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In vitro propagation of Royal Irises (section = *Oncocyclus*) in Palestine

By

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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In vitro propagation of Royal Irises (section = *Oncocyclus*) in Palestine

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Abstract

Palestine is a region rich in plant biodiversity. Despite that, several species are threatened due to various factors including overgrazing, human activities and intensive agriculture. As a part of the species, *Iris* species from section *Oncocyclus* are considered threatened species according to the IUCN red list. As a tool to protect and conserve these species, a protocol of *in vitro* propagation of Palestinian Royal Irises is developed. This study contains four phases: first, selection of suitable explants and establishment of irises from the embryos on an aseptic culture. Second, in *vitro* shoot proliferation and regeneration, followed by *in vitro* shoot multiplication. Third, *in vitro* rooting. Finally, hardening of plantlets in the greenhouse gradually. The highest embryo germination percentage was 100% on a half-strength MS medium without PGRs. The best shoot regeneration was obtained on half-strength MS medium supplemented with 0.5 mg/L or 1.0 mg/L of BA while the best rooting results were on half-strength MS medium supplemented with 0.5 mg/L IBA. Plantlets were acclimatized in peatmoss:sand (2:1) substrate with a survival rate of 75% - 100% after 4 weeks from transfer to pots. This method was efficient in overcoming seed dormancy in irises.

الملخص

اكثار نبات السوسن الفلسطيني (السوسن البني الداكن وسوسنة فقوعة) عن طريق زراعة الأنسجة.

فلسطين هي منطقة غنية بالموارد الطبيعية وبها تنوع بيولوجي عالي ولكن هناك العديد من النباتات المهددة بالانقراض في هذه المنطقة الجغرافية بسبب عدة عوامل منها الرعي الجائر والزراعة المكثفة و إزالة الغابات وأيضا عدم الوعي بندرة هذه النباتات. كل هذه العوامل تؤثر سلباً على أعداد النباتات المتواجدة في فلسطين والتي تتناقص باستمرار. من النباتات المهددة بالانقراض ، نبات السوسن الفلسطيني والذي ينتمي لفصيلة السوسنية ومن المهم إيجاد طريقة لاكثار نبات السوسن الفلسطيني بطريقة غير تقليدية وذلك بهدف إنتاج أكبر كمية ممكنة من نبات السوسن والمحافظة على أعدادها في الطبيعة. هذه الدراسة تهدف إلى اكثار نبات السوسن الفلسطيني عن طريقة زراعة الأنسجة للحفاظ على النبات ومادته الوراثية وتمت دراسة جنسين من الفصيلة السوسنية : السوسن البني الداكن *Iris atrofusca* وسوسن فقوعة *Iris hayeni* . في هذا البحث تم تأسيس بروتوكول اكثار يتضمن 4 مراحل، أولا: اختيار أجزاء النبات المناسبة للاكثار وتهيئة النبات لزراعة الأنسجة في ظروف معقمة. ثانيا، انبات و تكثير نبات السوسن عن طريق زراعة الأنوية . ثالثا، تجذير النبات ورابعا ، نقل النبات إلى البيت البلاستيكي لإنتاج أشتال. أعلى نسبة انبات للأنوية تمت على الوسط الغذائيMS تحتوي 0.5 ملغم\لتر بدون اضافة أي هرمونات نباتية طبيعية او صناعية. أعلى نسبة إنبات للأوراق الجديدة والتفرعات كانت على الوسط الغذائيMS والمدعم ب0.5 ملغم\لتر -1.0 ملغم\لتر من BA بينما أعلى نسبة تجذير كانت على الوسط الغذائيMS والمدعم ب 0.5 ملغم\لتر منIBA، تم نقل النباتات الى البيت البلاستيكي وكانت نسبة نجاة نبات السوسن تتراوح من 75%-100% وذلك بعد 4 أسابيع من النقل. هذا البروتوكول نجح في الحفاظ على نبات السوسن واكثاره وانتاج أشتال جديدة من هذا النبات, بالاضافة الى حل مشكلة سبات البذور التي تواجه نبات السوسن.

Declaration

I declare that the master thesis titled "***In vitro* propagation of Royal Irises (section = *Oncocyclus*) in Palestine.**" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgement is made in the text.

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Dedication

I dedicate this thesis to my family especially my dad, mum, father in law and mother in law for their encouragement, love and prayers.

I would like to express my gratitude to my husband Ribhi who was very supportive and encouraging. Thank you for being there for me. A special thanks to my son Mohammad and my baby girl Salma; you are a blessing in my life.

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Thanks to my friends and colleagues in the program for their friendship and kindness.

Abbreviations

Abbreviation	Word or sentence
ANOVA	Analysis of variance
et al.,	And others
BA	benzyl adenine
°C	Degree centigrade Celsius
DW	Distilled water
B5	Gamborg medium
g	Gram
IBA	indole-3-butyric acid
Kn	Kinetin
LSD	Least significant difference
L	Litre
mg	Milligram
ml	Millilitre
Min	Minute
M	Molar
MS	Murashige and Skoog medium
NAA	naphthalene acetic acid
NN	Nitsch and Nitsch medium
PGR	Plant growth regulator
Ph	Potential hydrogen
%	Percent
QL	Quoirin & lepoivre medium QL

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Chapter one

Introduction

Palestine as part of the Eastern Mediterranean region is a rich plant diversity and speciation spot at a global scale. This is due to its geographical position and diverse climate. It is an area with around 2750 species of plants from 138 families distributed across five different eco-geographical regions (Alkowni and Sawalha, 2012; Danin, 2004). The International Union for Conservation of Nature (IUCN) red list of endangered species updated the 'red list' of Palestine with 102 species, which indicates a high risk to the Palestinian threatened wild flora were 39.2% critically endangered, 3.72% endangered and 19.6% of species are vulnerable (Ali-shtayeh and Jamous, 2018). Moreover, the red list's species are facing threats in their natural habitats including expansion of urban areas, over-harvesting of wild plants and climatic and environmental changes (ARIJ, 2007).

The Royal Iris in Palestine is one of the threatened species particularly *Iris atrofusca* which is considered near threatened and *Iris haynei* is vulnerable according to IUCN. Many projects were focused to protect the royal irises, arising conservation efforts to maintain and increase populations of the threatened species such as: *in situ* conservation, *ex situ* conservation (Volis et al., 2008), seed banking (Barazani et al., 2016) and micropropagation.

There are several approaches to conserve endangered wild plants in Palestine, based on increasing their population annually. Some biotechnological methods have been implemented to conserve the royal irises like micropropagation which is an effective tool for rapid plant multiplication (Jevremović et al., 2013). On the other hand, plants can be conserved *in vitro* through short term preservation, medium-term preservation, cryopreservation and there are different techniques depending on the purpose and the plant species (Coelho et al., 2020). Other techniques include seed banks (Barazani et al., 2016) and *in situ* conservation and *ex-situ* conservation of irises (Volis et al., 2008). At present, current conservation actions include; protecting species by law in some regions and it is included in the red data book for Israeli endangered plants and added to the IUCN red list of threatened Palestinian wild flora (IUCN, 2016). Moreover, irises recently have been introduced to botanical gardens and their habitats are protected in some places in Palestine.

