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Date Palm (Phoenix dactylifera L.) Tissue Culture: Type of Contamination and Improvement of Disinfestation Techniques

Mouza Nasser Al-Shamisi

United Arab Emirates University
Deanship of Graduate Studies
M.Sc. Program in Environmental Sciences

DATE PALM (*Phoenix dactylifera*) CONTAMINATION AND IMPROVEMENT OF DISINFECTION TECHNIQUES

By

Mouza Nasser Al-Shamsi

Thesis
Submitted to

United Arab Emirates University
In partial fulfillment of the requirements
For the Degree of M.Sc. in Environmental Sciences

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Supervisors

Abdelouahhab ZAID
Professor
United Nations Development Program /
Date Palm Research &
Development Unit (Director)
UAE University

Ali KHALIL
Assistant Professor
College of Food and Agriculture
Department of Food Science
UAE University

2005-2006

The Thesis of Mouza Nasser Abdulla Al Shamsi is Approved and the Degree of Master of Science in Environmental Sciences is Granted.

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Chair of Examining Committee, Prof Abdelouahhab Zaid

.....

Internal Examiner, Dr.

.....

External Examiner, Hughes

.....

Director of the Program, Dr.

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.....

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DEDICATION

The present research work in the field of date palm tissue culture is dedicated to

His Highness Sheikh Nahayan Mabarak Al Nahayan

Minister of Higher Education and Scientific Research

Chancellor of UAE University

In recognition of His love to the Date Palm. His achievements in the development of date palm sector, are both a credit and an honor to the United Arab Emirates, and to all date growing countries.

ACKNOWLEDGMENTS

I would like to thank His Highness, Sheikh Nahayan Mabarak Al-Nahayan, Minister of higher education and scientific research, Chancellor of UAE University, for his continuous support to the University's objectives and goals.

My sincere thanks go to the Hon. Vice Chancellor of UAE University, Dr. Hadef Bin Jouan Al-Dhahiri, for authorizing me to conduct this important study.

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I am blessed to be surrounded by friends and colleagues from Date Palm Research and Development Unit of UAE University, who encouraged me during thesis through.

Last and not least, my mother for her continuous support and my family for their generosity of heart for always believing in me and giving me the additional strength to go on.

To all I offer heartfelt thanks.

MOUZA N. AL-SHAMSI

ABSTRACT

Date palm (*Phoenix dactylifera* L.) cultivation in the United Arab Emirates (UAE) is considered one of the most important economic supports for the agricultural sector. Propagation of date palm by offshoots can not satisfy the increasing demand of date palm trees, and high propagation rate can only be achieved using tissue culture techniques. However, one of the major problems of this technique is the failure of producing aseptic cultures, yielding to the loss of the explants at the initiation phase.

The objectives of this study were to develop a reliable disinfection technique of date palm explants at the initiation stage yielding a contamination rate less than 5 %, to identify tissue culture contaminants and propose respective control means.

To avoid contamination problems that are frequently experienced in the initiation stage, explants are to be handled and raised under specific aseptic conditions. Various concentrations of Sodium Hypochloride (NaOCl), Potassium permanganate ($KMnO_4^-$) and Aliette (fungicide) were tested during different periods of time. The recommended double disinfection technique is 40 % of NaOCl combined with 3 g/l of Aliette, and 30 % of NaOCl combined with 400 mg/l $KMnO_4^-$. The most frequent bacterial strains identified were *Cellulomonas cellulans*, *Enterobacter aerogenes*, *Bacillus fusiformis*, *Serratia marcescens*, *Kocuria rosea* and *Cellulomonas uda*.

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I. INTRODUCTION

I.1. Overview

The date palm (*Phoenix dactylifera* L.) is often described the "tree of life". The exact origin or gene centre of the date palm has been lost in antiquity. There seems to be a consensus that the earliest form of date palm cultivation coincided with the oldest civilizations [1]. However, evidence of date palm cultivation goes as far back as 4000 B.C. since it was used for the construction of the temple of the moon God near Ur, in southern Iraq – Mesopotamia (the area between the Euphrates and Tigris rivers) [1]. References to date palms have also been found in Egypt's Nile Valley where it was used as the symbol for a year in Egyptian hieroglyphics and its frond as a symbol for a month [2]. However, the culture of date palm did not become important in Egypt until somewhat later than that in Iraq, about 3000 - 2000 B.C [3]. The spreading of the date palm and its cultivation occurred during the past centuries following two distinct directions, one starting from Mesopotamia to Iran, to reach the Valley of the Indus and Pakistan; the other starting from Egypt towards Libya, North Africa and the Sahel countries [4].

The above is confirmed by the archaeological research into ancient historical remains of the Sumerians, Akadians and Babylonians [5]. Houses of these very ancient civilizations were roofed with date palm trunks and fronds. The uses of date for medicinal purposes, in addition to its nutritional value, were also documented [4].

Much later, the date palm became associated with three of the world's major religions: Islam, Jewish and Christianity. This was due to the influence of the Prophet Abraham, who was born and raised in the old city

of Ur where date palms were grown. Date palm fronds are used for the celebration of Palm Sunday among Christians and for the celebration of the Feast of Tabernacles among Jews, who consider the date as one of the seven holy fruits. Islam has mentioned date palm in the Holy Quran more than any other fruit-bearing plant. Throughout the month of Ramadan, dates are a common ingredient in the Muslims diet. Indeed, Muslims end their day of fasting with its sweet and nourishing flesh.

I.2. Date palm importance

With its simple structure, it is difficult to imagine all of the purposes a date palm can serve. The date palm usually has a simple trunk, called a stipe, with a network of roots in the soil and long or spatulate leaves, called fronds. Flowers are produced in clusters which arise from the axils of the leaves [5]. These may be male, female, or hermaphroditic. From the female flower parts, fruits arise which may have a fleshy or fibrous outer coat, a hard inner coat, and a somewhat hard, often hollow endosperm.

Date palm tree is an essential integral component of farming systems in dry and semi-arid regions and can be produced equally well in small farm units or as larger scale commercial plantations. The tremendous advantage of the tree is its resilience, its requirement for limited inputs, its long term productivity and its multiple purposes attributes [2].

In addition to producing a valuable dessert fruit, by-products such as building materials and versatile starting materials for handicrafts can be derived from its fronds and trunks making it an important multiple purpose tree and a significant earner of foreign currency for both small and large

farmers. The date palm also makes a significant contribution towards the creation of suitable microclimates within oasis ecosystems, thus enabling agricultural development to be sustained in many drought- and saline-affected regions.

Agro-industries and the date palm industry in particular, offer great job opportunities, particularly for women, through processing and packaging of products. The date palm also supports the introduction of modern practices, which consequently increases the agricultural contribution to gross domestic product (GDP) as well as to export earnings [4].

If date fruit packaging and processing is excluded, an average of seven working days are annually needed per ton produced based on fully mechanized field activities. Based on labor only (with no mechanization), date culture requires approximately 170 working days/ year/ hectare. In conclusion, a commercial date plantation of 100 ha requires 17,000 working days per year [4].

I.3. Date nutritional value

It is historically known that desert people lived on dates and milk for years without suffering any nutritional problems. In fact, date fruit is rich in nutrients, and due to its dietetic values it has always been held in high esteem by people. Compared to other fruits and foods (apricot: 520 calories/kg; banana: 970 calories/kg; orange: 480 calories/kg; cooked rice: 1,800 calories/kg; wheat bread: 2,295 calories/kg; meat (without fat): 2,245 calories/kg, dates give more than 3,000 calories per kilogram [4]. With the present uncertainty in the world food supply and the expected

increase in demand, the date palm could be a good source of food of high nutritional value.

The date fruit consists of 70% carbohydrates (mostly sugars), making it one of the most nourishing natural foods available to man. In most varieties, the sugar content of a date fruit is almost entirely of the inverted form (namely glucose and fructose), important for persons who cannot tolerate sucrose. The inverted sugar in dates is immediately absorbed by the human body without being subjected to the digestion that ordinary sugar undergoes. The flesh of dates contains 60 to 65% sugar, 2.5% fibre, 2% protein and less than 2% each of fat, minerals, and pectin substances [5]. Date fruits are also a good source of iron, potassium and calcium, with a very low sodium and fat content. In addition, moderate quantities of chlorine, phosphorous, copper, magnesium, silicon and sulphur along with various vitamins, including thiamine, riboflavin, biotin, folic and ascorbic acid are also found in the date fruit. The water content is between 15 to 30% depending on the variety and on the maturity stage of the fruit [6, 5].

The rich fruit is also used as a livestock feed supplement and gives the tree much added value. The secondary products generated from fruits are syrups, jams, ice creams, snacks and soft drinks [5]. Small- and intermediate-scale industries can therefore be supported over long term periods in both urban and rural situations. This importance is reflected in the date palm widely acknowledged sustainability value in social, economical and ecological terms.

I.4. Geographical distribution

Dates are cultivated mainly in the hot arid regions of South West Asia and North Africa. The fruit is also grown in some parts of Europe and the USA. The world total number of date palms at the present time is about 100 million, distributed in five regions. Asia has the greatest number with 60 million date palms (Saudi Arabia, Bahrain, United Arab Emirates (UAE), Iran, Iraq, Kuwait, Oman, Pakistan, Turkmenistan and Yemen); while Africa is second with 32.5 million date palms (Algeria, Egypt, Libya, Mali, Morocco, Mauritania, Niger, Somalia, Sudan, Chad and Tunisia). Mexico and the USA have 600,000 palms followed by Europe (Spain) with 32,000 and Australia with 30,000 [4, 5].

I.5. World production and trade

The date fruit is marketed all over the world as a high-value confectionery and fruit crop, and remains an extremely important subsistence crop in most of the desert regions. Worldwide date production has increased exponentially over the three decades (Figure 1). In 1963, production was 1.8 million tons. It increased to 2.6 and 6.7 millions by 1983 and 2003, respectively. The increase of 4.9 million tones since 1963 represents an annual expansion of about 6.8% [7].

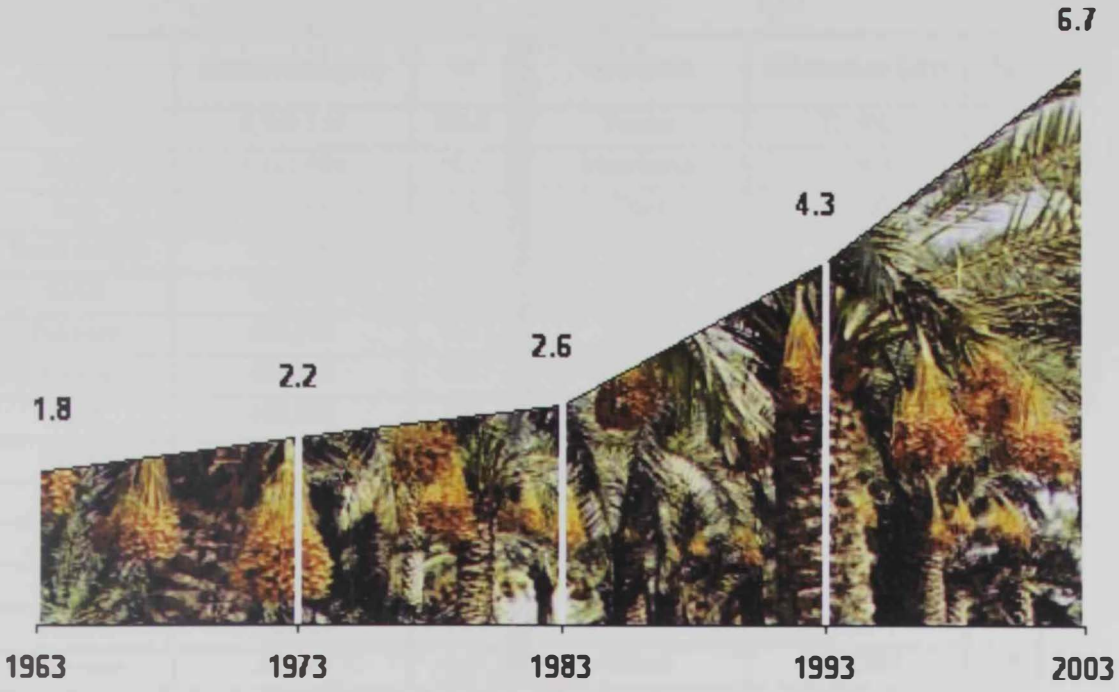

Figure 1: Global annual date production (Million Metric Tons) during the period from 1963 - 2003 (FAO Statistics, 2004).

However, most of the major dates producing countries have steadily expanded production over the last 10 years, with a 43% increase from 1994 to 2001. Date exports increased by only 25%, over the same period, especially in Oman, UAE, Egypt and Pakistan. Conversely, an output decrease has been observed in Iraq and Morocco, due mainly to war and Bayoud disease, respectively. In the Arab world, total date production is estimated to be about 4.5 million tons in 2003, representing 67% of the global date production (Table 1).

Table 1: Date palm production in leading countries (2003).

Countries	Production (Mt)	%	Countries	Production (Mt)	%
World	6,749,356	100.0	Yemen	32,500	0.5
Egypt	1,115,000	16.5	Mauritania	24,000	0.4
Iran	875,000	13.0	Chad	18,000	0.3
Saudi Arabia	830,000	12.3	USA	17,600	0.3
UAE	760,000	11.3	Bahrain	16,508	0.2
Pakistan	650,000	9.6	Qatar	16,500	0.2
Algeria	420,000	6.2	Kuwait	10,400	0.2
Iraq*	400,000	5.9	Turkey	9,400	0.1
Sudan	330,000	4.9	Niger	7,700	0.1
Oman	238,611	3.5	Palestine	5,500	0.1
Libyan	140,000	2.1	Spain	3,732	0.1
China	120,000	1.8	Mexico	3,600	0.1
Tunisia	115,000	1.7			
Morocco	54,000	0.8	Others	536,305	7.9

Source FAO statistics 2003, ** FAO estimate for 1997*

Mt: metric ton.

The Gulf Cooperation Council (GCC) countries produced 1.9 million tons in 2003, which represent 28% of the global production [8]. Saudi Arabia and UAE together produced 1.6 million tons in 2003. Oman has 7 million trees and a worldwide market acceptance. The remaining GCC countries (Bahrain, Qatar and Kuwait) have fewer date palm trees and lower production. During the last decade, the date palm production has increased by 86.7%, from 1.0 million tons in 1994 to 1.9 million tons by 2003 [7].

Relevant statistics indicate that only around 10% of dates produced in the world are traded. The global import of dates during the period from 1998 - 2000 averaged just over 560,000 tons per year. India is the largest importer of this fruit in the world (around 40% of dates exported). It imported 244,000 tons in 1998. After India, there is UAE which imported

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100,000 tons of dates in 1998 and 180,000 tons in 1999. In 2000, imports decreased to 43,000 tons [9].

I.6. Date palm importance in UAE

Date palm is considered the most important fruit crop tree in UAE. Despite its extreme aridity, nutrient-poor soil and high summer temperatures, the UAE's cultivated area now extends over 2.7 million donums (891,089 acres) which supports more than 40 million date palms, together with various types of fruits and vegetables [10].

The date palm has been cultivated in UAE for thousands of years. Date stones (seeds, also called pips) have been found in a 7000-year-old archeological site on Dalma Island [11]. Traditional cultivation of palms in UAE's desert and mountain oases, using sophisticated techniques for tapping underground water, provided more than the fruit itself. The date palm fronds and trunks were used for many purposes, including basket-making, house-building and boat-building. In the last thirty years, the availability of more water, through tapping of aquifers, desalination and recycling, has permitted massive expansion in UAE date cultivation. The date is not only part of the staple diet of the UAE's people; it is now the centre of a rapidly growing agricultural industry. The country is also a world leader in date palm propagation, using advanced tissue culture techniques. The UAE University tissue culture laboratory annually produces over 150,000 young plants of more than 20 date varieties. (http://datepalm.uaeu.ac.ae)

I.7. Date palm propagation

I.7.1. Seed propagation

Sexual propagation is the most convenient method to propagate date palms; seeds available in large numbers can be stored for years and germinate easily. However, this method cannot be used commercially for propagating the cultivars of interest in a true-to-type manner. The most obvious reason is the heterozygous characteristics of seedlings which are related to the dioecious nature of the date palm: half of the progeny are male, which produce no fruits, and large variations in phenotype can occur in the female progeny. It is not possible to detect (in the nursery) and then eliminate non-productive male trees prior to field planting. Another important drawback of seed propagation is that the growth and maturation of seedlings is extremely slow. A date palm seedling may take 8 to 10 years or even more before fruiting occurs. Seedlings differ considerably with regard to production potential, fruit quality and harvesting time, making them very difficult to market at one harvest. For all of these reasons, seed propagation results in waste of time, space and money. This method is not practiced by date growers and is used only in exceptional cases when supplies of offshoots are unavailable, or for breeding or research purposes.

I.7.2. Offshoot propagation

For centuries, the propagation of date palms by offshoots (also called suckers) was the only commercial method of vegetative propagation used in date growing regions of the world to multiply the best varieties. However, offshoots develop slowly and the numbers of these are limited and are produced only within a certain period in the mother palm's life. The low number of transplantable offshoots available in the life-time of a selected tree varies from 20 to 30 depending on the cultivar and the cultivation practices used [12]. No field-based methods are as yet available with which to increase the numbers of offshoots produced by each tree. Offshoots have to be large enough (i.e. 10 to 12 kg) to survive when transplanted in the field, a process of regeneration that can take up to 10 years. Moreover, the traditional sucker propagation method passes on pests and diseases and thus slows the substantial improvement of plantation quality.

