity and aeration space of the substrate. According to this author, the water holding capacity determines the irrigation frequency and is related to the presence of capillary pores. The opposite is the aeration space, which is determined by the distribution of no capillary pores (macropores).

Several substrates can be used for acclimatization of plants produced *in vitro*. Among them, peat is one of the most popular. This substrate is formed from partially decomposed vegetation, due to water excess or oxygen deficiency (Ballester-Olmos, 1993). Perlite is another substrate frequently used for growing plants under *ex vitro* conditions. It is an inorganic growing media component made from aluminium silicate volcanic rock that is mined, crushed, screened to size, then is heated to approximately 1000°C, which causes it to expand into a light-weight, white aggregate (Styer & Koranski, 1997).

2.2 Chlorophyll fluorescence

Chlorophyll fluorescence is a powerful tool that has been applied to study the effects of different stresses and environmental conditions on the photosynthetic process in plants. Therefore, this tool can be applied to investigate the effect of different culture conditions on the photosynthesis of micropropagated plants.

Measurements of chlorophyll fluorescence have been used in micropropagation to evaluate the photosynthetic capacity of plants under different *in vitro* conditions, as different light intensities (Genoud et al., 1999; Serret et al., 2001), sucrose concentrations (Hdider & Desjardins, 1994; Huylenbroeck & Debergh, 1996) and CO_2 availability (Serret et al., 2001). This method can be also used to evaluate the re-establishment of the photosynthetic capacity under *ex vitro* conditions.

Each quantum of light absorbed by a chlorophyll molecule raises an electron from the ground state to an excited state. This excited state is not stable and will relax back to its normal lower energy state (ground state), if there is no electron acceptor available. In this process, the extra energy from the excited state in relation to the ground state can be converted to heat, or released as light (fluorescence) (Harbinson & Rosenqvist, 2003). On the other hand, if the first acceptor in photosystem II ($Quinone_A$) is oxidized, energy transferred to another molecule may ultimately reach the reaction centers, where it is used to drive photochemistry.

In green photosynthetically active plant tissues, light absorbed by photosynthetic pigments (chlorophylls and carotenoids) is primarily used to drive photosynthesis. Under optimum growing conditions, nearly 90% of the absorbed light energy will be dissipated from excited chlorophyll *a* via photosynthetic quantum conversion (Krause & Weis, 1991). Under this condition, only a small proportion of the absorbed light will be de-excited as heat (ca. 5 to 15% of the absorbed energy) and as chlorophyll fluorescence (ca. 0.5 to 8% of the absorbed energy) (Lichtenthaler, 1990). Chlorophyll fluorescence is competing with both photosynthesis and heat dissipation. Therefore, a change in the yield of one process will lead to a corresponding change in the yield of the other two processes.

The chlorophyll fluorescence emission originates nearly exclusively from chlorophyll *a*, since the energy of excited states of chlorophyll *b* is transferred with a high efficiency to the chlorophyll *a* molecules. In addition, the chlorophyll

fluorescence emission at room temperature comes mainly from the chlorophyll *a* at or near the reaction center of photosystem II (Lichtenthaler & Rinderle, 1988).

Several chlorophyll fluorescence parameters are based on the measurement of the chlorophyll fluorescence induction kinetics termed *'Kautsky effect'*. Upon irradiation of a dark-adapted leaf with a saturating flash (> 10000 umol m^{-2} s^{-1}), a rise of chlorophyll fluorescence emission is induced from an initial minimum value termed F_o to a maximum level termed F_m, as shown in Figure 1.

In the dark-adapted state of photosynthesis, $Quinone_A$, the first electron acceptor of photosystem II, normally is fully oxidized and all reaction centers are in the "open state". Upon illumination with a very strong light pulse, $Quinone_A$ rapidly becomes fully reduced ("closed state") (Rohácek & Barták, 1999; Maxwell & Johnson, 2000). Consequently, fluorescence emission rises from the ground fluorescence F_o to a maximum F_m. This initial rise from F_o to F_m is termed "variable fluorescence" (F_v) of the "fast induction kinetics". The higher the variable fluorescence, the higher the photosynthetic capacity of the leaf (Lichtenthaler, 1990).

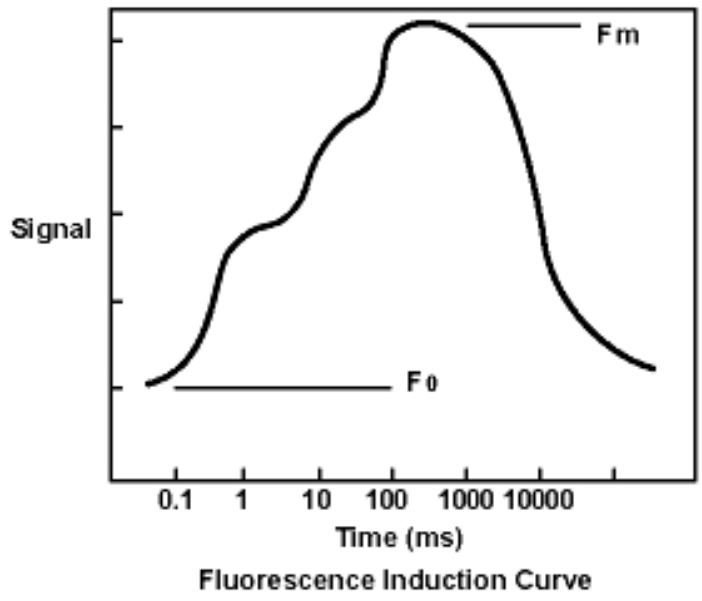

Figure 1. Chlorophyll fluorescence induction kinetics (*Kautsky effect*) upon irradiation of a dark-adapted leaf. Initial fluorescence (F_o) and maximal fluorescence (F_m) are represented (adapted from *http://www.kyokko.com*).

From the fast induction kinetic (Figure 1), several chlorophyll fluorescence parameters can be derived. These parameters reflect changes in the structure of the photosynthetic apparatus and are very sensitive indicators of plant stress. According

to Rohácek & Barták (1999), the analysis of the fast fluorescence induction kinetics can be used e.g. to describe the negative impact of the excessive radiation to the photosystem II functioning.

Dark-adapted values of the ratio F_v/F_m reflect the potential quantum efficiency of photosystem II and are used as an indicator of plant photosynthetic performance (Maxwell & Johnson, 2000).

3 Material and Methods

In order to study the micropropagation as well as the influence of different cultural conditions on this process in the species *Rosa* sp. L. (rose) and *Cynara scolymus* L. (globe artichoke), several experiments were carried out between 2001 and 2004 under controlled environmental conditions (temperature, air humidity and light intensity) in growth chamber and greenhouse.

The experiments were carried out in the Laboratory of Tissue Culture at the Department of Plant Sciences (Chair of Ornamental Plants) of the Technical University Munich (TUM) in Freising, Germany.

The present study was divided in two parts. The first part concerned studies of the micropropagation of rose (*Rosa* sp. L.) and the second one of the micropropagation of globe artichoke (*Cynara scolymus* L.).

3.1 Plant material

3.1.1 Rose (*Rosa* sp. L.)

Three commercial rose cultivars, namely 'Frisco', 'Kardinal' and 'Lambada' were used in this study. Plants of the three rose cultivars were grown in pots in a greenhouse, where stems were collected. The nodes with axillary buds were removed prior to disinfecting them. Afterwards, nodes were disinfected by immersion for 1 min in 70% alcohol, afterwards for 15 min in 1% sodium hypochlorite solution with some drops of Tween 20. This procedure was done in a becker glass using a plate with magnetic stirrer.

The following steps after desinfestation took place under aseptic conditions in a laminar air-flow cabinet in order to obtain an aseptic culture of the selected plant material. The nodes (approximately 2 cm) were rinsed three times with sterile deionized and distillated water and cut on Petri dishes using forceps and scalpel. Thereafter, nodes were put in culture medium for inducing the axillary bud to grow into shoots.

3.1.2 Globe artichoke (*Cynara scolymus* L.)

In the studies of globe artichoke, the cv. 'Green globe' was used. The starting material (axillary shoots) was micropropagated in the Laboratory of Tissue Culture at the Chair of Ornamental Plants (Technical University Munich).

3.2 Culture medium and vessel

The culture medium used was the modified Murashige & Skook (1962) medium. The composition of this medium is shown in Table 1.

Table 1. Composition of the Murashige & Skook (MS) medium.

Macronutrients	**Concentration**
	mg L^{-1}
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
NaFe-EDTA	36.7
Micronutrients	
$MnSO_4.4H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	8.3
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Other ingredients	
Myo-Inositol	10.0
Thiamine	0.1
Pyridoxine	0.5
Nicotinic acid	0.5
Glycine	2.0

Stock solutions for the macronutrients (10-fold concentration) as well as for the micronutrients (100-fold concentration) were used for the preparation of the culture medium. Stock solutions of other ingredients (as e.g. NaFe-EDTA and *myo*-Inositol) were also prepared and stored.

a) Rose

The MS medium was used in the initiation (phase 1), multiplication (phase 2) and rooting phases (phase 3). In all phases, the MS medium was supplemented with agar (6 g L^{-1}) and sucrose (40 g L^{-1}). In the initiation and multiplication phases, the following growth regulators were added to the culture medium: 0.004 mg L^{-1} of auxin (NAA, 1-naphtalene acetic acid), 2 mg L^{-1} of cytokinin (BA, 6-benzylaminopurine) and 0.1 mg L^{-1} of gibberellin (gibberellic acid, GA_3). In the rooting phase, only auxin (NAA) was supplemented to the MS medium at a concentration of 0.1 mg L^{-1}. In this phase, 1/3 of the concentration of macronutrients was used in the culture medium.

b) Globe artichoke

The MS medium was used in the multiplication (phase 2) and rooting phases (phase 3) and was supplemented with agar (6 g L^{-1}) and sucrose (30 g L^{-1}). In the multiplication phase, the following growth regulators were added to the culture medium: 0.2 mg L^{-1} of auxin (NAA, 1-naphtalene acetic acid) and 0.2 mg L^{-1} of cytokinin (BA, 6-benzylaminopurine). In the rooting phase, auxin (NAA) was supplemented to the MS medium at a concentration of 0.5 mg L^{-1}. In this phase, 1/2 of the concentration of macronutrients was used in the culture medium. Additionally, the MS medium for globe artichoke was supplemented with ascorbic acid (10 mg L^{-1}) to inhibit oxidation of phenolic compounds.

In all situations, the growth regulators were added to the culture medium prior to autoclaving. The pH of the medium was adjusted to 5.8 by addition of KOH (1 N) and HCl (1 N) before agar addition and autoclaved with 1.4 bar at 115°C for 15 min.

Explants were cultured in test tubes (height of 15 cm, diameter of 2.5 cm) containing 15 ml of MS medium and closed with glass lid.

The duration of the different micropropagation phases is presented in Table 2.

Table 2. Duration of the different micropropagation phases for rose (*Rosa* sp. L.) and globe artichoke (*Cynara scolymus* L.).

Species	**Duration (weeks)**		
	Initiation	**Multiplication**	**Rooting**
Rose	3	6	5
Globe artichoke	-(1)	8	6

(1) This phase was not carried out in our experiments.

3.3 Ambient conditions *in vitro*

The vessels containing the explants were kept in a growth chamber for cultivation. Cultures were maintained at about 24°C ± 1°C under a 16-h photoperiod of 100 μmol m^{-2} s^{-1}. Artificial light was provided by parallel fluorescent tubes (OSRAM L 36W/32-930) installed above the cultures (Figure 2).

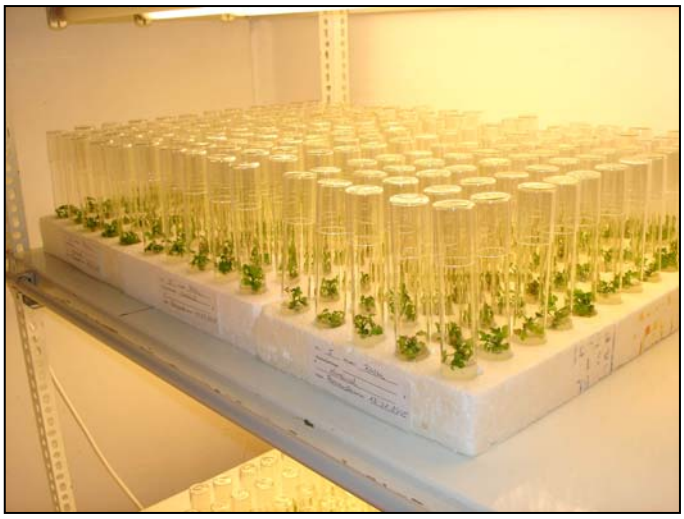

Figure 2. Growth chamber used for the cultivation *in vitro* (left) and culture of explants in the test tubes (right).

3.4 Acclimatization *ex vitro*

After five (rose) and six weeks (globe artichoke) in the rooting phase *in vitro*, plants were transferred to *ex vitro* growing conditions (greenhouse for globe artichoke or growth chamber for *ex vitro* cultivation for rose).

Prior to transplanting, rooted plants were dislodged from the agar medium and roots were washed with tap water. Furthermore, plants were rinsed once with a solution of the fungicide Previcur® N 1.5% (Propamocarb). Thereafter, dead leaves were removed and plants transplanted into the growing media (substrate).

For globe artichoke, the growing media (substrate) was constituted by peat (Floraton® 3, Floragard, Oldenburg, Germany), whereas for rose the substrate was constituted by a mixture of peat and perlite (Hortiperl®, Otavi Minen AG, Bülstringen, Germany), in a proportion of 1peat:3perlite (v/v).

Plants of both species were grown in trays (4 cm height) with 150 cells. Globe artichoke was acclimatizated in greenhouse under ambient daylight conditions with a temperature of about 25°C. Immediately after transferring the plants to the greenhouse, trays were initially covered for two weeks with a glass plate and shaded with paper for one week, in order to maintain high air humidity and to prevent photoinhibition, since light intensity under *ex vitro* conditions (greenhouse) is much higher than in the *in vitro* cultivation.

For rose, cultivation *ex vitro* (acclimatization) was performed in a growth chamber (Figure 3) with a constant room temperature of 22°C, light intensity of 60 μmol m^{-2} s^{-1} and photoperiod of 16h/8h day/night. The air humidity was maintained at about 85%.

The description of the cultural conditions showed in the Sections 3.2, 3.3 and 3.4 of this Chapter are standard conditions. However, these conditions may vary between the different experiments. In this case, the different conditions are presented in the description of each experiment (Section 3.5).

Figure 3. Growth chamber and tray used for the cultivation *ex vitro* of rose.

3.5 Description of experiments and treatments

The number of plants cultivated per repetition (block) varied between 8 and 15 plants and each treatment was repeated between 4 and 6 times.

3.5.1 Rose (*Rosa* sp. L.)

3.5.1.1 Gelling agent

In this experiment, treatments consisted of two different gelling agents (agar and gelrite) and two different concentrations of them in the culture medium. The concentrations of agar (Agar Merck 1614®) tested were 6 and 9 g L^{-1} and of gelrite (Phytagel Sigma 8169®) were 2 and 3 g L^{-1}. These treatments were applied to the cvs. 'Frisco' and 'Lambada'. This experiment was conducted during the phases 1 (initiation of culture), 2 (multiplication) and 3 (rooting) with the mentioned treatments. In the initiation phase, only the parameter number of shoots per plant was determined.

3.5.1.2 Type of closure of vessels and sucrose concentration

In order to study the effect of different types of closure of vessels and sucrose concentrations on the growth of rose, two experiments were carried out.

a) Type of closure of test tubes

In this experiment, four different types of closure for the test tubes (glass, aluminium, plastic film and steristop) (Figure 4) were tested in the rooting phase on three rose cultivars ('Frisco', 'Kardinal' and 'Lambada') in a factorial experiment 4x3. Evaluations were carried out only during the rooting phase (phase 3).

Figure 4. Types of closure tested in the test tubes.

b) Sucrose concentration and cover with filter

Baby food culture jars covered with Magenta B-CAP with filter (Sigma B-3031) or without filter (Sigma B-8648) were compared (Figure 5). Moreover, different sucrose concentrations (10, 20 and 40 g L^{-1}) in the culture medium in the rooting phase (phase 3) were tested on the cv. 'Kardinal', resulting in a factorial experiment 2x3. Evaluations were done in the rooting as well as in the acclimatization phases.

Figure 5. Baby food culture jars covered with Magenta B-CAP with filter (left) or without filter (right).

3.5.1.3 Room temperature

The treatments consisted of different air temperatures in the rooting phase (phase 3), namely 16, 20 and 24°C. The effect of room temperature was tested on three rose cultivars (‘Kardinal’, ‘Frisco’ and ‘Lambada’) (factorial 3x3). Evaluations were done in the rooting phase.

3.5.1.4 Duration of the rooting phase

Concerning the duration of the rooting phase, two experiments were carried out.

a) Two, three, four, five and six weeks on rooting medium

In this experiment, treatments consisted of five different durations of the rooting phase, namely two, three, four, five and six weeks. The rose cultivars studied were ‘Kardinal’, ‘Frisco’ and ‘Lambada’ (factorial experiment 5x3)

b) One, three and five weeks on rooting medium

In this experiment (only cv. ‘Kardinal’), treatments consisted of three different durations of the rooting phase, namely one, three and five weeks. Evaluations were carried out in the rooting as well as in the acclimatization phases. In the rooting phase, plants were grown in culture medium supplemented with 10 g L^{-1} of sucrose.

A schematic representation of the organization of this experiment is shown in Figure 6.

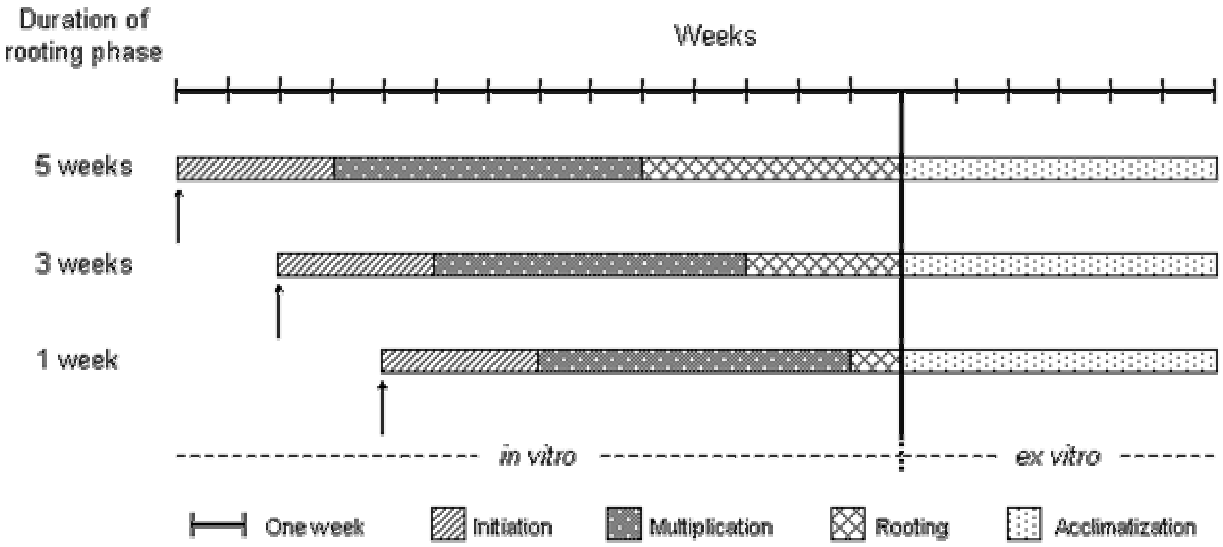

Figure 6. Schematic representation of the organization of the different treatments concerning the duration of the rooting phase. Arrows indicate the beginning of each treatment.

3.5.1.5 Consistency of the medium, auxin concentration and container type

In order to study the effect of medium consistency, auxin concentration and container type on the growth of rose cv. 'Kardinal' in the rooting phase, two experiments were carried out. The culture medium was supplemented with 10 g L^{-1} of sucrose.

a) Consistency of the medium and auxin concentration

Treatments consisted of two different consistencies of the culture medium (liquid or solidified with agar) and two concentrations of auxin (NAA, 1-naphtalene acetic acid) in the medium (0.1 and 0.5 g L^{-1}), resulting in a factorial experiment 2x2. Plants were grown in vessels with a capacity of 750 ml (Weck®, Germany) at a density of 5 plants per vessel. The volume of culture medium used in each vessel was 200 ml.

In this experiment, plants were grown in liquid medium using a hydroponic floating system described in Battke et al. (2003). This system is based on the use of polyethylene granulated (PE), as shown in Figure 7. In each vessel, 60 g of PE was used.

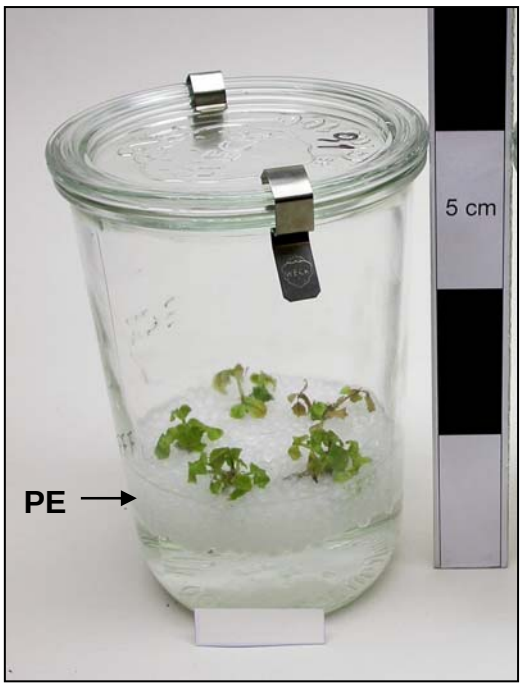

Figure 7. Plants growing in liquid medium with a hydroponic floating system based on the use of polyethylene granulated (PE).

b) Type of container

The performance of two different types of container, namely test tube and vessel with a capacity of 750 ml (Weck®, Germany), was compared in this experiment. Plants were grown in agar-solidified medium with a concentration of 0.1 mg L^{-1} NAA.

3.5.1.6 Substrates in the acclimatization phase

The treatments consisted of different substrates with different proportions of peat and perlite (per), namely 100% peat (pure peat) and three mixtures with different proportions of peat and perlite (v/v):

- 3peat:1per (75% peat, 25% perlite)
- 1peat:1per (50% peat, 50% perlite)
- 1peat:3per (25% peat, 75% perlite).

Moreover, two different light intensities were tested in the *ex vitro* environment (60 and 120 μmol m^{-2} s^{-1}). The rose cv. 'Kardinal' was used. The experiment was arranged in a 4x2 factorial.

The water retention function (water holding capacity) of the different substrate mixtures was determined in this study using the method described in Hartge & Horn (1989).

In this method, the substrates samples are placed on a ceramic plate in a pressure chamber. The samples are then consecutively submitted to different gas pressures and reweighed after each pressure step. The gas pressures used in the present study were 10, 50 and 100 hPa.

Based on the amount of water remaining in the substrate sample after the application of the respective pressure (determined by reweighing the samples), the following parameters (%) were calculated: solid material (100 – total porosity), air space (difference between total porosity and the moisture content at 10 hPa tension), easily available water (quantity of water released from the sample when the suction increases from 10 to 50 hPa), water buffering capacity (quantity of water released from the sample when the suction increases from 50 to 100 hPa) and less readily available water (quantity of water that remains in the sample after a tension of 100 hPa).

This determination was done at the Chair of Soil Science of the Technical University Munich (Freising, Germany).

3.5.2 Globe artichoke (*Cynara scolymus* L.)

3.5.2.1 Growth regulators: auxin and gibberellin

Treatments were arranged in a factorial 2x3 and consisted of different concentrations of auxin (NAA) and gibberellin (GA_3) in the culture medium in the rooting phase:

- 0.5 and 2.0 mg L^{-1} NAA (1-naphtalene acetic acid)
- without GA_3, 1.0 and 5.0 mg L^{-1} GA_3 (gibberellic acid)

The experiment was conducted in the rooting as well as in the acclimatization phase. The transfer of plants to a second rooting medium was necessary to promote the growth of plants, since plants did not show a satisfactory growth at the end of the "normal" rooting phase *in vitro* (first rooting medium). The composition of this second rooting medium was similar for all treatments and no growth regulator was supplemented to the culture medium. Plants were kept for five weeks in this medium. In the multiplication phase, plants were grown in culture medium supplemented with 2.0 mg L^{-1} BA + 2.0 mg L^{-1} NAA.

3.5.2.2 Growth regulators (auxin and cytokinins) and duration of the rooting phase

Treatments consisted of different auxin (NAA) and cytokinin (BA, Kinetin and 2iP) concentrations in the culture medium in the phase 2 (multiplication):

a) 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA (6-benzylaminopurine)

b) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA

c) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin

d) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP (2-isopentenyladenine)

Moreover, the effect of different durations of the rooting phase was tested (two, four and six weeks). In the rooting phase, plants were cultivated in the same culture medium with 0.1 mg L^{-1} NAA. This experiment was carried out during the multiplication, rooting and acclimatization phases.