Royal irises in Palestine

The family *Iridaceae* is an ornamental plant family in the Order Asparagales that consists of 60 genera and 800 species. *Iris* is the largest genus with the richest taxonomic diversity that represents six subgenera, 12 sections and 301 species (Okba et al., 2020). It is a genus of flowering plants that comprises 280 species and is distributed across the temperate northern hemisphere regions. Section *Oncocyclus* is found generally in small populations or are scattered locally across rocky hillsides, steppes and deserts. This section includes rhizomatous irises which are short and knobby. In addition, species of section *Oncocyclus* produce one single flower on the flower stalk (Wilson et al., 2016). (Figure 1.1)

Figure 1.1. Royal Irises in Palestine. (A) Represents *Iris atrofusca* species. (Sapir, 2016a). (B) Represents the *Iris haynei* species (Sapir, 2016b). (C) Represents rhizomes of *Iris atrofusca* (Sapir, 2016a).

This study focused on two closely related species of irises; *Iris atrofusca* and *Iris haynei*. The population of irises in the West Bank south of Jiftlik/Wadi Farah are considered *Iris atrofusca* and *Iris hayeni* are on the north of this region confirmed from genetic and morphological data (Arafeh et al., 2002) (figure 1.2).

Iris atrofusca is a perennial plant can be found in clones and grows in large patches of leaf fans that are connected to the rhizome (Volis et al., 2015). Plants flower from late February to early April (Avishai and Zohary, 1980). This plant is self-incompatible and is pollinated by solitary male bees (Sapir et al., 2005). In May, the plant produces seeds that are held in a capsule, each mature capsule holds up to 50 seeds. After the end of the vegetative season plants are dormant which means leaves are dry-out but rhizomes can stay dormant during summer until the next fall – to- spring growing season as observed by S. Volis et al (Volis et al., 2010). Seeds are mature when the capsule is brown and dry then splits from all sides (Samarah et al., 2009). (Figure 1.2)

Figure 1.2. Iris seeds in the field. (A) Dried seeds from mature capsule. (B) Opened capsule of mature iris. (C) Immature iris capsules. (Atrash, 2021)

All Royal Irises in Palestine are considered threatened and listed in the red list (Volis et al., 2015). According to the IUCN, *Iris atrofusca is considered* near threatened while *Iris haynei* is vulnerable. These species are highly vulnerable and their population size is decreasing continuously which makes these species more vulnerable. Some of the problems are urbanization, extensive agriculture, overgrazing, deforestation, Bedouin settlements, building infrastructure (Volis et al., 2010). Additionally, lack of awareness and being unprotected made these species' situation even worse.

The overall population size is decreasing. The population size of *Iris atrofusca* in the northern Negev and Rammoun is relatively larger, whereas the southern West bank has small subpopulations in Yatta and Bani-Naim (Sapir, 2016a). This population decline started in 2006 and declines in the Negev reached 50% while the West Bank population declines by 30% in the last ten years (IUCN, 2016; Sapir, 2016a). On the other hand, *Iris haynei* population size declined from thousands to tens of plants (IUCN, 2016; Sapir, 2016b). The reason behind this collapse is not clear whether it is due to overgrazing, pests or human activities but its population size will decline by 30% in the next 100 years due to habitat loss and ongoing development (Sapir, 2016b).

Seed dormancy and germination of *Oncocyclus* iris seeds

The majority of higher plants exhibit some degree of seed dormancy. From 50% to 90% of wild plant species worldwide produce dormant seeds with various dormancy traits driven by species' occurrence geography, genetic factors and growth form (Kildisheva et al., 2020). Most seeds consist of an embryo surrounded by covering layers. The covering layers has one to several layers that have a living endosperm, and testa which is a dead tissue (Hilhorst et al., 2010).

The timing of germination is influenced by the environmental conditions that occur during seed maturation and after seed dispersal (Carta et al., 2016) and it also affects the survival of seedlings (Donohue et al., 2010).

Five main classes of seed dormancy and can be divided into sublevels. 1) Physiological dormancy (PD), which is the most common form of seed dormancy in which embryos are fully developed but has low growth potential due to the presence of germination inhibitors. 2) Physical dormancy (PY) where the fruit or seed coat is water- impermeable. 3) Combinational dormancy (PY + PD) where the seed coat is water-impermeable and seed embryos are physiologically dormant. 4) Morphological dormancy (MD) where seeds readily imbibe water and embryos are underdeveloped but differentiated, it requires time to grow before germination. 5) Morph-physiological dormancy (MPD) seeds imbibe water but embryos are underdeveloped and/or undifferentiated and physiologically dormant (Baskin and Baskin, 2014; Finch-Savage and Leubner-Metzger, 2006).

Fresh seeds with fully developed embryos and a water-permeable seed coat could be non-dormant or have physiological dormancy (PD), while underdeveloped embryos which must have a period to embryo growth before germination could have morphological dormancy (MD) or morpho-physiological dormancy (MPD) (Fu et al., 2013; Martin, 1946). The seeds of the *Iridaceae* family don not have water-impermeable seed coats, thus they cannot have combinational dormancy (PY + PD) or physical dormancy (PY) (Baskin et al., 2000). To break seed dormancy, if seeds have MD, embryos growth would happen within a short period when the seeds are incubated suitable environmental conditions including controlled light and dark periods, moisture and temperature (Fu et al., 2013). Meanwhile, if seeds have MPD, the exposure to warm/cold temperatures would break physiological dormancy and induce embryo growth and germination (Fu et al., 2013).

Seeds germination depends on several factors that affect the germinating process such as how seeds respond to the biotic and abiotic environment along with the physical and physiological characteristics of seeds (Volis and Dorman, 2019). Generally, seeds can stay viable in soil for several years due to seed dormancy which prevents germination when environmental conditions are not favourable for seedling survival (Fenner and Thompson, 2005). To break seed dormancy, a point in time is determined when changes in the balance of endogenous hormones that prevent germination might happen or mechanical changes in the seed coat that will break seed dormancy, or changes in the rate of embryo development (Baskin and Baskin, 1998; Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). In conservation projects, it is critical to be able to predict the longevity of the seeds in the soil and the supporting environmental conditions that break dormancy and germination because poor regeneration with no emerging seedlings is common in these threatened species (Volis, 2019).

In general, seeds of *Iridaceae* e have linear embryos that could be as long as the seed (figure 1.3). *Iris* species have seeds with a very hard seed coat that develop during early summer which is the period when dispersed seeds shrink and dries (Avishai, 1977). These seeds possess strong dormancy and a slow rate of germination in their natural condition. In the first year only 30% of seeds will germinate (Shimshi, 1967). According to other studies, up to 15% of seeds germinate in the first year, the rest of the seeds will germinate in the next 5-6 years, this happens because of iris species habitat that is in semi-arid or arid climate (Avishai, 1977).