I.7.3. Tissue culture techniques

Plant tissue culture refers to the *in vitro* cultivation of all plant parts, whether a single cell, a tissue or an organ, under aseptic conditions [13]. Compared to the above two conventional propagation methods, the application of tissue culture techniques has many advantages, such as propagation of healthy selected female cultivars; multiplication at a large scale; multiplication under controlled conditions in the laboratory throughout the year; and production of genetically uniform plants. This *in vitro* technique ensures an easy and fast exchange of plant material between different regions of a country or between countries without risk of

the spread of diseases and pests; and it is economically reliable when large production is required [14, 5].

Therefore, several efforts have been exerted from researchers, scientists, and research institutions such as UAE University Date Palm Tissue Culture Unit to exploit the plant tissue culture technique to overcome constraints related to the defects of the above mentioned conventional methods. Tissue culture enables the rapid multiplication of selected cultivars and clones which is far more reliable than seed propagation and much quicker than the offshoots method.

There are three general kinds of date palm tissue culture used for rapid propagation. These are embryo culture, culture of meristematic tissues (shoot tips and buds) and the culture of highly differentiated somatic tissues (leaf, stem, inflorescence and root sections) [4].

I.7.3.1. Embryo culture

After pollination of an egg cell, an embryo develops which contains the genetic make-up of both parents. Embryo culture involves excising an embryo aseptically from the seed and planting it in a sterile nutrient medium [15]. Embryo culture is applied to save embryos that fail to develop naturally in the fruit or seed, or grow out hybrid embryos which arise through interspecific or intergeneric crosses (wide crosses) but die due to the failure of forming a normal endosperm [16]. Embryo culture may also be used to reduce lengthy dormancy periods due to physical and/or chemical inhibitors present in the fruit or seed [15]. Isolated embryos may also be used as explant material in metabolic studies [17].

Callus initiation and embryoid induction from oil palm embryos was first observed by Rabéchault [18]. Culturing date palm embryo- cotyledonary sheath tissue in media containing naphthalene acetic acid (NAA) produced callus and roots [19]. The presence of a piece of the cotyledon enhanced callus proliferation and differentiation of roots during subculturing. Moreover, Ammar and Bendadis [20] developed organic callus from the date palm cotyledonary sheath of zygotic embryo germinated *in vitro*.

Reynolds and Murashige [21] observed a creamy-coloured grainy callus on embryo explants *in vitro* in a medium enriched with 2,4-dichlorophenoxy acetic acid (2,4-D). Subculturing this callus into an auxin-free medium produced numerous asexual embryos. Tisserat [22] found that using nutrient media containing charcoal plus high auxins levels, 10 and 100 mg/l NAA, to culture mature zygotic embryos did produce nodular callus. Continuous callus subculture resulted in the formation of plantlets. By studying plantlet formation from date palm tissue cultures, Tisserat and DeMason [23] found that the morphological development of asexual embryos from callus closely paralleled excised zygotic embryo germination *in vitro*.

I.7.3.2. Meristematic tissues

Meristems (0.2 to 0.5 mm in size) consist of actively dividing cells that are present at the tip of the shoot or root as well as in the axillary buds. They are dissected under the microscope and can be regenerated on specific media.

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Shoot-tips and stem cuttings (0.5 to 1 cm in size) can also be cultivated *in vitro* to produce complete plantlets.

Culturing shoot-tips and lateral buds *in vitro* has many advantages as compared to other explant sources. They are protected by bud scales and leaves, and are usually easier to surface-sterilize than root or stem explants [24]. Moreover, entire shoots are already present in shoot-tips and/or buds; thus, only root induction is required to produce a whole plantlet [25, 26]. In addition, the cells of the shoot-tips and buds are more uniformly diploid than those derived from less meristematic regions [27].

Growth regulators play an important role in date palm shoot tip cultures. Optimal leaf formation will occur when media contain 0.1 mg/l NAA and 0.01 mg/l Kinetin [28]. In the presence of low concentrations of auxin and/or cytokinin, callus is formed at the cut surface of the tip cultures. In 1974, Reuveni and Kipnis [29] found that the callus was very short lived and its subculture was unsuccessful. Shoot-tip explants consisted of the apical dome with two to four leaf primordial, and varied in size from 0.5 to 1 mm^2.

El Hannawy and Wally [30] found that by using MS medium containing 200 mg/l "fermentol", that 60% bud differentiation occurred in date palm shoot tips cultured at 25° C.

After studying the conditions for bud development, Tisserat [22] and Zaid [31] observed that shoot-tips and lateral buds grew equally well on the same medium. They observed in 1983 that callus from subcultured lateral buds on nutrient media devoid of charcoal and supplemented with 0.1 mg/l NAA, produced adventitious plantlets [32]. Low concentrations of auxins added to MS half strength or Beauchesne

medium produced buds from the young leaves, soft tissues, shoot tips or axillary buds of date palm after 6 months of *in vitro* culture [33].

I.7.3.3. Highly differentiated somatic tissues

Highly differentiated somatic tissues such as leaf, stem, inflorescence and root, can be used as an explant to produce *in vitro* plantlets. According to Schroeder [34], callus was successfully produced through culturing of date palm leaf tissue and gave rise to roots several months later. Development of root initials occurred at a high rate in the presence of low levels of auxins [35]. Comparing date palm leaf explants obtained from adult trees, offshoots, seedlings and asexual plantlets, Zaid [31] found that only sub-cultured leaf callus from seedlings and asexual plantlets produced roots.

Tisserat [22] reported that date palm explants enlarged considerably in size during the first few weeks of culture. Repeated sub-cultures to fresh media resulted in the formation of non-friable nodular callus. Poulain *et al.* [36] also found that callus can be successfully initiated from date palm tissues. Both male and female inflorescences from oil palm were cultured on a variety of media and usually developed somewhat normally, but callus was not obtained [37]. A high auxin level was speculated to decrease normal development. This was subsequently confirmed in date palm by Eeuwens and Blake [35]. However, date palm ovules, carpel tissue, parthenogenetic endosperm, and the fruit stalk did fail in culture [29].

Startsky [38] and Schroder [34] were the first to investigate *in vitro* root cultures in palms. Callus was reported to form at the root tip region of

young date palm seedlings [37, 39]. This callus then produced leaves and shoots. Scharma *et al.* [40] reported no growth for cultured date palm roots. Usually, severe browning and death of root explants occurred within the first few weeks of culture.

I.8. Date palm tissue culture techniques

Date palm plantlets can be regenerated through organogenesis or embryogenesis, the route depending on the explant developmental stage, medium composition and incubation conditions. Organogenesis, in general, involves the differentiation of organs, e.g. root or shoot primordia from cells or tissues, while embryogenesis involves the development of an intact plant via somatic embryos [41].

I.8.1. Organogenesis

Organogenesis is based on the use of the naturally existing potential of the meristematic areas (meristems, axillary buds and the base of young leaves) to produce meristematic buds when the nutrient media and the incubation conditions are suitable [30, 4]. The advantage of organogenesis is that it avoids callus formation and consequently avoids the production of plantlets from mutations of callus cells.

The organogenesis technique consists of 4 steps: Initiation of meristematic buds, multiplication, elongation and rooting [5]. The success of the technique is dependent on the success of the first step (initiation).

Localized meristematic activity precedes the organized development of shoots and roots [42]. The original explant in date palm is taken from the apical dome area. During *in vitro* culture, the apical dome increases in size up to 50 fold within a three

month period. This proliferation is influenced by plant genotype with the bud initiation results from a direct neo-formation [43].

There is a strong relationship between the origins of meristematic buds and the uniformity of organogenesis-derived plants. If buds are pre-existent, then plants will be true-to-type, but if they are newly formed, the possibility exists for plant variation[4].

I.8.2. Somatic embryogenesis

Somatic (also called asexual) embryogenesis in date palm is based on callus production and multiplication as an initial step on an auxin-rich medium, followed by the germination and elongation of derived somatic embryos [44]. Once callus is transferred onto an auxin-free or low concentration medium, a reorganization and formation of somatic embryos occurs [45].

Up to now, this technique had been shown to be genotype-independent with a high rate of multiplication and a high survival rate upon transfer to soil [17]. For genetically stable species, somatic embryogenesis offers a fast scaling-up system, especially when it is possible to produce embryos in bioreactors. Unfortunately, this production via fermentors was not as simple as first envisaged, and today, only a few model plants are successfully produced by such technology. Several bottlenecks limit the use of this interesting technology. One of the main problems is genetic stability [46]. Another difficulty is the loss of embryogenic capacity over time [41]. It is a phenomenon observed in different plant species.

I.9. Problems in date palm tissue culture

I.9.1. Browning

During the course of *in vitro* growth and development, plant tissues not only deplete the nutrients that are furnished in the medium, but also release substances that can accumulate in the cultures. These substances, such as phenols, may have profound physiological effects on the cultured tissues [47]. Phenolic compounds are contained in plant tissues and consist of at least one hydroxy group on a benzene ring. These are strong reducing agents, and when phenolic compounds react with enzymes, usually separated by cell vacuoles, the hydroxy group is oxidized resulting in the formation of quinones and water [48]. This irreversible process of hydrogen bonding to proteins inhibits enzyme activity and leads to cell death. Oxidation and browning of tissue occurs instantaneously when the cut plant surfaces are exposed to air with damage appearing on and around the cut surface [4]. Other reported factors influencing tissue oxidation include age of parent material, time of collection and explant introduction. This poses major problems in the establishment of explants particularly with stem sections where a large surface area is exposed.

Date palm tissue culture, like that of many other plants, has been commonly observed to release discoloring substances into the medium which inhibit their own growth. Injury through cutting of date palm tissue is accompanied by secretion of the discoloring substance(s) into the medium. As explained above, browning of the date palm tissue and the adjacent medium is assumed to be due to the oxidation of polyphenols and formation of quinones which are toxic to the tissues [49, 50].

To minimize browning, Murashige [27] and later on Zaid and Tisserat [51] have suggested the pre-soaking of explants in ascorbic and citric acid solutions and adding

them to the culture medium. Zaid and Tisserat [32] soaked their date palm explants in an anti-oxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid) prior to the surface sterilization treatments. Addition of a combination of adsorbents including citrate, adenine and glutamine, retarded browning in date palm explants [52, 53]. On the other hand, addition of other adsorbents to nutrient media, such as dihydroxynaphtalene, dimethylsulfoxide, were ineffective against browning in date palm explants [53]. Apavatjrut and Blake [54] suggested that browning could be eliminated by a nutritionally balanced medium, while excision of brown explant parts during sub-culture was also advocated to prevent this problem [53].

The use of activated charcoal is preferred over cysteine and other adsorbents because the latter are often toxic to the plant tissues at higher concentrations [53, 55]. Addition of 3 % activated charcoal has favored substantial root and shoot growth of date palm embryos. Fridborg and Erikson [56] postulated that the addition of charcoal to a culture medium drastically alters the properties of this medium. Hence, growth regulator substances are tested at high levels (e.g. 10 and 100 mg/l) with activated charcoal included in the nutrient media to obtain beneficial effects on tissues [57, 58, 55].

I.9.2. Vitrification

Plant material suffering from vitrification (also called hyperhydricity) is described as being "glassy" and having a "translucent" appearance. It is a condition of metabolic and morphological derangement that leads to such abnormality [59, 60, 61].

Vitrification, a widespread problem in tissue culture, is associated with *in vitro* culture systems and partly caused by poor structural wax

development, non-functioning stomata and lack of mesophyll organisation preventing photosynthesis and impeding growth of cultures [61]. There are a number of factors that contribute to this phenomenon such as low potassium concentrations, high cytokinin levels and low concentrations of solidifying agents or sugar in the culture media [4]. This is a common problem with woody plant cultures and can be overcome by modification of culture conditions such as improving aeration [62], cooling and adjusting the gelling agent used [61].

I.9.3. Contamination

Plants growing *in vivo* may become systemically infected with fungal and bacterial diseases. To survive and grow *in vitro*, plant cultures need to be largely free of both fungal and bacterial infections. Contaminants may be introduced with the explants, during manipulations in the laboratory and/or by micro-arthropod vectors [63, 64, 65, 66]. Contaminations can cause large losses during micropropagation and their control is usually the most difficult problem encountered by commercial tissue culture laboratories. If contamination is to be avoided, it is important to detect and eliminate contaminating organisms before they are transferred to many culture vessels during routine subcultures. Simple surface sterilization does not always remove contamination or infection by bacteria, while internal infections do not become apparent until the culture has been maintained for a considerable period of time which may then become so severe that all infected cultures are lost.

Because customers are expecting plant material free from pests and diseases, the detection and elimination of microbial organisms should be high on the list of priorities of any tissue culture laboratory.

The phenomenon of microbial contamination in plant tissue culture has been studied by Cassells [63], Dodds *et al.* [67], George [14], Leifert *et al.* [65], Cole [68], and Herman [69]. Date palm explants suffer from contamination, which usually appears 2-3 days after the initial culture of explants. Tisserat [22] reported that date palm explants were difficult to free from initial contamination.

Culture media are rich in organic compounds such as sugar, amino acids and vitamins, and thus provide favorable conditions for the development of bacteria and fungi. Microbial contamination is a serious problem in plant tissue culture because of competition for nutrients, release of toxins and overgrowth of plant tissue. The loss of plant material can be very costly, especially if contamination rates go undetected at initiation. It is therefore necessary to eliminate micro-organisms with chemical disinfectants or sterilants before culture initiation. Achieving and maintaining asepsis throughout the entire culture process is essential if viable explants are to be established. The decontamination and preparation of explants is a vital step in the culture process and therefore good hygiene and laboratory procedures must be observed [70]. The induction of cultures from young actively growing tissue raised in greenhouses, laboratory or growth rooms is preferable as source material as compared to those directly collected from the field [14]. However, it is not always possible to obtain such material, and subsequently there is a greater degree of microbial contamination and therefore the development of suitable disinfestations protocols are necessary [60]. Sterilization routines should control all superficial contaminants, and are usually relied upon to set up aseptic cultures [14].

I.9.3.1. Types of contaminants

I.9.3.1.1. Bacteria

Bacteria constitute the most common and troublesome kind of contaminating micro-organisms in plant tissue culture. Bacterial contamination is not always detectable at the culture initiation stage; some internal contaminants (endophytic bacteria) become evident in later subcultures and are difficult to eliminate [71]. There are numerous reports on endophytic bacteria in various plant tissues, such as seeds and ovules [72], tubers [73], roots [74], stems and leaves [75], and fruits [76]. There are over 129 bacterial species representing over 54 genera that have been isolated from internal plant tissue of healthy plants, with *Pseudomonas, Bacillus, Enterobacter,* and *Agrobacterium* being the most commonly isolated bacterial genera [72, 77, 78, 79, 80, 81]. Contamination with bacteria can result in obvious adverse effects on growth [82, 83], lack of reproducibility of tissue culture protocols [83], possible hormone-mediated growth effects [84], possibility of carrying pathogens [85] and potential risk to *in vitro* gene banks [86]. All these lead to a decrease in the reliability of plant cell/tissue culture systems [87, 83]. Bacteria can be introduced to *in vitro* cultures with the plant material [88] during the handling of cultures [89] and with unsterile instruments [90] or culture vessels [91] as well.

I.9.3.1.2. Fungi and yeasts

Fungi and yeasts have been described as contaminants in plant tissue culture by many scientists such as Boxus and Terzi [89]. A filamentous fungus may carpet the tissue culture with mycelia after only a few days after culture initiation. Many fungal genera noted as contaminants in

tissue cultures include species of *Aspergillus, Candida (yeast), Cladosporium, Microsporium, Alternatia, Botrytis, Epicoccum, Mucor, Penicillium, Rhizopus, Rhodotorula (yeast), Trichoderma* and *Phialophora* which are generally aerial or dust-borne [14, 65, 68]. Fungal infections generally result in the death of the original explant, but sometimes a dual culture can be obtained, where growth of the cultured plant material can continue but at a very low rate [14].

Yeasts are commonly defined as fungi in which the unicellular form is predominant [92] and which ferment glucose and many other sugars under anaerobic conditions to end-products such as ethanol, glycerol, succinate and/or acetic acid [93]. In general, they are highly alcohol-tolerant [92]. Yeasts are insensitive to low pH (growing between pH 2.0 and 7.0). They also tolerate high sugar or salt concentrations (growing in media containing 70% (w/w) glucose or 20% (w/w) NaCl) and grow at low temperatures (<5°C) [94, 92]. Yeasts are widely distributed in nature and are generally found in habitats such as soil or water or on green leaves where simple carbon sources are present in low concentrations [95, 92, 96]. Contamination may, therefore, have originated from the stock plant or the environment.

I.9.3.2. Control of Contamination

In theory, any microorganisms which are commonly associated with explant tissue as an epiphyte, endophyte or pathogen represent a potential contaminant in plant tissue culture. Microorganisms may appear on all external plants surfaces; moreover, plants can harbour some kinds of bacterial contaminants interstitially between cells such as *Enterobacter*

asburiae and *Pseudomonas fluorescens* [97]. *P. fluorescens* may be present in intercellular spaces [98, 97], or systemically in vascular tissues [99, 100, 101]. Surface treatments using disinfectants will eliminate only surface contamination with no effect on endophytic contaminants. Furthermore, latent contaminants do not immediately reveal their presence by visible growth on the plant material or culture medium. Bacteria may not be adapted to *in vitro* conditions, or may not be able to multiply until cultures are transferred to a new nutrient medium more favorable to their growth.

For most plant cultures, superficial contamination needs to be controlled before the explants are introduced to the medium. Surface disinfection may be achieved with several different germicidal reagents. In general, the best products are those which are cheap, non-toxic to both plants and people, and effective on a wide range of plant material [102]. The most commonly used materials are: sodium hypochlorite [103, 77, 104, 105, 106], ethanol [107, 103, 100], hydrogen peroxide [108, 109], mercuric chloride [100, 98, 110] or a combination of two or more of these [111, 112].