3.5.2.3 Growth regulators: auxin

In this experiment, different auxin concentrations (NAA) in the rooting phase were tested:

- without NAA
- levels of 0.1, 0.5, 1.0 and 2.0 mg L^{-1} NAA.

This experiment was carried out during the rooting and acclimatization phases.

3.5.2.3 Sucrose concentration, type of vessel closure and light intensity

Two experiments were carried out to test the influence of different sucrose concentrations, type of vessel closure and light intensity on the growth of globe artichoke in the rooting phase.

a) Sucrose concentration and type of vessel closure

Plants were grown in baby food culture jars covered with Magenta B-CAP with filter (Sigma® B-3031) or without filter (Sigma® B-8648). Moreover, the effect of different sucrose concentrations in the culture medium (5, 30 and 60 g L^{-1}) was tested, resulting in a factorial experiment 2x3.

b) Sucrose concentration and light intensity

In this experiment, the sucrose concentrations tested in the rooting phase were 5 and 30 g L^{-1}. Moreover, the effect of two different light intensities was studied, namely 110 and 210 μmol m^{-2} s^{-1} (factorial experiment 2x2). In the first week of the rooting phase, all plants were grown under a light intensity of 110 μmol m^{-2} s^{-1}. Afterwards, the treatments of light intensity were applied.

3.6 Parameters evaluated

3.6.1 Description of the parameters

a) Quality score of shoots

The growth of shoots was evaluated by means of quality score degrees. The quality scores can vary between 1 and 9 and are described in Table 3.

Table 3. Description of the quality score degrees used for the evaluation of shoot growth of rose and globe artichoke.

	Quality score	Characteristics of shoots
worse	**1**	dead plant, hyperhydration symptoms
↓	**3**	many dead leaves, presence of yellow-green leaves, hyperhydration symptoms
↓	**5**	some dead leaves, presence of green leaves, reduced growth
↓	**7**	few dead leaves, presence of dark-green leaves, good shoot growth
better	**9**	few dead leaves, presence of dark-green leaves, very good shoot growth

Figures 8 (rose) and 9 (globe artichoke) show the quality score degrees used for the evaluation of shoot growth.

Figure 8. Quality score degrees of shoots in relation to the growth of rose.

Figure 9. Quality score degrees of shoots in relation to the growth of globe artichoke.

b) Number of leaves per plant

Average number of leaves per plant was determined by counting the number of fully developed leaves (with all three folioles unrolled) formed in each plant and then determining the mean number of leaves per plant for each treatment.

c) Chlorophyll content

The extractable chlorophyll content was spectrophotometrically determined. The photosynthetic pigments (chlorophylls) were extracted with a solution of acetone and bi-distillated water. The absorbance of the samples was measured at different wavelengths (646, 652 and 663 nm), using a photospectrometer (Biochrom 4060, Cambridge, UK).

The extractable chlorophyll content (expressed on leaf fresh weight basis) was calculated using the extinction coefficients and the following equations proposed by Schopfer (1989), where *'A'* represents the absorbance of the sample:

Total chlorophyll (mg L^{-1}) = $17.3*A_{646} + 7.18*A_{663}$

Chlorophyll *a* (mg L^{-1}) = $12.21*A_{663} – 2.81*A_{646}$

Chlorophyll *b* (mg L^{-1}) = $20.13*A_{646} – 5.03*A_{663}$

d) Hyperhydration rate

Hyperhydration rate was determined by counting the number of plants showing hyperhydration symptoms in comparison to the total number of plants of each treatment.

e) Dry weight determination of shoots and roots

Dry weight of shoots and roots was determined after drying the samples at 65^{o}C during 72 h.

f) Histological analysis of leaf anatomy

For studying leaf anatomy, leaves were prepared by the method described in Gutmann and Feucht (1991).

Immediately after cutting the leaves, samples were transferred into a fixation solution containing 2.5% glutaraldehyde in 0.1 *M* phosphate buffer at pH 7.5. The fixation vessels were exposed to a moderate vacuum of about 50 mbar for 30 minutes to remove the intercellular air. Afterwards, samples were dehydrated as follows:

1. Phosphate buffer
2. 50% ethanol
3. 70% ethanol
4. 99% ethanol

The treatment duration for each step was 30 min. After dehydration, samples were infiltrated with glycolmethacrylate (Historesin, Reichert-Jung) and ethanol in a proportion 1:1 (v/v) for 2 h and subsequently with glycolmethacrylate for 48 h.
Afterwards, transversal sections were cut on a Leitz microtom 1400 equipped with a carbide knife at a thickness of 2-3 μm at the Department of Plant Sciences (Unit of Fruit Science) of the Technical University Munich (Freising, Germany). All sections were stained with toluidine blue using the procedure described in Gerlach (1969).
The transversal sections were studied and photographed using a Zeiss light microscope equipped with a digital camera.

g) Chlorophyll fluorescence

Chlorophyll fluorescence was measured on the upper surface of the third leaf below the last leaf emitted by the plant using a pulse-modulated fluorometer (Photosynthesis Yield Analyzer Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). Fluorescence was excited by pulse modulated red light from a light-emitting-diode (LED). The pulse-width was 3 μs and pulse frequency is 0.6 kHz. The LED-light was passed through a cut-off filter, resulting in an excitation band peaking at 650 nm. Fluorescence was detected with a PIN-photodiode at wavelengths beyond 700 nm, as defined by a long-pass filter (type RG 9, Schott). This device was operated in conjunction with a PC and the software WinControl for automatic data acquisition.
Values of the parameters F_o (initial fluorescence) and F_m (maximum fluorescence) were determined on attached leaves placed into a leaf-clip holder (for defined positioning of the fiberoptics relative to the leaf plane) and a shutter plate was closed, so the leaf could be dark adapted.
The initial (or minimal) chlorophyll fluorescence (F_o) was measured after 15 min of dark-adaptation of the sample and recorded at very low photon flux density (0.15 μmol m^{-2} s^{-1}). The maximal chlorophyll fluorescence level (F_m) was obtained after a short saturation pulse of very high photon flux density (> 10000 μmol m^{-2} s^{-1}).
Maximum photochemical efficiency (potential quantum yield of PS II) of dark-adapted leaves was calculated using the following equation:
Maximum photochemical efficiency = F_v/F_m
with F_v being the variable fluorescence, calculated as F_m-F_o. F_o is the dark-level fluorescence (initial fluorescence) yield measured briefly before onset of the

saturation pulse, whereas F_m is the maximum fluorescence yield of a dark-adapted sample reached during the saturation pulse. The fluorescence ratio F_v/F_m is a measure of the potential quantum yield of the dark-adapted leaf.

h) Quality score of roots (only for rose)

The growth of roots was evaluated by means of quality score degrees. The quality scores can vary between 1 and 9 and are related to the length of roots formed. A description of the quality score of roots is presented in Table 4.

Table 4. Description of the quality score degrees used for the evaluation of root growth in rose.

	Quality score	Characteristic of roots (length)
worse	**1**	no root formation
↓	**3**	roots < 1 cm length
	5	roots between 1 and 2 cm length
	7	roots between 2 and 3 cm length
better	**9**	roots > 3 cm length

i) Number of roots per plant

Average number of roots was determined by counting the number of roots (> 1 mm length) formed in each plant and then determining the mean number of roots for that treatment.

j) Rooting percentage

Rooting was expressed in terms of rooting percentage. This percentage was determined by counting the number of plants that rooted compared with the total number of plants.

k) Callus formation

The formation of callus at the base of shoots in globe artichoke was evaluated by means of quality score degrees. The quality scores for callus formation varied between 1 and 5 (Table 5).

Table 5. Description of the quality score degrees used for the evaluation of callus formation in globe artichoke.

	Quality score	Characteristic of callus
worse	**1**	Formation of callus > 1 cm diameter
↓	**3**	Formation of callus < 1 cm diameter
better	**5**	no callus formation

l) Contamination rate of the culture medium

Contamination rate was determined by counting the number of vessels contaminated with fungi in comparison to the total number of vessels of each treatment.

m) Mortality rate of plants

Mortality rate was determined by counting the number of plants that died at the end of the respective phase in comparison to the total number of plants of each treatment.

3.6.2 Plant parameters determined in the experiments with rose (*Rosa* sp. L.)

Experiment	Parameters		
	Multiplication (phase 2)	**Rooting (phase 3)**	**Acclimatization (phase 4)**
Gelling agents	Quality score of shoots Number of shoots plant^{-1}	Quality score of shoots Hyperhydration rate	-
Type of closure	-	Quality score of shoots Quality score of roots Number of roots plant^{-1} Contamination rate Mortality rate of plants	-
Sucrose concentration and filter	-	Quality score of shoots Quality score of roots Number of roots plant^{-1} Chlorophyll content	Quality score of shoots Mortality rate of plants
Room temperature	-	Quality score of shoots Quality score of roots Number of roots plant^{-1} Chlorophyll content Histological analyses	-
Duration of the rooting phase (two up six weeks)	-	Quality score of shoots Quality score of roots Number of leaves plant^{-1} Number of roots plant^{-1}	-
Duration of the rooting phase (one up five weeks)	-	Quality score of shoots Quality score of roots Number of leaves plant^{-1} Number of roots plant^{-1}	Mortality rate of plants
Consistency of the medium and auxin concentration	-	Quality score of shoots Quality score of roots Number of roots plant^{-1} Chlorophyll content	Mortality rate of plants
Type of container	-	Quality score of shoots Quality score of roots Number of roots plant^{-1}	-
Substrates in the acclimatization	-	-	Quality score of shoots Dry biomass Chlorophyll content Mortality rate of plants

3.6.3 Plant parameters determined in the experiments with globe artichoke (*Cynara scolymus* L.)

Experiment	Parameters		
	Multiplication (phase 2)	**Rooting (phase 3)**	**Acclimatization (phase 4)**
Auxin and gibberellin	-	Quality score of shoots Rooting percentage	Mortality rate of plants
Auxin and cytokinin and duration of the rooting phase	Quality score of shoots Number of shoots plant^{-1} Quality score of callus	Quality score of shoots Rooting percentage Quality score of callus	Mortality rate of plants
Auxin	-	Quality score of shoots Rooting percentage Quality score of callus	Mortality rate of plants
Sucrose concentration and type of vessel closure	-	Quality score of shoots Rooting percentage Chlorophyll fluorescence	-
Sucrose concentration and light intensity	-	Quality score of shoots Rooting percentage Chlorophyll fluorescence	-

For both species, the evaluation of the different parameters in the multiplication and rooting phases was done always at the end of the respective phase. In the acclimatization phase, in turn, evaluations were done at 6 weeks after onset of this phase (if not, otherwise specified).

3.7 Statistical analysis

Analysis of variance (ANOVA) was performed to test the significance of the differences between treatments. When significant differences were found ($P \leq 0.05$), a multiple comparison test of means (Duncan's test) was calculated. Furthermore, correlation and regression analysis were performed to evaluate the relationship between the investigated variables. For the calculations, the software SPSS (Statistical Package for the Social Sciences) Version 12.0 (LEAD Technologies Inc., Chicago, USA) was used. For the statistical evaluation of frequencies (as e.g. mortality rate), data were analyzed by the chi-square test (χ^2), using the procedures described in Gomez & Gomez (1984) and Gomes (1990).

4 Results and Discussion

4.1 Rose (*Rosa* sp. L.)

4.1.1 Gelling agent

In this experiment, two types of gelling agents (agar and gelrite) and two different concentrations of them in the culture medium were tested. Gelrite have the strength equivalent to agar at approximately ½ - ¼ the concentration (George, 1993), so that the concentrations used in the experiment were 6 and 9 g L^{-1} of agar and 2 and 3 g L^{-1} of gelrite. These treatments were applied to two rose cultivars ('Frisco' and 'Lambada') and the experiment was carried out during the phases 1 (initiation of culture), 2 (multiplication) and 3 (rooting). This experiment was repeated and the observed results were similar. Therefore, the results here presented are from the first experiment.

a) Initiation and multiplication phases

In the initiation phase, the parameter number of shoots per plant was not significantly affected by the gelling agent present in the culture medium for both cultivars (data not shown). In contrast, in the multiplication phase, the gelling agent used in the medium significantly influenced the parameters number of shoots per plant and quality score in both cultivars studied (Figure 10).

For both parameters, the interaction between the factors gelling agent and cultivar was not significant ($P>0.05$). Consequently, only the main effect of gelling agent is shown (Figure 10).

The explants grown in medium solidified with agar produced more shoots per plant (Figure 10.a) and showed higher quality score (Figure 10.b) in comparison to explants grown in medium with gelrite. Plantlets grown in medium with agar had a quality score that was approximately three times higher than with gelrite (Figure 10.b).

In relation to the different agar concentrations tested, explants grown at 6 g L^{-1} produced more shoots per plant (Figure 10.a) and showed better quality score (Figure 10.b) when compared to explants grown at 9 g L^{-1} of agar in the medium.

Ghashgaie et al. (1991) studied the effect of different agar concentrations in the medium (0, 4, 5.5, 6, 7.5, 8, 9 and 15 g L^{-1}) on water status and growth of rose cv. 'Madame G. Delbard' cultured *in vitro*. These results support the use of moderate agar concentration (around 7.5 g L^{-1}), that allow good multiplication rate and good growth. Leaf osmotic potential also declined with increasing agar concentration. Simultaneous decrease of leaf water and osmotic potentials maintained leaf turgor pressure.

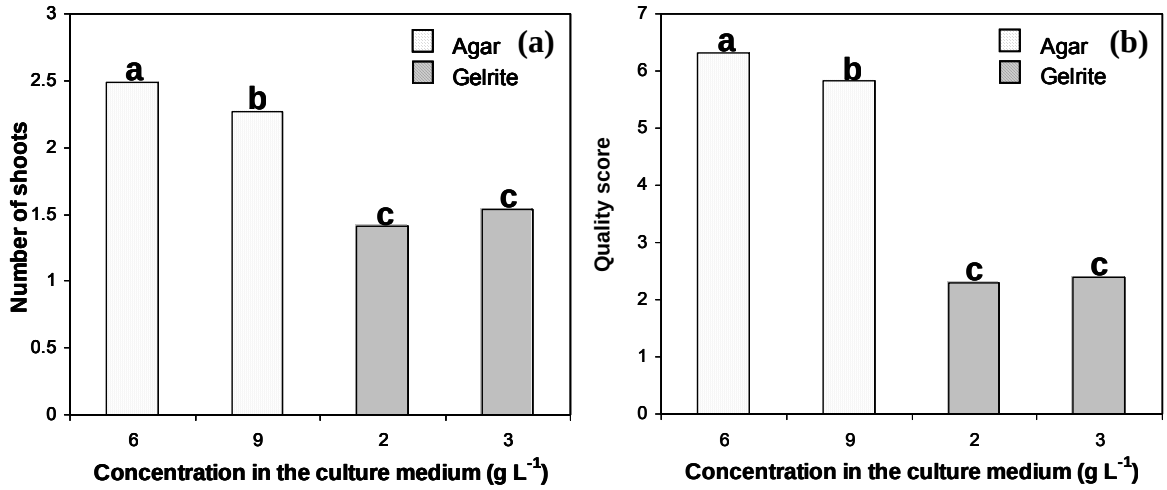

Figure 10. Number of shoots per plant (a) and quality score of shoots (b) in the multiplication phase of rose grown under different concentrations of the gelling agents agar and gelrite in the culture medium. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=120).

The higher quality score and number of shoots per plant for the concentration of 6 g L^{-1} of agar in relation to 9 g L^{-1} may be explained by an increase of medium strength with increasing agar concentration.

The diffusion of nutrients to shoots may decrease with increasing agar concentration in the medium (Torres et al., 2001), resulting in lower nutrient availability and, consequently, a reduction in growth.

Moreover, an agar-solidified medium has a lower water potential (more negative) than its liquid equivalent (Debergh et al., 1981). Consequently, the availability of

medium ingredients to tissues cultured on agar will be reduced by the medium having a decreased water potential, and an increased viscosity (which will limit diffusion). The decrease in water potential resulting from the matric potential of agar will limit uptake, since nutrient uptake is closely associated with the rate of water influx into tissues (George, 1993). The lower uptake of nutrients could explain the lower development of plants with increasing the agar concentration in the culture medium.
Another factor concerns the lowered availability of cytokinin in culture medium with high concentration of agar, resulting in a low propagation rate (Debergh et al., 1981). Furthermore, Romberger & Tabor (1971) suggested that the reduced growth might be caused by the immobilization of the enzyme invertase released from culture tissues, resulting in a reduced availability of glucose and fructose.

b) Rooting phase

In this phase, plants of both cultivars ('Frisco' and 'Lambada') grown in medium with agar showed better quality scores than shoots of plants grown in medium with gelrite. Furthermore, the quality score of shoots was not significantly different between the two agar as well as between the two gelrite concentrations tested (Table 6).
For the cultivar 'Frisco', the quality score of shoots grown in medium with agar was approximately 4.4 (mean value of the concentrations 6 and 9 g L^{-1}), whereas it decreased up to 1.4 when using gelrite as gelling agent in the culture medium (mean value of both gelrite concentrations). For 'Lambada', quality scores of shoots varied between approximately 5.5 in agar and 3.3 in gelrite (Table 6).

Table 6. Quality score of shoots in the rooting phase of two rose cultivars grown under different concentrations of the gelling agents agar and gelrite in the culture medium(1) (*n*=120).

Cultivar	**Gelling agent (g L^{-1})**			
	Agar		**Gelrite**	
	6	**9**	**2**	**3**
	quality score			
'Frisco'	B 4.57 a(2)	B 4.21 a	B 1.27 b	B 1.51 b
'Lambada'	A 5.68 a	A 5.38 a	A 3.25 b	A 3.32 b

(1) see Material and Methods for a description of the quality score of shoots (Table 3).
(2) Means followed by the same capital letter (A,B) in the column or by the same small letter (a,b) in the row are not significantly different according to Duncan's Test (P=0.05).

Furthermore, the number of plants showing hyperhydration symptoms was reduced with increasing the agar concentration in the growing medium (Table 7), although the reduction of the hyperhydration rate with increasing the agar concentration from 6 up to 9 g L^{-1} was significant only for the cultivar 'Lambada'. For 'Frisco', the occurrence of hyperhydration was not significantly affected by the agar concentration. In relation to the use of different concentrations of gelrite, no difference could be observed between the levels of 2 and 3 g L^{-1} on the hyperhydration rate in both cultivars (Table 7).

Raising the agar concentration reduced hyperhydration of *in vitro* plants, but may result in a drastically lower propagation ratio (number of shoots), as shown by Debergh et al. (1981). If the agar concentration is increased, it is more difficult for explants to make contact with the medium, which limits the uptake of nutrients (Pierik, 1997).

Table 7. Hyperhydration rate *in vitro* in the rooting phase of two rose cultivars grown under different concentrations of the gelling agents agar and gelrite in the culture medium (*n*=120).

Cultivar	**Gelling agent (g L^{-1})**					
	Agar		**Influence of**	**Gelrite**		**Influence of**
	6	**9**	**agar conc.**	**2**	**3**	**gelrite conc.**
	%	%	$\chi 2$	%	%	$\chi 2$
'Frisco'	20[(1)]	16	ns	97	97	ns
'Lambada'	8	2	*	65	60	ns
Influence of cultivar ($\chi 2$)	**	****		****	****	

[(1)] For the comparison of the hyperhydration rate between different concentrations of the same gelling agent at the same cultivar as well as between cultivars at the same gelling agent and concentration, the chi^2-test was used.

ns not significant

* Significant at 10%

** Significant at 5%

**** Significant at 0.1%

The treatments in which the plants were grown with gelrite as gelling agent in the culture medium showed a higher occurrence of hyperhydration in plants (Table 7) as well as the formation of shoots with lower quality score in the phases 2 (see

Figure 10.b) and 3 (see Table 6), in comparison to shoots grown in medium solidified with agar.

In some situations, the use of gelrite can increase the number of plants presenting hyperhydration symptoms (George, 1993; Debergh, 2000; Torres et al., 2001). In apple, Pasqualetto et al. (1988) showed that the use of gelrite increased the occurrence of hyperhydration of leaves and stems, when compared to agar, even though the apparent gel firmness was similar.

In relation to the gelrite concentration in the culture medium, no significant differences were observed for quality score of shoots between both concentrations tested in the phases 2 (see Figure 10.b) and 3 (see Table 6).

On the other hand, Cuzzuol et al. (1995) reported a decrease in the hyperhydration occurrence in *Dianthus caryophylus* with increasing the concentration of gelrite in the medium. They observed that the occurrence of abnormal shoots was reduced from 80% down to 8.4% with an increase in the gelrite concentration from 2.5 up to 9.0 g L^{-1}. In our study, only two gelrite concentrations were tested (2 and 3 g L^{-1}) and no significant difference was observed between both treatments. It is possible that the difference of strength of the culture medium between both concentrations of gelrite tested was not high enough to influence significantly the development of plantlets. An increase in the gelrite concentration beyond the levels used in our experiments (for example, up to 9 g L^{-1}) could show a positive effect on the growth of rose.

Another important parameter evaluated was the rooting *in vitro*. For this parameter, the interaction between the factors cultivar and gelling agent was significant, so that the simple effects of cultivar and gelling agent are shown (Table 8). Similarly as observed for the parameter quality score of shoots, plants grown in the medium solidified with agar presented better root development in comparison to plants grown in gelrite, as shown in Table 8. No significant differences in relation to root development were observed between the two agar concentrations used, neither between the two gelrite concentrations (Table 8).

Significant differences in relation to root development were observed between the cultivars ‘Lambada’ and ‘Frisco’ (Table 8). The cultivar ‘Lambada’ presented higher growth capacity and regeneration of roots in comparison to the cultivar ‘Frisco’ when using agar as gelling agent in the medium. Furthermore, the formation of roots in

both cultivars was negatively affected by the use of gelrite as gelling agent, independent of the concentration (Table 8).

The challenge is, in fact, the establishment of a concentration of gelling agent in the culture medium that optimizes nutrient absorption and plant growth, while avoiding the appearance of plants with hyperhydration symptoms. Agar is one of the most expensive components of the solid nutrient media, so that the use of 6 g L^{-1} of agar is recommended for roses. Moreover, one can state that gelrite (in the concentrations of 2 and 3 g L^{-1}) is not a good option as gelling agent in culture medium for the micropropagation of the rose cultivars tested in our experiment.

Taking into consideration the results obtained for the phases 2 and 3, the cv. 'Lambada' presented a better performance than the cv. 'Frisco' across all parameters evaluated. 'Lambada' showed higher number of shoots in the phase 2 (data not shown) as well as better quality of plants in the phases 2 (data not shown) and 3 (see Table 6) and lower hyperhydration rate (see Table 7) in comparison to 'Frisco'.

Table 8. Formation of roots *in vitro*, as evaluated by the quality score of roots, of two rose cultivars grown under different concentrations of the gelling agents agar and gelrite in the culture medium[(1)] (*n*=120).

Cultivar	**Gelling agent (g L^{-1})**			
	Agar		**Gelrite**	
	6	**9**	**2**	**3**
	quality score			
'Frisco'	B 2.4 a[(2)]	B 2.6 a	A 2.2 b	A 2.2 b
'Lambada'	A 3.4 a	A 3.6 a	A 2.1 b	A 2.1 b

[(1)] see Material and Methods for a description of the quality score of roots (Table 4).
[(2)] Means followed by the same capital letter (A,B) in the column or by the same small letter (a,b) in the row are not significantly different according to Duncan's Test (P=0.05).

4.1.2 Type of closure of vessels and sucrose concentration

When cultivated under *in vitro* conditions, plants grow under special conditions of high relative air humidity and low radiation. Moreover, sucrose is added to the culture medium (Pospíšilová et al., 1999). These factors promote an increase in the multiplication rate, but may induce the occurrence of anatomical, morphological and physiological disorders. Furthermore, plants grown under these conditions are usually unable to become autotrophic.

In order to study the effect of different types of closure of vessels and sucrose concentrations on the quality score of shoots and roots as well as on the number of roots per plant, two experiments were carried out.

a) Type of closure of test tubes

In 2003, some problems related to the attack of thrips in the growth chambers of our Department occurred. These insects were capable to enter the test tubes, thus contaminating the culture medium.

In this sense, an experiment was carried out with the objective to find a reliable closure type to avoid the entrance of thrips in the test tubes, without affecting the growth of plants. Furthermore, the use of a reliable lid in the test tubes may increase gaseous changes, having a positive effect in the acclimatization of plants, since plants may become more autotrophic.

In this experiment, four different types of closure for the test tubes (glass, aluminium, plastic film and steristop) were tested on three cultivars (‘Frisco’, ‘Kardinal’ and ‘Lambada’). The cv. ‘Kardinal’ was included in this experiment for comparison to the two other cultivars, since ‘Kardinal’ presents a good development *in vitro*. Evaluations were carried out only during the rooting phase.