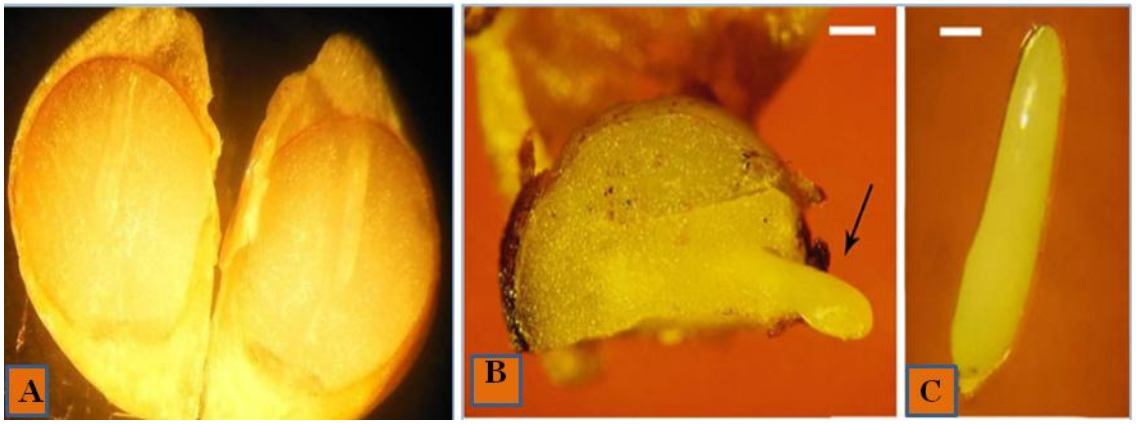

Figure 1.3. (A) A longitudinal section of *Iris sibirica* seed (Tikhomirova, 2020). (b) Isolation of zygotic embryos of *Iris reichenbachii* (arrow). (c) Isolated zygotic embryo of *Iris pumila* placed on agar medium (Jevremović et al., 2013).

Several studies showed that the germination rate of *Oncocyclus* irises is species-specific (Blumenthal et al., 1986). For example, *I. lortetii* germinated seeds is 1.0% in the first year whereas *I. atropurpurea* germinated seeds is 60% in the first year (Volis and Dorman, 2019). Moreover, extracts from the outer integument of the seed coat is toxic for germinating embryo thus, the main cause of deep dormancy in *Oncocyclus* is a high mechanical resistance of the seed coat at the micropylar area (Blumenthal et al., 1986). Two factors can be responsible for deep dormancy and slow rate of seed germination; water impermeability of the seed coat and the accumulation of inhibiting substances in the seed coat that prevents germinating during or shortly after seed maturation (Volis and Dorman, 2019).

In order to germinate iris seeds , three methods were proposed; first, *in vitro* germination of embryos that include the use of fresh seeds, removal of its seed coat and *in vitro* culture on a suitable growth medium (Shimshi, 1967). The second method was proposed by Dorman et al, (2009), *in vitro* and *in vivo* seed germination after scarification (any process that involves weakening and altering the seed coat to encourage germination). Mechanical scarification on *in vitro* seed germination which broke the innate dormancy germinated successfully three *Oncocyclus* iris species, and *in vivo* germination by controlling soil type, shading and amount of water but this method despite sterilization was affected by fungal contamination which reduced the production of seedlings (Dorman et al., 2009). The third method was proposed by Volis and Dorman (2019) in which seeds should be sowed 3.0 cm deep in their natural soil with supplementary watering will increase seed germination not only in the first year but also the second and third year (Volis and Dorman, 2019).

1.1. *In vitro* propagation

In vitro propagation is a term used to describe vegetative propagation of plants through an aseptic culture of plant part (explant) such as shoot tips, root tips, leaf tissues, anthers, nodes, embryos and meristems which result in producing a whole plant. Micropropagation is achieved on artificial growth mediums which contains nutrients to support the living tissues under controlled environmental conditions such as humidity, photoperiod, temperature and light (Bridgen et al., 2018). In addition, micropropagation must be conducted under sterile conditions to ensure having healthy plantlets free from bacterial, fungal and viral infection. This technique has various advantages including the production of plants in large quantities,

producing genetically identical plants, producing plants within a short time, the ability to generate new genetically modified plants, cloning of endangered species safely and finally, the ability to produce pathogen, disease and pest free plants (Pierik, 1987; Yildiz, 2012). On the other hand, this method has many disadvantages such; it requires trained staff, sophisticated faculties and expensive materials. Moreover there are some challenges as contamination, some plants are difficult to maintain by tissue culture while other plants produce inhibitory substances that make culture media become brown.

The five stages of micropropagation are; stock plant selection, the establishment of aseptic culture, multiplication or shoot proliferation, *in vitro* rooting and finally transplanting to *ex vitro* conditions and acclimatization or hardening (Kyte, 2013).

As for explants selection, donor plants must be healthy, disease-free, pest-free and infection-free as mentioned previously. The plant material plays an important role in tissue culture success. Several factors affect the regeneration capacity of the explants including the explants size, age, genotype, source, density and physiological stage. All these factors affect the response of the explants to tissue culture (Yildiz, 2012).

In vitro propagation of royal irises

Royal irises are rhizomatous irises that have been mentioned in several studies (Gilmour, 2006). Propagation by the splitting of rhizomes is used but slow and time-consuming procedure since this method gives maximum ten plants per year (Jéhan et al., 1994). Therefore, micropropagation is considered a good alternative for mass propagation in this case (Hussey, 1975). In recent years, several studies have reported the *in vitro* propagation of *Iris* species such as *I. germanica, I. nigricans, I. pumila, I. atrofusca, I. petrana, I. vartanii* (Al-Gabbiesh et al., 2006; Jevremovic and Radojevic, 2002; Shibli and Ajlouni, 2000) from a different range of explants.

De Munk et al. concluded that for shoot regeneration of rhizomatous irises, full or half-strength MS medium supplemented with 2-4% of sucrose can be used (Munk and Schipper, 1993), another study suggested modified LS medium (Linsmaier and Skoog Medium) can be used for rhizomatous irises (Meyer, 1984). Comparatively, NAA was proposed as a necessary

component for optimal shoot regeneration at 0.03-2.0 mg/L) or supplement medium with equal amounts of NAA and BAP or Kinetin (0.5-1.0 mg/L) (Hussey, 1975).

On the other hand, embryo culture is one of the earliest techniques used in tissue culture; it depends on isolating the embryo without injury followed by embryogenic growth and seedlings formation (Bridgen et al., 2018). This tool is difficult because it needs specific growth mediums and tedious dissection. Moreover, this tool's success depends on the developmental stage of the embryo when it is isolated (Bridgen, 1994).