NaOCl is soluble in water and although an aqueous solution can be obtained from laboratory suppliers, most laboratories use household or industrial bleach solutions as a convenient and cheap source. The bactericidal action of hypochlorite solutions is due to both hypochlorous acid (HOCl) and the OCl^- ion [14]. Commercial bleach solutions vary in their respective NaOCl concentrations. Clorox, Javex and Purex contain 5.25 % w/v NaOCl (5% available chlorine) [102, 113], but some other brands of bleach solution contain a less active ingredient. Domestos has only 4% available chlorine (4.2% w/v NaOCl) [114].

The hypochlorite ion is usually obtained from NaOCl or calcium hypochlorite (CaOCl). Calcium hypochlorite is sold in powder form and consequently is less conveniently handled than sodium hypochlorite, but frequently it is cheaper. Scientists have thought calcium hypochlorite to be less effective than sodium hypochlorite in removing contaminants [115], but others have found it equal in activity and less liable to induce tissue browning or injury [116], possibly due to the high concentration of calcium ions in solution.

Alcohols are not only germicidal, but also remove surface waxes from plant tissue [14]. Ethanol is the most widely used alcohol for disinfection, but only rarely can explant material be disinfested in ethanol alone. Dips in 70-95% aqueous solution are frequently combined with soaks in other disinfectants. Thus a preliminary dip in ethanol permits plant tissues to be more effectively wetted and facilitate penetration by another germicide. The duration of such a pre-treatment needs to be varied according to the type of tissue or organ (30 sec to 1 min is often recommended for soft material, 1 to 2 min for seeds [14].

Alternative disinfectants, that have been reported to be suitable antimicrobial agents for explants, are hydrogen peroxide and potassium permanganate. Hydrogen peroxide solutions are unstable and even concentrates have a limited life. McCulloch and Briggs [117] found that potassium permanganate (at 0.01% solution for 4-32 min) was a much better disinfectant than hydrogen peroxide [14].

Antibiotics were found to work as a preventative measure against the spread of contaminants. Antibiotics can be added to plant culture media or sprayed on the mother plants before explants are removed or explants can be dipped in a solution of antibiotics before initial culture [14]. The use of antibiotics in the control of bacterial contaminants of date palm tissue has been reported [118].

Although there are several published reports of successfully using antibiotics in plant tissue cultures, Debergh and Vanderschaeghe [119] were skeptical of the results because no evidence is usually provided that the treatment had eliminated the contaminant. Fisse *et al.* [120] also stated that if antibiotics had a bacteristatic or fungistatic action, bacterial growth could re-occur once the compound is removed. Moreover, antibiotics are not suitable for the routine removal of superficial contaminants because they are expensive, and most of them are only able to restrain a narrow spectrum of micro-organisms and may eventually lead to the development of resistant strains.

Contamination control is therefore much better with the use of simple disinfectants combined with good isolation practices. Antibiotics should only be employed when explanted material contains concealed micro-organisms or superficial contaminants that prove impossible to eliminate by other means.

It is important that a disinfectant makes good contact with all surfaces of the plant tissue. Penetration is considerably assisted by a short prior immersion in 70% ethanol or by adding a non-phytotoxic wetting agent to

the sterilant solution. Many detergents are suitable such as Teepol® or Lissapol F® (0.05-0.1%), Tween 20® or Tween 80® (0.01-0.05%) and Alconox® (0.2%) [14]. To eliminate endophytic contaminants it may be necessary to withdraw air trapped within the explant. This may be done by applying a partial vaccuum to the disinfecting solution containing the original plant material [121, 14].

II. OBJECTIVES

The aims of the present study are to:

- Develop a reliable disinfection technique for date palm explants at initiation yielding a contamination rate less than 5 %.
- Identify tissue culture bacterial contaminants of date palm and propose some control means.

The expected outcomes of this study will impact the success of tissue culture of date palm varieties as well as to improve the reproducibility of date palm *in vitro* introduction protocols.

III. MATERIALS & METHODS

III. 1. Plant material

The two cultivars "Khissab" and "Khenezi"of date palm (*Phoenix dactylifera* L.) from Al Ain oasis (UAE) were used in the present study. Their selection was based on their market importance and on availability of their offshoots' (Figure 2).

III. 2. Offshoots preparation

Selected offshoots were 4-5 years old with a minimum weight of 8 kg, and were collected from healthy, disease-free mother palm trees. The offshoots bases were cleaned with running water and the outer large leaves and fibers were carefully and gradually removed with a sharp knife until the appearance of the soft white meristematic tissues (Figure 3). Special care was taken not to injure the soft meristematic region. Shoot tips and their surrounding primordial leaves were then carefully excised and reduced to approximately 5-10 cm in length and 3-5 cm in width. Excised explants were then soaked in an antioxidant made of 100 mg/L of ascorbic acid and 150 mg/L of citric acid, for a period of up to 1 hour in order to minimize the browning phenomenon caused by oxidation of phenols.

Khissab cv. : Its cultivation is spread between UAE, Oman and KSA. The fruits are of good quality with a late to very late ripening period. Khissab's date fruit is also characterized by tangy flavor and dark red color at the Rutab stage. The fruit shape is oval-round with soft to dry texture and a medium size.

Khenezi cv. : Its cultivation is from Al Katif / Saudi Arabia and was largely propagated in the Gulf region. It is of good fruit quality but of medium season ripening. The fruit flavor is sweet close to Barhee's. The fruit color is red at the Rutab stage with an oval-long shape and a soft texture with a medium size.

Figure 2: Fruit and tree of "Khissab" and "Khenezi" date cultivars and their characteristics.

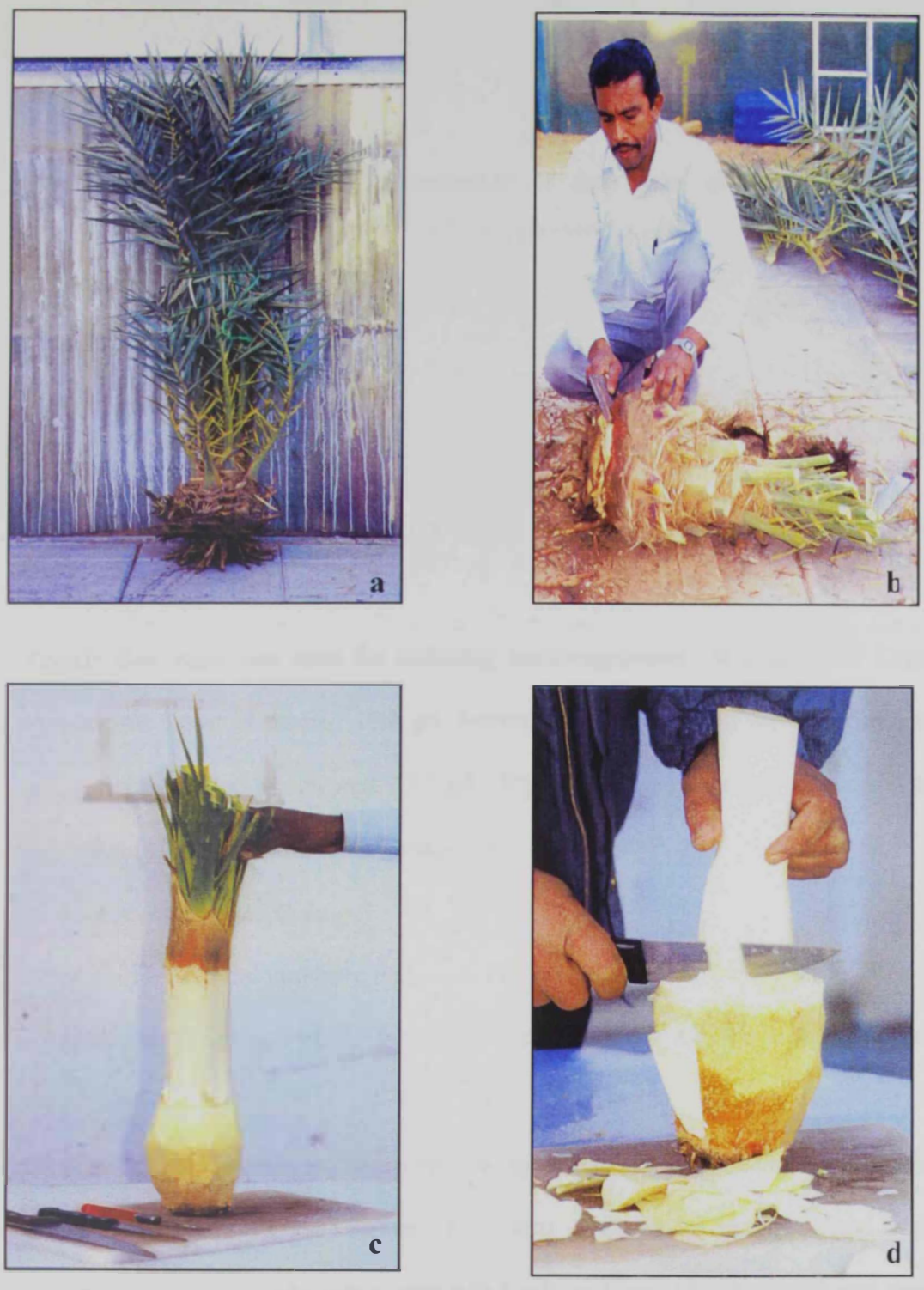

Figure 3: Date palm offshoot preparation before starting the introduction procedures:
a. Healthy and disease-free offshoot candidate for propagation.
b. The outer large leaves, roots and fibers were carefully and gradually removed.
c. Cleaned offshoot: the outer large leaves and fibers have been removed.
d. The soft white meristematic tissues used as source of explants.

III. 3. Nutrient media

The nutrient medium used for initiation of date palm explants was modified Murashige and Skoog medium [122] (MS) supplemented with:

- Thiamine-HCL: 0.4 mg/l;
- I-inositol: 100 mg/l;
- Sucrose: 30,000 mg/l;
- Phytoagar: 8,000 mg/l; and
- Neutralized activated charcoal: 3,000 mg/l.

Tryptic Soy Agar was used for culturing microorganisms. It consists of Tryptone (pancreatic digest of casein) 15.0 g/l, Soytone (Papiac soybean meal digest) 5.0 g/l, sodium chloride 5.0 g/l and agar 15.0 g/l. Tryptic soy agar is mainly used as an initial growth medium for the following purposes:

- Observe colony morphology,
- Determine bacterial numbers, and
- Achieve sufficient growth for further biochemical testing and for culture storage.

A Nutrient agar medium that supported the growth of a wide range of microorganisms was used to develop a pure culture. It consists of a peptic digest of animal tissue (5 g/l), beef extract (1.5 g/l), yeast extract (1.5 g/l), sodium chloride (5 g/l) and agar (15 g/l).

III. 4. Explants disinfection

The aims of this study were to develop a reliable disinfection technique of date palm explants at the initiation phase yielding a contamination rate of less than 5 %, and to identify any bacterial contaminants so as to better propose a means of control. Various concentrations of sodium hypochloride (NaOCl), potassium permanganate ($KMnO_4$) and Aliette (Fosetyl-Al /$C_6H_{18}AlO_9P_3$) (fungicide) were tested. The duration of each disinfection period was fixed to 20 min in experiment I and II. The best combination of disinfectant solution in both experiments was tested for 15, 20 and 25 min in experiment III. Three offshoots per cultivar were used per treatment, which corresponds to 54 offshoots from each cultivar in experiments I and II, and 18 offshoots in experiment III. The total number of offshoots used in this study was 126.

III. 4. 1. Experiment I: Effect of NaOCl and Aliette on the efficiency of surface explants disinfection

Various concentrations of NaOCl and Aliette have been tested. NaOCl at different concentrations (30, 40, 50 %) was combined with different amounts of Aliette (1, 2 and 3 g/l) Table (2).

Table 2: Different concentrations of NaOCl and amounts of Aliette used to disinfect shoot-tips of date palm.

Factors tested	NaOCl**	Aliette
Treatment *	(%)	(g/L)
I_1	30	1
I_2	40	
I_3	50	
I_4	30	2
I_5	40	
I_6	50	
I_7	30	3
I_8	40	
I_9	50	

* 1: refers to Experiment 1.
1-9: refers to different concentrations of the tested disinfection solution.
** 5 % available chlorine.

After soaking the explant in an antioxidant solution and before starting the disinfection trial, a sample (1 g) was taken from the surface of the shoot tip using cleaned scalpel and forceps. The explant was then surface-sterilized by immersion into 30 % of NaOCl and 1 g/L of Aliette solution and shaken well for 20 min. One set of leaves was removed after rinsing the explant in two successive changes of sterile distilled water, and then a second sample was taken. The explant was then immersed into 35 % of NaOCl and 200 mg/L of $KMnO_4$. The emulsifiant Tween 20 was used at a rate of 10 drops / liter in the disinfection solution. The explant was subjected to frequent pressure changes using a vacuum pump (high performance vacuum pump; model: 15601; SPX Robinair Corp.) in order to make sure that the disinfecting solution penetrates well between the leaves surrounding the shoot tip area [121, 14]. The excised explant was then aseptically transferred to the culture medium. Samples were crushed and diluted in sterile distilled water (1/10, 1/100 and 1/1000). This procedure

was followed in all treatments in experiment I with both cultivars Khissab and Khenezi. Three offshoots were used in each treatment (27 offshoots for each cultivar; 54 offshoots in total).

III. 4. 2. Experiment II: Effect of NaOCl and $KMnO_4^-$ on endophytic contamination.

Different concentrations of NaOCl combined with different amounts of $KMnO_4^-$ were tested. NaOCl at 30, 40 and 50 % combined with 200, 300 and 400 mg/l of $KMnO_4^-$ (Table 3). The emulsifiant (Tween 20) was used at a rate of 10 drops / liter of the disinfecting solution in order to increase the surface contact between the explant and the disinfecting solution.

Table 3: Different concentrations of NaOCl and amounts of $KMnO_4^-$ used to disinfect endophytic contamination of date palm shoot-tips.

Factors tested	NaOCl**	$KMnO_4^-$
Treatment *	(%)	(mg/L)
II_1	30	200
II_2	40	
II_3	50	
II_4	30	300
II_5	40	
II_6	50	
II_7	30	400
II_8	40	
II_9	50	

* 1: refers to Experiment 2.
1-9: refers to different concentrations of the tested disinfection solution (30, 40 and 50 %).
** 5 % available chlorine.

the results in experiment I were obtained (which is represented by the t bacterial contamination rate), experiment II was initiated. The new :d explants were soaked in an antioxidant solution, and then erred to a first disinfection solution which gave the lowest bacterial nination rate in experiment I. Sterile distilled water was used twice se the explant and one set of leaves was removed. The second ction solution was then prepared by mixing 30% of NaOCl and 200 f $KMnO_4$. Tween 20 was added to the solution in the range of 10 liter. Under frequent vacuum shocks, explants were disinfected for 20 and then rinsed twice in sterilized distilled water under aseptic conditions, by a laminar airflow hood, to remove any residual disinfectant. The ed explants were then soaked in a presterilized container containing an nt solution to minimize production of phenols that cause browning, and to ıe explant from desiccation. The antioxidant solution consisted of 2 g/l pyrolydone (PVP, Mwt = 40,000), 100 mg/l sodium diethydithiocarbonate, ıg/l anhydrous caffeine. Explants were then maintained in this solution for until culture.

ry xylem and bases of leaves of the explant were cut off and the rest of the ıs sectioned in half at right angles around the apical dome. The apical ic area was divided into about 0.5 to 1 cm^3 pieces. One of these pieces was as a second sample and the remaining were cultured on a 20 ml initiation 24 x 200 mm test tubes. The sample was crushed and diluted in sterile ıter (1/10 and 1/100). This procedure was followed through out all ı experiment II.

ce the results in experiment I were obtained (which is represented by the vest bacterial contamination rate), experiment II was initiated. The new :ised explants were soaked in an antioxidant solution, and then nsferred to a first disinfection solution which gave the lowest bacterial ıtamination rate in experiment I. Sterile distilled water was used twice rinse the explant and one set of leaves was removed. The second infection solution was then prepared by mixing 30% of NaOCl and 200 /L of $KMnO_4^-$. Tween 20 was added to the solution in the range of 10 ıps / liter. Under frequent vacuum shocks, explants were disinfected for 20 ıutes, and then rinsed twice in sterilized distilled water under aseptic conditions, vided by a laminar airflow hood, to remove any residual disinfectant. The infected explants were then soaked in a presterilized container containing an ioxidant solution to minimize production of phenols that cause browning, and to tect the explant from desiccation. The antioxidant solution consisted of 2 g/l yvinylpyrolydone (PVP, Mwt = 40,000), 100 mg/l sodium diethydithiocarbonate, l 200 mg/l anhydrous caffeine. Explants were then maintained in this solution for 3 min until culture.

: primary xylem and bases of leaves of the explant were cut off and the rest of the ılant was sectioned in half at right angles around the apical dome. The apical ristematic area was divided into about 0.5 to 1 cm^3 pieces. One of these pieces was n taken as a second sample and the remaining were cultured on a 20 ml initiation dium in 24 x 200 mm test tubes. The sample was crushed and diluted in sterile ıilled water (1/10 and 1/100). This procedure was followed through out all ıtments in experiment II.