The interaction between the factors cultivar and type of closure was significant for the parameter quality score of shoots (Table 9). The best results were observed when using glass, steristop or aluminium as closing material for the cultivars ‘Kardinal’ and ‘Lambada’. For the cultivar ‘Frisco’, the best results related to the quality score of shoots were found by using glass or steristop as closure (Table 9).

The plants grown in test tubes closed with glass lids showed the highest quality of shoots, though not significantly different from steristop and aluminium closures

(except for the cultivar 'Frisco'), as shown in Table 9. On the other hand, the greatest occurrence of fungal contamination (most likely due to thrips attack) was observed when using glass as closure (Table 10).

The greater contamination of the test tubes closed with glass may be related to the fact that the penetration of the insects was facilitated, since the glass lid was adjusted in the test tubes without being sealed with parafilm. Our supposition is that the insects were able to move the glass lid and enter the tubes or that the penetration of insects occurred due to irregularities in the contact between the glass lid and the upper part of the tubes. The contaminated test tubes were progressively removed from the growth chambers, in order to control the proliferation of thrips.

Table 9. Quality score of shoots in the rooting phase of three rose cultivars as affected by the type of closure used in the test tubes[1] (*n*=50).

Cultivar	Type of closure			
	Glass	**Steristop**	**Aluminium**	**Plastic film**
	quality score			
'Frisco'	C 2.69 a[2]	C 2.47 a	C 2.25 ab	C 1.90 b
'Kardinal'	A 6.31 a	A 5.86 a	A 5.81 a	A 4.02 b
'Lambada'	B 4.92 a	B 4.82 a	B 4.72 a	B 3.43 b

[1] see Material and Methods for a description of the quality score of shoots (Table 3).
[2] Means followed by the same capital letter (A,B,C) in the column or by the same small letter (a,b) in the row are not significantly different according to Duncan's Test (P=0.05).

The lowest contamination rate by fungi was observed when using plastic film as closure (Table 10). Actually, no contamination was observed in this situation, since the plastic film prevented the entrance of thrips, due to the tightly closure. However, plants growing in test tubes closed with plastic film showed lower quality scores for shoots (Table 9) and early senescence symptoms, when compared to the other types of material. It may indicate that ethylene accumulated inside the test tubes closed with plastic film.

The increase in the ethylene level in glass tubes with sealed caps was shown by Horn et al. (1988). They found that sealing inhibited the exchange of ethylene, promoting an accumulation of this gas. In fact, an ethylene concentration of 0.8 pmol ml^{-1} was measured in unsealed glass tubes and increased up to

6.5 pmol ml^{-1} by sealing with nescofilm. In gas-proof stopper, ethylene accumulated up to 92.5 pmol ml^{-1} (Horn et al., 1988).

Enhanced ethylene production is associated with chlorophyll loss and color fading, which are characteristic features of leaf senescence (Taiz & Zeiger, 1991). For instance, rose plants grown in glass tubes with caps sealed with nescofilm produced shoots with a higher number of yellow leaves and lower quality score in comparison to unsealed caps (Horn et al., 1988).

Table 10. Contamination rate of the culture medium for three rose cultivars as affected by the type of closure used in the test tubes (*n*=60).

Cultivar	**Type of closure**				**Influence**
	Glass	**Steristop**	**Aluminium**	**Plastic**	**of closure**
	%	%	%	%	χ^2
'Frisco'	22[(1)]	2	7	0	****
'Kardinal'	12	2	8	0	****
'Lambada'	10	3	3	0	****
Influence of cultivar (χ2)	**	ns	ns	ns	

[(1)] For the comparison of the contamination rate between different types of closure at the same cultivar as well as between cultivars at the same closure type, the chi^2-test was used.
ns not significant
** Significant at 5%
**** Significant at 0.1%

Ethylene is a growth regulator produced even by cells growing under *in vitro* conditions. This hormone can influence the pattern of culture development and its presence is regulated by the closing type used in the vessel and its tightness (Caldas et al., 1998).

Our hypothesis concerning the higher accumulation of ethylene in containers tightly closed is supported by the work of Park et al. (2004), which tested different types of ventilation of the culture vessel. Shoots of potato showed strong symptoms of hyperhydration when cultivated in completely sealed vessels, whereas shoots grown in gas permeable vessels showed normal shoot proliferation. When an ethylene inhibitor was applied to the completely sealed vessels, shoots exhibited normal growth, indicating that hyperhydration was related to the ethylene accumulated during the *in vitro* culture (Park et al., 2004).

Furthermore, if the closure is too tight, the transpiration flow may be decreased due to a reduction in the gradient in water vapor pressure between outside and the air spaces in the leaf. Consequently, symptoms of mineral deficiency (as e.g. calcium deficiency) may appear, due to the lowered translocation of water inside the plant and, consequently, a reduced flow of nutrients, resulting in necrosis in the growing tissues (Grattapaglia & Machado, 1998).

Therefore, the use of steristop could be recommended, since it permitted a good development of shoots (see Table 9) as well as prevented the penetration of thrips, as shown by the low contamination rates of the culture medium (see Table 10).

Figure 11 shows the development of the three rose cultivars at the end of the rooting phase as affected by the type of closure.

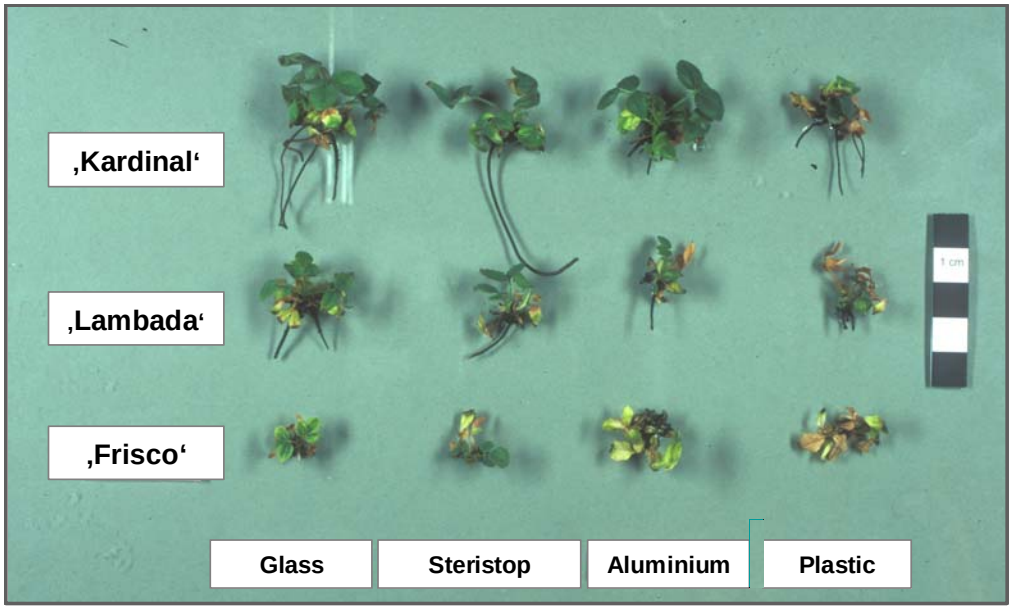

Figure 11. Development of plants of three rose cultivars (‘Kardinal’, ‘Lambada’ and ‘Frisco’) grown in test tubes with different types of closure (glass, steristop, aluminium and plastic film).

One can see in the Figure 11 the greater *in vitro* shoot development of the cultivar ‘Kardinal’ in comparison to ‘Lambada’ and ‘Frisco’. The cultivar ‘Frisco’ presented the worst performance *in vitro* among the three rose cultivars studied. Moreover, the use of a plastic film as closure resulted in a decreased growth of plantlets, when compared to the other types of closure (Figure 11).

For the parameters number of roots per plant and quality score of roots, the interaction between the factors closure type and cultivar was not significant ($P>0.05$)

(Figure 12). The best results were obtained by using glass as closure, with plants presenting greater root development and more roots (Figure 12). Furthermore, no significance differences were observed between steristop, aluminium or plastic, as shown in Figure 12.

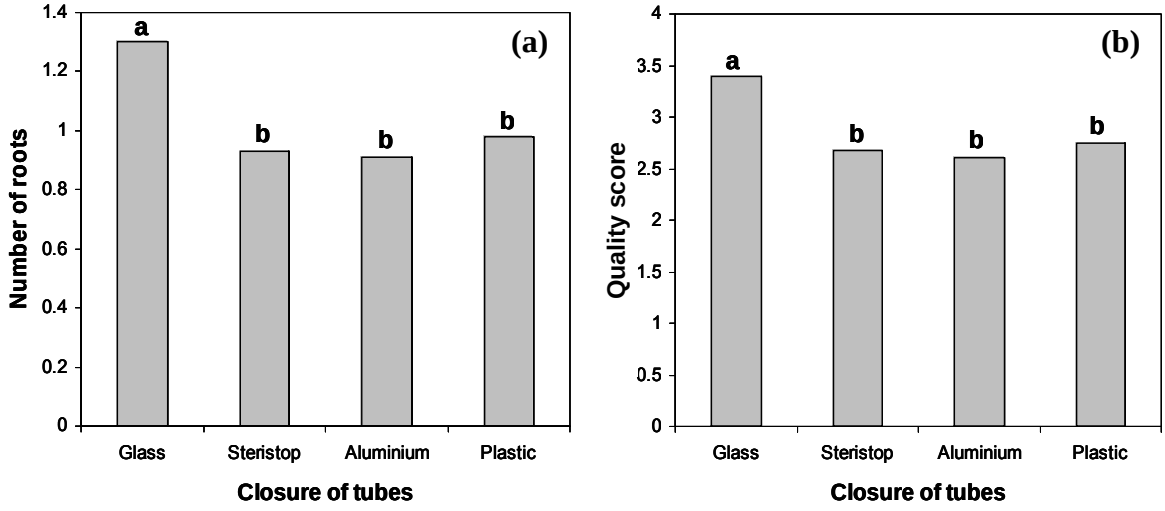

Figure 12. Number of roots per plant (a) and quality score of roots (a) of rose in the rooting phase grown in test tubes with different types of closure. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 4) (*n*=50).

At the end of the rooting phase, a high mortality rate of plantlets (Table 11) was observed in the treatment with plastic film, confirming the negative effect of this closure type. This effect was found in all cultivars studied. Moreover, plantlets grown in test tubes closed with glass presented lower mortality rates at the end of the rooting phase (except for the cultivar 'Lambada') (Table 11).

The worse performance of steristop in comparison to glass (except for the cultivar 'Lambada') may be related to a greater loss of water from test tubes closed with steristop, resulting in a strong drying of the *in vitro* environment.

Especially in cv. 'Frisco', the use of aluminium was not suitable, since a great number of plantlets did not survive up to the end of the rooting phase. It is possible that the use of aluminium reduced the gaseous exchanges between the *in vitro* and the *ex vitro* environments. Furthermore, aluminium may have caused a higher shadowing of the plantlets, since approximately 1/4 of the height of test tubes was covered by this material, preventing the penetration of part of the supplied light.

Concerning the effect of cultivar, 'Frisco' had significantly higher mortality rates than the other two cultivars for all closure types tested. 'Kardinal', in turn, had the lowest mortality rate (Table 11).

Table 11. Mortality rate of plants in the rooting phase of three rose cultivars grown in test tubes with different types of closure (*n*=50).

Cultivar	Type of closure				Influence
	Glass	Steristop	Aluminium	Plastic	of closure
	%	%	%	%	χ^2
'Frisco'	29[(1)]	37	43	46	***
'Kardinal'	0	4	6	14	****
'Lambada'	11	9	11	18	**
Influence of cultivar (χ2)	****	****	****	****	

[(1)] For the comparison of the mortality rate between different types of closure at the same cultivar as well as between cultivars at the same closure type, the chi^2-test was used.
** Significant at 5%
*** Significant at 1%
**** Significant at 0.1%

b) Sucrose concentration and cover with filter

This experiment was installed with the objective to study the combined effect of different sucrose concentrations and gaseous changes, with the use of covers with and without filter. Baby food culture jars covered with Magenta B-CAP with filter (Sigma B-3031) or without filter (Sigma B-8648) were used. Moreover, sucrose concentrations of 10, 20 and 40 g L^{-1} were tested on the cv. 'Kardinal'. Evaluations were done in the rooting as well as in the acclimatization phases.

Rooting phase

The quality score of shoots was not significantly influenced by the sucrose concentration in the culture medium and by the presence or absence of filter in the baby food culture jars (data not shown).

On the other hand, the formation of roots, as evaluated by the number and length (quality score) of roots, was significantly affected by the different sucrose concentrations (Figure 13). The increase in the sucrose concentration in the medium up to 40 g L^{-1} promoted the formation of longer roots (higher quality score) in

comparison to 10 and 20 g L^{-1} (Figure 13.a). However, the number of roots per plant linearly decreased with the increment in sucrose concentration (Figure 13.b).

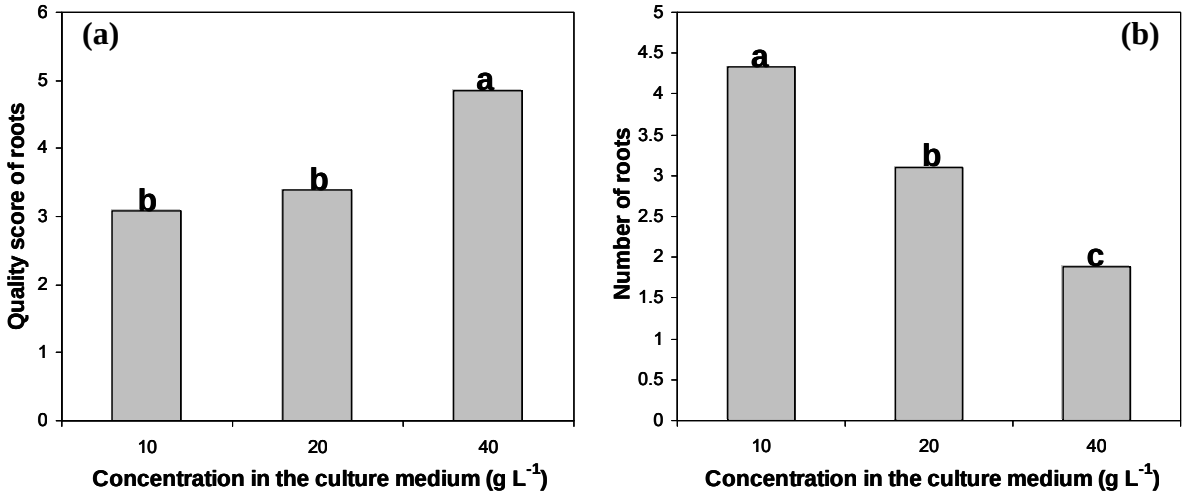

Figure 13. Quality score of roots (a) and number of roots per plant (b) of rose in the rooting phase grown under different sucrose concentrations in the culture medium. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 4) (*n*=50).

Shorter roots (i.e. lower quality score) were observed in the treatments with lower concentrations of sucrose in the culture medium, as shown in Figure 13.a, which is a desirable characteristic according to Grattapaglia & Machado (1998), since it facilitates the washing and the removal of the culture medium from the roots. Moreover, shorter roots are in a phase of active growth, facilitating the acclimatization process.

Ault (1984) found a comparative effect of increasing the root length of *Eriostemon* 'Stardust' by an increase in the sucrose concentration in the medium (from 25 up to 50 g L^{-1}). Furthermore, Végvári (2003) studied the occurrence of morphological changes of *in vitro* apple plants during acclimatization. Roots developed *in vitro* lost their hairs and the elongation stopped when the plant was transferred from the *in vitro* to the *ex vitro* environment (Végvári, 2003). Such roots are possibly not able to absorb nutrients from the soil, but these roots are still important because new fully functional roots develop from them. In this case, the number of roots may be even more important than their length.

The effect of sucrose concentration in the culture medium on the root formation was studied by Khan et al. (1999). They found that the increase in sucrose concentration (from 10 to 30 g L^{-1}) was positively correlated with the rooting percentage, root number per shoot and root length in *Syzygium alternifolium*. Concentrations of sucrose higher than 40 g L^{-1} inhibited overall rooting response.

Our results are in agreement with previous results reported by Calvete et al. (2002). They did not observe any *in vitro* root development in strawberry plants grown in the absence of sucrose. According to their results, plants grown at a concentration of 45 g L^{-1} of sucrose presented greater formation of roots. The use of higher sucrose concentrations (60 g L^{-1}) reduced root number (Calvete et al., 2002).

In relation to the use of filter in the lid, no significant difference was found between both treatments for the parameters quality score and number of roots per plant (data not shown).

The concentration of photosynthetic pigments was also affected by the different sucrose concentrations as well as by the use of filter (Table 12). No interaction between the factors sucrose concentration and filter was observed.

The total chlorophyll content varied between 1.17 mg g^{-1} FW (at the concentration of 40 g L^{-1} of sucrose) and 1.76 (at the concentration of 10 g L^{-1} of sucrose). Similar behavior was observed for the chlorophyll *a* and *b* contents (Table 12).

The high levels of sucrose normally used to support the growth of tissue cultures may cause a reduction in chlorophyll biosynthesis (Edelman & Hanson, 1972). Huylenbroeck & Debergh (1996) observed this phenomenon in *Spathiphyllum* plantlets, where the total chlorophyll content at the end of the *in vitro* phase decreased from 0.38 up to 0.35 mg g^{-1} FW with increasing the sucrose concentration in the medium from 30 up to 60 g L^{-1}.

Table 12. Total chlorophyll (*a*+*b*), chlorophyll *a* and chlorophyll *b* contents as affected by the sucrose concentration and the use of filter (*n*=6).

Treatment	Total chlorophyll (*a*+*b*)	Chlorophyll *a*	Chlorophyll *b*
	mg g^{-1} FW		
Sucrose			
10 g L^{-1}	1.759 a[(1)]	1.399 a	0.361 a
20 g L^{-1}	1.211 b	0.972 b	0.239 b
40 g L^{-1}	1.168 b	0.937 b	0.231 b
Closure			
With filter	1.325 b	1.064 b	0.261 b
Without filter	1.499 a	1.196 a	0.304 a

[(1)] Means followed by the same letter in the column within the same factor (sucrose concentration or filter) are not significantly different according to Duncan's Test (P=0.05).
FW=fresh weight

Contrasting with our results, Langford & Wainwright (1987) reported for the rose cv. 'Peace' an increase in total chlorophyll content (from 0.95 to 1.79 mg g^{-1} FW) when sucrose concentration was increased from 10 up to 20 g L^{-1}. In shoots grown at 40 g L^{-1}, chlorophyll content reached a value of 1.70 mg g^{-1} FW. This contrast between different studies may be related to the fact that different cultivars were used. In our study, the use of filter promoted a decrease in total chlorophyll content as well as in chlorophyll *a* and *b* contents, when compared to the treatment without filter (Table 12). This result is not in agreement with previous results reported by Haisel et al. (1999), who verified an increase in chlorophyll *a* and *b* contents in tobacco plants grown at higher CO_2 supply. Based on the studies from Haisel et al. (1999), one could expect higher chlorophyll content in plantlets growing in vessels with filter, since it is expected a higher supply of CO_2 for the plantlets in this situation. This behavior was not found in our study. A possible explanation concerns higher loss of water from vessels with filter, what could have reduced the synthesis of plant pigments.

Acclimatization phase

The quality score of shoot and the mortality rate was evaluated in plants that were grown under different sucrose concentrations (10 up to 40 g L^{-1}) and in baby food culture jars with or without filter in the rooting phase.

A significant effect of sucrose concentration on the quality of shoots was observed. The best quality of shoots was observed at the concentration of 10 g L^{-1}. The continuous increase in sucrose concentration up to 40 g L^{-1} resulted in a decrease in this parameter (Figure 14).

On the other hand, the quality score of shoots was not significantly influenced by the use or absence of filter in the cover (data not shown).

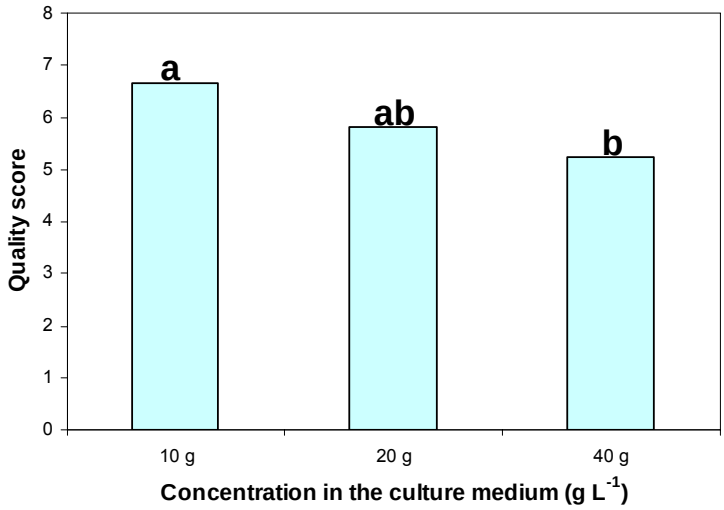

Figure 14. Quality score of shoots of rose in the acclimatization phase grown under different sucrose concentrations in the culture medium in the rooting phase. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=40).

Our results indicated that plants submitted to low concentrations of sucrose in the culture medium in the rooting phase may have developed a greater autotrophic capacity in this phase, even though the quality score of shoots in the phase 3 was not significantly affected by the sucrose concentration. This indicates that plants grown at 10 g L^{-1} of sucrose were partly capable of supplying their carbohydrate needs by photosynthetic carbon assimilation, whereas plants grown at 40 g L^{-1} of sucrose probably used the carbon source (sucrose) available in the culture medium.

The greater autotrophic capacity of plants grown at 10 g L^{-1} of sucrose may have benefited the process of acclimatization. The larger chlorophyll content observed in the rooting phase in plants growing at lower sucrose concentrations (see Table 12) may indicate that such plants could present a higher photosynthetic capacity, facilitating the acclimatization.

Supporting results were found by Hdider and Desjardins (1994). They investigated the photosynthetic process in strawberry plants grown under different sucrose concentrations (without sucrose, 10, 30 and 50 g L^{-1}) during the rooting phase *in vitro*. The photosynthetic capacity was influenced by the sucrose level in the medium. Plants grown in a medium with lower sucrose levels (without sucrose and 10 g L^{-1}) expressed higher photosynthetic rates. Similarly, our study showed that lower concentrations of sucrose promoted a better development of plants in the acclimatization phase, as evaluated by the quality score of shoots (Figure 14).

Although a decrease in the sucrose concentration in the medium may increase the photosynthetic ability of rose, it is obvious that a minimum amount of this carbohydrate is necessary for a normal growth of plantlets. Langford & Wainwright (1987) also studied the effect of reducing the sucrose concentration in the medium on the micropropagation of rose cvs. 'Iceberg' and 'Peace'. According to their results, a concentration of 10 g L^{-1} of sucrose seemed to be limiting, below which the growth and chlorophyll levels of shoots declined.

Another hypothesis to explain the better growth of plants in the acclimatization previously submitted to low sucrose concentration (10 g L^{-1}) concerns a positive effect of the lower sucrose concentration on the root formation (number of roots) in the rooting phase (see Figure 13.b), which could have facilitated the adaptation of plants to the *ex vitro* environment.

Concerning the type of closure, the use of filters did not significantly influence the quality score of shoots (data not shown).

The evaluation of the mortality rate (Table 13) showed that the low sucrose concentration in the rooting phase resulted in plants with a higher survival capacity in the acclimatization, which may be related to a higher autotrophic capacity. In our study, the highest mortality rate (38%) was observed in plants grown *in vitro* at 40 g L^{-1} of sucrose and with filter in the cover (Table 13).

Table 13. Mortality rate of plants in the acclimatization phase of the rose cv. 'Kardinal' grown under different sucrose concentrations and types of closure of baby food culture jars in the rooting phase (*n*=40).

Filter	**Sucrose concentration (g L^{-1})**			**Influence of**
	10	**20**	**40**	**sucrose conc.**
	%	%	%	$\chi 2$
Without	9[(1)]	24	18	***
With	14	19	38	****
Influence of filter ($\chi 2$)	ns	ns	***	

[(1)] For the comparison of the mortality rate between different sucrose concentrations at the same type of closure as well as between types of closure at the same sucrose concentration, the chi^2-test was used.
[ns] not significant
*** Significant at 1%
**** Significant at 0.1%

Contrasting with our study, Capellades et al. (1991) found a higher survival rate of shootlets of rose in the acclimatization phase in plants submitted to higher sucrose concentration (50 g L^{-1}), although the treatments with the lowest amount of sucrose (10 and 30 g L^{-1}) in the culture medium presented the highest photosynthetic rate and the lowest starch content. They supposed a better re-establishment of the photosynthetic capacity in plants grown at 50 g L^{-1} of sucrose due to the translocation of the stored assimilates.

No significant effect of the use of filter in the rooting phase was observed on the mortality rate of plants grown at 10 or 20 g L^{-1} of sucrose. Only at 40 g L^{-1} a significant effect of the use of filter was observed (Table 13).