- **Embryo culture of irises.**

The embryo culture of iris is one of the earliest techniques for growing irises. In 1936, Werckmeister reported growing malformed embryos from mature capsules in certain iris crosses (Werckmeister, 1936). Moreover, some scientists have used the embryo culture method for growing mature iris embryos; to overcome the slow germination that occurs in irises (Dahl, 1949; Randolph, 1945). In 1954, a study was conducted on iris embryos to determine how early the embryos can be removed from the seeds and grown on nutrient agar successfully, this study indicated that a growth factor or factors may be necessary for young embryos to grow *in vitro* successfully (Lenz, 1954). Another study of iris embryo culture that was cultured on Randolph's nutrient agar (Appendix 1 Table 3) and kept at room temperature 25°C, which concluded that most of the embryos that are going to germinate will take 1-2 weeks and once germinated the seedlings, will grow gradually and slowly (figure 1.4) (Lenz, 1955).

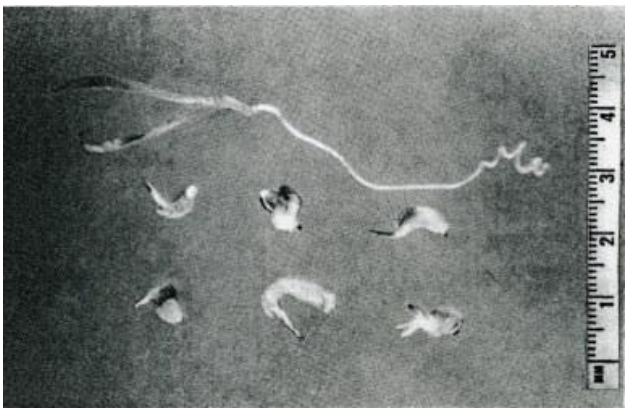

Figure 1.4. Embryos and seedling of Iris hybrid (*I.hoogiana* 'Bronze Beauty' X *I. stolonifera*) at the end of three months at room temperature (Lenz, 1955).

- **Establishment of Iris species.** The collected plant material usually has microorganisms that are considered contaminated. These microorganisms can affect the success of plant tissue culture. So, first, the plant material must have a decontamination process without being harmful to the living tissues. Some procedures could be harmful and damage the surface of plant material which affect negatively on *in vitro* micropropagation. Second, the choice of the starting material plays an important role depending on the purpose of tissue culture (Bridgen et al., 2018). The starting material could be anthers, leaves, roots, pollen, shoot apices, embryos, rhizomes, small bulblets, leaf base and rhizomes apices and other parts (Gilmour, 2006). The common surface sterilization procedure is by washing the explants under running water followed by sterilization with a bleach solution or sodium hypochlorite bleach. Using this method the plant shall be contamination-free and ready for tissue culture. To ensure that, explants are cultured in a growing medium for 3-5 days to observe contamination to guarantee tissue culture success (Bridgen et al., 2018). The rhizomatous explants have a high degree of contamination and require sterilization of the vegetative buds, even in successful sterilization process contamination can be manifested in all stages of micropropagation (Tikhomirova, 2020). If the rhizome tissue had the presence of the bacterium after (7-10) days that indicates internal contamination therefore the sterilization method is inefficient (Tikhomirova, 2020).

- **Shoot proliferation and regeneration.** This stage of micropropagation aims to multiplicate plantlets and produce a mass amount of plantlets by repeat culturing and by supplementing the growth medium to cytokinin. The growth medium composition plays a huge role in tissue culture success depending on the purpose of culture and the species**.** Moreover, cytokinin levels alter plant multiplication and regeneration (Millam, 2005), many cytokinins can be used in micropropagation including TDZ (thidiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea) kinetin, zeatin, BA (6-Benzylaminopurine), 2-ip (6-(γ,γ-Dimethylallylamino) purine and others. The response of cytokinins differs from species to species and concentration must be experimented with to determine the optimum growth regulator and its concentration regarding the species (Bridgen et al., 2018). Additionally, the ratio between cytokinin and auxin determines the fate of regeneration of explants (Skoog and Miller, 1957), high cytokinin to auxin ratio stimulates shoot regeneration while high auxin to cytokinin induces root formation and regeneration. Generally, cytokinins promote cell division and induce axillary shoot proliferation and shoot formation (Altaf Hussain, 2012). The kinetin commonly used to induce shoot regeneration in plant tissues and promotes organogenesis from immature explants (Çöçü et al., 2004; Uranbey, 2011).

- ***In vitro* rooting of the regenerated shoots.** The rooting stage is pre-transplanting the shoots that have been regenerated and multiplied *in vitro*, then followed with auxin treatment to induce rooting of the explants. Several auxins can be used to induce rooting including the auxins NAA, IBA and IAA. Some plants tend to have problems with root initiation which make them a choice for *in vitro* micropropagation. Thus, auxin induces rooting *in vitro* to ensure speeding the rooting phase and having a higher survival rate of transplants (Bridgen et al., 2018). NAA and IBA are commonly used to stimulate root growth when taking cuttings from plants. Moreover, auxin positively influences cell enlargement, bud formation and root initiation (Agboola et al., 2014).

- **Acclimatization.** The final stage of micropropagation is acclimatization and transplanting. The main purpose of this stage is to harden plantlets. To illustrate, transplanting is obtained through moving plantlets gradually from *in vitro* growing conditions to the greenhouse unit where the environment is considered harsh compared to the tissue culture unit.

The plantlets should be kept in a high humidity area for few days before transplanting to the greenhouse (George et al., 2008). Several factors contribute to the acclimatization process success: first, water loss and moving plantlets from *in vitro* culture where high humidity to *ex vitro* conditions where humidity is low. Second, the effect of light exposure and difference in light intensity from *in vitro* artificial light to *ex vitro* sunlight would cause stress in the plantlets and a shock. If the plantlets were kept in an area that contains shades and high humidity, this will decrease stress and gradually plantlets will be able to maintain living in the greenhouse.

1.2. Effect of Basal salts in tissue culture

To establish a new protocol in tissue culture, experiments must be conducted using various basal salts to determine the suitable medium for the species or purpose of tissue culture. For some species, the full strength of basal salts is more effective than other concentrations but other species show better growth results *in vitro* when the strength of salts is reduced (Abobkar and Ahmed, 2012; Altaf Hussain, 2012). According to the nutrient composition of basal salts, optimal growth, shoot regeneration, plant proliferation and morphogenesis of tissues may vary**.** for example, Murashige and Skoog MS media is commonly used in tissue culture and it contains desired salt concentration (Murashige and Skoog, 1962), while Gamborg B5 media is used for cell suspension or callus culture and contain a greater proportion of Ammonium and Nitrate ions(Gamborg et al., 1968). The Nitsch and Nitsch NN media is commonly used for another culture and contains low salt concentration (Nitsch and Nitsch, 1969).

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2. Problem statement and objectives

2.1 Problem statement

Royal iris is an endangered species and its seeds have morphological dormancy and morpho-physiological dormancy that hinder seed germination, which reduce population size of plants in their natural habitat. Moreover, the propagation of those plants in fields is slow, limited and time-consuming, so *in vitro* propagation of plants offers a promising alternative for rapid propagation and break dormancy of seeds in irises.