Once the results in experiment I were obtained (which is represented by the lowest bacterial contamination rate), experiment II was initiated. The new excised explants were soaked in an antioxidant solution, and then transferred to a first disinfection solution which gave the lowest bacterial contamination rate in experiment I. Sterile distilled water was used twice to rinse the explant and one set of leaves was removed. The second disinfection solution was then prepared by mixing 30% of NaOCl and 200 mg/L of $KMnO_4^-$. Tween 20 was added to the solution in the range of 10 drops / liter. Under frequent vacuum shocks, explants were disinfected for 20 minutes, and then rinsed twice in sterilized distilled water under aseptic conditions, provided by a laminar airflow hood, to remove any residual disinfectant. The disinfected explants were then soaked in a presterilized container containing an antioxidant solution to minimize production of phenols that cause browning, and to protect the explant from desiccation. The antioxidant solution consisted of 2 g/l polyvinylpyrolydone (PVP, Mwt = 40,000), 100 mg/l sodium diethydithiocarbonate, and 200 mg/l anhydrous caffeine. Explants were then maintained in this solution for 2 – 3 min until culture.

The primary xylem and bases of leaves of the explant were cut off and the rest of the explant was sectioned in half at right angles around the apical dome. The apical meristematic area was divided into about 0.5 to 1 cm^3 pieces. One of these pieces was then taken as a second sample and the remaining were cultured on a 20 ml initiation medium in 24 x 200 mm test tubes. The sample was crushed and diluted in sterile distilled water (1/10 and 1/100). This procedure was followed through out all treatments in experiment II.

III. 4. 3. Experiment III: Effect of time on the efficiency of the best combinations obtained from experiment I and II

After determining the best two combinations from experiments I and II that yielded the least contamination, they were re-tested for 15, 20 and 25 min. The previous disinfection process was followed in experiment III. Three different samples were taken during the disinfection procedure. The first two samplings were before and after applying the first disinfection solution with samples diluted in sterile distilled water (1/10, 1/100 and 1/1000). The last sample was taken after the second disinfection solution. Samples were crushed and diluted in sterile distilled water (1/10 and 1/100). Three offshoots were tested from both cultivars (Khissab and Khenezi) at 15, 20 and 25 min (18 offshoots in total).

III. 5. Bacteriological analysis

In the present study, date palm tissues were collected in a sterile container using aseptic techniques and processed as rapidly as possible. To avoid sampling errors, the solid tissue samples were homogenized using a sterilized mortar and pestle.

III. 5. 1. Total bacterial count

Total bacterial counts were enumerated on standard plate count (TSA) agar [123].

III. 5. 2. Isolation of pure culture

Isolation of bacterial colonies was obtained by the streak plate method using nutrient agar media. The Gram stain was used to distinguish between two groups of bacteria by the identification of differences in the structure of their cell walls.

III. 5. 3. Bacterial identification

Pure strains were subjected to classical bacteriological tests at Tawam hospital / AL Ain and the Environmental Agency / Abu Dhabi, UAE: Gram's stain, shape, motility and catalase tests were used for initial characterization. Identification of Gram-negative strains to species level was made using the API E20 test strips (API-system Bio-Merieux, France). The results were analyzed by the API computer identification software and further analysis was carried out by matching the 16S ribosomal DNA sequence of the unknown isolates with the bacterial database at the Advance Biotechnology Lab in Dubai, UAE. The 16S rRNA genes (rDNAs) from bacterial DNAs [124, 125] were amplified by using universal primers [126]. The PCR products were cloned [127] and sequenced (ABI Prism 377 DNA sequencer; Perkin-Elmer). The 16S rDNA sequences were aligned with all accessible sequences, obtained through the Ribosomal Database Project (RDP), with the program sequence Alignment [128]. (Further details about this procedure are in Appendix VI).

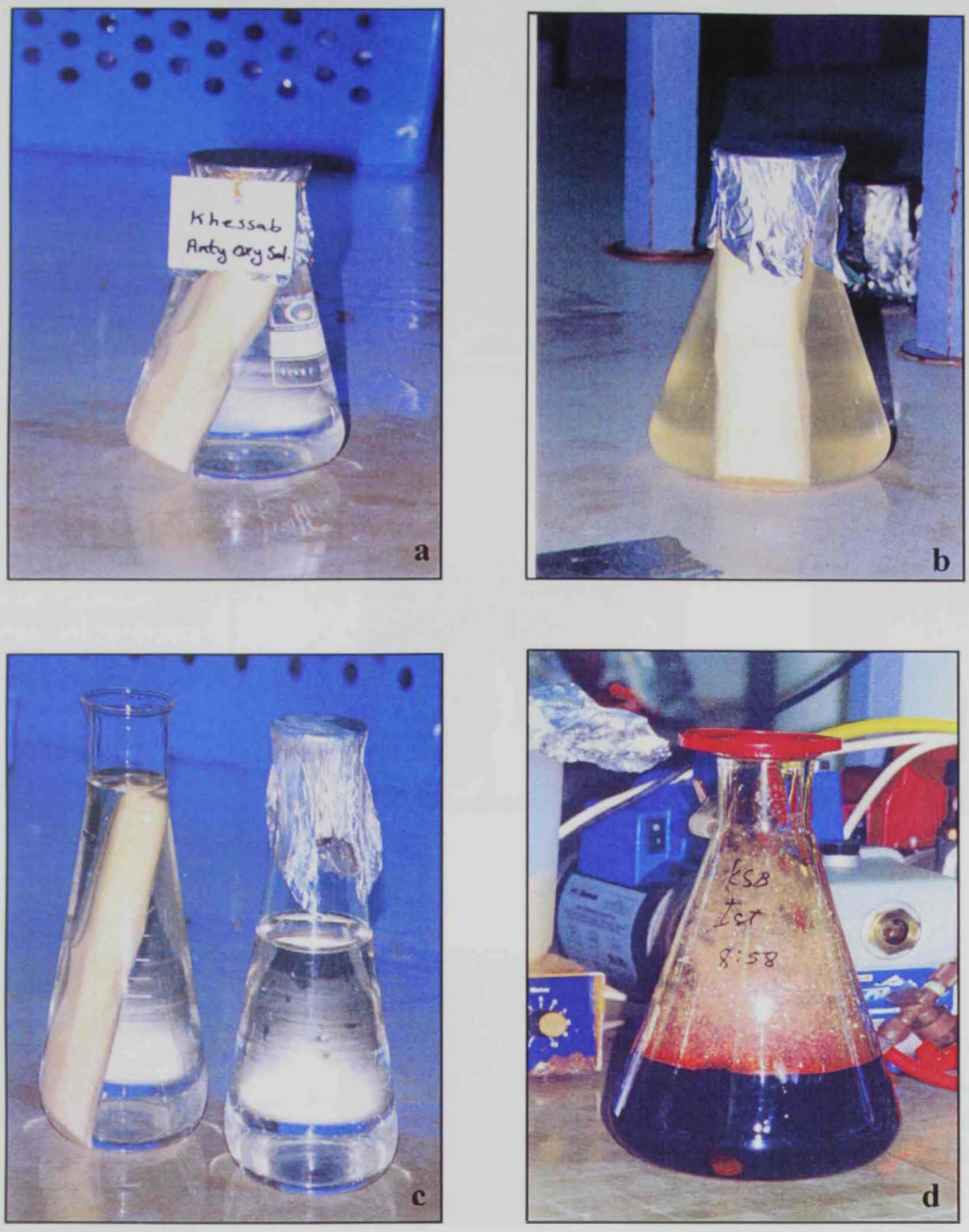

Figure 4: Various steps of meristematic tissue disinfection used during experiments I, II and III:

- **a.** Storage of the explant source in an antioxidant solution till disinfection.
- **b.** First disinfection step in fungicide solution (NaOCl and Alitte).
- **c.** Rinsing the explant twice to remove any residual disinfectant solution.
- **d.** Second disinfection step using NaOCl and $KMnO_4^-$ under pressure ensured by a vacuum pump.

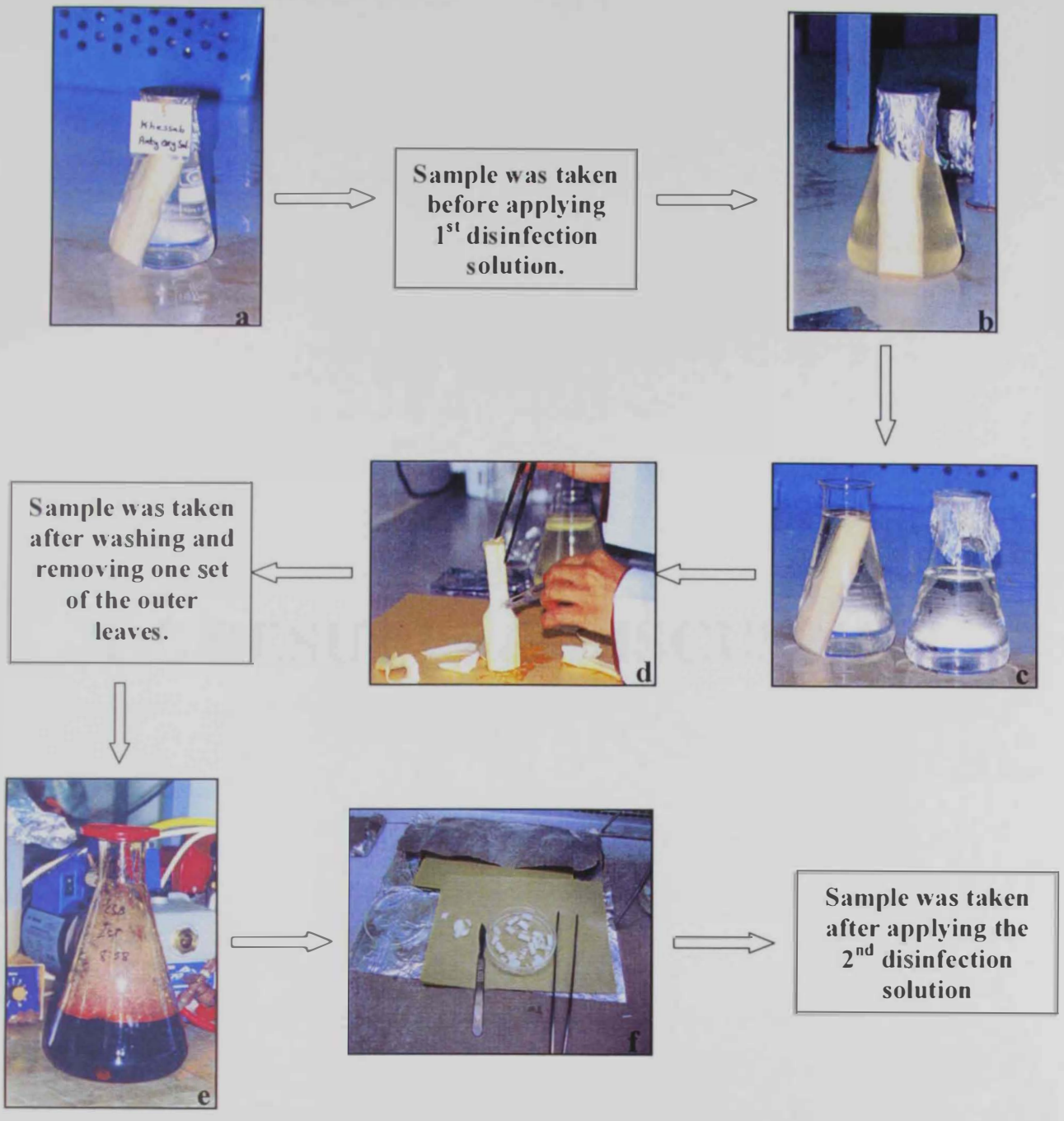

Figure 5: Sampling stages before and after the first and second disinfection solutions:

a. Explant in the anti-oxydant solution;
b. First surface disinfection of the explant;
c. Rinsing the explant twice with autoclaved distilled water;
d. Removal of one set of the outer leaves;
e. Explant soaked in 2nd disinfection solution and subjected to frequent pressure change provided by a vacuum pump.
f. Explant divided into small pieces under aseptic condition provided by a laminar air flow hood.

IV. RESULTS & DISCUSSION

The disinfection of a species is strongly dependent on the explant size, source, disinfectant concentration and time of sterilization [129]. Several chemical and physical treatments have been applied to obtain clean cultures. The most common procedure involves immersion of explants in sodium hypochlorite (NaOCl) solution, containing a few drops of emulsifier (Tween 20) for 15 to 30 min. The explant is then usually rinsed 2 or 3 times with sterile distilled water to remove any residual disinfectant. In the current study, different concentrations of NaOCl combined with different amounts of Aliette (fungicide) or potassium permanganate ($KMnO_4^-$) have been tested on shoot tips of two date palm cultivars (Khissab and Khenezi). The size of the explants was approximately 5 - 10 cm in length and 3 - 5 cm in width. The best combination from NaOCl and Aliette were then combined with the best combination from NaOCl and $KMnO_4^-$ and retested for different time periods of 15, 20 and 25 min.

IV. 1. Effect of NaOCl and Aliette on the surface contamination

Introduction of bacteria with insufficiently sterilized plant material is estimated to cause between a quarter and half of observed contamination [130]. Plant surfaces carry a wide range of microbial contaminants. Their origins, identities, and population densities are, in many cases, unknown [131]. Therefore, the plant material must be thoroughly sterilized before being inoculated. To our knowledge, most of the date palm tissue culture laboratories believe in the fact that existing procedures do not guarantee complete exterior sterilization of the explant.

Total bacterial contamination after disinfection used in experiment I for 20 min in different concentrations for both Khissab and Khenezi cultivars is shown in tables 4 and 5, respectively.

Table (4) illustrates the effect of combination of different concentrations of NaOCl with various amounts of Aliette on total bacterial count (log) in date palm shoot tips culture on Khenezi cv. The results obtained show that the Total bacterial count before disinfection ranged from 3.36 to 4.68 $\log_{10}$ cfu. After sterilizing the explants using the above mentioned disinfectant solution, the total bacterial count ranged between 0 and 1.12 $\log_{10}$ cfu. This reduction in the total bacterial count was attributed to the bactericidal action of hypochlorite solutions, i.e both hypochlorous acid (HOCl) and hypochlorite (OCl^-) ion [14]. The highest reduction rate of bacterial number, which is represented by 100 %, was achieved in four different concentration of the disinfection solution. These concentrations were 30% of NaOCl with 1 g/l of Aliette, 50 % of NaOCl with 2 g/l of Aliette and 40 and 50 % of NaOCl with 3 g/l of Aliette (for further details see appendix I).

Table 4: Effect of combinations of different NaOCl concentrations with various amounts of Aliette on total bacterial count (log_{10} cfu) in date palm shoot tip cultures (Khenezi cv.).

Treatment		TBC ± SD**	TBC ± SD	Reduction Rate (%)
Aliette (g/l)	NaOCl * (%)	Before treatment	After treatment	
1	30	4.68 ± 0.26	0	100
	40	4.64 ± 0.12	0.47 ± 0.81	89.87
	50	4.45 ± 0.17	0.80 ± 1.39	82.02
2	30	4.17 ± 0.75	1.00 ± 1.73	76.02
	40	3.87 ± 0.85	0.57 ± 0.98	85.27
	50	3.78 ± 0.84	0	100
3	30	3.36 ± 0.14	1.12 ± 1.94	66.67
	40	4.49 ± 0.11	0	100
	50	4.17 ± 0.45	0	100

(*) 5 % available chlorine.
(**) TBC ± SD: Total bacterial count ± Standard deviation (log).

Table 5: Effect of combinations of different NaOCl concentrations with various amounts of Aliette on total bacterial count (log_{10} cfu) in date palm shoot tip cultures (Khissab cv.).

Treatment		TBC ± SD**	TBC ± SD	Reduction Rate (%)
Aliette (g/l)	NaOCl* (%)	Before treatment	After treatment	
1	30	4.17 ± 0.20	0	100
	40	4.36 ± 0.24	0.93 ± 0.81	78.67
	50	4.47 ± 0.25	0	100
2	30	4.71 ± 0.51	0.47 ± 0.81	90.02
	40	4.30 ± 0.40	0	100
	50	4.32 ± 0.13	0.47 ± 0.81	89.12
3	30	4.62 ± 0.06	0.47 ± 0.81	89.83
	40	4.52 ± 0.25	0	100
	50	3.76 ± 0.40	0	100

(*) 5 % available chlorine.
(**) TBC ± SD: Total bacterial count ± Standard deviation (log).

Results obtained with Khissab cv. (Table 5) using the same disinfection treatments were similar to Khenezi cv. These treatments were found appropriate and the disinfection solution had almost a complete minimizing effect on the surface Total bacterial count.

The Total bacterial count on the surface of the Khissab explant before the disinfection trial ranged from 3.76 to 4.71 $\log_{10}$ cfu. This number has reduced after applying different combination of disinfection solutions. The best result (100 % reduction) was obtained by applying 30 and 50 % of NaOCl with 1 g/l Aliette; 40 % of NaOCl with 2 g/l Aliette and in 40 and 50 % of NaOCl with 3 g/l Aliette (for further details see appendix II). Based on these data, 40 % of NaOCl with 3 g/l Aliette has been selected as the best disinfection solution for eliminating surface bacteria. Indeed, surface contaminations which result in losses during the *in vitro* stages of plant tissue culture [87, 83] may be substantially reduced by using various concentrations of NaOCl combined with Aliette.

IV. 2. Effect of NaOCl and $KMnO_4^-$ on the endophytic contamination

While most of the yeast and fungal microorganisms are eliminated during surface sterilization [132], endophytic bacteria survive through successive multiplication, as they are not exposed to the sterilant during treatment [133]. Cassells [134] explained such delays in appearance of bacterial contamination as due to the presence of tannins in sap extracts, which may inhibit or delay the growth of microorganisms. Some endophytic bacteria are latent, producing no visible plant symptoms or growth in the medium through many subculture cycles. Others are non-latent endophytic bacteria which are initially detected from a halo or cloudiness around the base of the explant in the medium in surface-treated

explants [133]. Propagation of explants infected with latent endophytic bacteria can cause severe losses at later stages of tissue culture or after weaning of plants [135, 136, 137]. This endophytic contaminant can be controlled through use of specific concentration of NaOCl combined with $KMnO_4^-$ plus Tween 20 for 20 min under frequent vacuum shocks.