4.1.3 Room temperature

The temperature may influence the development of micropropagated plants, especially in the rooting phase. In order to determine the effect of different air temperatures in this phase on the vegetative growth (quality score of shoots) and root formation, three rose cultivars (‘Kardinal’, ‘Frisco’ and ‘Lambada’) were grown under different room temperatures (16, 20 and 24°C).

The factors temperature and cultivar did not exhibit significant interaction for quality score of shoots and number of roots per plant. Concerning the quality score of shoots, a negative effect of the room temperature of 16°C on shoot growth was found (Figure 15.a). On the other hand, plants grown at 16°C produced more roots than at 20°C and 24°C (Figure 15.b). It indicates that different temperatures are required for the optimum growth of shoots and roots.

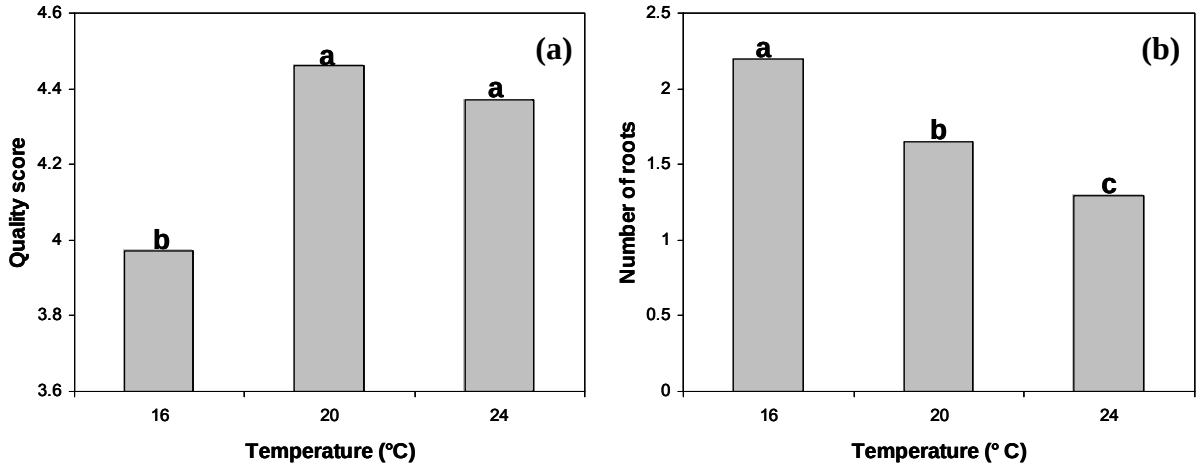

Figure 15. Quality score of shoots (a) and number of roots per plant (b) of rose in the rooting phase grown under different room temperatures. Means followed by the same letter are not significantly different according to Duncan’s Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=55).

Figure 16 shows the development of three rose cultivars grown under different room temperatures at the end of the rooting phase *in vitro*. One can see the greater *in vitro* shoot development of the cultivar ‘Kardinal’ in comparison to ‘Lambada’ and ‘Frisco’. Moreover, the use of 16°C resulted in a decreased growth of plantlets, when compared to the other temperatures tested (Figure 16).

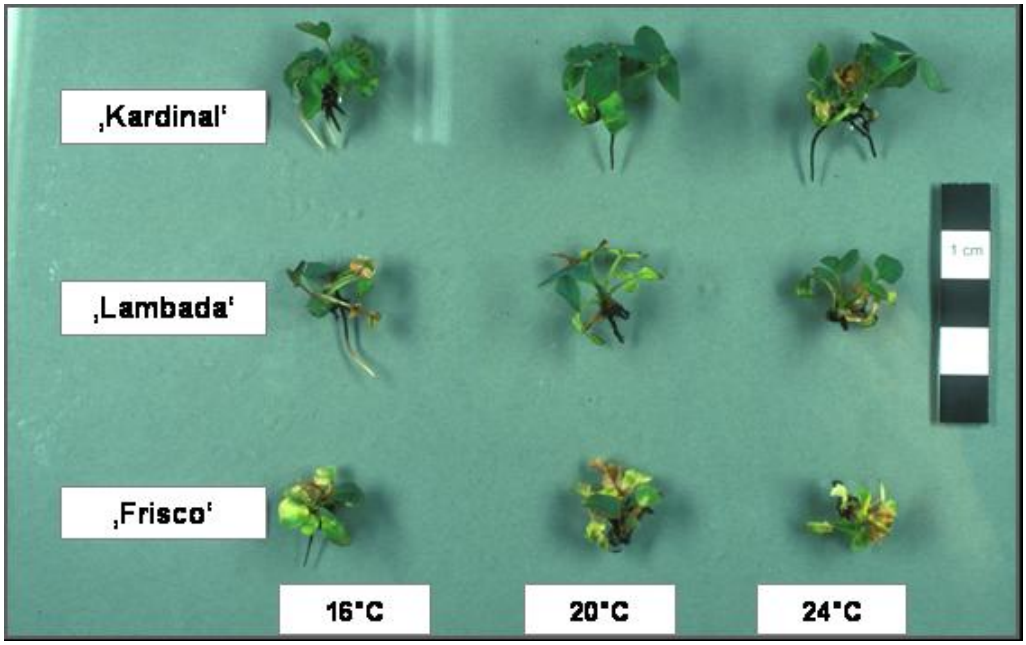

Figure 16. Development of plants of three rose cultivars ('Kardinal', 'Lambada' and 'Frisco') at the end of the rooting phase grown under different room temperatures.

Our results are in agreement with previous results reported by Leyhe & Horn (1994) for six rose cultivars (including the cultivars 'Kardinal' and 'Frisco'). The optimal growth of shoots was obtained at 21°C (except for the cultivar 'Europa'), whereas root development (number and length of roots) was optimal at 15°C for 'Kardinal' and 18°C for 'Frisco'. Pierik et al. (1988) found that optimal rooting (percentage of rooting and number of roots per plant) of *Alstroemeria* hybrids occurred at 21°C in comparison to 25°C and 27°C. In fact, root induction on shoots in many species produced *in vitro* requires a slightly lower temperature than the temperature necessary for shoot multiplication and growth (George, 1993).

The temperature is considered one of the key factors controlling plant development. The physiologic processes in the plant have an optimum interval for their performance. In fact, the optimum temperature curve for growth of an organism effectively represents a composition of the temperature curves for photosynthesis, respiration, and other critical metabolic processes (Hopkins & Hüner, 2004).

The results of the quality score of roots (root length) showed that only the cv. 'Frisco' was significantly influenced by the room temperature (Table 14). For this cultivar, the best quality score was observed at 16°C. Increasing the room temperature up to

20°C or 24°C resulted in the formation of shorter roots (as shown by decreased values for the quality score). The quality score of roots for the cultivars 'Kardinal' and 'Lambada', in turn, was not significantly affected by the room temperature (Table 14). Concerning the cultivars studies, one can see that 'Kardinal' had the highest rooting capacity *in vitro*. The cv. 'Frisco', in turn, presented a low rooting capacity (Table 14). Based on the results showed in Figure 15.b (number of roots) as well as on the results showed in the Table 14 (root length), it can be concluded that the room temperature influenced more strongly the root formation (number of roots) than its growing in length.

Table 14. Quality score of roots of three rose cultivars grown under different room temperatures in the rooting phase[(1)] (*n*=55).

Cultivar	**Temperature (°C)**		
	16	**20**	**24**
	quality score		
'Frisco'	B 2.4 a[(2)]	C 1.8 b	C 1.5 b
'Kardinal'	A 3.8 a	A 4.2 a	A 4.5 a
'Lambada'	A 3.7 a	B 3.4 a	B 3.0 a

[(1)] see Material and Methods for a description of the quality score of roots (Table 4).
[(2)] Means followed by the same capital letter (A,B,C) in the column or by the same small letter (a,b) in the row are not significantly different according to Duncan's Test (P=0.05).

An important topic concerns the effect of varying temperatures in the light and dark periods, i.e. the difference between day and night temperatures. The growth of many plants is improved when both temperatures are different from each other. The effect of different temperatures between day and night period is due to the effect of temperature on the processes of photosynthesis and respiration. Even though, different kind of responses can be found between different species as well as between different cultivars within the same species.

Different kinds of plant can be successful multiplied in growth chambers with the same dark and light temperatures. Many small micropropagation laboratories have only a single growth room, in which temperature is fairly uniform along the growing period (George, 1993).

The cultivars tested in our study may respond positively to differences between day and night temperatures. As observed by Bressan et al. (1982) in rose cv. 'Improved Blaze', a constant temperature of 21°C resulted in the highest rate of shoot multiplication and root initiation. A reduction in the night temperature from 21°C down to 11°C did not increase shoot multiplication, although root initiation was enhanced by lowering the night temperature to 11°C or 16°C.

The extractable chlorophyll content was also evaluated and the interaction between temperature and cultivar was not significant (Table 15). The total chlorophyll as well as chlorophyll *a* and *b* contents were significantly higher in plants grown at 20°C.

The higher chlorophyll content at 20°C confirms the results previously showed in this section, which demonstrated that plants grown at this temperature had the highest quality score of shoots (see Figure 15.a).

Table 15. Total chlorophyll (*a*+*b*), chlorophyll *a* and chlorophyll *b* contents in the rooting phase as affected by the room temperature (*n*=6).

Temperature	Total chlorophyll (*a*+*b*)	Chlorophyll *a*	Chlorophyll *b*
	mg g^{-1} FW		
16°C	0.53 b[(1)]	0.41 b	0.12 b
20°C	0.71 a	0.56 a	0.15 a
24°C	0.45 b	0.37 b	0.08 c

[(1)] Means followed by the same letter in the column are not significantly different according to Duncan's Test (P=0.05).
FW=fresh weight

At the end of this phase, leaves were collected from plants submitted to the different temperature treatments and analyzed by light microscopy techniques.

Examination of histological sections prepared for light microscopy revealed that leaves of plants grown *in vitro* had a disorganized palisade layer (Figure 17.d) when compared to a leaf from a greenhouse plant (Figure 17.a). Furthermore, stomata were open in the *in vitro* plants (Figure 17.b, 17.c and 17.d).

Palisade cells were densely packed together in leaves of the cv. 'Lambada' grown *ex vitro* (Figure 17.a). In the Figure 17.b, one can observe that the palisade cells in leaves of the cv. 'Lambada' grown *in vitro* at room temperature of 16°C are not so

elongated and, consequently, more disorganized, when compared to leaves of rose plants grown *ex vitro* (greenhouse plants).

Leaves of the cv. 'Lambada' cultured *in vitro* at 24°C room temperature presented a better organization of the palisade parenchyma layers (Figure 17.c). Cells of the palisade parenchyma were elongated, similarly as observed for greenhouse plants. On the other hand, leaves of the cv. 'Frisco' cultured at 24°C (Figure 17.d) presented a poorly developed and disorganized palisade parenchyma layer (only one layer) as well as larger air space when compared to 'Lambada' grown at the same room temperature (Figure 17.c).

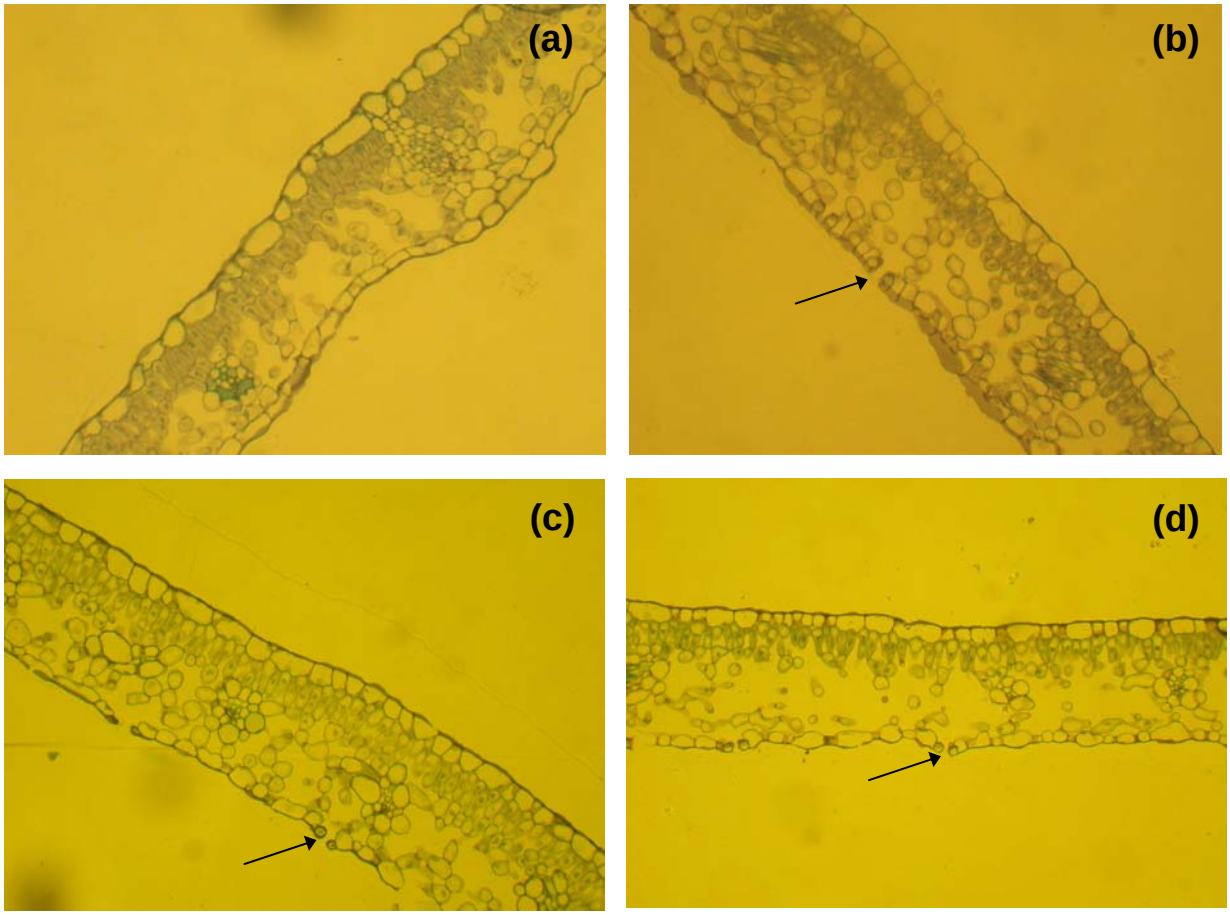

Figure 17. Transverse sections of rose leaves (semi-thin sections stained with toluidine blue). (a) Greenhouse plant, cv. 'Lambada'. (b) Cv. 'Lambada' cultured *in vitro* at 16°C room temperature. (c) Cv. 'Lambada' cultured *in vitro* at 24°C room temperature. (d) Cv. 'Frisco' cultured *in vitro* at 24°C room temperature. Arrows indicate open stomata.

Anatomical differences between leaves of plants grown under *ex vitro* versus *in vitro* culture conditions were also studied by Wetzstein & He (2000). They found that plants cultivated *in vitro* form more extensive intercellular spaces and often a poorly differentiated palisade parenchyma as well as a reduced or defective stomatal function. Environmental factors *in vitro* can be controlled for the production of plantlets with fewer morphological disorders, as seen in leaves of the cv. ‘Lambada’ grown at a room temperature of 24°C.

4.1.4 Duration of the rooting phase

In order to characterize the growth of rose under different durations of the rooting phase *in vitro* and to determine the optimal duration of this phase to be used in future studies on micropropagation, two experiments were carried out.

a) Two up to six weeks on rooting medium

In this experiment, five different durations of the rooting phase (phase 3) were tested (two, three, four, five and six weeks) on three rose cultivars (‘Kardinal’, ‘Frisco’ and ‘Lambada’).

The cvs. ‘Kardinal’ and ‘Lambada’ showed a quality score of shoots around 5.0 after two weeks in rooting medium (Figure 18.a). Thereafter, the quality score decreased after three and four weeks down to values near 4.0. Afterwards, this parameter increased again up to six weeks (Figure 18.a).

This behavior can be explained by the fact that the leaves developed during the previous multiplication phase progressively died as the duration of the rooting phase increased.

The number of leaves per plant decreased from two up to four weeks for the cvs. ‘Kardinal’ and ‘Lambada’. Simultaneously, new leaves are formed as the duration of this phase increased. For this reason, the quality score of shoots was higher after two weeks, due to the presence of leaves formed in the multiplication phase, and decreased up to three weeks, due to senescence of these leaves (Figures 18.a and 19). Afterwards, quality score of shoots increased from four up to six weeks (Figures 18.a and 19) due to the formation of new leaves during the rooting phase (Figure 18.b).

The cv. 'Frisco' showed, in turn, a continuous decrease in the quality of shoots as the duration of the rooting phase increased (Figure 18.a and 19). It was caused by the fact that leaves developed in the multiplication phase progressively died and new leaves were not formed in the rooting phase. This observation is supported by the results shown in Figure 18.b. The number of leaves per plant in this cultivar decreased with increasing the duration of the rooting phase. The results observed for this cultivar are supported by previous results reported in this study, which showed that ‘Frisco’ is a cultivar of difficult micropropagation.

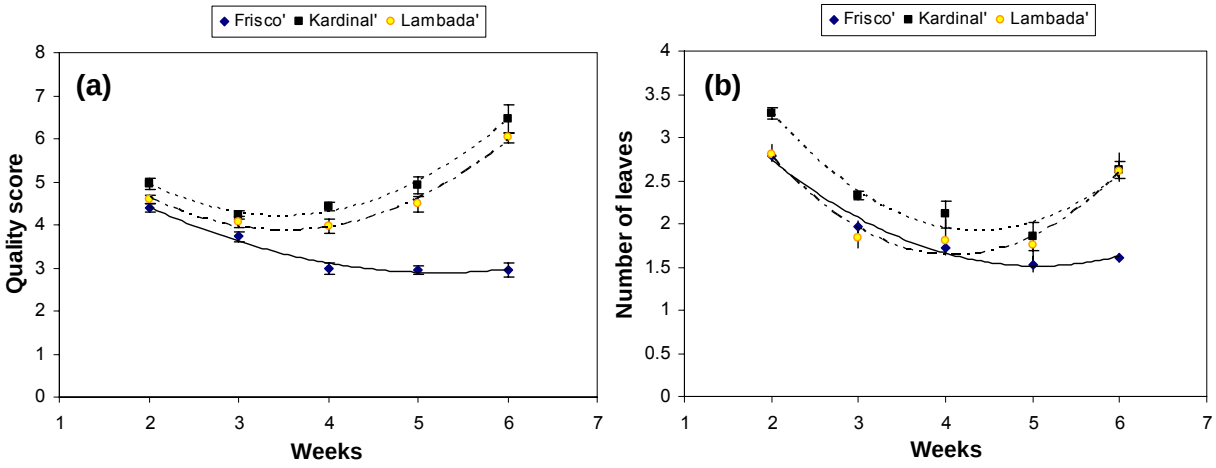

Figure 18. Quality score of shoots (a) and number of leaves per plant (b) of three cultivars of rose as affected by the duration of the rooting phase. Lines represent fitting regression curves. A description of the quality score is presented in the chapter Material and Methods (Table 3). Regression equations were as follows: Figure (a): 'Frisco' y= 6.91 -1.54x + 0.15x^2, 'Kardinal' y= 8.37 – 2.40x + 0.35x^2, 'Lambada' y= 8.05 – 2.38x + 0.334x^2; Figure (b) 'Frisco' y= 4.89 – 1.34x + 0.13x^2, 'Kardinal' y= 6.57 – 2.13x + 0.24x^2, 'Lambada' y= 6.01 – 2.14x + 0.26x^2 (*n*=55).

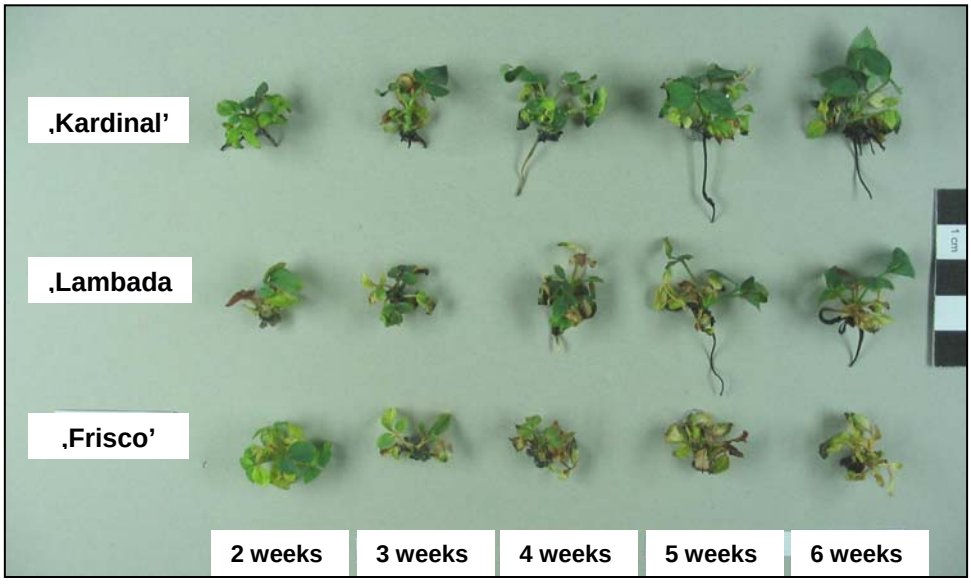

Figure 19. Development of plants of three rose cultivars ('Kardinal', 'Lambada' and 'Frisco') as affected by the duration of the rooting phase.

In relation to the root formation in the cvs. 'Kardinal' and 'Lambada', the quality score of roots linearly increased as the duration of the rooting phase increased (Figure 20.a). The cv. 'Frisco', in turn, showed a low efficiency in the formation and growth of new roots, since the quality score of roots was little affected by the duration

of the rooting phase. For all treatments, the quality score for ‘Frisco’ was lower than for the other two cultivars (Figure 20.a). After six weeks, only 28% of plants of the cv. ‘Frisco’ rooted, whereas 78% of plants of 'Kardinal' formed roots (data not shown).

The number of roots emitted per plant was also influenced by the duration of the rooting phase. For ‘Kardinal’ and ‘Lambada’, number of roots increased from two up to six weeks (Figure 20.b).

For both cultivars, the highest increment in the number of roots was observed between two and four weeks. Thereafter, the formation of new roots was nearly stopped (Figure 20.b).

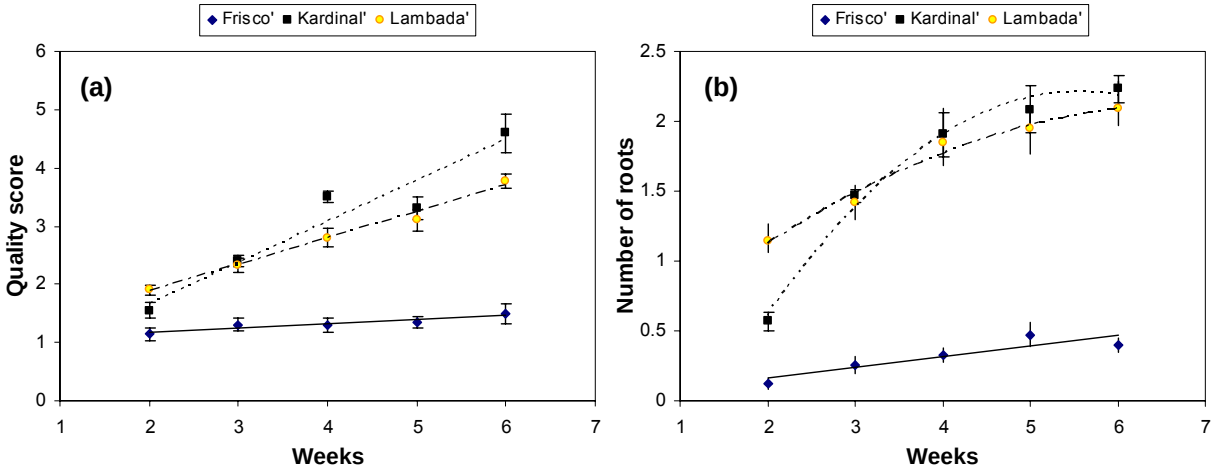

Figure 20. Quality score of roots (a) and number of roots per plant (b) of three rose cultivars as affected by the duration of the rooting phase. Lines represent fitting regression curves. A description of the quality score is presented in the chapter Material and Methods (Table 4). Regression equations were as follows: Figure (a): ‘Frisco’ y=1.02 + 0.07x, ‘Kardinal’ y=0.26 + 0.72x, ‘Lambada’ y=0.98 + 0.45x; Figure (b) ‘Frisco’ y=0.01 + 0.08x, ‘Kardinal’ y=-1.69 + 1.41x - 0.13x^2, ‘Lambada’ y=0.15 + 0.57x – 0.04x^2 (*n*=55).