2.2 Main objective

The main objective of this study is to outline an appropriate protocol for *in vitro* propagation of the endangered *Iris atrofusca* and *Iris haynei* (section *Oncocyclus* = the royal irises) in Palestine to conserve irises from seeds.

Specific objectives:

A. To evaluate proper starting material (explants) for in vitro establishment.

B. To determine suitable cytokinin PGRs for shoot proliferation.

C. To determine suitable auxin PGRs for royal irises *in vitro* rooting.

D. To select a listing of proper conditions for plant hardening and acclimatization in the greenhouse to make sure having healthy plants are able to grow in fields.

Chapter two

Materials and methods

This study was conducted in the plant tissue culture laboratory in the Palestine-Korea Biotechnology Center at Palestine Polytechnic University, Hebron, Palestine.

2.1 Chemicals and reagents used in micropropagation of royal irises

All plant basal salt media, plant growth regulators (PGRs) and chemical reagents which were used in this study were purchased from Sigma-Aldrich chemical company. (Appendix 1, Table 1).

2.2 Media preparation and plant growth regulators

Plant growth regulators Kinetin (KN), 6-Benzylaminopurine (BA), Indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) were prepared as a concentrated stock solution at 1.0 mg/ml concentration. Half strength Murashige and Skoog (MS) media was used for *in vitro* embryo germination experiments. To prepare one litre of full strength MS, 30 g/l of sucrose and 4.408 g/l of MS media were dissolved in 900 ml of distilled water. Depending on each experiment's purpose, plant growth regulators were added. Medium then was brought to the final volume and pH was adjusted to 5.8 with 1.0M KOH or 1.0M HCl. Finally, to solidify the media 7.0 g/l of agar was added. Media was poured into bottles closed with screw caps and autoclaved at 121°C for 20 min and then 25 ml were poured into sterile Petri dishes of 9.0 cm. As for shoot regeneration experiments, media was poured into jars closed with aluminium foil and autoclaved at 121°C and 15psi (= 104 kPs) for 20 min. Detailed media components are listed in appendix 1 table 1.

2.3 Plant material

Plant material was collected from different areas in the West Bank, Palestine (Figure 2.1). Samples from Rashaydeh, Bani-Naim, Faqoua and Rammoun were collected from mature non-dry capsules; seeds are oval-shaped with an average diameter equal to 0.5 cm. Below table 2.1 shows geographical coordinates for the localities from which the plants were collected.

Table 2.1. Localities, the geographical coordinates and explants type of collected iris samples from the West Bank.

Area	Latitude (N)	Longitude (E)	Explant type	Collecting date
Faqoua'	31°30'55.6	35°09'54.8	Seed	2018
Bani-Naim	31°30'36.0	35°10'12.0	Seed	2018
Rashaydeh	31° 45′ 11	35° 20′ 51	Seed	2019
Ramon	31°55'43.4	35°17'58.4	Nodes from underground rhizomes	2019
Ramon	31°55'43.4	35°17'58.4	Seed	2020

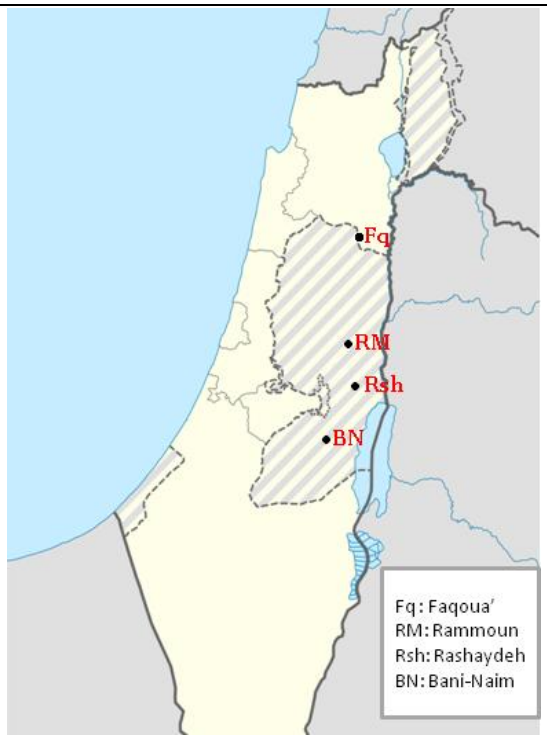


Figure.2.1. Location of the samples of iris population in this study.

2.4 Explants collection and surface sterilization

❖ **Establishment of mother stock material:**

- **Whole seeds:** immature capsules were collected and stored at 4°C for two days. Seeds were sterilized in the laminar flow hood with 70% ethanol, they were immersed in distilled water and 20% chlorine solution for 15 min, after that capsules were washed twice with sterilized distilled water. Capsules were immersed again in 70% ethanol for 5 minutes and finally washed twice with distilled water. Following that, capsules were opened with a blade. Although seeds were already sterilized since they were in a closed capsule, an additional sterilization step with 70% ethanol solution was obtained and seeds were rinsed with distilled water (figure 2.2). In this experiment, number of seeds from faqoua population was 40 seed whereas Rashaydeh population was 52 seeds.