Before starting experiment II, the total number of endophytic bacteria was determined in surface sterilized shoot tips from both date palm cultivars. The Total bacterial count was 1.41 $\log_{10}$ cfu. The effect of different concentrations of NaOCl and varying amounts of $KMnO_4^-$ on the endophytic bacteria were tested in Khenezi and Khissab explants, see results in Tables 6 and 7.

In Khenezi cv., the Total bacterial count was reduced from 1.41 $\log_{10}$ cfu before treatment to 0.13 ± 0.23 $\log_{10}$ cfu after. The highest reduction rate (90.78 %) was accomplished when 40 % of NaOCl was combined with 400 mg/l of $KMnO_4^-$. While the lowest reduction rate was obtained when 30 % of NaOCl was combined with 300 mg/l of $KMnO_4^-$ (for further details see appendix III).

Table 6: Effect of combinations of different NaOCl concentrations with various amounts of $KMnO_4^-$ on total bacterial count (log_{10} cfu) in date palm shoot tip cultures (Khenezi cv.).

Treatment		TBC ± SD **	Reduction Rate (%)
$KMnO_4^-$ (mg/l)	NaOCl * (%)	After treatment	
200	30	0.37 ± 0.35	73.76
	40	0.57 ± 0.51	59.57
	50	0.53 ± 0.46	62.41
300	30	1.37 ± 0.35	2.84
	40	0.76 ± 0.79	46.10
	50	0.50 ± 0.87	64.54
400	30	0.27 ± 0.23	80.85
	40	0.13 ± 0.23	90.78
	50	0.89 ± 0.50	36.88

(*) 5 % available chlorine.
(**) TBC ± SD: Total bacterial count ± Standard deviation (log).

Table 7: Effect of combinations of different NaOCl concentrations with various amounts of $KMnO_4^-$ on total bacterial count (log_{10} cfu) in date palm shoot tip cultures (Khissab cv.).

Treatment		TBC ± SD **	Reduction Rate (%)
$KMnO_4^-$ (mg/l)	NaOCl * (%)	After treatment	
200	30	0.13 ± 0.23	90.78
	40	1.31 ± 0.38	7.09
	50	0.53 ± 0.46	62.41
300	30	0.53 ± 0.46	62.41
	40	1.22 ± 1.04	13.48
	50	1.09 ± 0.60	22.70
400	30	0.47 ± 0.50	66.67
	40	0.45 ± 0.78	68.09
	50	0.57 ± 0.98	59.57

(*) 5 % available chlorine.
(**) TBC ± SD: Total bacterial count ± Standard deviation (log).

In "Khissab" cv., the highest reduction rate (90.78 %), and the lowest endophytic bacterial numbers (0.13 ± 0.23 log_{10} cfu), have been achieved when 30 % of NaOCl was combined with 200 mg/l $KMnO_4^-$. On the other hand, the lowest reduction rate (7.09 %), yielding the highest survival of endophytic bacterial (1.31 ± 0.38 log_{10} cfu) was when 40 % of NaOCl was combined with 200 mg/l of $KMnO_4^-$ (for further details see appendix IV).

In consultation with the thesis advisor, it was decided to eliminate the concentration 40 % NaOCl and 400 mg/l KMnO4$^-$ since its original data was not reliable. Therefore, 30 % of NaOCl with 400 mg/l of $KMnO_4^-$ was selected as the best disinfection solution for eliminating endophytic bacteria. Although the reduction rate of concentration by 200 mg/l of $KMnO_4^-$ is higher than 400 mg/l of $KMnO_4^-$ concentration; we selected this last combination because it had the low average of total bacterial count after treatments.

IV. 3. Effect of the best combination of disinfection solution during different periods of time

The best two combinations from experiment I and the best two combinations from experiment II were combined and retested using 15, 20 and 25 min to determine the optimum time for both experiments.

IV. 3. 1. Effect of the 1st disinfection solution during different periods of time

The results of the effect of the 1st disinfection solution (NaOCl + Aliette) on the surface bacterial count during different time periods are shown in Figures 6, 7 and 8.

The average total bacterial number found on the surface of both cultivars of date palm shoot tips before starting treatment for 15 min was 3.75 ± 0.26 and 3.65 ± 0.22 log_{10} cfu on Khenezi cv. and on Khissab cv., respectively (Figure 6). These numbers have been markedly declined reaching 0.79 ± 0.77 log_{10} cfu with 78.93 % reduction rate on Khenezi cv. and 0.96 ± 0.85 log_{10} cfu with 73.70 % reduction rate on Khissab cv.

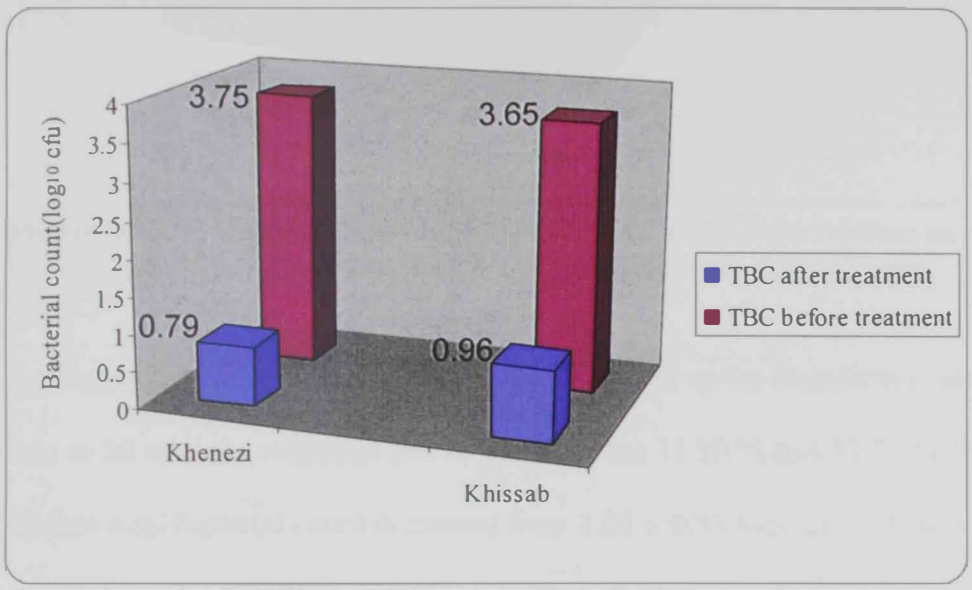

Figure 6: Effect of the best combination of NaOCl and Aliette solution on surface bacterial number on shoot-tips of both date palm cultivars during 15 min.

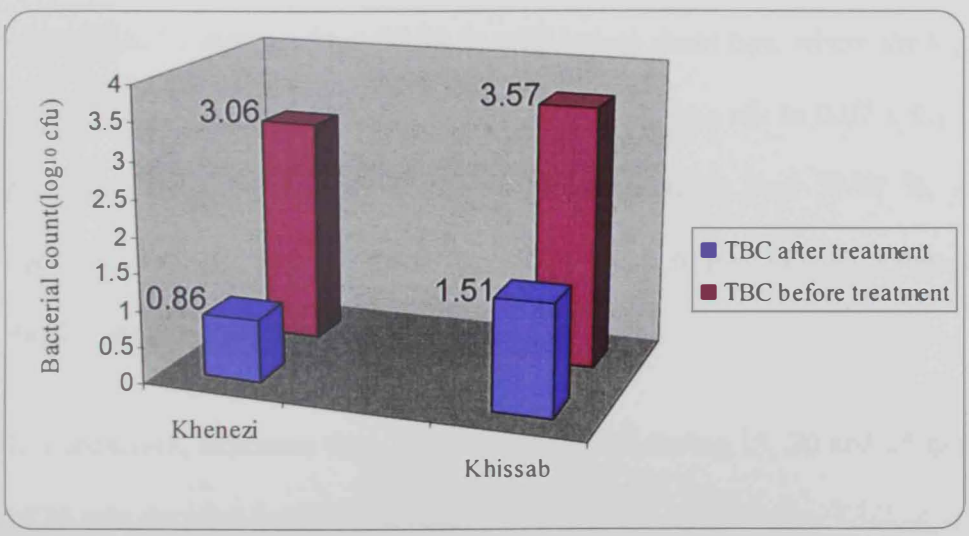

Figure 7: Effect of the best combination of NaOCl and Aliette solution on surface bacterial number on shoot-tips of both date palm cultivars during 20 min.

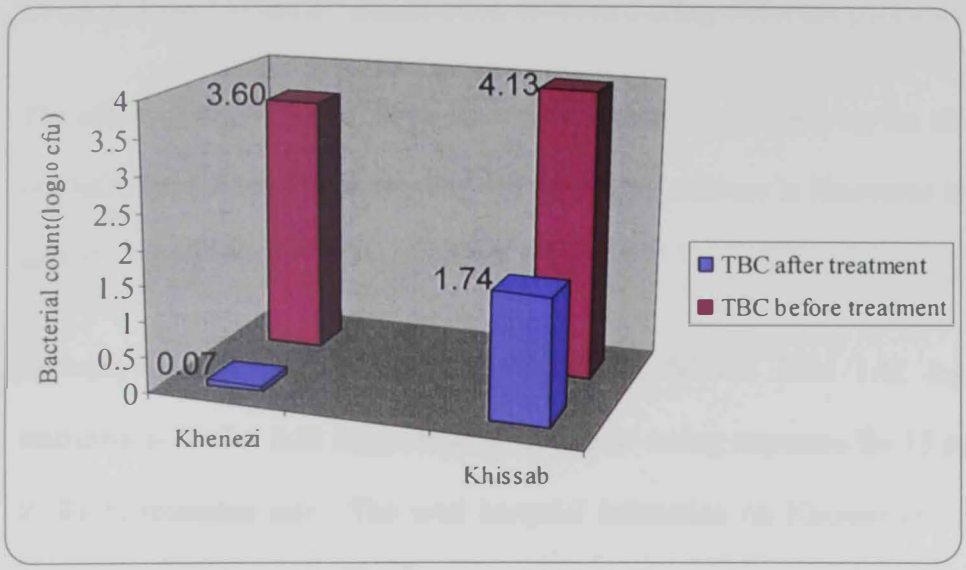

Figure 8: Effect of the best combination of NaOCl and Aliette solution on surface bacterial number on shoot-tips of both date palm cultivars during 25 min.

By increasing the exposure period of the shoot tips to the disinfection solution from 15 min to 20 min, the reduction rate ranged between 71.90 % and 57.70 % (Figure 7). The surface total bacterial count decreased from 3.06 ± 0.95 log_{10} cfu to 0.86 ± 0.96 log_{10} cfu and from 3.57 ± 0.32 log_{10} cfu to 1.51 ± 0.92 log_{10} cfu in Khenezi and Khissab, respectively. The optimum reduction rate was achieved when the exposure period was elevated to 25 min reaching 98.06 % on Khenezi shoot tips, where the log total bacterial count had a massive depression from 3.60 ± 0.30 log_{10} cfu to 0.07 ± 0.13 log_{10} cfu. On the other hand, the reduction rate on Khissab variety was 57.70 %, where the total bacterial number decreased from 4.13 ± 0.22 log_{10} cfu to 1.74 ± 0.57 log_{10} cfu (for further details see appendix V).

In conclusion, exposure time has no major effect during 15, 20 and 25 min. But, the use of 25 min duration is recommended.

IV. 3. 2. Effect of the 2nd disinfection solution during different periods of time

The effect of time on the endophytic contaminations while applying the best combination of NaOCl and $KMnO_4$ solution in both date palm cultivars is illustrated in Figures 9, 10 and 11.

Endophytic contamination on Khenezi cv. did fall-off from 1.41 log_{10} cfu before treatment to 0.27 ± 0.23 log_{10} cfu after treatment during exposure for 15 min, realizing a 80.85 % reduction rate. The total bacterial infestation on Khissab cv. has completely disappeared achieving a reduction rate of 100 % (Figure 9).

Using the same concentration of disinfection solution during exposure for 20 min did not show any significant effect on the total bacterial count on Khenezi shoot tips (Figure 10). However, the total bacterial count showed a slight decrease on Khissab shoot tips, reaching 0.37 ± 0.35 log_{10} cfu, accomplishing a reduction rate of 73.76 %.

During the 25 min exposure, bacterial numbers on Khenezi explants showed a massive decline accomplishing 100 % reduction rate (Figure 11). On the other hand, the disinfection concentration, during the same time has insignificant effect on the bacterial count on Khissab explants as compared with Khenezi explants (for further details see appendix VI).

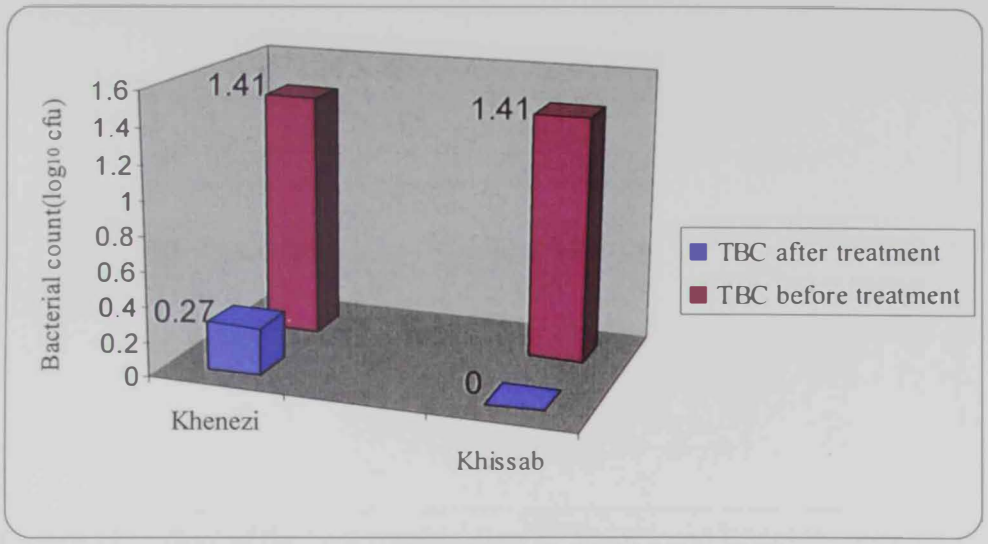

Figure 9: Effect of the best combination of NaOCl and $KMnO_4^-$ solution on endophytic bacterial number on shoot-tips of both date palm cultivars during 15 min.

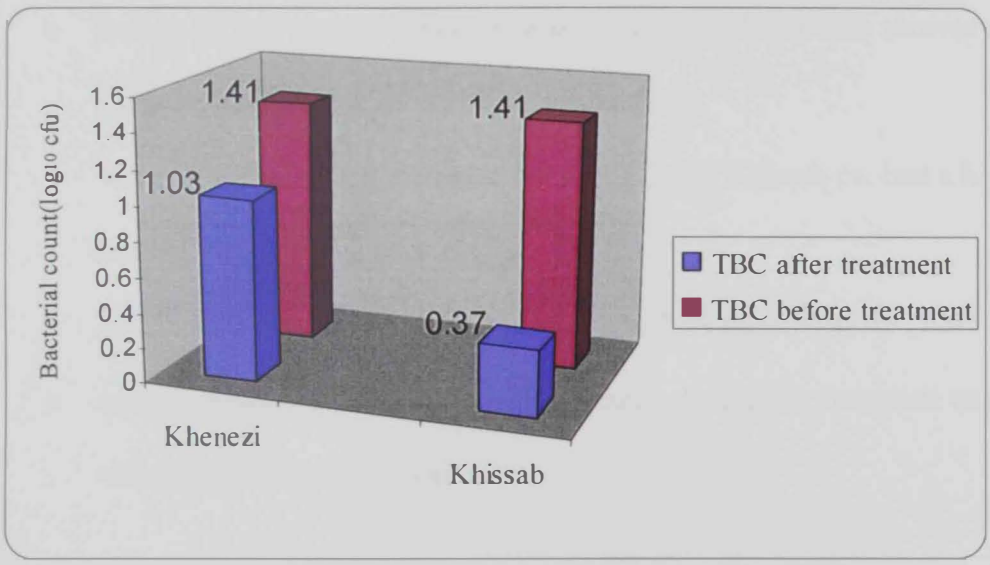

Figure 10: Effect of the best combination of NaOCl and KMnO4- solution on endophytic bacterial number on shoot-tips of both date palm cultivars during 20 min

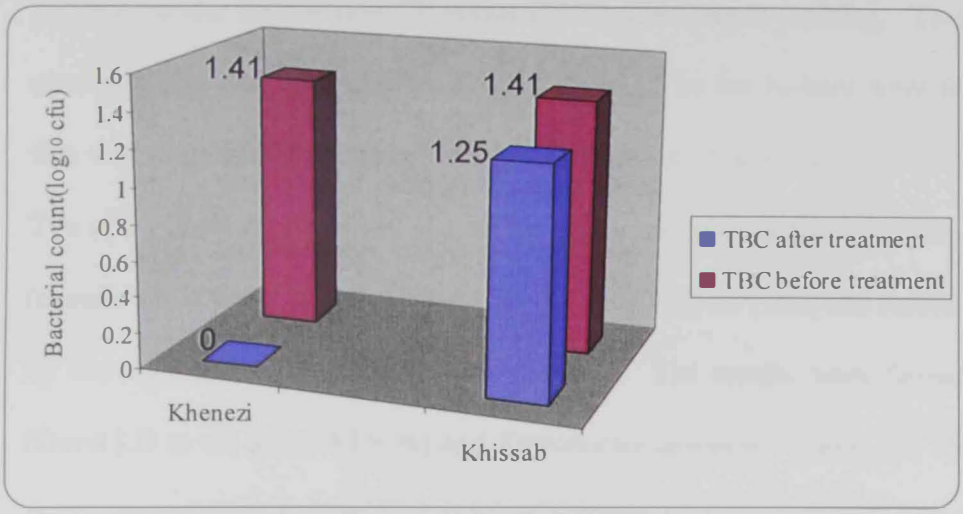

Figure 11: Effect of the best combination of NaOCl and KMnO4- solution on endophytic bacterial number on shoot-tips of both date palm cultivars during 25 min.