The formation of roots can be divided in three different phases, namely the induction, initiation and elongation phases. The first two phases (induction and initiation) are responsive or depend on the presence of auxin, whereas the growth of roots may be inhibited by this growth regulator (Grattapaglia & Machado, 1998). In our study, the length of roots (as evaluated by the quality score) continuously increased up to six weeks (see Figure 20.a). It shows that the growth of roots was not inhibited by the prolonged contact of roots with auxin in the culture medium. Therefore, the inhibition

of root growth by low concentrations of auxin (0.1 mg L^{-1}) could not be found in our study.

b) One up to five weeks on rooting medium

In this experiment (only cv. 'Kardinal'), treatments consisted of three different durations of the rooting phase, namely one, three and five weeks. The experiment was carried out with the objective to characterize the ideal time (smallest possible time) for plants to reach an appropriate growth in order to ensure the survival of plants in the acclimatization, since the long contact of plants in the phase 3 with the rooting medium containing auxin can be a negative factor in the development of the plant in the further phase 4 (acclimatization).

Rooting phase

The quality score of shoots was not significantly different between one and three weeks and significantly increased after five weeks (Figure 21.a). This increase can be explained by the formation of new leaves in this phase.

Furthermore, the number of leaves per plant was higher after one and five weeks (Figure 21.b). When comparing the Figures 21.a and 21.b, one can see that the higher number of leaves observed after one week (Figure 21.b) did not correspond to a higher quality score of shoots (Figure 21.a).

The number of leaves decreased from one up to three weeks, indicating that the leaves present in the plant at the beginning of the rooting phase died. Thereafter, the further increase in the number of leaves observed after five weeks is due to the formation of new leaves (Figure 21.b).

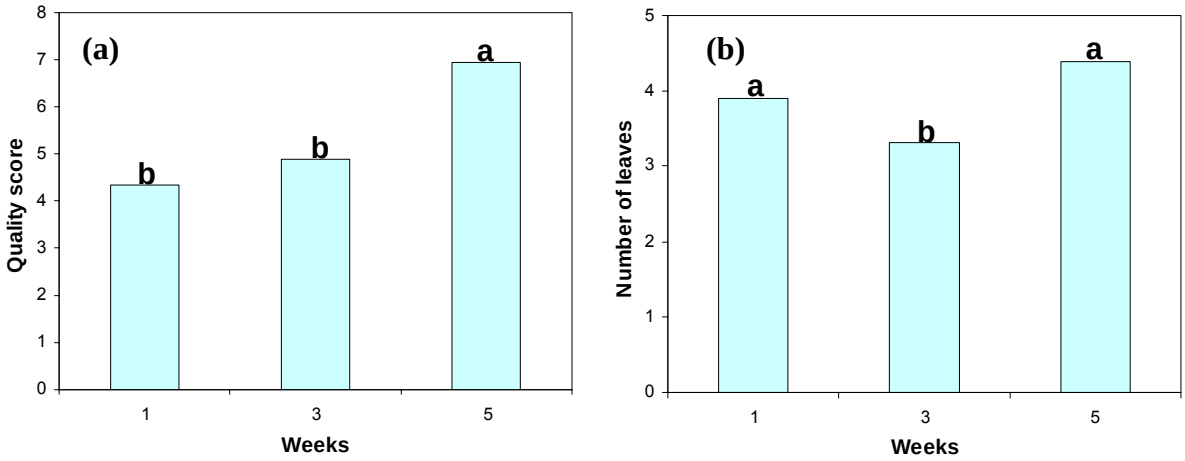

Figure 21. Quality score of shoots (a) and number of leaves per plant (b) of rose as affected by the duration of the rooting phase. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=60).

In relation to root formation, the quality score as well as the number of roots per plant increased significantly from one up to five weeks (Figures 22.a and 22.b). The first roots appeared approximately one week after transferring the explants to the rooting medium (Figure 22.b). Thereafter, the number of roots rapidly increased after three weeks, indicating that the greater part of roots was formed up to the third week in the rooting medium (Figure 22.b). Between the third and the fifth week, the formation of new roots slowed down.

Several authors showed that the appearance of the first roots occurred in the first week after transferring the explants to the rooting medium. In sweet potato, the appearance of roots was observed after seven days (Castro & Andrade, 1995), whereas in *Gypsophila paniculata* the emission of roots started after eight days in the rooting medium (Bosa et al., 2003b).

According to Hartmann & Kester (1990), the process of root formation can be divided in two periods. The first one is the initiation period, with duration of four to eight days. This first period is subdivided in two phases, namely the formation of the meristem (with intense auxin activity) and the beginning of root formation. The second period is the root elongation, in which auxin is not necessary. This period is characterized by the development of the root primordium and its emergence on epidermis.

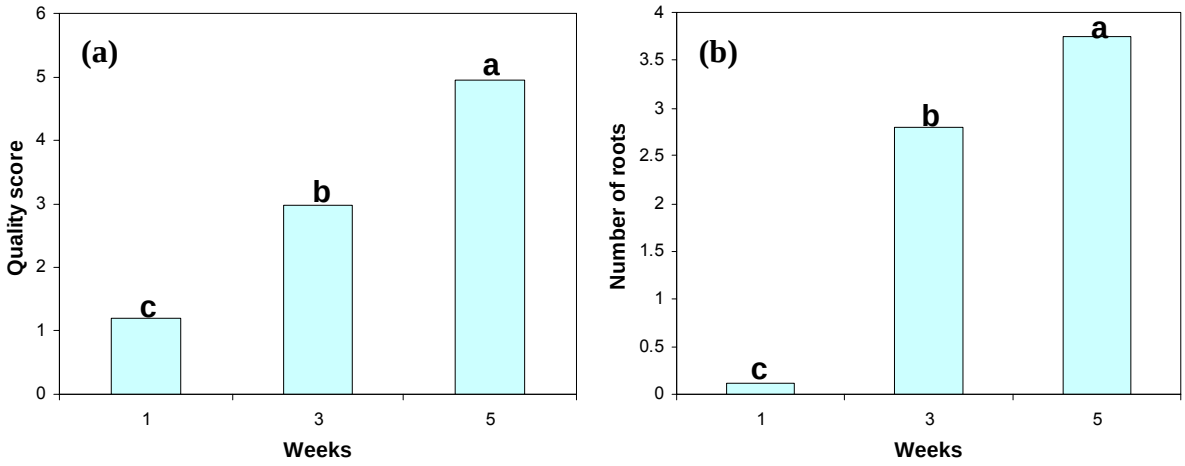

Figure 22. Quality score of roots (a) and number of roots per plant (b) of rose as affected by the duration of the rooting phase. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 4) (*n*=60).

Acclimatization phase

The mortality rate of plants was influenced by the duration of the rooting phase. The lowest mortality rate (2%) was found after five weeks in the rooting medium (Table 16). On the other hand, an increase on the mortality rate up to 18% was observed after three or one week (Table 16).

The results of the mortality rate evaluation (Table 16) showed that rose plants were not negatively affected by a prolonged contact with auxin in the rooting medium up to five weeks. The most decisive factor concerning the lower mortality after five weeks seems to be associated with the better development of shoots and roots of these plants in the rooting phase *in vitro*.

Supporting results were found by Silva et al. (1995) for explants of cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*) and blackberry (*Rubus idaeus*). The increase in the duration of the rooting phase resulted in a lower mortality rate of plants (Silva et al., 1995).

Based on the results showed in the present study, a minimum duration of the rooting phase of five weeks should be used in the micropropagation of rose cvs. 'Kardinal' and 'Lambada'.

Table 16. Mortality rate of rose plants in the acclimatization phase as affected by the duration of the rooting phase (*n*=60).

Duration of rooting phase	**Mortality rate**
	%
1 week	18[(1)]
3 weeks	19
5 weeks	2
Influence of duration (χ^2)	****

[(1)] For the comparison of the mortality rate between different durations of the rooting phase, the chi^2-test was used.
**** Significant at 0.1%.

4.1.5 Consistency of the culture medium, auxin concentration and container type

Concerning the consistency, the culture medium used for micropropagation can be classified as liquid or solid. Normally, the culture in liquid medium requires some type of support for the plants.

In this experiment, a supporting system for the plants described in Battke et al. (2003) for hydroponically grown barley was used. This system is based on the use of a polyethylene granulate as floating body. The plants are anchored in the polyethylene granulate and roots grow into the liquid medium.

The use of liquid media often results in faster growth rates than on semi-solid media. This is due to the fact that nutrient absorption may be facilitated in this kind of medium (George, 1993). It was found that the propagation ratio of artichoke was higher in liquid than in solid medium (Debergh et al., 1981) as well as that the use of a liquid medium gave more vigorous seedlings in *Epidendrum radicalis* (Pateli et al., 2003).

Furthermore, the costs for preparing a liquid medium are lower than an agar-solidified one, since agar is one of the most expensive components of the culture medium. In the experiments reported before, the culture medium used was always solidified with agar or gelrite (Chapter 4.1.1).

In a pre-experiment, it was found that plants grown in liquid medium showed a low formation of roots. Consequently, two auxins concentrations were used in the present experiment to promote the root formation.

In order to study the effect of medium consistency, auxin concentration and container type on the growth of rose cv. ‘Kardinal’, two experiments were carried out.

a) Consistency of the medium and auxin concentration

In this experiment, two different consistencies of the culture medium (liquid and solidified with agar) and two concentrations of auxin (0.1 and 0.5 g L^{-1} of NAA) were tested on the cv. ‘Kardinal’. Plants were grown in vessels with a volume of 750 ml at a density of 5 plants per vessel.

Rooting phase

According to the analysis of variance, the interaction between the factors consistency of the medium and auxin concentration was significant for quality score of shoots and roots ($P<0.05$).

The explants grown in medium solidified with agar showed higher quality of shoots when compared to explants grown in liquid medium with 0.1 mg L^{-1} of NAA (Figure 23.a). Plantlets grown in agar-solidified medium had a quality score that was two times higher than in liquid medium. On the other hand, the quality score of shoots at 0.5 mg L^{-1} NAA was not significantly different between both consistencies (Figure 23.a).

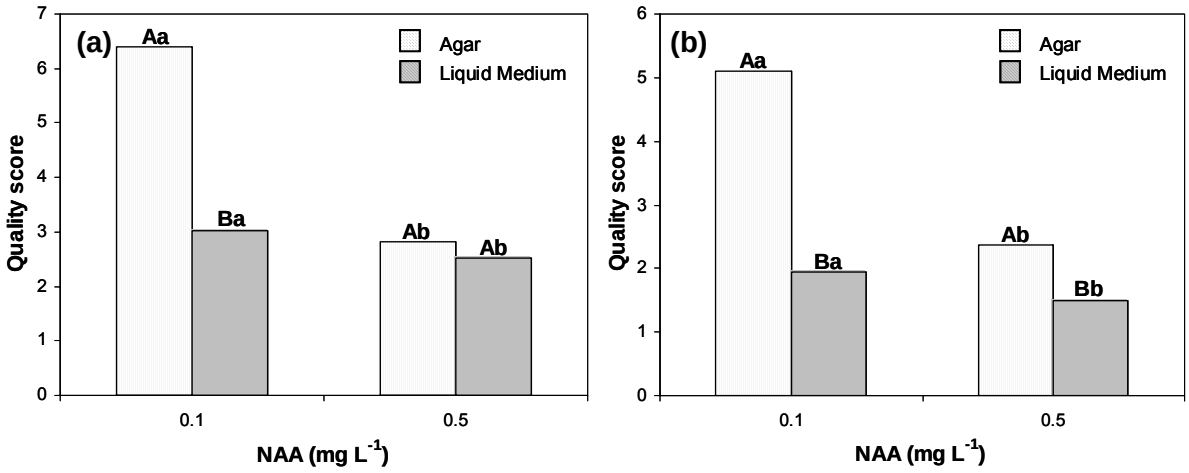

Figure 23. Quality score of shoots (a) and roots (b) in the rooting phase of rose as affected by the concentration of NAA and the consistency of the culture medium. Means followed by the same capital letter (A,B) within the same NAA-level or by the same small letter (a,b) within the same medium consistency are not significantly different according to Duncan's Test ($P<0.05$). A description of the quality scores is presented in the chapter Material and Methods (Tables 3 and 4) (n=60).

Although the use of liquid mediums can be economically favorable, due to a reduction of costs, it may result in a higher hyperhydration occurrence in plants grown *in vitro* (Grattapaglia & Machado, 1998). In the present study, plants grown in liquid medium showed hyperhydration symptoms, which resulted in lower values for the quality score of shoots. Plants in agar-solidified medium, in turn, did not show any hyperhydration symptoms.

Shoots which are perpetually submerged in liquid cultures may have a water-soaked appearance (hyperhydration) and will then be useless for micropropagation (George, 1993). In this case, the use of agar as a gelling agent can prevent the occurrence of hyperhydration.

One alternative to prevent the occurrence of hyperhydration when using liquid medium can be the alternation of physical states of the culture medium, as proposed by Drew (1988). The use of the liquid medium resulted in a rapid growth of axillary branches of papaya. However, an intermediate step on agar-solidified medium was essential, because hyperhydration and leaf abscission occurred when shoots were left in the liquid medium for longer than 14 days (Drew, 1988).

In relation to the different auxin concentrations tested, explants grown at 0.1 mg L^{-1} NAA showed better quality score of shoots when compared to explants grown at 0.5 mg L^{-1} NAA in the medium (Figure 23.a). This behavior was observed for the agar-solidified as well as for the liquid medium. This result indicates that rose does not tolerate an auxin concentration of 0.5 mg L^{-1} NAA, independent of the consistency of the culture medium.

Some of the phenotypical effects produced by auxin are similar to those produced by ethylene. Auxin treatment was found to increase the biosynthesis of the enzyme ACC synthase, thus increasing the production of ethylene from S-adenosylmethionine (George, 1993). This suggests that some of the responses of plants to auxin are ultimately caused by the ethylene produced in response to an auxin treatment (George, 1993). Therefore, the low quality score of shoots at higher concentration of auxin (0.5 mg L^{-1} NAA) observed in our study (see Figure 23.a) may be explained by the effect of auxin on the synthesis of ethylene, since ethylene is a strong promoter of leaf senescence and abscission (Hopkins & Hüner, 2004).

Figure 24 shows the development of plants in agar-solidified and liquid medium at different auxin concentrations. Plants grown in liquid medium supplemented with 0.5 mg L^{-1} NAA showed the worst performance *in vitro*. The use of liquid medium resulted in a decreased growth of plantlets in comparison to the agar-solidified medium (Figure 24).

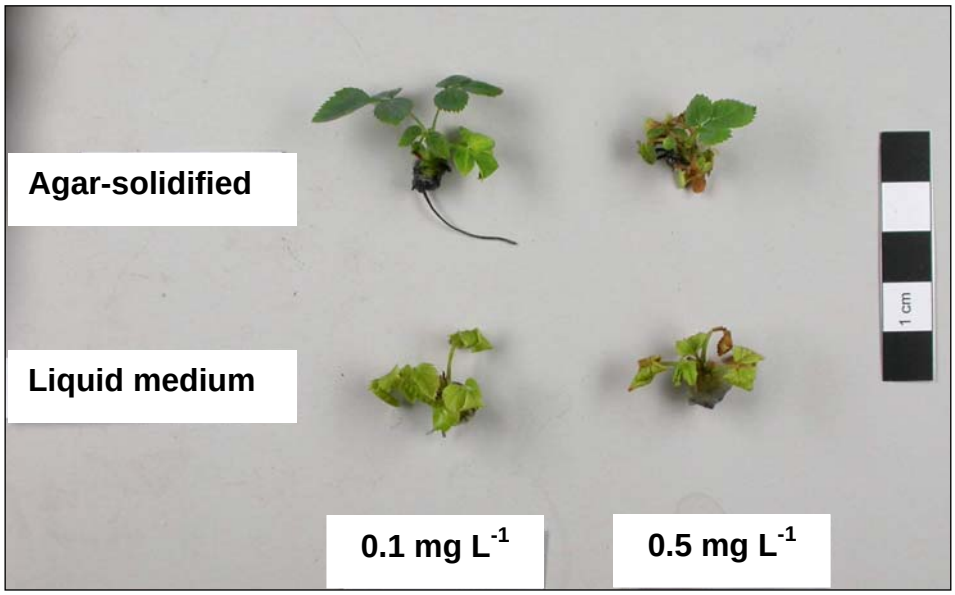

Figure 24. Development of plants of rose cv. 'Kardinal' in the rooting phase as affected by the concentrations of NAA and the consistency of the culture medium.

The quality score of roots was also affected by medium consistency and auxin concentration. Roots grown in culture medium solidified with agar presented higher quality score in comparison to liquid medium (Figure 23.b). This effect was observed at 0.1 mg L^{-1} NAA as well as at 0.5 mg L^{-1} NAA. The best quality score of roots was observed in plants grown in agar-solidified medium with an auxin concentration of 0.1 mg L^{-1} NAA (Figure 23.b).

In relation to the auxin concentrations tested, explants grown at 0.1 mg L^{-1} showed higher quality score of roots in comparison to explants grown at 0.5 mg L^{-1} NAA in both media tested (Figure 23.b).

Our results showed that plants grown at lower auxin concentrations (as e.g. at 0.1 mg L^{-1} NAA) had longer roots (as shown by the higher quality score of roots) than plants grown at 0.5 mg L^{-1} NAA.

Two factors may have contributed to the observed results. The presence of auxin at high concentrations may have inhibited the elongation of roots. In fact, the presence of auxin is necessary only in the induction as well as in the initiation phases of rhizogenesis, which occur in the first days after transferring the plants to the rooting medium. Afterwards, auxin may inhibit root elongation (Grattapaglia & Machado, 1998).

Another factor concerns the induction of callus formation by high concentrations of auxin in the medium. With low auxin concentrations, adventious root formation predominates, whereas with high auxin concentrations root formation fails to occur and callus formation takes place (Pierik, 1997).

In fact, the use of a higher concentration of auxin in the culture medium (0.5 mg L^{-1} NAA) resulted in the formation of bigger callus (data not shown). In addition, more callus were formed in plants grown in liquid in comparison to the agar-solidified medium.

The higher occurence of callus formation in the liquid medium in comparison to the agar-solidified medium may be explained by the higher availability of auxin in the medium. Liquid media may promote a lower resistency to the diffusion of nutrients and auxin in comparison to solid media (Pereira & Fortes, 2004). In potato, the formation of callus was increased in liquid medium, indicating that the effect of auxin was maximized in this medium (Pereira & Fortes, 2004).

The impaired root formation, as a consequence of the formation of callus, may have caused a reduction in shoot growth in plants grown in liquid medium. Furthermore, this effect may have occurred in plants grown at 0.5 mg L^{-1} NAA.

The number of roots per plant was significantly affected only by the consistency of the culture medium. The number of roots decreased from approximately 2.6 roots per plant in the agar-solidified medium up to 0.8 root per plant in the liquid medium (data not shown).

The chlorophyll content was also evaluated. The interaction between medium consistency and auxin concentration was significant (Table 17). Plants grown in agar-solidified medium had significantly higher total chlorophyll as well as chlorophyll *a* and *b* contents than in liquid medium, independent of the auxin concentration. Moreover, higher chlorophyll contents were found in plants grown in agar-solidified medium at 0.1 mg L^{-1} in comparison to 0.5 mg L^{-1} NAA. On the other hand, no significant differences between both auxin concentrations were found when plants were grown in liquid medium (Table 17).

The higher chlorophyll content of plants grown in agar-solidified medium and at low auxin concentration (0.1 mg L^{-1} NAA) confirms the results previously presented in this section, which showed that plants grown under these conditions had the highest quality score of shoots (see Figure 23.a).

Table 17. Total chlorophyll (*a*+*b*), chlorophyll *a* and chlorophyll *b* contents of rose cv. 'Kardinal' as affected by the concentration of NAA and the consistency of the culture medium (*n*=6).

Consistency of the medium	**NAA (mg L^{-1})**	
	0.1	**0.5**
	mg g^{-1} FW	
	Total Chlorophyll *a*+*b*	
Agar-solidified	A 1.66 a[(1)]	A 0.48 b
Liquid	B 0.26 a	B 0.25 a
	Chlorophyll *a*	
Agar-solidified	A 1.34 a	A 0.38 b
Liquid	B 0.20 a	B 0.21 a
	Chlorophyll *b*	
Agar-solidified	A 0.32 a	A 0.11 b
Liquid	B 0.06 a	B 0.04 a

[(1)] Means followed by the same capital letter in the column (A,B) or by the same small letter (a,b) in the row within the same parameter are not significantly different according to Duncan's Test (P=0.05).
FW=fresh weight

Acclimatization phase

The mortality rate of plants was also influenced by the medium consistency and auxin concentration in the rooting phase. The lowest mortality rate (12%) was found when plants were grown in agar-solidified medium with 0.1 mg L^{-1} NAA (Table 18). An increase in the mortality rate up to 56% was observed when the auxin concentration was increased up to 0.5 mg L^{-1} (Table 18).

Higher mortality was observed for plants grown in liquid in comparison to agar-solidified medium. The combined effect of liquid medium and 0.5 mg L^{-1} NAA in the rooting phase resulted in a mortality rate of plants of 100% (Table 18), confirming the negative effect of this culture medium on the growth of rose cv. 'Kardinal'.

Table 18. Mortality rate of plants in the acclimatization phase of rose cv. 'Kardinal' as affected by the concentrations of NAA and the consistency of the culture medium in the rooting phase (*n*=35).

Consistency of the medium	NAA (mg L^{-1})		Influence of NAA conc.
	0.1	0.5	
	%	%	χ^2
Agar-solidified	12[(1)]	56	****
Liquid	86	100	****
Influence of consistency (χ^2)	****	****	

[(1)] For the comparison of the mortality rate between different NAA concentrations at the same medium consistency as well as between different medium consistencies at the same NAA concentration, the chi^2-test was used.
**** Significant at 0.1%

b) Type of container

The growth of micropropagated plants may be influenced by the size of the container in which plants are cultivated. Therefore, the performance of two different types of container (test tube and vessel with a capacity of 750 ml) was compared in an experiment. Plants were grown in agar-solidified medium with a concentration of 0.1 mg L^{-1} NAA. The objective of this study was to compare the method used in our Department, namely the use of test tubes, with the use of vessels with higher capacity (750 ml).

No significant differences between both types of container were observed on the quality score of shoots and roots and number of roots per plant. Since the growth of rose was not influenced by the container type, other factors may be taken into account when choosing the vessel to be used in micropropagation, namely costs for material and labor.

One disadvantage in using bigger vessels is the higher risk of contamination of plants. In bigger vessels, several plants are cultivated next to each other. When one plant in the vessel is contaminated, this contamination may be rapidly transmitted to the other plants in the same vessel.

According to Kozai (1991), vessels used for micropropagation must be relatively small in size in order to avoid a sudden large loss of plantlets *in vitro* due to contamination of any single plantlet at one time. In contrast, for individualized plants in test tubes, the contamination is restricted to one plant.

Furthermore, the amount of culture medium used in the vessel with a capacity of 750 ml (120 ml for 5 plants, i.e. approximately 24 ml per plant) is higher than the amount used in test tubes (15 ml per test tube) and, consequently, per plant. On the other hand, the use of bigger recipients may facilitate the subculturing process, because a lower number of vessels have to be manipulated.

4.1.6 Substrates in the acclimatization phase

The acclimatization phase is one of the most critical and expensive phases in the production of micropropagated plants. Consequently, it can be a limiting factor for the cultivation of ornamental plants in large scale (Lewandowski, 1991).

The substrate used for growing plants in the acclimatization phase is one of the key factors that can be manipulated to optimize plant growth, since substrate may define the patterns of drainage and development of new roots.

Furthermore, in the experiments carried out before, a high mortality rate of plants during acclimatization was observed. In all situations, peat was used as substrate. Thus, the present experiment was carried out with the objective to evaluate the performance of alternative substrates (peat mixed with perlite in different proportions) in the acclimatization of rose, in order to optimize the growth and to increase the survival of plants in this phase.

Four different substrates with different proportions of peat and perlite (per) were tested: 100% peat (pure peat) and three different mixtures with different proportions of peat and perlite (v/v), namely 3peat:1per, 1peat:1per and 1peat:3per. Moreover, two different light intensities were tested in the *ex vitro* environment (60 and 120 μmol m^{-2} s^{-1}). The cv. 'Kardinal' was used.

The results of the determination of the water retention capacity of the different mixtures of peat and perlite are shown in Figure 25. All substrates evaluated presented high total porosity, with values that varied between 94% in pure peat and 97% in the mixture 1peat:3per, what is a desirable characteristic for the substrates used in the cultivation of plants in trays.

According to Ballester-Olmos (1993), a total porosity in the substrate between 70% and 90% is optimal for growth of horticultural plants. However, the value of total porosity includes macroporos as well as microporos and the proportion between both sizes of pores will actually define the drainage pattern and the water retention capacity of a given substrate.

An increase in the air space was observed with increasing the proportion of perlite in the substrate mixture, with values that varied from 23% (100% peat) up to 26% (in the mixture 1peat:3per) (Figure 25). Referential values for air space in substrates

vary between 20% and 30% (Boodt & Verdonck, 1972). Therefore, the substrates tested in our experiment were within the recommended values.