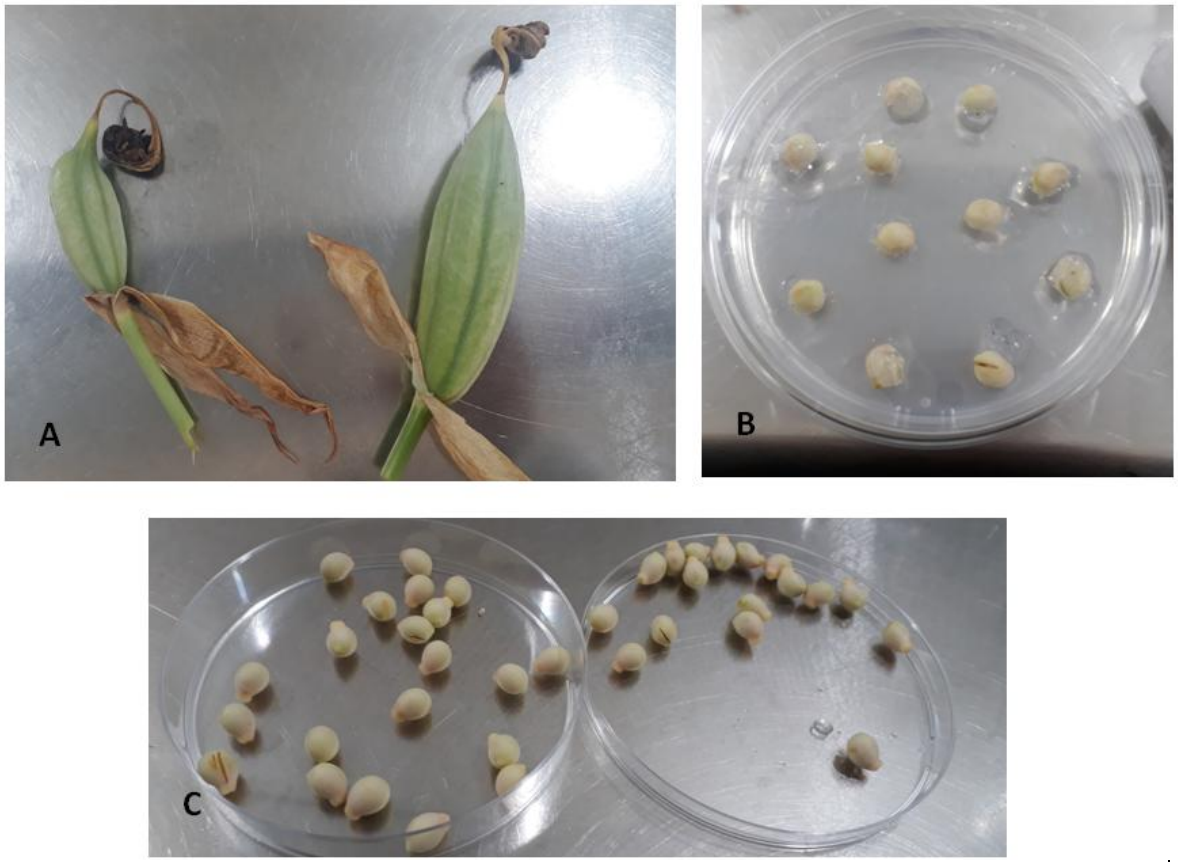


Figure 2.2. Collected iris seeds from immature capsules. (A) Represents immature capsules of irises. (B) Cultured seeds on MS medium. (C) Seeds were obtained from one capsule.

- **Excised embryos:** Under the laminar flow cabinet, the seed coat was cut longitudinally to extract the embryo; seeds were pressed gently from the backside of the seed to push the embryo out. Collected embryos were cultured on PGR-free, half-strength MS medium (figure 2.3). Petri dishes were closed with parafilm tape and incubated at 25°C for 4 weeks. After 4 weeks, germinated embryos were transferred into a regeneration medium. Germination percentage was recorded after 4 weeks of culture. In this experiment, the number of embryos from Rammoun population was 30 embryo whereas the number of embryos from Faqoua population was 25 embryo.

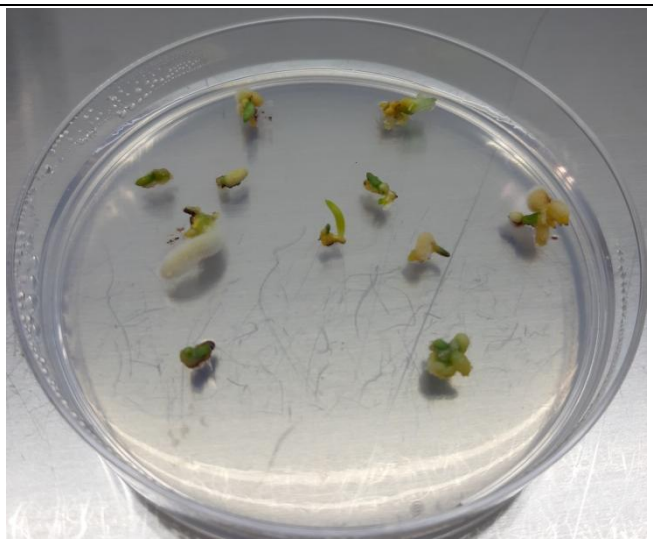


Figure 2.3. Excised iris embryos from Faqoua' population.

- **Vegetative nodes:** plant seedlings were collected from Rammoun fields; plant was cleaned and washed with tap water to remove soil from plant leaves and rhizomes, then sterilized with 70% ethanol followed with 20% chlorine wash. As a final step, plants were sterilized with 70% ethanol. Under the laminar flow cabinet, the plants were cut, a 3.0 cm nodes were taken as explants from underground rhizomes. Collected explants were cultured on PGR-free, Half-strength MS medium and incubated at 25°C for 4 weeks. (Figure 2.4)

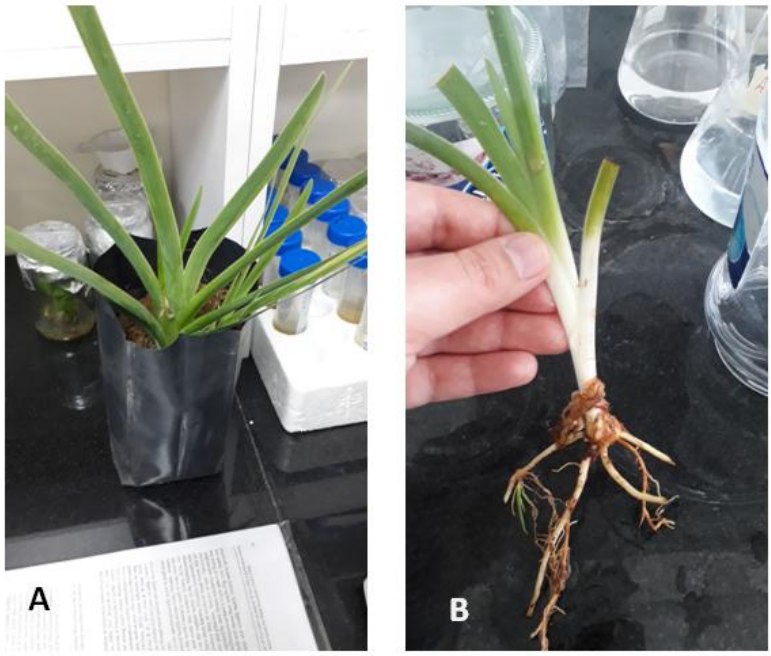


Figure 2.4. Iris rhizome from Rammoun population. (A) Represents plants from the field. (B) Cleaned and washed plantlets.

2.5 Micropropagation

2.5.1 Effect of basal salts on *in vitro* germinated embryos

Four different basal media were used to test shoot growth and proliferation of the germinated embryos. Full strength MS, QL, B5 and NN media, all were PGRs-free were used. Each treatment had 5 replicates. Explants were transferred from Faqoua' germinated embryos. After that, cultured embryos were incubated at the growth room where temperature = 23° C temperature and light regime of 16 hour light: 8 hours dark ; data collected from this experiment was the number of shoots and the number of roots after two, four and six weeks.

2.6.2 Effect of PGR-free MS media on shoot regeneration

The effect of the half-strength MS medium was tested on shoot regeneration of royal irises, mainly on Faqoua' and Rammoun populations. The experiment was repeated four times from Aug-Nov 2020. Explants were taken from germinated embryos that were cultured previously. Explants then were cultured on half-strength MS medium and kept in the growth room at 23°C. After 4 weeks, the following data were collected: number of shoot multiplication, shoot length, number of shoots, number of root, root length and root diameter.

2.6.3 Effect of Cytokinin concentrations on shoot regeneration and multiplication.

Half strength MS medium was supplemented with two types of cytokinin PGRs at three concentrations each. BA and KN were used at 0.25 mg/L, 0.5 mg/L and 1.0 mg/L resulting in 6 treatments. (Table 2.2) Each treatment is composed of 10 replicates. The starting material of shoots was cultured previously on PGR-free, half-strength MS media. Each shoot explant was 3.0 cm long and cultured in jars. Data were collected as the number of shoots, shoots multiplications, and the number of roots after 4 weeks and 8 weeks.