The data obtained can be summarized as follows:

- During 15 min exposure, both Khenezi and Khissab explants showed a significant reduction rate of 80.85 and 100 %, respectively.
- When this exposure is increased to 20 min., only Khissab cv. had a high reduction rate (73.76 %).
- At 25 min., only Khenezi cv. showed the highest reduction rate (100 %).
- As a conclusion, in both date palm explants, the highest reduction rate is obtained with 15 min disinfection exposure.

IV.4. Identification of bacteria

Colonies for most bacterial microorganisms were visible on Tryptic Soy Agar plates in only 3 days of incubation. The most frequent ten bacterial colonies were isolated and purified on nutrient agar media. Colony pigmentation varied from cream to light cream, yellow to whitish yellow, pink to pink-red and orange colored. Purified bacteria were

observed under microscope (Olympus PX50) after proper staining. Two of them were gram negative and the rest were gram positive. The ten isolates were found to contain five that were identical; which brings the samples of analyzed isolates to six samples. The cell morphology was rod shaped for all the samples except two were spherical cells (cocci). Only Gram-negative were further tested with API 20E and results were analyzed by the API computer identification software. The results were *Serratia marcesceus* (Good I.D to the genus 83.9 %) and *Entrobacter aerogenes* (Good I.D 96 %). Bacterial strains were then transfered to Advanced Biotechnology Lab (Dubai) for identification by 16S rDNAs. The obtained results are shown in Table 8.

Table 8: Morphological and biochemical characteristics of identified selected isolates.

No.	Bacteria	Colony Color	Gram stain	Rods	Motility	Catalase
1	*Cellulomonas cellulans*	Yellow	+	+	+ / -	+
2	*Cellulomonas uda*	Whitish yellow	+	+	+ / -	+
3	*Bacillus fusiformis*	Light cream	+	+	+	+
4	*Serratia marcescens*	Rose	-	+	+	+
5	*Kocuria rosea*	Orange	+	-	+	+
6	*Enterobacter aerogenes*	Cream	-	+	+	+

Rafferty *et al.* [138], reported that *Serratia marcescens* may become pathogenic to the *in vitro* micro-propagated cultures of cabbage. *Enterobacteriaceae* contaminant is also found by Leifert and Woodward [139] in *in vitro* plant propagation due to inefficient disinfection of explants. Many scientists such as Trick and Lingens [91] and Cornu and Michel [88] reported that *Bacillus* and *Enterobacter* species have frequently been found in plant tissue cultures. Habiba *et al.* [140] found *Cellulomonas uda* during their study of endogenous bacterial contamination in *in vitro* culture of table Banana.

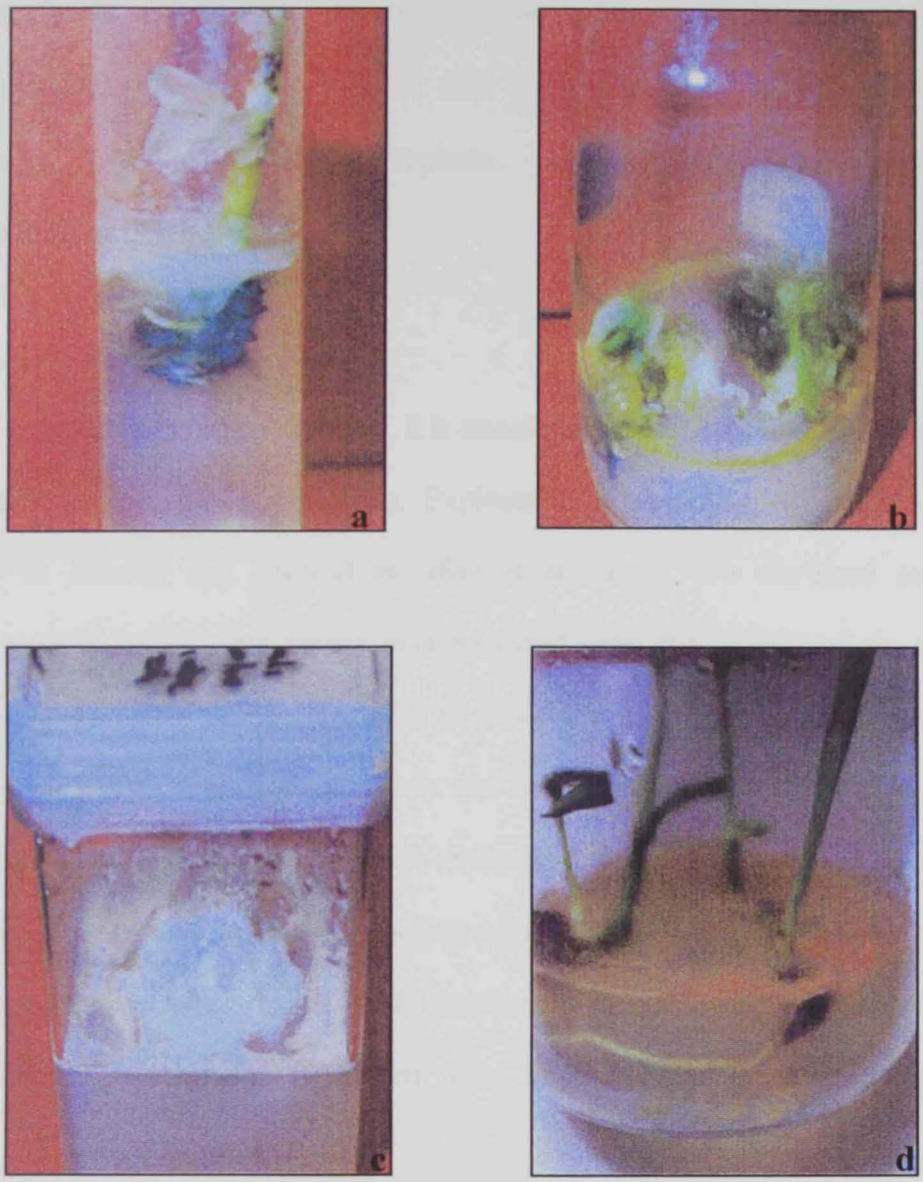

Figure 12: Different types of endophytic bacteria found at various stages of *in vitro* multiplication of date palm:

- **a.** Veil bacteria occur usually at the surrounding area of the explants base. It appears mostly at the multiplication, elongation and rooting stages.
- **b.** Yellow bacteria commonly found at the surface of the nutrient media.
- **c.** White creamy bacteria commonly found at the surface of the nutrient media but rapidly invading the cultured explants.
- **d.** Rose bacteria at both the surface and inside the nutrient media.

IV. 5. Organogenesis technique

Organogenesis technique has the advantage of omitting both the callus and embryoid phases and significantly reducing the total number of stages in culture by the direct formation of new shoots from the explants. Organogenesis process is made from the following four (4) stages:

IV. 5. 1. Initiation stage

Once the explant is fully disinfected, it is transferred to the laminar airflow hood in order to initiate the culture of first explants. Explants were rinsed twice with sterilized distilled water to remove any residual disinfectant solution. On sterilized surface, the meristematic tissues were placed in a sterilized petri dish containing an antioxidant solution (mentioned in experiment II) to avoid browning and desiccation of explants during culturing. The primary xylem and bases of leaves were then cut off using sterilized surgical blades. Meristematic explants were then divided into small pieces each of about 3-5 mm^3. Each explant was then cultured on a 20 ml initiation medium in 24 x 200 mm culture tubes, capped with aluminum foil and Nalagin plastic cover. The explants were kept in a special growth room, in complete darkness, at a temperature of 28° C ± 1, during around 4 months.

The medium which have been used for initiation of date palm explants is the modified Murashige and Skoog [122] as detailed in Materials and Methods.

IV. 5. 2. Multiplication stage

After seven to twelve months on initiation medium, first buds are initiated and were transferred to a multiplication medium containing the same components as in initiation medium, but devoid of activated charcoal and supplemented with 30 g/l sucrose instead of 40 g/l. Cultures were then maintained under light conditions of 16/8-hour photoperiod. Cultures were subcultured every four to five weeks, and consequently several thousand buds true-to-type to the mother plant are produced.

IV. 5. 3. Elongation stage:

Formed buds were isolated and individually cultured on an elongation medium. This medium contained the same components as in the initiation medium, but without activated charcoal and growth regulators, and supplemented with 30 g/l sucrose. The culture would take one month in this stage under a 16/8-hour photoperiod regime, at 30 $\mu mol\ m^{-2}\ sec^{-1}$ before being transferred to the rooting stage.

IV. 5. 4. Rooting stage:

This is the last stage *in vitro* before transferring to the hardening process. As soon as the elongated shoots reach 13-18 cm in length, they were transferred to a rooting medium containing the same basic components as in the initiation medium, but without charcoal, and supplemented with 30 g/l sucrose and 1 mg/l NAA. Cultures were kept under the same light regime as in the multiplication and elongation stages, until they were ready to transfer to the greenhouse, this may take about four weeks.

IV. 5. 5. Hardening-off:

Date palm as most species grown *in vitro* require an acclimatization process before transferring to the open field. *In vitro* environments produce plants which cannot survive under harsh field conditions because of many reasons, such as the *in vitro* high relative humidity. More over, the *in vitro* plants are having a heterotrophic growth since a carbon source is provided in the media.

Because of the above physiological abnormalities *in vitro* plants will not survive if they are directly transferred from the tissue culture laboratory to the field. Therefore, several intermediate stages are necessary in which vitroplants are gradually acclimatized to the outside environment by reducing relative humidity and increasing progressively the temperature (from 27° C to 35° C). After transferring the plant to *in vivo* conditions, a gradual reduction in relative humidity levels and as increasing of temperature will assist the plantlets to begin a new autotrophic growth. In this way, the plants become acclimatized to the new growth soil conditions and can overcome the shock of transplantation before they are planted in there final holes in the open field.

Figure 13: Various stages of the organogenesis tissue culture technique:

- **a.** Initiation stage;
- b. Multiplication stage;
- **c.** Elongation stage; and
- **d.** Rooting stage.

Figure 14: Various steps of hardening:

- **a.** Vitro plant 1 (VP1) at the first acclimatization outside the test tubes (6 months).
- **b.** Vitro plant 2 (VP2) transfer from Geffry pots to 1L plastic bag (1 year).
- **c.** Vitro plant 3 (VP3) transfer to 7L plastic bag (1 year).

N.B: Each stage is having a special irrigation and nutritional requirement.

V. CONCLUSIONS

The aims of this study were to develop a reliable disinfection technique of date palm explants at the initiation phase yielding a contamination rate of less than 5 %, and to identify any bacterial contaminants so as to better propose control mean.

These goals were approached through three experiments. In experiment I, different concentrations of NaOCl (30, 40 and 50 %) and Aliette (1, 2 and 3 g/l) were tested in order to eliminate surface contamination. In experiment II, various concentrations of NaOCl (30, 40 and 50 %) and $KMnO4^-$ (200, 300 and 400 mg/l) were tested to reduce the endophytic contaminations. The best two combinations from experiments I and II that yielded the least contamination, were re-tested for 15, 20 and 25 min. Two date palm cultivars (Khissab and Khenezi) were used in this study in a total of 126 offshoots. Bacterial identification was carried out by using classical biochemical test and by matching the 16S Ribosomal DNA sequence of unknown isolate with the bacterial database.

The best disinfection concentration that can eliminate surface contamination is 40 % of NaOCl and 3 g/l Aliette during 25 min. The best disinfection concentration that can reduce endophytic contamination is 30 % of NaOCl and 400 mg/l of $KMnO4^-$ during 15 min. The most frequent bacterial strains identified were *Cellulomonas cellulans*, *Enterobacter aerogenes*, *Bacillus fusiformis*, *Serratia marcescens*, *Kocuria rosea* and *Cellulomonas uda*.

The future aims of this study are to apply the present selected disinfection technique to all date palm cultivars under multiplication in the Date Palm Research and Development Unit of UAE University, and to strengthen the achievement of less than 5% contamination rate of the Unit and consequently bring it down to less than 1%.

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VII. APPENDICES

Appendix I: Experiment I: Total bacterial count on the shoot tip surface of Khenezi cv. before and after applying 1st disinfection solution during 20 min.

		Before treatment				*After treatment*			
Treatment	**Samples**	**1/100**		**1/1000**		**1/100**		**1/1000**	
I1	S1	116	181	40	50	0	0	0	0
	S2	154	410	158	154	0	0	0	0
	S3	61	100	53	85	0	0	0	0
I2	S1	150	15	54	60	0	0	0	0
	S2	119	232	82	70	0	0	0	0
	S3	214	205	100	79	1	0	0	0
I3	S1	125	210	32	95	0	0	0	0
	S2	194	140	50	43	0	0	1	0
	S3	30	95	29	31	0	0	0	0
I4	S1	38	49	8	14	0	0	0	0
	S2	138	525	7	95	10	10	1	1
	S3	25	42	26	62	0	0	0	0
I5	S1	324	214	18	40	2	0	0	0
	S2	77	34	45	33	0	0	0	0
	S3	11	19	1	0	0	0	0	0
I6	S1	67	60	10	6	0	0	0	0
	S2	9	52	2	0	0	0	0	0
	S3	327	305	62	84	0	0	0	0
I7	S1	14	72	3	6	0	0	0	0
	S2	75	56	8	20	0	0	0	0
	S3	4	79	35	5	1	0	9	0
I8	S1	227	126	60	70	0	0	0	0
	S2	282	240	32	33	0	0	0	0
	S3	55	180	43	41	0	0	0	0
I9	S1	100	44	27	18	0	0	0	0
	S2	343	206	75	43	0	0	0	0
	S3	148	90	13	30	0	0	0	0

Appendix (I) continue: Exp. I:Total bacterial count on the shoot tip surface of Khenezi cv. before applying 1st disinfection solution during 20 min.

Treatment	Samples	1/100		Average*100	1/1000		Average*1000	[(Aver*100)+(Aver*1000)]/2	log	Average	SD*
I1	S1	116	181	14850	40	50	45000	29925	4.48	4.68	0.26
	S2	154	410	28200	158	154	156000	92100	4.97		
	S3	61	100	8050	53	85	69000	38525	4.59		
I2	S1	150	15	8250	54	60	57000	32625	4.51	4.64	0.12
	S2	119	232	17550	82	70	76000	46775	4.67		
	S3	214	205	20950	100	79	89500	55225	4.74		
I3	S1	125	210	16750	32	95	63500	40125	4.60	4.45	0.17
	S2	194	140	16700	50	43	46500	31600	4.50		
	S3	30	95	6250	29	31	30000	18125	4.26		
I4	S1	38	49	4350	0	0	0	2175	3.34	4.17	0.75
	S2	138	525	33150	0	95	95000	64075	4.81		
	S3	0	42	2100	26	62	44000	23050	4.36		
I5	S1	324	214	26900	0	40	20000	23450	4.37	3.87	0.85
	S2	77	34	5550	45	33	39000	22275	4.35		
	S3	11	19	1500	0	0	0	750	2.88		
I6	S1	67	60	6350	0	0	0	3175	3.50	3.78	0.84
	S2	0	52	2600	0	0	0	1300	3.11		
	S3	327	305	31600	62	84	73000	52300	4.72		
I7	S1	0	72	3600	0	0	0	1800	3.26	3.36	0.14
	S2	75	56	6550	0	0	0	3275	3.52		
	S3	0	79	3950	0	0	0	1975	3.30		
I8	S1	227	126	17650	60	70	65000	41325	4.62	4.49	0.11
	S2	282	240	26100	32	33	32500	29300	4.42		
	S3	55	180	11750	43	41	42000	26875	4.43		
I9	S1	100	44	7200	0	0	0	3600	3.75	4.17	0.45
	S2	343	206	27450	75	43	59000	43225	4.64		
	S3	148	90	11900	0	30	15000	13450	4.13		

* Standard deviation.

Appendix (I) continue: Exp. I:Total bacterial count on the shoot tip surface of Khenezi cv. after applying 1st disinfection solution during 20 min.

Treatment	Samples	1/100		Average*100	1/1000		Average*1000	[(Aver*100)+(Aver*1000)]/2	log	Average	SD*
I1	S1	0	0	0	0	0	0	0	0	0.00	0.00
	S2	0	0	0	0	0	0	0	0		
	S3	0	0	0	0	0	0	0	0		
I2	S1	0	0	0	0	0	0	0	0	0.47	0.81
	S2	0	0	0	0	0	0	0	0		
	S3	1	0	50	0	0	0	25	1.4		
I3	S1	0	0	0	0	0	0	0	0	0.80	1.39
	S2	0	0	0	1	0	500	250	2.4		
	S3	0	0	0	0	0	0	0	0		
I4	S1	0	0	0	0	0	0	0	0	1.00	1.73
	S2	10	10	1000	1	1	1000	1000	3		
	S3	0	0	0	0	0	0	0	0		
I5	S1	2	0	100	0	0	0	50	1.7	0.57	0.98
	S2	0	0	0	0	0	0	0	0		
	S3	0	0	0	0	0	0	0	0		
I6	S1	0	0	0	0	0	0	0	0	0.00	0.00
	S2	0	0	0	0	0	0	0	0		
	S3	0	0	0	0	0	0	0	0		
I7	S1	0	0	0	0	0	0	0	0	1.12	1.94
	S2	0	0	0	0	0	0	0	0		
	S3	1	0	50	9	0	4500	2275	3.36		
I8	S1	0	0	0	0	0	0	0	0	0.00	0.00
	S2	0	0	0	0	0	0	0	0		
	S3	0	0	0	0	0	0	0	0		
I9	S1	0	0	0	0	0	0	0	0	0.00	0.00
	S2	0	0	0	0	0	0	0	0		
	S3	0	0	0	0	0	0	0	0		

* Standard deviation.