The portion of available water (easily available water + water buffering capacity) was higher in the mixtures 1peat:1per and 1peat:3per (approximately 48%), in comparison to pure peat (about 33%). Pure peat, in turn, presented a high value for water retention after drainage at 100 hPa (less readily available water) (approximately 38%), corresponding to the amount of water retained in the microporos. The increasing addition of perlite to the substrate mixture reduced the water volume retained at 100 hPa (less readily available water), with about 21% of water remaining after drainage at 100 hPa in the mixture 1peat:3per (Figure 25).

In materials constituted of very small particles (as e.g. in peat), the remaining water after drainage at 100 hPa is high, and, in consequence, drainage may be reduced (Kämpf, 2000), especially when such materials are used as substrate in containers (plugs) with low height. The use of plugs with low height may reduce water drainage, promoting a reduction in the air space. Therefore, plants grown under these conditions usually require a substrate with lower water retention capacity, to prevent the excessive accumulation of water in the root area.

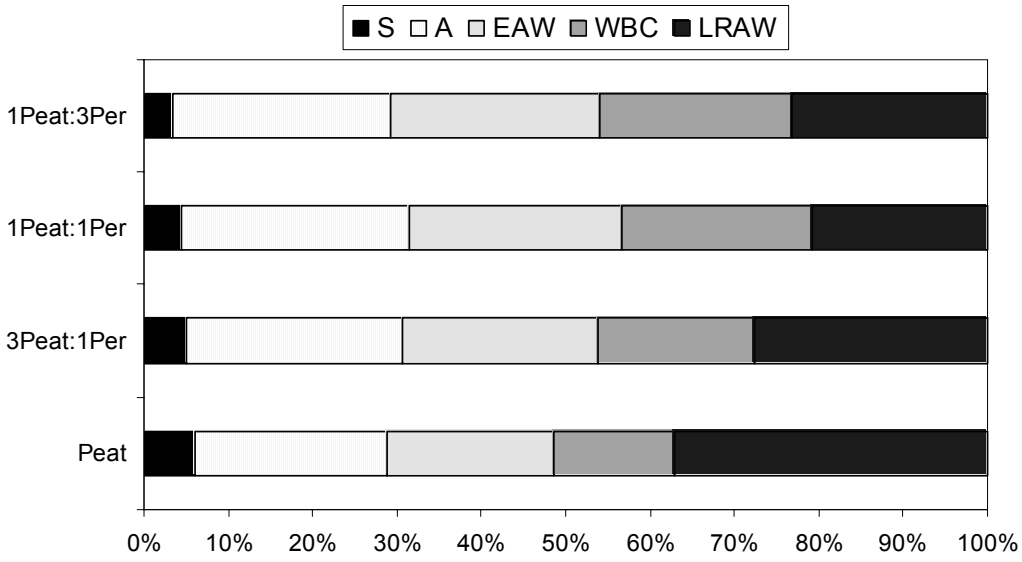

Figure 25. Variation in the volume of solid material (S), air space (A), easily available water (EAW), water buffering capacity (WBC) and less readily available water (LRAW) for the substrates tested. Per = Perlite.

Comparable values for total porosity in samples of black peat and carbonized rice hulls were observed by Kämpf (2000). However, the proportion of microporos was

higher for peat, whereas macroporos were the predominating size of pores in carbonized rice hulls (Kämpf, 2000). Consequently, the irrigation should be carried out in different ways when comparing both substrates. Carbonized rice hulls have a lower water retention capacity, requiring a smaller interval between irrigations. On the other hand, peat shows a larger water retention capacity and the interval between irrigations may be increased. Rice hulls are an easy available industrial residue in rice-producing countries. After the carbonization process, rice hulls have a neutral pH, a low bulk density and a high total porosity, being a good alternative mixture material (Kämpf & Jung, 1991).

After evaluation of the physical characteristics of the substrates, the parameters quality score of shoots and dry biomass of shoot and roots were also evaluated. Accordingly to the results of the analysis of variance, no significant effect of the substrate and light intensity tested was verified on these parameters (data not shown).

In the other hand, the mortality rate of plants in the acclimatization was significantly influenced by the substrate and light intensity. The mortality rate was evaluated at four weeks after transferring the plants from the *in vitro* to the *ex vitro* environment and the results are shown in Table 19.

Table 19. Mortality rate of plants at four weeks after onset of acclimatization as affected by the proportion of peat in the substrate and by light intensity (*n*=40).

Light	**Substrate**				**Influence**
intensity	**1peat:3per**	**1peat:1per**	**3peat:1per**	**Pure peat**	**of substr.**
μmol m^{-2} s^{-1}	%	%	%	%	$\chi 2$
60	15	35	35	35	****
120	25	25	30	27.5	ns
Influence of light ($\chi 2$)	*	ns	ns	ns	

(1) For the comparison of the mortality rate between different substrates at the same light intensity level as well as between different light intensities at the same substrate, the chi^2-test was used.
ns not significant
* Significant at 10%
**** Significant at 0.1%

Plants cultivated in the mixture 1peat:3per under the light intensity of 60 μmol m^{-2} s^{-1} showed the lowest mortality rate (15%). At this light intensity, the mortality rate of plants increased up to 35% as the proportion of peat in the substrate mixture increased (1peat:1per). Afterwards, the mortality rate remained constant at 35% with increasing the proportion of peat in the mixture (Table 19). At 120 μmol m^{-2} s^{-1}, in turn, the mortality rate was not significantly affected by the substrate (Table 19).

The results showed that rose cv. 'Kardinal' prefers substrates with a larger air space and lower proportion of microporos, when using trays with 4 cm height for acclimatization. Our results are supported by results reported by Offord & Campbell (1992) for *Telopea speciosissima*. The plants were acclimatizated under high humidity conditions in three different substrates: sand, sand + peat (1:1 v/v) and peat + perlite (1:1 v/v). The mixture peat + perlite showed the largest number of explants rooted *ex vitro* as well as the largest survival of plantlets (80-90%, in comparison to sand with 50%).

The better performance of this substrate appears to be correlated with the increase in air-filled porosity of the substrate with the addition of perlite. Moreover, Avanzato & Cherubin (1993) studied the rooting process of microcuttings of apple in substrates with varying water retention capacities. Larger survival and rooting rates were found for substrates constituted by perlite (rough perlite), which had larger air space (19%) and smaller water retention capacity.

The use of perlite mixed with organic materials (as e.g. peat) in the substrate may be recommended in order to increase the looseness and permeability of the substrate as well as to improve the aeration, being a good option to be used in a mixture of substrates (Ballester-Olmos, 1993). In our study, the use of perlite in the mixture with peat (in a proportion of 1peat:3per) showed the best results concerning the decrease on the mortality rate of plantlets in the acclimatization (see Table 19).

For *Limonium platyphyllum*, it was found that plants grew better at the beginning of the acclimatization in trays that were 5 cm high filled with a substrate with larger air space (carbonized rice hulls). After acclimatization, plants were transferred to trays that were 11 cm high. In this situation, the substrate may have a lower air space and higher water retention capacity (carbonized rice hulls + peat) (Schneider, 2000), since drainage of water is enhanced by increasing the height of the plug.

The effect of the light intensity on the mortality rate was not significant for all substrates evaluated, except for the substrate 1peat:3per. For this substrate, the mortality rate of plantlets was significantly higher at 120 μmol m^{-2} s^{-1} than at 60 μmol m^{-2} s^{-1} (Table 19).

The extractable chlorophyll content was also determined in plants grown under the light intensity of 120 μmol m^{-2} s^{-1}. Total chlorophyll as well as chlorophyll *a* and *b* contents were higher for plants grown in the substrate with a larger proportion of perlite (1peat:3per) (Table 20).

Table 20. Total chlorophyll (*a*+*b*), chlorophyll *a* and chlorophyll *b* contents at four weeks after onset of acclimatization as affected by the proportion of peat in the substrate (*n*=6).

Substrate	Total chlorophyll (*a*+*b*)	Chlorophyll *a*	Chlorophyll *b*
	mg g^{-1} FW		
1peat : 3per	3.01 a[(1)]	2.33 a	0.68 a
1peat : 1per	1.84 c	1.48 b	0.36 c
3peat : 1per	2.30 b	1.81 b	0.49 b
Pure peat	2.12 bc	1.69 b	0.43 bc

[(1)] Means followed by the same letter in the column are not significantly different according to Duncan's Test (P=0.05).
FW=fresh weight

Our results showed that the acclimatization process of rose cv. 'Kardinal' was improved by using substrates with higher drainage capacity (as e.g. in the mixture 1peat:3per) in the initial phase of acclimatization. However, after this initial acclimatization phase, plants must be transferred to bigger containers. Therefore, the substrate with more perlite may be not reliable for acclimatization after the initial phase, since plants require higher water availability (due to higher transpiration and number of roots than in the initial phase) and the substrate with more perlite has a lower water retention capacity and, consequently, a lower water supply capability.

4.1.7 Rose: conclusions and outlook

Based on the results found in the present study concerning the effect of different cultural conditions on the micropropagation of *Rosa* sp. (rose), the following considerations can be done.

The growth of rose plants *in vitro* was affected by the type of gelling agent added to the culture medium. The best results concerning the formation of shoots were found by using agar as gelling agent at the concentration of 6 mg L^{-1} in the multiplication phase, whereas in the rooting phase no differences concerning the formation of shoots and roots were found between the concentrations of 6 and 9 mg L^{-1} of agar. Since agar is one of the most expensive components of the culture medium, the use of 6 g L^{-1} is recommended. The use of gelrite as gelling agent in the culture medium in the concentrations tested in the present study (2 and 3 mg L^{-1}), cannot be recommended for the micropropagation of *Rosa* sp. cv. 'Frisco' and 'Lambada', because shoot and root formation was impaired in this medium.

The type of closure used in the test tubes was important for the micropropagation. In the rooting phase, the use of glass, steristop or aluminium as closure improved the growth of shoots in comparison to plastic film for all cultivars tested ('Frisco', 'Kardinal' and 'Lambada'). Concerning the formation of roots, optimum results were obtained by using glass as closing material for all cultivars. However, the glass cover did not prevent the penetration of thrips, resulting in higher fungal contamination of the culture medium. Based on the results of the present study, the development of plants was impaired by the use of plastic film as closure.

Concerning the effect of the sucrose concentration in the culture medium, the results showed that plants of rose cv. 'Kardinal' grown at 10 g L^{-1} in the rooting phase produced more roots than plants grown at 20 or 40 g L^{-1} of sucrose. Furthermore, plants grown in culture medium supplemented with 10 g L^{-1} of sucrose showed higher survival rates in the acclimatization phase than with the other sucrose concentrations tested. The results indicated that a reduction in the sucrose concentration in the culture medium up to 10 g L^{-1} is favorable to the micropropagation of rose. In addition, the growth of this species was not influenced by the use of Magenta B-CAP covers with or without filter in baby food culture jars.

A significant effect of the room temperature on *in vitro* development of rose plants was found for temperatures between 16°C and 24°C. The formation of roots was improved at 16°C, whereas shoot growth was better at higher temperatures (20°C or 24°C) for all cultivars tested (‘Frisco’, ‘Kardinal’ and ‘Lambada’). It indicates that different temperatures are required for the optimum growth of shoots and roots.

The influence of the duration of the rooting phase on the growth and mortality rate of plants was also evaluated. A minimum duration of the rooting phase of five weeks should be used in the micropropagation of rose.

Explants of the cv. ‘Kardinal’ grown in culture medium solidified with agar showed higher quality of shoots and roots in the rooting phase as well as lower mortality of plants in the acclimatization when compared to explants grown in liquid medium.

Moreover, explants grown at 0.1 mg L^{-1} of NAA (auxin) in the rooting phase presented better quality score of shoots in this phase as well as higher survival in the acclimatization phase when compared to explants grown at 0.5 mg L^{-1} of NAA in the medium. Therefore, it can be concluded that the liquid medium is not appropriate for the micropropagation of rose as well as that this species does not tolerate an auxin concentration of 0.5 mg L^{-1} NAA, independent of the consistency of the medium.

No significant effect of the container size on the quality score of shoots and roots and on the number of roots per plant was found. Other factors such costs for material and labor should be taken into account when choosing the appropriate vessel to be used in the micropropagation of rose.

In relation to the initial phase of acclimatization, substrates with higher drainage capacity (as e.g. a mixture of peat and perlite in the proportion 1peat:3perlite v/v) should be used, since the results showed that rose prefers substrates with a larger air space and lower proportion of microporos, when using trays with 4 cm height.

The use of perlite mixed with peat may be recommended in order to increase the looseness and permeability of the substrate as well as to improve the aeration. When using trays that are 4 cm high, the following physical characteristics of the substrate concerning its water retention capacity can be recommended: 26% air space, 48% portion available water and 21% less readily available water.

Concerning the micropropagation of the different cultivars used in our experiments, it was shown that the cv. ‘Kardinal’ is a cultivar easy to root under *in vitro* conditions. Furthermore, it showed a better growth of shoots *in vitro* in comparison to the cvs.

‘Frisco’ and ‘Lambada’, being a cultivar easy to micropropagate. In contrast, the cv. ‘Frisco’ can be considered a cultivar difficult to root *in vitro* and, in general, a cultivar of difficult micropropagation. The cv. ‘Lambada’ can be considered as intermediate concerning the rooting and the growth under *in vitro* conditions.

Further experiments should be carried out with the objective to investigate the effect of different cultural conditions in the acclimatization *ex vitro* on the growth and mortality of rose in this phase. In the present study, only the effects of substrate and light intensity *ex vitro* were studied. Other important topics to be investigated could be type and frequency of irrigation, CO_2 enrichment, air humidity and the use of alternative substrates.

4.2 Globe artichoke (*Cynara scolymus* L. cv. 'Green globe')

4.2.1 Growth regulators: auxin and gibberellin

Globe artichocke has been shown to be a plant of difficult propagation under *in vitro* conditions. For instance, explants have been found to be difficult to root (Rossi & Paoli, 1992; Kanakis & Demetriou, 1993). With the objective to study and to optimize the formation of roots and shoots in globe artichoke, the following experiment was carried out.

The treatments consisted of different concentrations of auxin (NAA) and gibberellin (GA_3) in the culture medium. Two levels of NAA (0.5 and 2.0 mg L^{-1}) and three levels of GA_3 (without GA_3, 1.0 and 5.0 mg L^{-1}) were tested. The experiment was carried out in the rooting and in the acclimatization phases.

In this experiment, the transfer of plants to a second rooting medium was necessary to promote the growth of plants, since plants did not show a satisfactory growth at the end of the first rooting phase *in vitro*.

Rooting phase

a) First rooting medium

The quality score of shoots was influenced by the use of different auxin and gibberellin concentrations in the culture medium. The interaction between the factors auxin and gibberellin concentration was not significant ($P>0.05$).

The results showed that the quality score of shoots decreased from 4.3 up to 2.6 with increasing the concentration of NAA in the culture medium from 0.5 mg L^{-1} up to 2.0 mg L^{-1}, showing the negative effect of an increment of the auxin concentration on the growth of artichoke shoots (data not shown). The decrease in the quality score of shoots with increasing the auxin concentration was caused, in part, by the occurrence of plants with hyperhydration symptoms. This may be explained by the influence of auxin on the mechanical properties of cell walls.

Auxin may indirectly influence cell enlargement, through its effects on a number of biophysical parameters, including cell wall mechanical properties, turgor pressure,

osmotic pressure and water permeability (Taiz & Zeiger, 1991). Due to the influence of auxin, cells may become more plastic.

Since one of the main characteristics of vitrified leaves is the occurrence of cells filled with water (Debergh et al., 1981), one can hypothesize that the use of higher concentration of auxin in the medium promoted hyperhydration in plants due to the positive effect of this growth regulator on cell enlargement and water permeability of cell walls, facilitating the uptake of water by cells.

In relation to the different concentrations of GA_3 tested, one can see that the use of this growth regulator promoted a decrease in the quality score of shoots. The supplementation of the culture medium with gibberellic acid (1.0 or 5.0 mg L^{-1} GA_3) resulted in the formation of shoots with lower quality scores than in absence of this growth regulator (Figure 26).

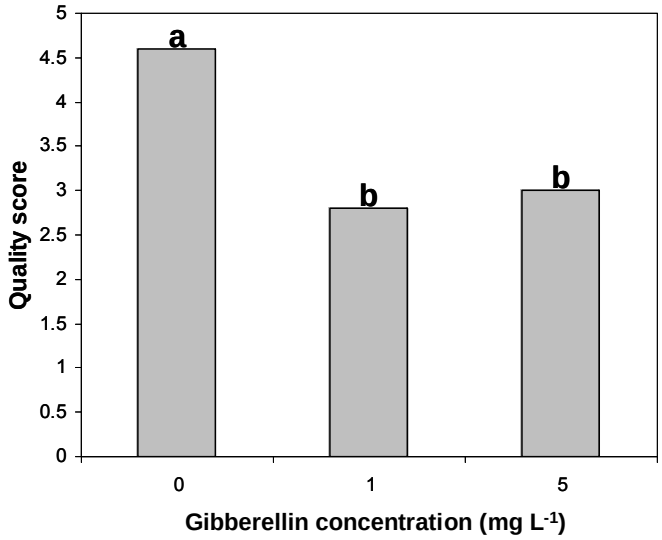

Figure 26. Quality score of shoots of globe artichoke in the rooting phase grown under different concentrations of GA_3. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=60).

The results concerning the rooting percentage were comparable to that of the quality score of shoots. The rooting percentage significantly decreased with increasing the concentration of NAA from 0.5 up to 2.0 mg L^{-1} for all levels of GA_3 (Table 21). This decrease is, in part, explained by the higher formation of callus observed in plants grown at the highest auxin concentration (2.0 mg L^{-1}) (visual observation).

Table 21. Rooting percentage of globe artichoke in the rooting phase grown under different concentrations of GA_3 and NAA (first rooting medium) (*n*=60).

GA_3	**NAA (mg L^{-1})**		**Influence of**
	0.5	**2.0**	**NAA conc.**
mg L^{-1}	%	%	$\chi 2$
0	32	18	***
1.0	17	8	*
5.0	13	3	***
Influence of GA_3 conc. ($\chi 2$)	****	****	

(1) For the comparison of the rooting percentage between different NAA concentrations at the same GA_3 level as well as between different GA_3 concentrations at the same NAA level, the chi^2-test was used.
* Significant at 10%
*** Significant at 1%
**** Significant at 0.1%

The results that have been published concerning the effect of different auxin concentrations on the growth of different species are conflicting.

Several plant species showed a decreased development of shoots and roots in presence of high NAA concentrations in the rooting medium, as e.g. rose grown under auxin concentrations higher than 0.1 mg L^{-1} (Horn, 1992) and apple cv. 'Jork 9' (Klerk et al., 1997) and cv. 'Fred Hough' (Centellas et al., 1999) grown in medium with more than 0.5 mg L^{-1} of NAA, as well as in *Limonium latifolium* grown at auxin concentrations that exceeded 1.0 mg L^{-1} (Fior et al., 2000).

Some species, in turn, may not respond to increments in the auxin concentration in the rooting medium. Studying the rooting of axillary buds of *Minthostachys andina*, Castillo & Jordan (1997) found that the rooting was similar in presence of NAA at the concentrations of 0.3, 0.5 or 1.0 mg L^{-1}, combined with 2.0 mg L^{-1} IBA (auxin). In plantlets of carnation, no increase in root formation was found when the concentration of NAA in the culture medium was incremented from 0 up to 1.0 mg L^{-1} (Cuzzuol et al., 1996).

On the other hand, it has been shown that an increase in the concentration of NAA may be even beneficial to some plant species. In explants of *Swietenia macrophylla* King, it was found that concentrations of 2.0 mg L^{-1} and 5.0 mg L^{-1} NAA in the culture

medium were more efficient for induction of rooting than 1.0 mg L^{-1} NAA (Lopes et al., 2001).

This differential response between different species shows that the effect of growth regulators is specific for each species. In fact, the responses of tissues grown *in vitro* to growth regulators can vary according to cultural conditions, the type of explant and the plant genotype (George, 1993).

The supplementation of the culture medium with GA_3 resulted in a significant decrease in the rooting percentage for both levels of NAA (Table 21). Rooting percentage of 32% was found for plants grown in absence of GA_3, combined with 0.5 mg L^{-1} NAA. The supplementation of the culture medium with gibberellin (1.0 or 5.0 mg L^{-1} GA_3) at this level of NAA resulted in the formation of roots in approximately only 15% of plants (Table 21). This same negative effect of gibberellin was also observed when plants were grown at 2.0 mg L^{-1} NAA. Under this NAA concentration, 18% of plants rooted in absence of GA_3, whereas only 3% of plants rooted with 5.0 mg L^{-1} GA_3 (Table 21).

This observation is not supported by the results found by Morzadec & Hourmant (1997). They observed a significant increment in rooting and leaf growth of globe artichoke cv. 'Camus de Bretagne' grown on rooting media containing 1.0 or 5.0 mg L^{-1} GA_3. This differential response may be explained by the intrinsic characteristics of the different cultivars tested.

b) Second rooting medium

The composition of the second rooting medium was the same for all treatments and no growth regulator was supplemented.

The quality score of shoots grown in the second rooting medium was not significantly influenced by the different auxin and gibberellin concentrations used in the first rooting medium (data not shown).

On the other hand, the formation of roots (as evaluated by the rooting percentage) was significantly affected by the different gibberellin and auxin concentrations used in the first rooting medium. Plants grown in absence of GA_3 and with 0.5 mg L^{-1} NAA produced, in the second rooting medium, plants with the highest rooting percentage (39% of plants rooted) (Table 22). The increase in the GA_3 concentration up to

5.0 mg L^{-1} resulted in a significant decrease in the rooting percentage independent of the NAA concentration (Table 22).

Table 22. Rooting percentage of globe artichoke in the rooting phase grown under different concentrations of GA_3 and NAA (second rooting medium) (*n*=40).

GA_3	**NAA (mg L^{-1})**		**Influence of**
	0.5	**2.0**	**NAA conc.**
mg L^{-1}	%	%	$\chi 2$
0	39	21	***
1.0	20	11	*
5.0	16	8	*
Influence of GA_3 conc. ($\chi 2$)	****	***	

(1) For the comparison of the rooting percentage between different NAA concentrations at the same GA_3 level as well as between different GA_3 concentrations at the same NAA level, the chi^2-test was used.
* Significant at 10%
*** Significant at 1%
**** Significant at 0.1%

The slightly increase in rooting percentage in the second rooting medium (Table 22) in comparison to the first rooting medium (see Table 21) shows that the transfer of plants to a second rooting medium without growth regulators was, to some extent, efficient to promote a better root formation. Normally, GA_3 is an inhibitor of root formation (George, 1993). On the other hand, a pre-treating of the plant material with GA_3 may enhance root formation when cuttings are afterwards placed on a root-inducing medium (without GA_3).

In the present experiment, the rooting percentage slightly increased between the first and the second rooting media (in absence of growth regulators) for all GA_3 concentrations. However, the negative effect of the supplementation of the first rooting medium with GA_3 was maintained when plants were transferred to the second rooting medium without gibberellin (Table 22). The best results concerning rooting percentage in the second medium were found in absence of GA_3 in the first rooting medium for both levels of NAA.

Furthermore, the negative effect of high auxin concentration (2.0 mg L^{-1} NAA) in the first rooting medium on the rooting percentage was maintained for all levels of GA_3 (Table 22).

In mahogany (*Swietenia macrophylla* King), positive effects of higher NAA concentrations (up to 5.0 mg L^{-1}) were found when explants remained for five days in the culture medium in contact with auxins and, afterwards, were transferred to new medium without growth regulators. In this case, the concentrations of 2.0 and 5.0 mg L^{-1} NAA were found to be more efficient for induction of rooting than 0.1 mg L^{-1} NAA (Lopes et al., 2001).

In our experiment, roots remained in contact with NAA in the first rooting medium for six weeks. Under these conditions, the prolonged contact of roots with higher concentrations of this growth regulator (2.0 mg L^{-1} NAA in comparison to 0.5 mg L^{-1} NAA) promoted a reduction in the number of plants that rooted. This negative effect of high auxin was maintained when plants were grown in a second rooting medium in absence of growth regulators for five weeks.

Acclimatization phase

The lowest mortality rate (55%) was observed in plants grown in the rooting phase in culture medium (first rooting medium) supplemented with 0.5 mg L^{-1} of NAA and in absence of GA_3 (Table 23).

The supplementation of GA_3 to the first rooting medium significantly increased the mortality rate at both NAA levels (Table 23). When the medium was supplemented with 0.5 mg L^{-1} of NAA, an increase in GA_3 concentration up to 5.0 mg L^{-1} resulted in an increment in the mortality rate from 55% (in absence of GA_3) up to 87%. In the treatments supplemented with 2.0 mg L^{-1} of NAA, the mortality rate increased from 78% (in absence of GA_3) up to values around 93% by adding GA_3 to the culture medium (Table 23).