Table 2.2. Cytokinin Trial's symbol and concentration

Symbol	Media type	Cytokinin	Concentration
A	1/2 MS	BA	0.25 mg/L
B	1/2 MS	BA	0.5 mg/L
C	1/2 MS	BA	1.0 mg/L
D	1/2 MS	KN	0.25 mg/L
E	1/2 MS	KN	0.5 mg/L
F	1/2 MS	KN	1.0 mg/L

2.7 *In vitro* rooting

Half strength MS medium was supplemented with two auxins PGRs with three concentrations each. NAA and IBA were used at 0.25, 0.5 and 1.0 mg/L resulting in 6 treatments (Table 2.3). Each treatment is composed of 10 replicates. Shoot explants were obtained from half-strength MS cultures. Each shoot explant was 3 cm long and cultured in Petri dishes. Data was collected as the number of roots after 4 weeks and 8 weeks.

Table 2.3. Auxin Trial's symbol and concentration

Symbol	Media type	Auxin	Concentration
A'	1/2 MS	NAA	0.25 mg/L
B'	1/2 MS	NAA	0.5 mg/L
C'	1/2 MS	NAA	1.0 mg/L
D'	1/2 MS	IBA	0.25 mg/L
E'	1/2 MS	IBA	0.5 mg/L
F'	1/2 MS	IBA	1.0 mg/L

2.8 Acclimatization and hardening of royal irises.

To harden rooted shoots, explants were transferred to peatmoss and sand mixture (2:1) and planted in the greenhouse, considering controlled conditions in the greenhouse such as soil moisture, temperature, light intensity, airflow in the greenhouse and humidity. Three groups of explants were transferred in November, December and February. Data was recorded as the survival percentage of explants in the greenhouse.

2.9 Statistical analysis.

Experimental units were organized at completely randomized design (CRD). Data from each experiment were analyzed using SPSS. Analysis of variance (ANOVA) was used to test the statistical significance of means in the following experiments: cytokinin experiment, auxin experiment, MS experiment and the effect of basal salts. The mean separation was carried out using the least significant difference (LSD) test for mean separation at p-value ≤ 0.05. Pearson chi-square test was used for categorical variables such as length of shoots and root diameter in the cytokinin experiment.

Chapter three

Results and discussion

Micropropagtion

1) *In Vitro* culture of iris seeds and embryos.

In all *in vitro* culture experiments and in all populations, seeds in which the aril is intact showed no germination or any signs of root emergence or embryo growth. This result indicates strongly seed physical dormancy during this stage where seed coat and aril hinder water from passing to the embryo.

Immature seeds which were collected from Rashaydeh and Faqoua populations and cultured on full strength MS medium in which the aril is removed showed successful germination. Germination percentage in the Rashaydeh was 22% and in Faqoua was 39.3%.

In the second experiment where the embryo is removed out of the seed, embryos from Faqoua population were cultured on full strength MS showed growth percentage of 100% and callogenesis. Germination percentage of embryos is high compared to seed culture as shown in figure 3.1. On the other hand, embryos from Rammoun population have also germinated successfully on half-strength MS with 100% but without callogenesis.

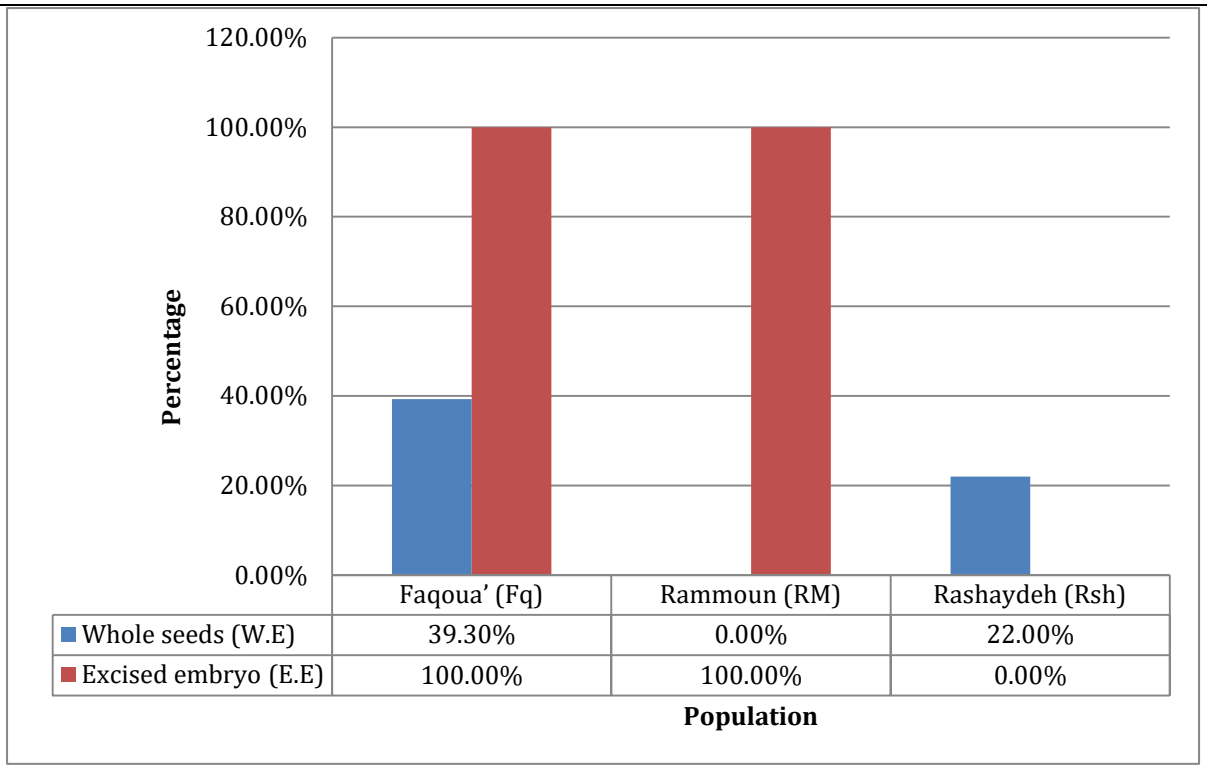


Figure 3.1. Germination percentage in different iris populations from seeds and excised embryos.

The difference in the germination percentage between unripe intact seeds, aril-removed seeds and excised embryos can be explained by the formation of physical dormancy and impermeable seed coat which prevents germination process by hindering water imbibition through the seed coat and reaching the embryo. For this reason, it can be concluded that royal irises possess physical seed dormancy in the seeds premature stage that prevents seed germination (Avishai, 1977).