Appendix II: Experiment I: Total bacterial count on the shoot tip surface of Khissab cv. before and after applying 1st disinfection solution during 20 min.

		Before treatment				*After treatment*			
Treatment	**Samples**	**1/100**		**1/1000**		**1/100**		**1/1000**	
I1	S1	106	95	20	22	0	0	0	0
	S2	85	70	33	43	0	0	0	0
	S3	130	63	9	8	0	0	0	0
I2	S1	135	25	55	13	1	0	0	0
	S2	70	0	70	86	0	0	0	0
	S3	0	0	34	21	1	0	0	0
I3	S1	187	92	44	40	0	0	0	0
	S2	310	90	51	120	0	0	0	0
	S3	41	160	19	29	0	0	0	0
I4	S1	379	332	276	450	1	0	0	0
	S2	390	210	19	20	0	0	0	0
	S3	29	96	51	49	0	0	0	0
I5	S1	81	165	25	31	0	0	0	0
	S2	43	13	7	20	0	0	0	0
	S3	300	225	24	120	0	0	0	0
I6	S1	94	105	35	37	0	0	0	0
	S2	100	45	8	36	1	0	0	0
	S3	150	150	38	38	0	0	0	0
I7	S1	57	250	110	22	1	0	0	0
	S2	59	87	63	67	0	0	0	0
	S3	155	122	70	95	0	0	0	0
I8	S1	391	321	48	59	0	0	0	0
	S2	116	103	18	28	0	0	0	0
	S3	315	59	94	62	0	0	0	0
I9	S1	13	93	9	11	0	0	0	0
	S2	118	58	22	9	0	0	0	0
	S3	9	23	2	3	0	0	0	0

Appendix (II) continue: Exp. I:Total bacterial count on the shoot tip surface of Khissab cv. before applying 1st disinfection solution during 20 min.

Treatment	Samples	1/100		Average*100	1/1000		Average*1000	[(Aver*100)+(Aver*1000)]/2	log	Average	SD*
I1	S1	106	95	10050	20	22	21000	15525	4.19	4.17	0.20
	S2	85	70	7750	33	43	38000	22875	4.36		
	S3	130	63	9650	9	8	8500	9075	3.96		
I2	S1	135	25	8000	55	13	34000	21000	4.33	4.36	0.24
	S2	70	0	3500	70	86	78000	40750	4.61		
	S3	0	0	0	34	21	27500	13750	4.14		
I3	S1	187	92	13950	44	40	42000	27975	4.45	4.47	0.25
	S2	310	90	20000	51	120	85500	52750	4.72		
	S3	41	160	10050	19	29	24000	17025	4.23		
I4	S1	379	332	35550	276	450	363000	199275	5.3	4.71	0.51
	S2	390	210	30000	19	20	19500	24750	4.39		
	S3	29	96	6250	51	49	50000	28125	4.45		
I5	S1	81	165	12300	25	31	28000	20150	4.3	4.30	0.40
	S2	43	13	2800	7	20	13500	8150	3.91		
	S3	300	225	26250	24	120	72000	49125	4.7		
I6	S1	94	105	9950	35	37	36000	22975	4.36	4.32	0.13
	S2	100	45	7250	8	36	22000	14625	4.17		
	S3	150	150	15000	38	38	38000	26500	4.42		
I7	S1	57	250	15350	110	22	66000	40675	4.61	4.62	0.06
	S2	59	87	7300	63	67	65000	36150	4.56		
	S3	155	122	13850	70	95	82500	48175	4.68		
I8	S1	391	321	35600	48	59	53500	44550	4.65	4.52	0.25
	S2	116	103	10950	18	28	23000	16975	4.23		
	S3	315	59	18700	94	62	78000	48350	4.69		
I9	S1	13	93	5300	9	11	10000	7650	3.88	3.76	0.40
	S2	118	58	8800	22	9	15500	12150	4.09		
	S3	9	23	1600	2	3	2500	2050	3.31		

* Standard deviation.

Appendix (II) continue: Exp. I:Total bacterial count on the shoot tip surface of Khissab cv. after applying 1st disinfection solution during 20 min.

Treatment	Samples	1/100		Average*100	1/1000		Average*1000	[(Aver*100)+(Aver*1000)]/2	log	Average	SD*
I1	S1	0	0	0	0	0	0	0	0.00	0.00	0.00
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
I2	S1	1	0	50	0	0	0	25	1.40	0.93	0.81
	S2	0	0	0	0	0	0	0	0.00		
	S3	1	0	50	0	0	0	25	1.40		
I3	S1	0	0	0	0	0	0	0	0.00	0.00	0.00
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
I4	S1	1	0	50	0	0	0	25	1.40	0.47	0.81
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
I5	S1	0	0	0	0	0	0	0	0.00	0.00	0.00
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
I6	S1	0	0	0	0	0	0	0	0.00	0.47	0.81
	S2	1	0	50	0	0	0	25	1.40		
	S3	0	0	0	0	0	0	0	0.00		
I7	S1	1	0	50	0	0	0	25	1.40	0.47	0.81
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
I8	S1	0	0	0	0	0	0	0	0.00	0.00	0.00
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
I9	S1	0	0	0	0	0	0	0	0.00	0.00	0.00
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		

* Standard deviation.

Appendix III: Experiment II: Total bacterial count of the endophytic contamination of Khenezi cv. after applying the 2nd disinfection solution during 20 min.

		After treatment			
Treatment	**Samples**	**1/10**		**1/100**	
II1	S1	1	1	0	0
	S2	1	0	0	0
	S3	0	0	0	0
II2	S1	0	0	0	0
	S2	3	1	0	0
	S3	2	0	0	0
II3	S1	2	0	0	0
	S2	0	0	0	0
	S3	2	1	0	0
II4	S1	0	0	1	0
	S2	0	0	2	0
	S3	4	0	0	0
II5	S1	2	0	0	0
	S2	0	0	0	0
	S3	2	3	1	0
II6	S1	0	0	0	0
	S2	0	3	1	0
	S3	0	0	0	0
II7	S1	1	0	0	0
	S2	1	0	0	0
	S3	0	0	0	0
II8	S1	1	0	0	0
	S2	0	0	0	0
	S3	0	0	0	0
II9	S1	0	0	1	0
	S2	1	0	0	0
	S3	1	2	0	0

Appendix III: continue: Exp. II: Total bacterial count of the endophytic contamination of Khenezi cv. After applying 2nd disinfection solution during 20 min.

Treatment	Samples	1/10		Average*10	1/100		Average*100	[(Aver*10)+(Aver*100)]/2	log	Average	SD
II1	S1	1	1	10	0	0	0	5	0.70	0.37	0.35
	S2	1	0	5	0	0	0	2.5	0.40		
	S3	0	0	0	0	0	0	0	0.00		
II2	S1	0	0	0	0	0	0	0	0.00	0.57	0.51
	S2	3	1	20	0	0	0	10	1.00		
	S3	2	0	10	0	0	0	5	0.70		
II3	S1	2	0	10	0	0	0	5	0.70	0.53	0.46
	S2	0	0	0	0	0	0	0	0.00		
	S3	2	1	15	0	0	0	7.5	0.88		
II4	S1	0	0	0	1	0	50	25	1.40	1.37	0.35
	S2	0	0	0	2	0	100	50	1.70		
	S3	4	0	20	0	0	0	10	1.00		
II5	S1	2	0	10	0	0	0	5	0.70	0.76	0.79
	S2	0	0	0	0	0	0	0	0.00		
	S3	2	3	25	1	0	50	37.5	1.57		
II6	S1	0	0	0	0	0	0	0	0.00	0.50	0.87
	S2	0	3	15	1	0	50	32.5	1.51		
	S3	0	0	0	0	0	0	0	0.00		
II7	S1	1	0	5	0	0	0	2.5	0.40	0.27	0.23
	S2	1	0	5	0	0	0	2.5	0.40		
	S3	0	0	0	0	0	0	0	0.00		
II8	S1	1	0	5	0	0	0	2.5	0.40	0.13	0.23
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		
II9	S1	0	0	0	1	0	50	25	1.40	0.89	0.50
	S2	1	0	5	0	0	0	2.5	0.40		
	S3	1	2	15	0	0	0	7.5	0.88		

* Standard deviation.

Appendix IV: Experiment II:Total bacterial count of the endophytic contamination of Khissab cv. after applying the 2nd disinfection solution during 20 min.

		After treatment			
Treatment	**Samples**	**1/10**		**1/100**	
II1	S1	0	0	0	0
	S2	1	0	0	0
	S3	0	0	0	0
II2	S1	1	2	0	0
	S2	3	2	1	0
	S3	1	1	1	0
II3	S1	0	0	0	0
	S2	3	0	0	0
	S3	2	0	0	0
II4	S1	0	0	0	0
	S2	2	1	0	0
	S3	1	1	0	0
II5	S1	17	31	2	3
	S2	3	0	0	0
	S3	1	0	0	0
II6	S1	0	0	1	0
	S2	1	1	0	1
	S3	1	0	0	0
II7	S1	1	3	0	0
	S2	1	0	0	0
	S3	0	0	0	0
II8	S1	0	0	0	0
	S2	3	6	0	0
	S3	0	0	0	0
II9	S1	0	0	2	0
	S2	0	0	0	0
	S3	0	0	0	0

Appendix IV: continue: Exp. II: Total bacterial count of the endophytic contamination of Khissab cv. after applying 2nd disinfection solution during 20 min.

Treatment	Samples	1/10		Average*10	1/100		Average*100	[(Aver*10)+(Aver*100)]/2	log	Average	SD
II1	S1	0	0	0	0	0	0	0	0.00	0.13	0.23
	S2	1	0	5	0	0	0	2.5	0.40		
	S3	0	0	0	0	0	0	0	0.00		
II2	S1	1	2	15	0	0	0	7.5	0.88	1.31	0.38
	S2	3	2	25	1	0	50	37.5	1.58		
	S3	1	1	10	1	0	50	30	1.48		
II3	S1	0	0	0	0	0	0	0	0.00	0.53	0.46
	S2	3	0	15	0	0	0	7.5	0.88		
	S3	2	0	10	0	0	0	5	0.70		
II4	S1	0	0	0	0	0	0	0	0.00	0.53	0.46
	S2	2	1	15	0	0	0	7.5	0.88		
	S3	1	1	10	0	0	0	5	0.70		
II5	S1	17	31	240	2	3	250	245	2.39	1.22	1.04
	S2	3	0	15	0	0	0	7.5	0.88		
	S3	1	0	5	0	0	0	2.5	0.40		
II6	S1	0	0	0	1	0	50	25	1.40	1.09	0.60
	S2	1	1	10	0	1	50	30	1.48		
	S3	1	0	5	0	0	0	2.5	0.40		
II7	S1	1	3	20	0	0	0	10	1.00	0.47	0.50
	S2	1	0	5	0	0	0	2.5	0.40		
	S3	0	0	0	0	0	0	0	0.00		
II8	S1	0	0	0	0	0	0	0	0.00	0.45	0.78
	S2	3	6	45	0	0	0	22.5	1.35		
	S3	0	0	0	0	0	0	0	0.00		
II9	S1	0	0	0	2	0	100	50	1.70	0.57	0.98
	S2	0	0	0	0	0	0	0	0.00		
	S3	0	0	0	0	0	0	0	0.00		

* Standard deviation.

Appendix V: Experiment III - Effect of time on the efficiency of the best combinations obtained from experiment I & II.

Total bacterial count before and after applying the best combinations obtained from experiments I and II during 15 min.

Experiment: III **Khenezi cv.** **Before 1st treatment** **Duration time:15 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	Average	SD*
1	278-381	3295	87-115	10100	14-13	13500	8965	3.95		
2	277-109	1930	44-46	4500	2-2	2000	2810	3.45	3.75	0.26
3	335-320	3275	100-98	9900	8-7	7500	6891.67	3.84		

Experiment: III **Khenezi cv.** **After 1st treatment** **Duration time:15 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	Average	SD
1	4-0	20	0-0	0	0-0	0	6.67	0.82		
2	6-5	55	1-0	50	0-0	0	35	1.54	0.79	0.77
3	0-0	0	0-0	0	0-0	0	0	0		

Experiment: III **Khissab cv.** **Before 1st treatment** **Duration time:15 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	Average	SD
1	158-154	1560	37-87	6200	1-11	6000	4586.67	3.66		
2	265-280	2725	35-32	3350	16-18	17000	7691.67	3.87	3.65	0.22
3	75-86	800	39-29	3350	5-3	4000	2716.67	3.43		

Experiment: III **Khissab cv.** **After 1st treatment** **Duration time:15 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	Average	SD
1	10-5	75	1-0	50	0-0	0	41.67	1.62		
2	0-0	0	0-0	0	0-0	0	0	0	0.96	0.85
3	6-5	55	0-0	0	0-0	0	18.33	1.26		

Experiment: III **Khenezi cv.** **After 2nd treatment** **Duration time:15 min**

sample	1/10	average*10	1/100	average*100			[(Aver*100)+(Aver*1000)]/2	log	Average	SD
1	1-0	5	0-0	0			2.5	0.4		
2	1-0	5	0-0	0			2.5	0.4	0.27	0.23
3	0-0	0	0-0	0			0	0		

Experiment: III **Khissab cv.** **After 2nd treatment** **Duration time:15 min**

sample	1/10	average*10	1/100	average*100			[(Aver*100)+(Aver*1000)]/2	log	Average	SD
1	0-0	0	0-0	0			0	0		
2	0-0	0	0-0	0			0	0	0.00	0.00
3	0-0	0	0-0	0			0	0		

*SD = Standard deviation

Appendix V: Experiment III - Effect of time on the efficiency of the best combinations obtained from experiment I & II.

Total bacterial count before and after applying the best combinations obtained from experiments I and II during 20 min.

Experiment: III **Khenezi cv.** **Before 1st treatment** **Duration time:20 min**

sample	1/10	averagex10	1/100	averagex100	1/1000	averagex1000	[(Averx100)+(Averx1000)+(Averx1000)]/3	log	Average	SD*
1	100-29	500	16-22	1900	1-1	1000	1133.33	2.06		
2	160-103	1315	119-68	93500	19-14	16500	9055	3.96	3.06	0.95
3	44-38	410	19-40	2950	2-0	1000	1453.33	3.16		

Experiment: III **Khenezi cv.** **After 1st treatment** **Duration time:20 min**

sample	1/10	averagex10	1/100	averagex100	1/1000	averagex1000	[(Averx100)+(Averx1000)+(Averx1000)]/3	log	Average	SD
1	2-1	15	0-0	0	0-0	0	5	0.70		
2	0-0	0	0-0	0	0-0	0	0	0.00	0.86	0.96
3	5-1	30	3-1	200	0-0	0	76.67	1.89		

Experiment: III **Khissab cv.** **Before 1st treatment** **Duration time:20 min**

sample	1/10	averagex10	1/100	averagex100	1/1000	averagex1000	[(Averx100)+(Averx1000)+(Averx1000)]/3	log	Average	SD
1	105-39	525	30-29	2950	24-19	21500	8325	3.92		
2	24-14	190	53-5	2650	8-6	7000	3280	3.52	3.57	0.32
3	98-62	800	7-28	1400	1-6	3500	1900	3.28		

Experiment: III **Khissab cv.** **After 1st treatment** **Duration time:20 min**

sample	1/10	averagex10	1/100	averagex100	1/1000	averagex1000	[(Averx100)+(Averx1000)+(Averx1000)]/3	log	Average	SD
1	2-3	25	0-0	0	1-0	500	175	2.24		
2	2-4	30	0-0	0	0-0	0	3	0.48	1.51	0.92
3	3-4	35	1-2	150	0-0	0	61.67	1.80		

Experiment: III **Khenezi cv.** **After 2nd treatment** **Duration time:20 min**

sample	1/10	averagex10	1/100	averagex100			[(Averx100)+(Averx1000)]/2	log	Average	SD
1	0-0	0	0-0	0			0.00	0		
2	0-0	0	1-0	50			25.00	140	24.17	0.9
3	5-4	45	1-0	50			47.50	1.68		

Experiment: III **Khissab cv.** **After 2nd treatment** **Duration time:20 min**

sample	1/10	averagex10	1/100	averagex100			[(Averx100)+(Averx1000)]/2	log	Average	SD
1	1-0	5	0-0	0			2.50	0.4		
2	0-0	0	0-0	0			0.00	0	2.5	0.35
3	1-1	10	0-0	0			5.00	0.7		

*SD = Standard deviation

Appendix V: Experiment III - Effect of time on the efficiency of the best combinations obtained from experiment I & II.

Total bacterial count before and after applying the best combinations obtained from experiments I and II during 25 min.