The difficulty in obtaining a good survival percentage in globe artichoke has been reported in other studies (Ordas et al., 1990; Iapichino, 1996). This may be caused by the excessive water loss through the leaves during acclimatization, poor vascular conetions between shoots and roots, and limited uptake of water (Johnson et al., 1992).

Table 23. Mortality rate of globe artichoke in the acclimatization phase grown under different concentrations of GA_3 and NAA in the rooting phase (first rooting medium) (*n*=35).

GA_3	**NAA (mg L^{-1})**		**Influence of**
	0.5	**2.0**	**NAA conc.**
mg L^{-1}	%	%	$\chi 2$
0	55[(1)]	78	****
1.0	82	95	***
5.0	87	91	ns
Influence of GA_3 conc. ($\chi 2$)	****	****	

[(1)] For the comparison of the mortality rate between different NAA concentrations at the same GA_3 level as well as between different GA_3 concentrations at the same NAA level, the chi^2-test was used.

[ns] not significant

*** Significant at 1%

**** Significant at 0.1%

Based on the results presented, one can conclude that the supplementation of the culture medium in the rooting phase with GA_3 cannot be recommended for the micropropagation of globe artichoke cv. ‘Green globe’. Moreover, the use of lower concentrations of NAA (0.5 mg L^{-1} in comparison to 2.0 mg L^{-1}) is beneficial to the micropropagation of this cultivar.

4.2.2 Growth regulators (auxin and cytokinins) and duration of the rooting phase

Globe artichoke is a plant of difficult propagation *in vitro*, especially in the rooting phase, due to problems concerning the formation of roots. Moreover, the formation of roots may be influenced by the previous phase, namely the multiplication.

In order to study the effect of different concentrations and types of the growth regulators auxin and cytokinin in the culture medium in the multiplication phase as well as of the duration of the rooting phase, this experiment was carried out during the multiplication, rooting and acclimatization phases. Treatments consisted of different NAA and cytokinin (BA, Kinetin and 2iP) concentrations in the culture medium in the multiplication phase and were as follows:

a) 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA

b) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA

c) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin (Kin)

d) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP

Moreover, different durations of the rooting phase were tested (two, four and six weeks). In this phase, plants were cultivated in the same culture medium supplemented with 0.1 mg L^{-1} NAA.

Multiplication phase

The number of shoots per plant was affected by the type and concentration of cytokinin and auxin in the culture medium (Figure 27.a).

The highest number of shoots per plant (approximately seven) was found with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA (Figure 27.a). In contrast, plants grown in culture medium supplemented with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP produced on average only one shoot. Intermediate results were observed with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA and with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin (Figure 27.a).

As shown in Figure 27.a, the use of 2.0 mg L^{-1} of NAA and BA in the multiplication phase was more effective in relation to the formation of shoots than 0.2 mg L^{-1} of both growth regulators.

Supporting results were found for *Gypsophila paniculata*. Adventitious shoots formed only at high concentration of the cytokinins Kinetin, Zeatin and BA. The

supplementation of the culture medium with 6.0 mg L^{-1} BA led to a more than two fold increase in the number of explants forming shoots as compared to 3.0 mg L^{-1} BA (Ahroni et al., 1997).

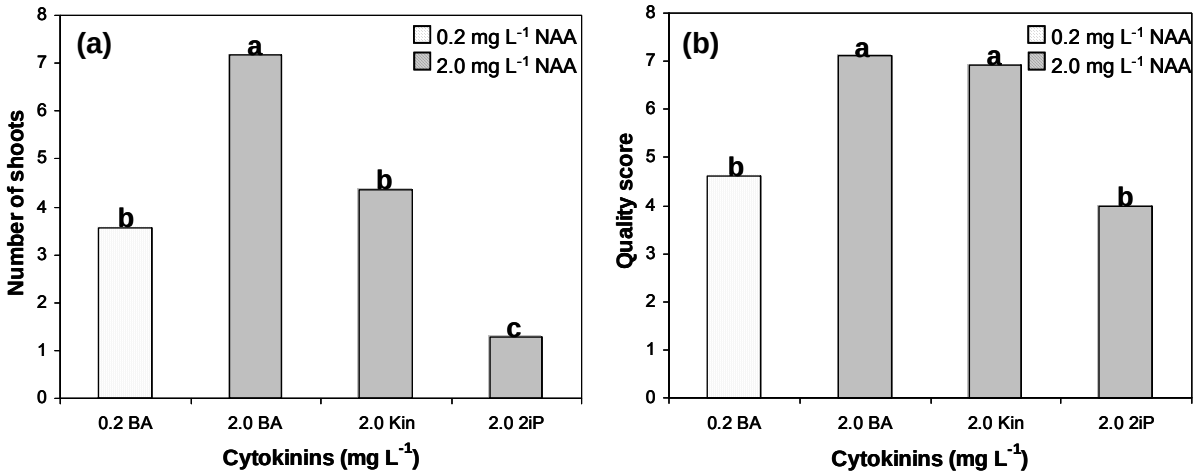

Figure 27. Number of shoots per plant (a) and quality score of shoots (b) of globe artichoke in the multiplication phase grown under different concentrations and types of auxin and cytokinins. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality scores is presented in the chapter Material and Methods (Table 3) (*n*=60).

The quality score of shoots was also influenced by the concentration and type of growth regulators in the culture medium. The highest quality scores were found with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA as well as with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin (Figure 27.b). On the other hand, the quality score of shoots was reduced in plants grown with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP and with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA (Figure 27.b).

Comparable results were found by Sarwar et al. (1998) for apple. They verified that the cytokinin 2iP was not as useful for stem elongation as the cytokinin BA. Furthermore, it was found for Greek olive (*Olea europaea* L.) that Zeatin was the most effective cytokinin among those tested (namely BA, Zeatin and 2iP) for shoot proliferation, while 2iP was the least effective (Grigoriadou et al., 2002).

The formation of callus was also evaluated by means of quality score values. Higher values indicate the formation of smaller callus or no formation, since the formation of callus is not desirable in the rooting phase. The use of 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin resulted in higher quality score, whereas 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP

promoted a decrease in this parameter, indicating the formation of bigger callus in plants grown in medium supplemented with 2iP (Figure 28). Moreover, a reduction in the concentration of NAA and BA from 2.0 down to 0.2 mg L^{-1} did not prevent the formation of callus (Figure 28).

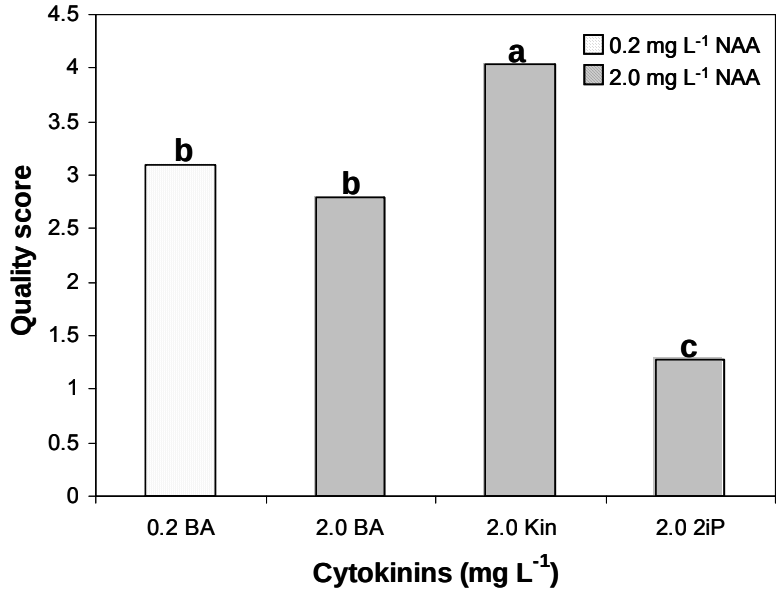

Figure 28. Quality score of callus of globe artichoke in the multiplication phase grown under different concentrations and types of auxin and cytokinins. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 5) (*n*=60).

Based on the results concerning the multiplication phase, one can state that the combination 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA is reliable for the multiplication of globe artichoke cv. 'Green globe'. In contrast, the supplementation of the culture medium with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP cannot be recommended for the multiplication of this cultivar.

In the case of globe artichoke, the kind of cytokinin used in the medium had an important role. Artichoke shoot development was improved by BA in comparison to Kinetin and 2iP. In fact, the requirement for a particular cytokinin has been shown for different species and for different cultivars within the same species (Paek et al., 1987; George, 1993; Sarwar et al., 1998).

Supporting results were found for globe artichoke cv. 'Violetto di Sicilia' by Iapichino (1996), who found that BA was the most effective in promoting shoot proliferation in comparison to Zeatin, 2iP and Kinetin, as well as by the studies carried out by

Kanakis & Demetriou (1993) in the cv. 'Argos'. They found that BA at 3.0 mg L^{-1} was more effective than kinetin (3.0 mg L^{-1}) in the regeneration of adventitious shoots.

The fact that shoot development was improved by the cytokinin BA in comparison to Kinetin and 2iP may indicate a differential tolerance of the artichoke cultivar used in our study to different types of cytokinins. Several studies have shown that different cultivars within the same species may have different degrees of tolerance to some types of cytokinins (George, 1993, Iapichino, 1996, Sarwar et al., 1998). In apple, the strain 'Wijcik' exhibited more tolerance to BA and 2iP than 'McIntosh' (Sarwar et al., 1998).

Concerning our results, one can hypothesize that the cv. 'Green globe' is more tolerant to the presence of BA than of 2iP in the culture medium. This differential behavior can be related, in part, to a better ability of this cultivar to metabolize or compartmentalize BA in comparison to 2iP (Sarwar et al., 1998).

Actually, the reliable concentration of each type of growth regulator in the culture medium may differ greatly according to the kind of plant or cultivar being cultured, the culture conditions and the compounds used. Interactions between auxins and cytokinins are often complex, and more than one combination of substances is likely to produce optimum results (George, 1993, Sarwar et al., 1998). For instance, the combination of the cytokinins BA with Zeatin was found to improve the shoot proliferation rate for olive (*Olea europaea* L.) compared to BA alone (Grigoriadou et al., 2002), indicating that the mixture of compounds may give better results than either compound alone.

Rooting phase

The highest quality score of shoots was found in plants grown in culture medium supplemented with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA as well as with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin in the multiplication phase after six weeks in the rooting medium (Table 24). Furthermore, the supplementation of the culture medium with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP or 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA resulted in reduced values for this parameter (Table 24).

After two and four weeks of the rooting phase, in turn, the quality score of shoots was similar for all combinations of growth regulators tested (Table 24).

Concerning the duration of the rooting phase, plants grown with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA or 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin in the multiplication phase produced better quality shoots when the rooting phase was extended up to six weeks (Table 24). For the medium supplemented with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA, in turn, the optimal duration of the rooting phase was four or six weeks. On the other hand, the quality score of shoots was not affected by the duration of the rooting phase when the culture medium was supplemented with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP (Table 24).

Table 24. Quality score of shoots of globe artichoke in the rooting phase grown under different concentrations and types of auxin and cytokinin in the multiplication phase[(1)] (*n*=50).

Auxin + Cytokinin	**Duration of the rooting phase (weeks)**		
	2	**4**	**6**
mg L^{-1}	quality score		
0.2 NAA + 0.2 BA	A 3.0 b[(2)]	A 3.7 b	A 4.9 a
2.0 NAA + 2.0 BA	A 2.9 b	A 3.6 a	B 3.7 a
2.0 NAA + 2.0 Kin	A 2.9 c	A 3.9 b	A 5.0 a
2.0 NAA + 2.0 2iP	A 3.0 a	A 4.1 a	B 3.8 a

[(1)] see Material and Methods for a description of the quality score of shoots (Table 3).
[(2)] Means followed by the same capital letter (A,B) in the column or by the same small letter (a,b,c) in the row are not significantly different according to Duncan's Test (P=0.05).

Based on the results concerning the multiplication as well as the rooting phases, one can conclude that the medium supplemented with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA was appropriate for the cultivation of globe artichoke cv. 'Green globe' in the multiplication phase (see Figure 27). However, in the rooting phase, plants grown with this combination of growth regulators produced shoots with lower quality when compared to the other combinations, especially when the rooting phase was extended up to six weeks (see Table 24).

The rooting percentage was also evaluated. The best results were found with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin and 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA in the multiplication phase and after six weeks in rooting medium (Table 25). In this situation, the rooting percentage reached values that varied between 48% and 45%.

Among the different NAA and BA concentrations tested, the combination 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA gave better results concerning the rooting percentage than the combination 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA after four and six weeks in rooting medium. In fact, 45% of shoots produced roots when grown at the lower concentration of both growth regulators, whereas the highest concentration gave 32% of rooted plants after six weeks of the rooting phase (Table 25). This effect may be ascribed to the persistence of cytokinins in the tissues, creating an unsuitable hormonal balance in this phase (Kanakis & Demetriou, 1993).

Table 25. Rooting percentage of globe artichoke in the rooting phase grown under different concentrations and types of auxin and cytokinin in the multiplication phase and different durations of the rooting phase (*n*=50).

Auxin + Cytokinin	**Duration of the rooting phase (weeks)**			**Influence of**
	2	**4**	**6**	**duration**
mg L^{-1}	%	%	%	$\chi 2$
0.2 NAA + 0.2 BA	9	20	45	****
2.0 NAA + 2.0 BA	11	17	32	****
2.0 NAA + 2.0 Kin	19	41	48	****
2.0 NAA + 2.0 2iP	9	16	15	ns
Influence of growth regulators ($\chi 2$)	**	****	***	

(1) For the comparison of the rooting percentage between different durations of the rooting phase at the same combination of growth regulators as well as different growth regulators at the same duration of the rooting phase, the chi^2-test was used.
ns not significant
** Significant at 5%
*** Significant at 1%
**** Significant at 0.1%

Bressan et al. (1982) found that root initiation for rose was affected by the culture conditions during shoot multiplication just prior to transfer to rooting medium. The number of roots per explant decreased with increasing the concentration of BA in the multiplication phase. They attributed this effect to the BA accumulated in the tissue, since root initiation may be inhibited if the endogenous cytokinin level is too high.
Furthermore, only 15% of shoots produced roots after six weeks in rooting medium when grown with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP in the multiplication phase (Table 25).

For all combinations of the growth regulators NAA (auxin), BA and Kinetin (cytokinins), the percentage of shoots that produced roots increased significantly with increasing the duration of the rooting phase from two up to six weeks (Table 25). In contrast, the percentage of rooting in plants grown with 2.0 mg L^{-1} NAA + 2.0 mg 2iP was not influenced by the duration of this phase.

The low rooting percentage in plants grown with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP (Table 25) may be associated with a higher occurrence of callus formation under these conditions (Figure 29), which affected negatively the formation of roots.

The formation of callus was evaluated by means of quality score values. Higher values for quality score indicate the formation of smaller callus or no formation. The use of 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA in the culture medium in the multiplication phase resulted in higher values for the quality score, whereas the use of 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP promoted a decrease in this parameter, indicating the formation of bigger callus in plants grown with this combination of auxin and cytokinin (Figure 29).

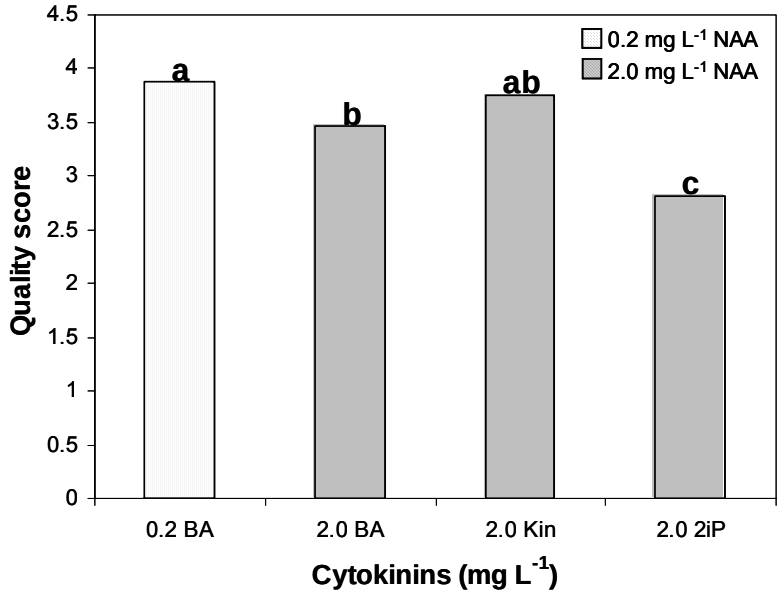

Figure 29. Quality score of callus of globe artichoke in the rooting phase grown under different concentrations and types of auxin and cytokinins. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 5) (*n*=50).

Acclimatization phase

The establishment of plants *ex vitro* was affected by the composition of the culture medium in the multiplication phase as well as by the duration of the rooting phase.

The lowest mortality rate was found when plants were grown in culture medium supplemented with of 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA in the multiplication phase after six weeks in rooting medium (Table 26). The supplementation of the culture medium with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA promoted an increase in the mortality rate in comparison to the combination 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA for all durations of the rooting phase (Table 26).

Table 26. Mortality rate of globe artichoke in the acclimatization phase grown under different concentrations and types of auxin and cytokinin in the multiplication phase and different durations of the rooting phase (*n*=45).

Auxin + Cytokinin	**Duration of the rooting phase (weeks)**			**Influence of**
	2	**4**	**6**	**duration**
mg L^{-1}	%	%	%	$\chi 2$
0.2 NAA + 0.2 BA	81[(1)]	72	65	**
2.0 NAA + 2.0 BA	92	87	88	ns
2.0 NAA + 2.0 Kin	86	70	70	***
2.0 NAA + 2.0 2iP	100	82	93	****
Influence of growth regulators ($\chi 2$)	****	****	****	

[(1)] For the comparison of the mortality rate between different durations of the rooting phase at the same combination of growth regulators as well as between different growth regulators at the same duration of the rooting phase, the chi^2-test was used.
[ns] not significant
** Significant at 5%
*** Significant at 1%
**** Significant at 0.1%

In fact, the use of 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA in the culture medium was appropriate only in the multiplication phase. In the subsequent phases (rooting and acclimatization), the best performance of plants observed in this treatment was not maintained.

Higher mortality rates were found for plants grown with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP in the multiplication phase. Taking into account the results obtained in the different phases, the supplementation of the culture medium in the multiplication

phase with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP cannot be recommended for the micropropagation of globe artichoke cv. ‘Green globe’.

Concerning the duration of the rooting phase, higher mortality rates were found after two in comparison to six weeks in rooting medium for all combinations of growth regulators (Table 26).

4.2.3 Growth regulators: Auxins

In the experiment presented in the chapter 4.2.1, the effect of the growth regulators auxin and gibberellin in the rooting phase was studied. The best results were obtained in absence of gibberellin in the rooting medium. Moreover, the use of lower concentrations of auxin (0.5 mg L^{-1} in comparison to 2.0 mg L^{-1} NAA) was beneficial to the micropropagation process.

This experiment had the objective to determine the optimal concentration of NAA to be used in the rooting medium for globe artichoke cv. 'Green globe'. The following concentrations were tested: absence of NAA, 0.1, 0.5, 1.0 and 2.0 mg L^{-1} NAA. The culture medium was not supplemented with gibberellin.

Rooting phase

A significant quadratic relationship existed between quality score of shoots and NAA concentration in the culture medium (Figure 30).

The addition of NAA promoted an increase in the quality score of shoots up to the concentration of 0.5 mg L^{-1}. Increasing the NAA concentration from 0.5 up to 1.0 mg L^{-1} did not result in a further increment of the quality score. Afterwards, the increase in the concentration up to 2.0 mg L^{-1} promoted a decrease in this parameter (Figure 30).

The negative response of plants to higher NAA concentrations (as e.g. 2.0 mg L^{-1}) may be caused by the ethylene produced in response to the supplementation of the culture medium with auxin (George, 1993).

The results also showed that plants grown in medium with 0.5 or 1.0 mg L^{-1} NAA showed a better shoot development (higher quality score) in comparison to plants grown in absence of NAA. It indicates that the presence of a minimum amount of auxin in the culture medium is important for the development of globe artichoke shoots *in vitro*.

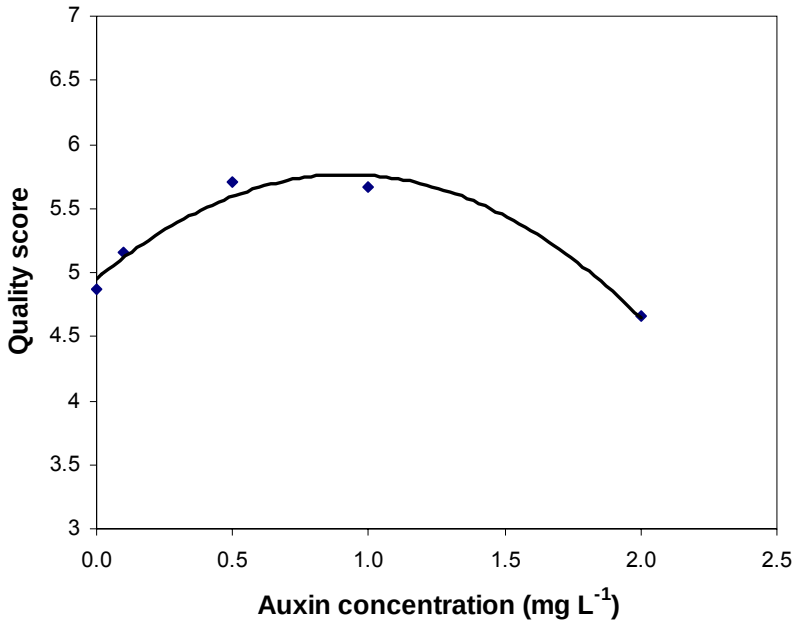

Figure 30. Quality score of shoots of globe artichoke in the rooting phase grown under different concentrations of NAA. Line represents fitting regression curve. Regression equation was as follows: $y=4.95 + 1.77x - 0.96x^2$ ($r^2=0.97$). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=60).

Normally, auxins promote cell elongation, swelling of tissues, cell division, and the formation of adventious roots (low auxin concentration), as well as callus formation and the inhibition of adventious axillary shoot formation (high auxin concentration) (Pierik, 1997).

Figure 31.a shows the effect of different NAA concentrations on the formation of roots (rooting percentage). Even in absence of NAA, some plants rooted (9% of plants).

Several species, especially herbaceous, are able to root in the presence of reduced levels of auxin or even in culture medium without this growth regulator (Cuzzuol et al., 1996). In this case, auxin is produced by shoots and translocated to its base, stimulating rhizogenesis (Grattapaglia & Machado, 1998).

In our study, the addition of NAA to the culture medium up to 1.0 mg L^{-1} promoted an increase in the percentage of plants that rooted. In this condition, approximately 35% of shoots produced roots. Thereafter, the percentage of plants producing roots decreased down to approximately 25% with increasing the NAA concentration up to 2.0 mg L^{-1} (Figure 31.a).

Contrasting results were found for globe artichoke cv. 'Romanesco' (Ancora et al., 1981) and cv. 'Argos' (Kanakis & Demetriou, 1993). In both studies, optimal results concerning the production of roots were obtained in plants grown at higher NAA concentrations, namely 2.0 mg L^{-1} in comparison to 1.0 mg L^{-1}. These differences indicate that the genotype is important in relation to the response to the concentration of growth regulators.

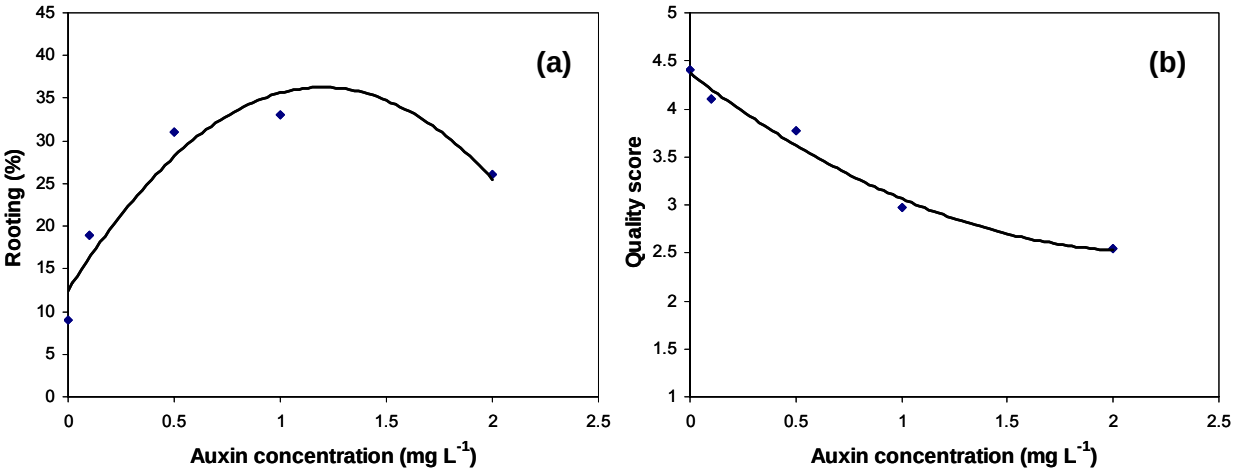

Figure 31. Rooting percentage (a) and quality score of callus (b) of globe artichoke in the rooting phase grown under different concentrations of NAA. Lines represent fitting regression curves. Regression equations were as follows: (a) $y=12.42 + 39.88x – 16.66x^2$ ($r^2=0.91$) and (b) $y= 4.37 - 1.7x + 0.39x^2$ ($r^2=0.98$). A description of the quality score is presented in the chapter Material and Methods (Table 5) (n=60).