Experiment: III **Khenezi cv.** **Before 1st treatment** **Duration time:25 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	average	SD*
1	136-172	1540	66-81	7350	12-10	11000	6630	3.82		
2	17-40	285	24-10	1700	2-5	3500	1828.33	3.26	3.60	0.30
3	130-146	1380	15-12	1350	12-15	13500	5410	3.73		

Experiment: III **Khenezi cv.** **After 1st treatment** **Duration time:25 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	average	SD
1	1-0	5	0-0	0	0-0	0	1.67	0.22		
2	0-0	0	0-0	0	0-0	0	0	0	0.07	0.13
3	0-0	0	0-0	0	0-0	0	0	0		

Experiment: III **Khissab cv.** **Before 1st treatment** **Duration time:25 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	average	SD
1	137-111	1240	37-13	2500	25-13	19000	7580	3.88		
2	30-17	235	93-118	10550	30-40	35000	15261.67	4.18	4.13	0.22
3	223-0	1115	158-123	14050	39-55	47000	20721.67	4.32		

Experiment: III **Khissab cv.** **After 1st treatment** **Duration time:25 min**

sample	1/10	average*10	1/100	average*100	1/1000	average*1000	[(Aver*100)+(Aver*1000)+(Aver*1000)]/3	log	average	SD
1	1-0	5	1-0	50	0-0	0	18.33	1.26		
2	12-9	105	5-7	600	0-0	0	235	2.37	1.74	0.57
3	6-7	65	1-0	50	0-0	0	38.33	1.58		

Experiment: III **Khenezi cv.** **After 2nd treatment** **Duration time:25 min**

sample	1/10	average*10	1/100	average*100			[(Aver*100)+(Aver*1000)]/2	log	average	SD
1	0-0	0	0-0	0			0	0		
2	0-0	0	0-0	0			0	0	0.00	0
3	0-0	0	0-0	0			0	0		

Experiment: III **Khissab cv.** **After 2nd treatment** **Duration time:25 min**

sample	1/10	average*10	1/100	average*100			[(Aver*100)+(Aver*1000)]/2	log	average	SD
1	4-2	30	0-0	0			15	1.18		
2	3-2	25	0-0	0			12.5	1.1	1.25	0.20
3	2-0	10	1-0	50			30	1.48		

* SD = Standard deviation

Appendix VI: Procedure used for the identification of selected bacteria isolates(Advanced Biotechnology Center / Dubai)

A) Pure bacterial colony picked from the nutrient agar plate and proceeded for DNA extraction

I-DNA extraction protocol using DNA Extraction using QIAGEN DNA mini kit

Steps	Action
1	Pick a full grown colony and suspend in 200 µl of distilled water, vortex and centrifuge for 5 min at 5000 x g (7500 rpm) **Note: Perform Step 1 and 2 in the Bio-Safety Class II cabinet.**
2	Calculate the volume of the pellet or concentrate and add buffer ATL to a total volume of 180µL
3	Add 20 µl of the proteinase K and Incubate at 56°C with shaking until the tissue is completely lysed.
7	Briefly centrifuge the tubes to remove the drops from the inside of the lid.
8	Add the **Internal Control** (IC) at the volume of 0.1 µl per 1 µl elution volume. The IC is added to check for the isolation procedure.
8	Add 200 µL buffer AL to the sample, mix by pulse-vortexing for 15 sec and incubate at 70°C for 10 min. Briefly centrifuge the tubes to remove the drops from the inside of the lid.
	Add 200 µL ethanol to the sample. Mix by pulse-vortexing for 15 sec. Briefly centrifuge the tubes to remove the drops from the inside of the lid.
9	Apply the mixture to the QIAamp spin column without wetting the rim.
10	Close the cap and centrifuge at 6000 x g for 1min. Place the QIAamp spin column in clean 2 ml collection tube and discard the tube containing the filtrate
11	Add 500µl of AW1 buffer. Close the caps and centrifuge at 6000 x g for 1min. Discard the filtrate.
12	Place the column in fresh 2 ml vial. Add 500 µl of AW2 buffer. Close the caps and centrifuge at 20000 x g for 3min. Discard the filtrate.
13	Keep the spin column in the 2 ml collection tube and centrifuge at 20000 x g for 1min. This step will remove the remaining ethanol.
14	Place the spin column in 1.5 ml microcentrifuge tubes and add 200 µl of AE buffer or distilled water. Incubate at room temperature for 1min and centrifuge at 6000 x g for 1min.

II-PCR Amplification of the Extracted DNA

<table>
<tr><th>Steps</th><th colspan="4">Action</th></tr>
<tr><td>1</td><td colspan="4">Prepare the sample sheet to perform the PCR reaction. Perform the PCR in 0.2ml tubes as follows</td></tr>
<tr><td></td><td>If preparing</td><td colspan="3">Then combine the following</td></tr>
<tr><td></td><td>Negative Control</td><td colspan="3">• 25µ l of PCR Master Mix
• 25µ l of sterile deionised water</td></tr>
<tr><td></td><td>Positive control</td><td colspan="3">• 25µ l of PCR Master Mix
• 25µ l of 1 ng/µ l Positive control DNA</td></tr>
<tr><td></td><td>Sample</td><td colspan="3">• 25µ l of PCR Master Mix
• 25µ l of Extracted 1:4 dil. DNA</td></tr>
<tr><td>2</td><td colspan="4">Cap the tubes and placed them in the Thermal cycler and perform the PCR reaction as per the following conditions;</td></tr>
<tr><td></td><td colspan="4"><table>
<tr><th rowspan="2">Initial incubation</th><th colspan="3">Each of 30 cycles</th><th rowspan="2">Final extension</th><th rowspan="2">Final step</th></tr>
<tr><th>Melt</th><th>Anneal</th><th>Extend</th></tr>
<tr><td>Hold</td><td colspan="3">Cycle</td><td>Hold</td><td>Hold</td></tr>
<tr><td>95°C
10 min</td><td>96°C
30 Sec</td><td>60°C
30 sec</td><td>72°C
45 sec</td><td>72°C
10 min</td><td>4°C
forever</td></tr>
</table></td></tr>
<tr><td>3</td><td colspan="4">Check the PCR product using 1.5% Agarose gel electrophoresis with suitable DNA marker.</td></tr>
<tr><td></td><td colspan="4">Note: If not proceeding to cycle sequencing then store the product at-20°C for further use</td></tr>
</table>

III- Analyzing the PCR Products:

Steps	Action
1	Prepare 1.5% of the Agarose in 1X TBE buffer (1.5gm Agarose n 100ml 1X TBE buffer).
2	Dissolve the Agarose using Microwave oven.
3	Cool the gel to 55°C and add 10 ul of ethidium bromide (10mg/ml) & pour the gel in gel forming tray. Put the comb and allow the gel to solidify.
4	After the gel solidifies carefully remove the combs and put the tray in electrophoresis apparatus containing 1X TBE buffer.
5	Mix 5µ l of the PCR products with 3 ul of the gel loading dye and load the samples in appropriate wells.
6	In one of the well load the DNA ladder for locating the product size (mix 5µlof the ladder DNA and 2µl of the gel loading dye).
7	Close the assembly with proper positive and negative terminus and run the gel at 100V for 30-35 min.
8	After electrophoresis put the gel tray on UV transilluminator. Wear eye safety goggles.
9	Locate the 500bp product with the positive and unknown samples. No product should be visible in the negative control.
10	Proceed for the PCR product purification step.
11	**Note: If not proceeding to cycle sequencing then store the product at -20°C for further use**

IV- Purifying PCR product

IV.1. Using Exo I/SAP enzyme treatment

Steps	Action
1	For the remaining PCR product (~35µl) add 2µl of the Exo I (10U/µl) /SAP (1U/µl) enzyme mix.
2	Incubate the tubes at 37^{0}C for 2hrs I the thermal cycler.
3	Incubate at 85^{0}C for 15min to inactivate the enzymes.
4	The PCR product is ready for sequencing.

IV.2. Purifying using Microcon 100 Microconcentrator columns:

Steps	Action
1	Assemble the Microcon-100 column and label the tubes properly as per the protocol.
2	Add 500µl deionized water to the column.
3	Spin the column at 500 x g in fixed angle rotor for 6 min.
4	Add 400µl deionized water to the column.
5	Add the entire PCR product to the column.
6	Spin the column at 500 x g in fixed angle rotor for 15 min.
7	Remove the collection tube and discard it
8	Add 25µl of deionized water to the column and tap briefly.
9	Invert the column and attach it to a new collection tube.
10	Spin the inverted column at 1000 x g in fixed angle rotor for 3 min to collect the sample in the collection vial.
11	Discard the column and proceed to cycle sequencing.
	Note: If not used the product should be kept at -20^{0}C till further use.

V- Cycle sequencing of the 500bp product:

<table>
<tr><th>Steps</th><th>Action</th></tr>
<tr><td>1</td><td>Combine the following in two separate 0.2-mL PCR tube:
7 µL of purified PCR product (Approx 5-20ng)
13 µL of Forward or Reverse Sequencing Mix</td></tr>
<tr><td>2</td><td>Cap the tubes and place them in the thermal cycler, perform the thermal cycling using the following program
<table>
<tr><th colspan="3">Each of 25 cycles</th><th rowspan="2">Final step</th></tr>
<tr><th>Melt</th><th>Anneal</th><th>Extend</th></tr>
<tr><td colspan="3">Cycle</td><td>Hold</td></tr>
<tr><td>96^{0}C
10 Sec</td><td>50^{0}C
5 sec</td><td>60^{0}C
4 min</td><td>4^{0}C
forever</td></tr>
</table></td></tr>
<tr><td>4</td><td>Perform thermal cycling using the parameters above.</td></tr>
<tr><td>5</td><td>Upon completion of thermal cycling, briefly spin the tubes to collect the liquid at the bottom of the tube.</td></tr>
<tr><td>6</td><td>After the program is finished, proceed to “Purifying Extension Products”</td></tr>
</table>

VI- Purifying Extension Products (Precipitation in Microcentrifuge Tubes)

Steps	Action
1	For each sequencing reaction, prepare a 1.5-mL microcentrifuge tube containing the following: • 2.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 • 2.0 µL of 125mM EDTA (If using Big dye v 1.1.) • 50 µL of 95% ethanol (EtOH) **Note: Make sure that the EDTA and Na-Acetate reaches to the bottom of the tube.**
2	Pipette the entire contents of each extension reaction into a tube of Sodium acetate/ethanol mixture. Mix thoroughly.
3	Vortex the tubes and leave at room temperature for 15 minutes to precipitate the extension products. Precipitation times <15 minutes will result in the loss of very short extension products.
4	Spin the tubes in a microcentrifuge for 20 minutes at maximum speed.
5	Discard the supernatant by inverting the tube carefully and soak the last drop of the liquid on tissue paper.
6	Rinse the pellet with 70 µL of 70% ethanol.
7	Spin for 5 minutes in a microcentrifuge at maximum speed. Again, discard the supernatant by inverting the tube carefully and soak the last drop of the liquid on tissue paper.
8	Repeat step 5 and 6 one more time
9	Dry the pellet at room temperature for 30-45 min, till all the alcohol evaporates.
10	Add 15µL of Hi-Di formamide, vortex mix and briefly spin the tubes.
11	Denature the tubes at 95^0C for 2 min and chill at 4^0C for 3-4 min. Spin briefly and pipette the entire volume to the corresponding 96-well plate (if using 3100 Avant) or the 0.5ml sequencing tubes(if using 310 genetic analyzer).
10	Proceed to "Sample Electrophoresis" on 310 or 3100 genetic analyser as per the instruction.

Instructions to switch on 3100 Avant

Steps	Action
1	Switch on the computer.
2	Switch on the machine. **Note: Wait till the green light stabilizes.**
3	On the desktop click on 3100 **Avant data collection** icon. Wait till all the colour on the service console window turns green.
4	Click on gh 3100 Avant icon> Plate manager> New
5	On the new plate dialogue window; ➢ **Name** : Microseq_B500_Expt No._ Date of Exprt. ➢ **Description**: Sample type e.g. blood..... ➢ **Application**: Sequencing analysis ➢ **Plate type** : 96-well ➢ **Owner Name**: source of the lab ➢ **Operator** : Initials of the operating person Click OK the plate view will come in the 96-well format.
6	In the sequence analysis plate editor window: ➢ **Well** Position of the sample A1, B1, C1... etc. ➢ **Sample Name**: Give the name for each sample. ➢ **Result Group**: Seq_Result_Group. ➢ **Instrument Protocol**: give the name of the protocol.
7	Click **OK**
8	Click 3100 avant-1> Run Scheduler>Plate view > Find all
9	Highlight your plate. Click on the plate icon having yellow background.
10	After linking the plate, the colour changes to yellow and the green button at the top left hand corner gets activated.
11	Start the sequencer by click on this green button.
12	For checking the run status, go to 3100 Avant-1, Instrument status. Check the status for current, temperature etc. **Note: After the run is completed the data is automatically save in the data folder i.e. My computer>E drive>Applied Biosystem> UDC>Data collection>Data>Choose file.**
13	After the run completed switch off the data collection software, then the machine and then shut down the computer if not required.

MicroSeq ID Analysis Software Version 1.0

Steps	Action
1	Click on to the MicroSeq v1.0 analysis icon on the desktop.
2	Give the password
3	Select **File>New project** to open the select project setting dialog box in the New Project wizard.
4	Complete the filed in the dialog box: • **Project Name**: Type name for the project. • **Matches to display**: You can choose to display from 3 to 20 matches. • **Kit**: Select the kit used to generate the sample data in the project. • **Analysis Protocol:** Select the analysis protocol to use for the analysis. • **Library available:** Select the libraries to use for the analysis.
5	Click **Next**. The Create Specimen dialog box appears.
6	To navigate the library for the sample; **My computer>E drive>Applied Biosystem> UDC>Data collection>Data>Choose file.**
7	Select the samples to be analyzed: • Click the **New Specimen** • Select the sample file(s) of interest • Click **Add Sample**
8	Click **Next** to view the project setup summary. If you want to make any changes to the project settings, click **Back**.
9	After the confirmation click on **Finish and Analyze**
10	The analysis QC report provides the following information; • **Summary**: display project information and the specimen in the report. • **Specimen Analysis**: Display the specimen name, no. of samples in the specimen, status of the analysis steps, specimen score, the top library match, and the **%** Match. • **Sample Analysis**: Display sample analysis errors and details.
11	To evaluate results in the Analysis QC report Select: **Analysis>Report Manager** then select QC report; • Check the analysis step columns for unsuccessful analysis (yellow or red icons) • Check the values in the specimen score column. Values above 30 indicate high-quality values. • Check values displayed in the **%** match column.
12	To evaluate results in the Library search report Select: **Analysis>Report Manager** then select Library search report; • **Summary**: Display project information and the specimens in the report. • **Library**: Display the libraries searched. • **Hit list**: Displays the library matches found; their % match, the no. of bases searched, and the no. of bases in the consensus sequence that did not matched the library sequence. • **Concise Alignment**: For each specimen with a % Match <100, displays the position each mismatch occurred, the base called in the consensus sequence for that position, and the base present for that position in the library. • **Phylogenetic tree**: For each specimen listed in the hit list, displays a phylogenetic tree diagram.
13	For exporting the result in the *.PDF format Use the following; **File> Export >Result** Give the name

الملخص

تُعتبر زراعة النخيل في دولة الإمارات العربية المتحدة من أهم مصادر الدعم الإقتصادي في المجال الزراعي بالإضافة إلى دورها في المجالين البيئي والإجتماعي. إكثار النخيل بواسطة طريقة الفسائل التقليدية لا تفي لإنتاج الأعداد المطلوبة لإكثار المساحات المزروعة بالنخيل , لذلك تعتبر تقنية إكثار النخيل بواسطة زراعة الأنسجة المصدر الوحيد لتحقيق الدعم المنشود عن طريق إنتاج أعداد هائلة من الشتلات النسيجية. ومن أهم المشكلات التي تواجه تقنية زراعة أنسجة النخيل وخاصة في مرحلة الإدخال هي الإخفاق في الحصول على نباتات خالية من التلوث البكتيري داخل المختبر والذي يؤدي بدوره إلى خسائر فادحة.

لذلك جاءت هذه الدراسة لدراسة هذا الموضوع ولاقتراح حلول فعالة من خلال تطوير عمليات تعقيم خاصة للتقليل من التلوث البكتيري قبل البدء في عملية إكثار النخيل, كما تهدف الدراسة إلى التعرف على الأنواع البكتيرية المصاحبة لأنسجة النبات.

ومن أجل تحقيق الأهداف المبتغاة, استخدمت تراكيز مختلفة من هيبوكلوريد الصوديوم وبرمنجنات البوتاسيوم بالإضافة إلى مبيد الفطريات (Aliette) في عملية التعقيم خلال فترات زمنية مختلفة. وتبين من خلال التجربة أن 40 % من هيبوكلوريد الصوديوم مضافاً إليه 3 غ / لتر مبيد الفطريات, و 30 % من هيبوكلوريد الصوديوم مضافاً إليه 400 ملغ / لتر من برمنجنات البوتاسيوم, تعتبر من أفضل التراكيز المستعملة في مرحلتين متتاليتين للتقليل من الأعداد البكتيرية في النخيل. وقد تم التعرف على أكثر الأنواع البكتيرية انتشارا وكانت كالتالي: *Cellulomonas cellulans*, *Enterobacter aerogenes* *Bacillus fusiformis*, *Serratia marcescens*, *Kocuria rosea* and *Cellulomonas uda*.

جامعة الإمارات العربية المتحدة
عمادة الدراسات العليا
برنامج ماجستير علوم البيئة

عنوان الرسالة

زراعة أنسجة نخيل التمر: أنواع التلوث وتحسين طريقة التعقيم

رسالة مقدمة من الطالبة

موزه ناصر الشامسي

إلى

جامعة الإمارات العربية المتحدة
إستكمالاً لمتطلبات الحصول على درجة الماجستير في علوم البيئة

2006-2005