The increase in the NAA concentration in the culture medium up to a given level (namely 1.0 mg L^{-1}) stimulated the emission of roots. Afterwards, an increase in the concentration of NAA beyond this level inhibited root formation.

The formation of roots often increases in proportion to the concentration of auxin applied, but when the concentration becomes supra-optimal, callus formation is promoted, and roots have a abnormal appearance and their average length, and subsequent shoot growth may be decreased (George, 1996). In fact, the presence of auxin is necessary only in the induction as well as in the initiation phases of rhizogenesis, which occur in the first days after transferring the plants to the rooting medium (Grattapaglia & Machado, 1998).

In the present experiment, plants probably remained for to long time in contact with high levels of NAA (as e.g. 2.0 mg L^{-1}), which may decrease the formation of roots. Moreover, high levels of auxin promote the formation of callus at expense of roots.

In fact, the addition of NAA to the culture medium promoted a reduction in the quality score of callus, which indicates an increase in callus formation with an increment in the NAA concentration (Figure 31.b). A significant quadratic relationship was found between both variables.

Previous results obtained in apple by Centellas et al. (1999) support this observation. They found that the increase in the NAA concentration from 0.5 to 1.0 mg L^{-1} resulted in an increase in the formation of callus.

Figure 32 shows the development of globe artichoke plants at the end of the rooting phase grown in culture medium supplemented with different NAA concentrations. The supplementation with 0.5 mg L^{-1} resulted in the formation of shoots with better quality in comparison to other NAA concentrations.

Figure 32. Development of plants of globe artichoke in the rooting phase as affected by the concentration of NAA in the culture medium.

Acclimatization phase

The results in this phase showed that the mortality rate was influenced by the concentration of auxin in the culture medium in the rooting phase. The lowest mortality rate was found for plants grown in medium supplemented with 0.5 mg L^{-1} NAA (Table 27).

Moreover, an increment in the mortality rate was found by reducing the concentration of NAA in the rooting phase down to 0.1 mg L^{-1} or in absence of this growth regulator as well as by increasing the NAA concentration up to 2.0 mg L^{-1} (Table 27).

The increase in the mortality rate with increasing the NAA concentration from 0.5 up to 2.0 mg L^{-1} can be related to the effect of excess auxin. The excess of this growth regulator *in vitro* will result in the production of callus rather than roots. Excessive callus production prevents the formation of direct vascular connections between shoots and roots, so that after transplanting to soil (acclimatization) the plant will die as a result of transpiration loss (Collin & Edwards, 1998).

Table 27. Mortality rate of globe artichoke as affected by the concentration of NAA in the rooting phase (*n*=40).

NAA	**Mortality rate**
mg L^{-1}	%
absence	81[(1)]
0.1	79
0.5	58
1.0	70
2.0	83
Influence of auxin concentration	****

[(1)] For the comparison of the mortality rate between different NAA concentrations, the chi^2-test was used.

**** Significant at 0.1%.

4.2.4 Sucrose concentration, type of vessel closure and light intensity

Several authors have suggested that the use of a source of carbohydrate in the culture medium may inhibit the carbon photosynthetic metabolism (Reuther, 1991; Huylenbroeck & Debergh, 1996). On the other hand, alterations in the chemical composition of the culture medium and in the gaseous exchanges between the growing environment inside the vessels and the atmosphere may improve the photosynthetic efficiency, reducing the loss of plants during acclimatization. Moreover, the light intensity is one key factor to plants become autotrophic.

Two experiments were carried out to test the effect of different sucrose concentrations and two types of vessel closure (with and without filter) as well as two light intensities on the growth of globe artichoke *in vitro*.

a) Sucrose concentration and type of closure of vessel

This experiment was installed with the objective to study the combined effect of different sucrose concentrations in the culture medium and gaseous changes, with the use of covers with and without filter. Baby food culture jars covered with Magenta B-CAP with filter (B-3031) or without filter (B-8648) were used. Moreover, sucrose concentrations of 5, 30 and 60 g L^{-1} were tested.

Rooting phase

A significant effect of sucrose concentration on the quality score of shoots was observed. The effect of sucrose concentration was independent of the presence or absence of filter in the baby food culture jars.

The best quality of shoots was found at the concentration of 5 g L^{-1} of sucrose (Figure 33). The increase in sucrose concentration from 5 up to 30 g L^{-1} led to a significant decrease in the quality score of shoots. Furthermore, the quality score remained constant when the sucrose concentration was increased from 30 up to 60 g L^{-1} (Figure 33).

Concerning the effect of the type of vessel closure, the quality score of shoots was not significantly influenced by the use or absence of filter in the cover (data not shown).

In *in vitro*-grown *Pelargonium* and *Vitis* plantlets, it was shown that a low sucrose concentration in the culture medium (namely 5 g L^{-1}) favored photoautotrophic and vegetative growth of plants (Reuther, 1991). For instance, *Spathiphyllum* plantlets grown at 30 g L^{-1} of sucrose in the culture medium showed an increased net photosynthesis than plantlets grown at 60 g L^{-1}, indicating that the supplementation of the medium with 60 g L^{-1} of sucrose led to a more mixotrophic metabolism, while the supplementation with 30 g L^{-1} led to a autotrophic metabolism (Huylenbroeck & Debergh, 1996). When sugar is present at high concentrations in the culture medium, the plantlets do not develop photoautotrophy, which may cause slow growth (Kozai, 1991).

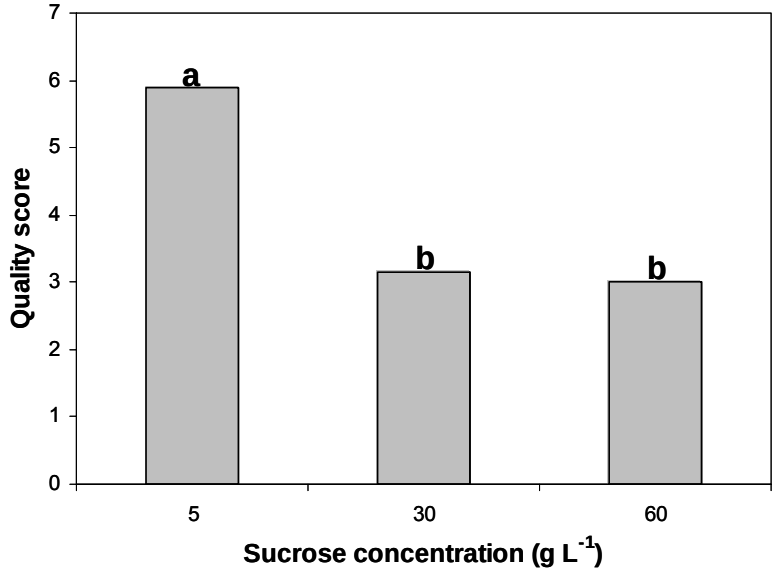

Figure 33. Quality score of shoots of globe artichoke in the rooting phase grown under different sucrose concentrations. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05). A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=50).

In the present study, comparative results were found, since plants grown at low concentrations of sucrose showed a better growth of shoots (higher quality score), as shown in Figure 33. According to Lees (1994), it may be possible to manipulate the photosynthetic capacity of plants by decreasing the medium carbohydrate concentration.

Another hypothesis to explain the lower growth of plants at higher sucrose concentrations concerns the osmotic effect of sucrose in the culture medium. Sugars

are responsible for much of the osmotic potential of normal plant culture media. When the concentration of sucrose in a high salt medium (such as the MS medium) is increased above 40-50 mg L^{-1}, there begins to be a progressive inhibition of cell growth in many types of culture, and this appears to be an osmotic effect (George, 1993). For instance, a culture medium supplemented with 5 g L^{-1} of sucrose has an osmotic potential of -0.037 MPa. The medium supplemented with 60 g L^{-1} of sucrose, in turn, has an osmotic potential of -0.461 MPa (George, 1993). In consequence of this increase in osmotic potential, the water content of tissues decreases (George, 1993). In artichoke cv. 'Violet d'Huyeres', the increase in sucrose concentration from 10 up to 20 g L^{-1} reduced the propagation ratio in the multiplication phase (Debergh et al., 1981). This effect was related to the decreased osmotic and water potential (more negative) in the medium with increasing sucrose concentration.

A significant effect of sucrose concentration on the chlorophyll fluorescence measurements was observed. Maximum photochemical efficiency (potential quantum yield of Photosystem II) of dark-adapted leaves was calculated by the equation F_v/F_m, with F_v being the variable fluorescence, calculated as F_m-F_o. F_o is the dark-level fluorescence (initial fluorescence) yield measured briefly before onset of the saturation pulse, whereas F_m is the maximum fluorescence yield of a dark-adapted sample reached during the saturation pulse.

The value of the potential quantum yield (ratio F_v/F_m) was maximal in plants grown at 5 g L^{-1} of sucrose in the culture medium (value around 0.73), even though not significantly different from plants grown at 30 g L^{-1} of sucrose (Figure 34). Moreover, the ratio F_v/F_m declined to approximately 0.66 in plants grown at 60 g L^{-1} of sucrose (Figure 34).

A decrease in the ratio F_v/F_m with increasing sucrose concentration *in vitro* was also shown by Hdider & Desjardins (1994) in strawberry plantlets. They found that the ratio F_v/F_m was highest in plantlets cultured in medium containing the lower amount of sucrose, namely in absence or at 10 g L^{-1}. According to these authors, the lower F_v/F_m ratio of plants cultured on media containing high levels of sucrose could be explained either by an inhibition of the dark reactions of photosynthesis due to starch accumulation or to a low activity of Rubisco, due to feedback inhibition.

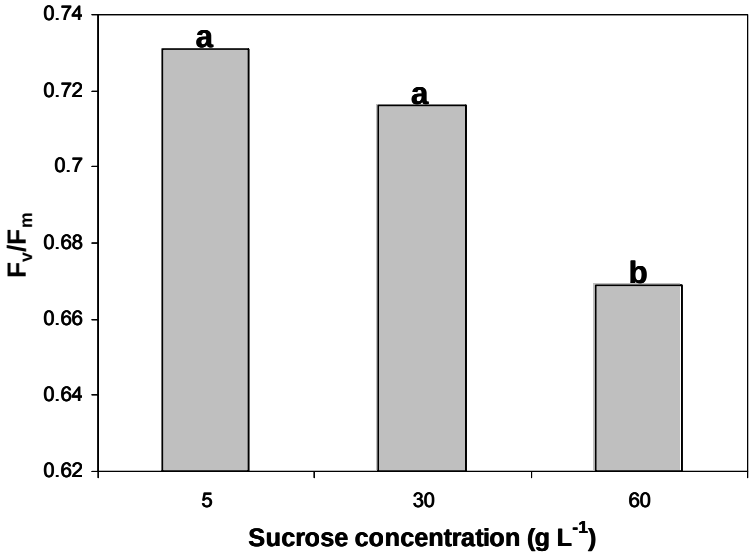

Figure 34. Chlorophyll fluorescence ratio F_v/F_m of globe artichoke in the rooting phase grown under different sucrose concentrations. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05) (*n*=6).

In fact, a decline in the F_v/F_m ratio may reflect a reduction in light energy utilization by chloroplasts in the photosynthesis (Huylenbroeck & Debergh, 1996; Pompodakis et al., 2005) and can be attributed to stresses that affect the energy transfer pathway from antennae to the reaction centers (Krause & Weis, 1991).

The decrease in the ratio F_v/F_m with increasing sucrose concentration suggests that carbon metabolism and/or electron transport was being adversely affected by high amounts of sucrose in the culture medium. For instance, higher rates of photosynthesis (O_2 evolution per area) were found for strawberry plants grown in absence or at 10 g L^{-1} of sucrose in comparison to plants grown at 30 or 50 g L^{-1} of sucrose (Hdider & Desjardins, 1994). The lower F_v/F_m for the higher sucrose-treated plants reveals an unfunctional photosynthetic apparatus with a low oxidation rate of the primary quinone acceptor (Huylenbroeck & Debergh, 1996).

According to Lazár (1999), optimal values for the ratio F_v/F_m are around 0.832 for most plant species. In the present study, maximal values of 0.73 were reached for this ratio (Figure 34). Values lower than 0.83 for the F_v/F_m ratio may indicate that plants have been exposed to stress (Maxwell & Johnson, 2000).

Comparable values for the F_v/F_m ratio were shown by Huylenbroeck & Debergh (1996) in *Spathyphyllu*m plantlets. They found that at the end of the *in vitro* period,

F_v/F_m was significantly lower for plants grown at 60 g L^{-1} sucrose (ratio of 0.67) in comparison to plants grown at 30 g L^{-1} (ratio of 0.74).

Concerning the rooting percentage of plants, no significant influence of the type of closure (with or without filter) as well as of the sucrose concentration in the culture medium was found (data not shown).

b) Sucrose concentration and light intensity

This experiment was installed with the objective to study the combined effect of different sucrose concentrations (5 and 30 g L^{-1}) and light intensities (110 and 210 μmol m^{-2} s^{-1}) in the rooting phase.

Rooting phase

The use of different sucrose concentrations in the medium as well as different light intensities significantly influenced the development of shoots (as evaluated by the quality score of shoots) (Figure 35.a and 35.b). The interaction between both factors was not significant ($P<0.05$).

In relation to the different sucrose levels tested, explants grown in culture medium supplemented with 5 g L^{-1} showed higher quality score of shoots in comparison to explants grown at 30 g L^{-1} (Figure 35.a). This behavior was similar to that found in the experiment previously described in the present chapter.

Concerning the influence of light intensity, one can observe that the use of the higher light intensity (210 μmol m^{-2} s^{-1}) was not suitable for the cultivation of globe artichoke in the rooting phase (Figure 35.b). The quality score of shoots decreased significantly with an increment in the light intensity from 110 up to 210 μmol m^{-2} s^{-1}.

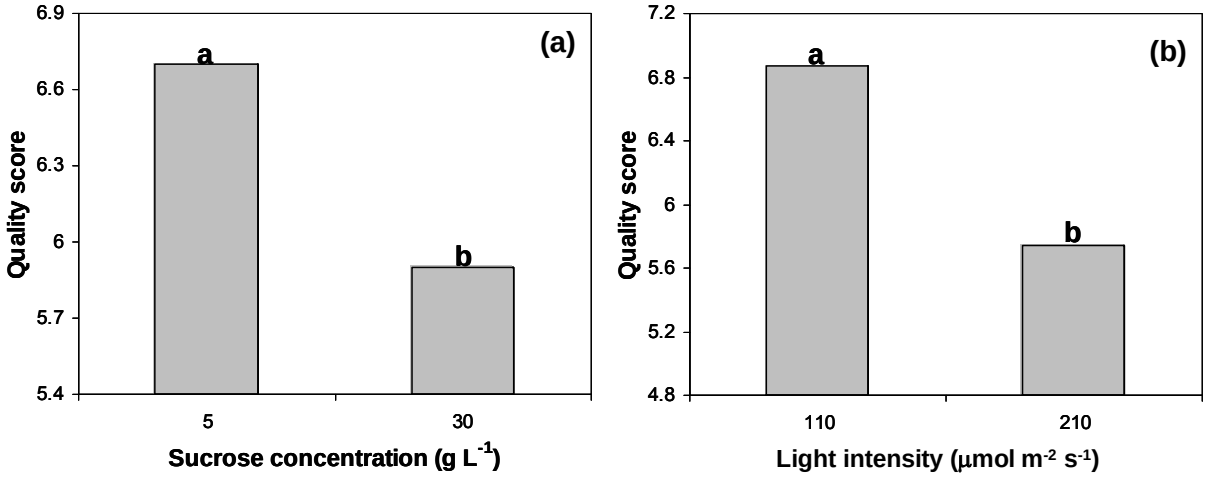

Figure 35. Quality score of shoots of globe artichoke in the rooting phase grown under different concentrations of sucrose (a) (means of both light intensities) and different light intensities (b) (means of both sucrose concentrations). Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05) A description of the quality score is presented in the chapter Material and Methods (Table 3) (*n*=30).

One hypothesis to explain the lower growth of plants at higher light intensity concerns the occurrence of photoinhibition under this condition, since photoinhibition lowers photosynthetic rates and impairs electron transport and photophosphorylation (Taiz & Zeiger, 1991). Plants growing under *in vitro* conditions are submitted to low light intensities in comparison to plants grown under greenhouse or field conditions. Light intensities used for *in vitro* micropropagation of globe artichoke normally vary between 30 μmol m^{-2} s^{-1} (Debergh, 1983) and 60 μmol m^{-2} s^{-1} (Rossi & Paoli, 1992). Consequently, plants are more susceptible to the occurrence of photoinhibition by high light intensities, since leaves formed under low light conditions are thin and thus resemble shade leaves (Preece & Sutter, 1991).

Langford & Wainwright (1987) found that plants were able to become more autotrophic with increasing light intensity, associated with a reduction in the sugar concentration in the growing medium. In our study, plants grown under the highest light intensity (210 μmol m^{-2} s^{-1}) showed a lower growth of shoots (lower quality score) (Figure 35.b). It shows that the highest light intensity was prejudicial to the growth of globe artichoke *in vitro*, which may be associated to the occurrence of photoinhibition.

The occurrence of photoinhibition in plants grown at 210 μmol m^{-2} s^{-1} can be demonstrated by the chlorophyll fluorescence measurements.

The value of the F_v/F_m ratio was maximal and significantly different in plants grown at 110 μmol m^{-2} s^{-1} in comparison to 210 μmol m^{-2} s^{-1} (Figure 36). In plants grown at 110 μmol m^{-2} s^{-1}, the ratio F_v/F_m reached a value of 0.74, while at 210 μmol m^{-2} s^{-1} the F_v/F_m ratio was 0.54 (Figure 36).

According to Maxwell & Johnson (2000), lower values of the F_v/F_m ratio may indicate the occurrence of photoinhibition due to damage to photosystem II reaction centers in response to excess irradiance. Upon exposure to excess light, the D1 protein of photosystem II reaction center gets inactivated by phosphorylation and then degraded, leading to an inactive photosystem II center (Fracheboud, 2003).

In fact, the values of the F_v/F_m ratio are an indicator of the extent to which photosystem II is using the energy absorbed by chlorophyll and the extent to which it is being damaged by excess light (Maxwell & Johnson, 2000). Changes in F_v/F_m ratio are accepted as reliable diagnostic indicator of photoinhibition (Valladares & Pearcy, 1997) or other kind of injury caused to the photosystem II complexes.

The occurrence of photoinhibitory effects due to an increase in the light intensity could be demonstrated by a decrease of the ratio F_v/F_m (Figure 36). According to Critchley (1998), a decrease of this ratio is considered to constitute photoinhibition and is equivalent to the decrease in CO_2 fixation.

The F_v/F_m ratio value of 0.74 is lower than the value of 0.832 suggested by Maxwell & Johnson (2000) as being the maximum quantum yield of photosystem II photochemistry. On the other hand, the value of 0.74 is higher than the value of 0.725 suggested by Critchley (1998) as a clear indication of photoinhibition. Therefore, it seems that a light intensity of 110 μmol m^{-2} s^{-1} can be used for the micropropagation of globe artichoke cv. 'Green globe', since plants were not photoinhibited under this condition.

The response of *Clematis* plants to increasing light intensities was studied by Lees (1994). The best results concerning the multiplication rate as well as shoot elongation were found when plants were grown under 20 μmol m^{-2} s^{-1} in comparison to 80 μmol m^{-2} s^{-1}. These results demonstrated that an irradiance of only 80 μmol m^{-2} s^{-1} was high enough to cause some damage to the photosynthetic apparatus and photoinhibition of photosynthesis. Further experiments with globe artichoke are

necessary in order to determine the optimum light intensity to be used in the micropropagation of this species.

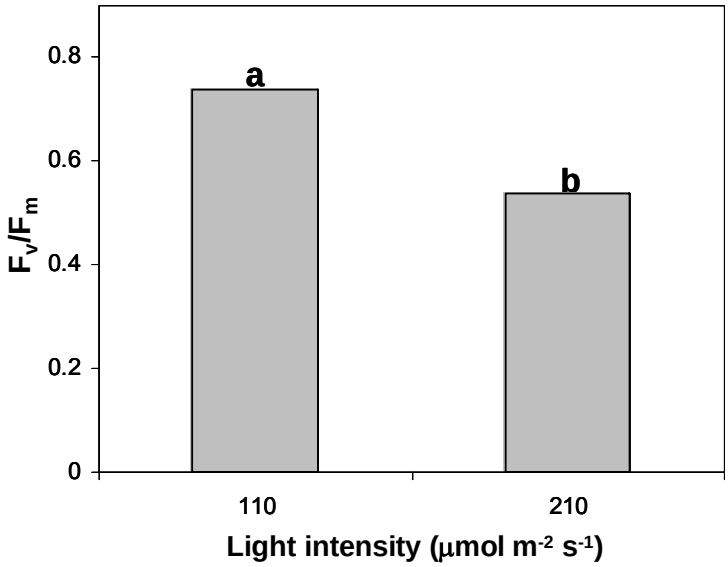

Figure 36. Chlorophyll fluorescence ratio F_v/F_m of globe artichoke in the rooting phase grown under different light intensities. Means followed by the same letter are not significantly different according to Duncan's Test (P=0.05) (*n*=6).

Concerning the rooting percentage of plants, no significant influence of the light intensity as well as of the sucrose concentration in the culture medium was found (data not shown).

4.2.5 Globe artichoke: conclusions and outlook

Based on the results found in the present study concerning the effect of different cultural conditions on the micropropagation of globe artichoke (*Cynara scolymus* L. cv. 'Green globe'), the following considerations can be done.

The growth of globe artichoke plants *in vitro* was affected by the type and concentration of growth regulators supplemented to the culture medium.

The use of 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA in the culture medium promoted a better growth of shoots in the multiplication phase in comparison to the cytokinin 2iP at the same concentration.

In the subsequent phases (rooting and acclimatization), the best performance of the combination 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA was not maintained. In these phases, higher quality score of shoots and rooting percentage as well as lower mortality rate of plants were found when plants were grown in culture medium supplemented with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA or with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin in the multiplication phase. The supplementation of the culture medium in the multiplication phase with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2iP cannot be recommended for the micropropagation of globe artichoke cv. 'Green globe'.

The objective of the micropropagation must be taken into account when choosing the reliable growth regulator combination to be used. If the objective is the continuous subculturing (multiplication) of propagules, in which a high number of shoots per plant is desirable, the culture medium should be supplemented with the combination 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BA. On the other hand, if the objective is the production of plants for acclimatization, the supplementation of the culture medium with 0.2 mg L^{-1} NAA + 0.2 mg L^{-1} BA or with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kinetin in the multiplication phase can be recommended.

Concerning the rooting phase, the addition of auxin (NAA) to the rooting medium at a concentration of 0.5 mg L^{-1} is suitable for the micropropagation of globe artichoke cv. 'Green globe'. Moreover, the supplementation of the rooting medium with gibberellic acid (GA_3) is not recommended. The results also showed that a minimum duration of the rooting phase of six weeks should be used in the micropropagation of this species.

A significant effect of the sucrose concentration in the culture medium on the growth of globe artichoke cv. 'Green globe' was found for concentrations between 5 and 60 g L^{-1}. The best results were obtained by using the lowest sucrose concentration (5 g L^{-1}). The observed decrease in the ratio F_v/F_m with increasing the sucrose concentration suggests that carbon metabolism and/or electron transport was being adversely affected by the sucrose in the culture medium. In addition, the growth of this species was not influenced by the use of Magenta B-CAP covers with or without filter in baby food culture jars.

Concerning the influence of light intensity on the growth of shoots, it seems that a light intensity of 110 μmol m^{-2} s^{-1} can be used for the micropropagation of globe artichoke, since plants were not photoinhibited under this condition.

The value of the F_v/F_m ratio was higher in plants grown at 110 μmol m^{-2} s^{-1} in comparison to 210 μmol m^{-2} s^{-1}, which indicate the occurrence of photoinhibitory effects in plants grown at 210 μmol m^{-2} s^{-1}. Lower values of the F_v/F_m ratio may indicate the occurrence of photoinhibition due to damage to photosystem II reaction centers in response to excess irradiance. Therefore, the use of the light intensity of 210 μmol m^{-2} s^{-1} is not suitable for the cultivation of globe artichoke in the rooting phase.

Further experiments should be carried out to study the response of different globe artichoke cultivars to different cultural conditions *in vitro* and *ex vitro*. Moreover, investigations concerning the acclimatization *ex vitro* could be carried out in order to improve the survival of plants in this phase, as e.g. the effect of substrate.

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