To overcome seed dormancy, Dorman et al. (2009) studied *in vitro* germination of cut and uncut seeds. They found that exposing the embryo to nutrient media by removing the arils helped to break the innate dormancy. Germination results were for *I. atrofusca* 41%, *I. mariae*56%, and *I. petrana* 46%. On the other hand, uncut seeds germination percentage was about 20% (Dorman et al., 2009). Likewise, this study results indicate that germination percentage of Rashaydeh and Faqoua were 22% and 39.3% respectively (figure 3.2). Excised embryo culture from Faqoua' and Rammoun populations gave 100%. As a result, cut seeds and excised embryo culture gave much higher germination percentage when the seeds were uncut. Accordingly, seed scarification might help in increasing germination and overcome the

physical dormancy. Moreover, germination percentage varies between populations which indicates that each posses different dormancy level and different seed coat properties.

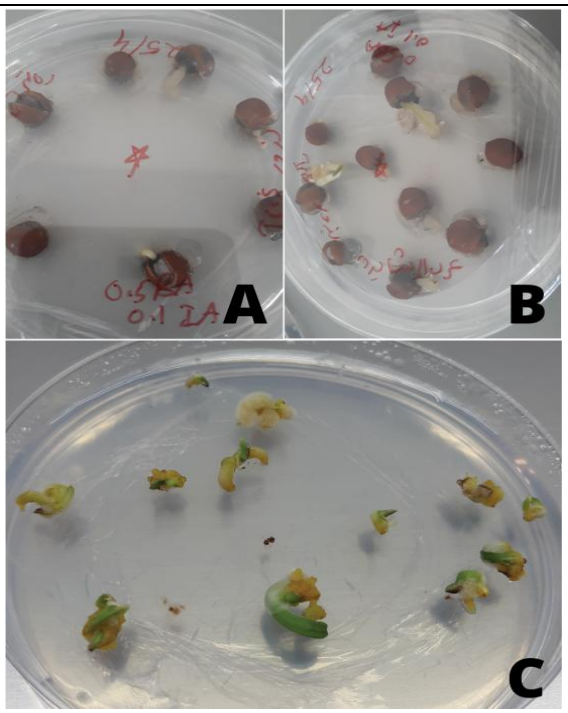


Figure 3.2. *In vitro* germination of *Oncocyclus* irises. (A + B) seed germination after two weeks. (C) Embryos cultured from Faqoua' population after two weeks.

Excised (rescued) embryos were grown on MS medium successfully; some embryos were injured during the process of extraction from seeds. Injured embryos showed low growth rate and abnormalities in growth. On the other hand, most embryos were uninjured and have high germination percentage without growth abnormalities (figure 3.3).

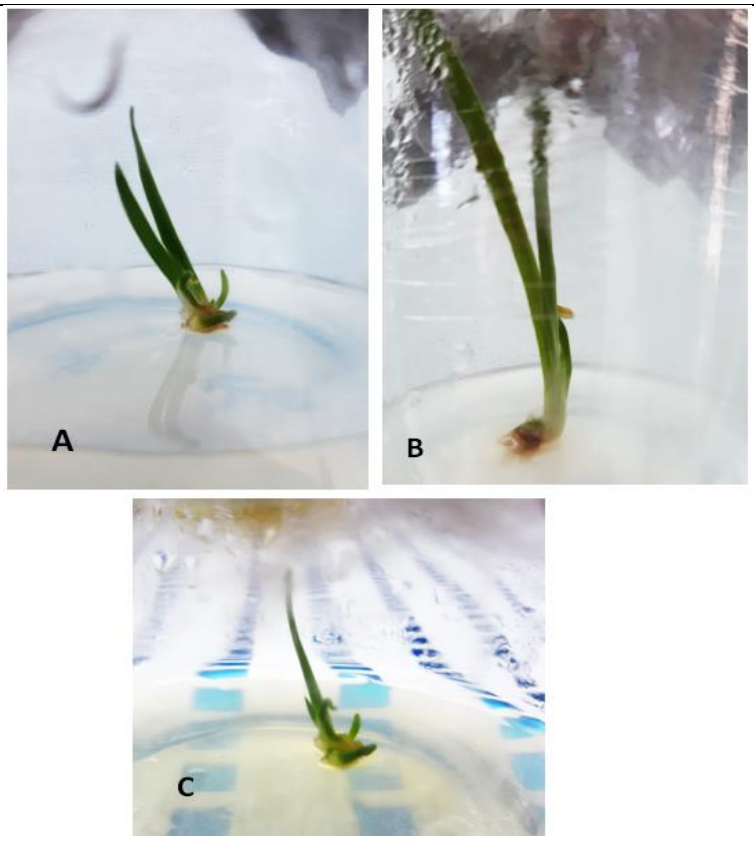


Figure 3.3 Shoot growth after embryo rescue and culture.

2) *In vitro* culture of vegetative nodes from rhizomes.

As for Rammoun in vitro shoot growth from field material (vegetative nodes), all explants showed bacterial contamination in spite of surface disinfection so eventually, contaminated shoots were discarded.

A proposed solution to overcome the bacterial contamination in field material is the procedure of Berger et al. (1994) who proposed a disinfection protocol starts by holding irrigation of stock plants for 2 months before the excision of explants, then disinfect explants with ethanol before excision from the rhizome then soaked immediately in 1.0% (v/v) sodium hypochlorite followed by mercuric chloride. As a result, this method decreased infection of bacterial soft rot up to 90% of explants (Berger et **al**., 1994).

Effect of PGR-free MS medium on shoot regeneration

The following part shows the descriptive statistics of half-strength the supplemented MS culture trials obtained in Aug-Sep-Oct of 2020 on Rammoun and Faqoua' populations. The results showed that the mean of shoot multiplications equals 0.5588 which is low. As for shoots: the mean of the shoot length equals 9.54 and the mean of shoot number equals 6.68. As for roots: the mean of the root number is 8.55 and the mean of the root length is 18.95, which is high (Table 3.1).

Table 3.1.The Descriptive Statistics of MS culture's experiments.

Variable	Mean	Std. Deviation
Shoot multiplication	0.5588	1.15971
Shoot Length	9.5417	7.74816
Shoot Number	6.8611	7.69101
Root Number	8.5556	10.21608
Root Length	18.9500	10.05179
Root Diameter	0.8611	1.22247

As mentioned previously in a study for *Iris lactea Pall. var. chinensis* micropropagation, half-strength MS has a better impact on root growth than full strength MS. Additionally, half-strength MS supplemented with 0.5 mg/L IBA were reported to have better impacts on rooting growth (Meng et al., 2009). On the other hand, shoots cultured in medium without PGRs, rooting success was only 52.5% in both species (Uzun et al., 2014). Boltenkov et al. (2007) suggested that PGRs were required for root initiation in *Iris ensata* (Boltenkov et al., 2007). This was clear in our first experiments (figure 3.4). For instance, Faqoua' embryos were cultured in full strength MS whereas Rammoun embryos were cultured in half-strength MS. Both had high germination percentages of 100% but full strength MS showed abnormalities of embryo growth and formed twisted shapes of germinated embryos resulted in abnormal plants.

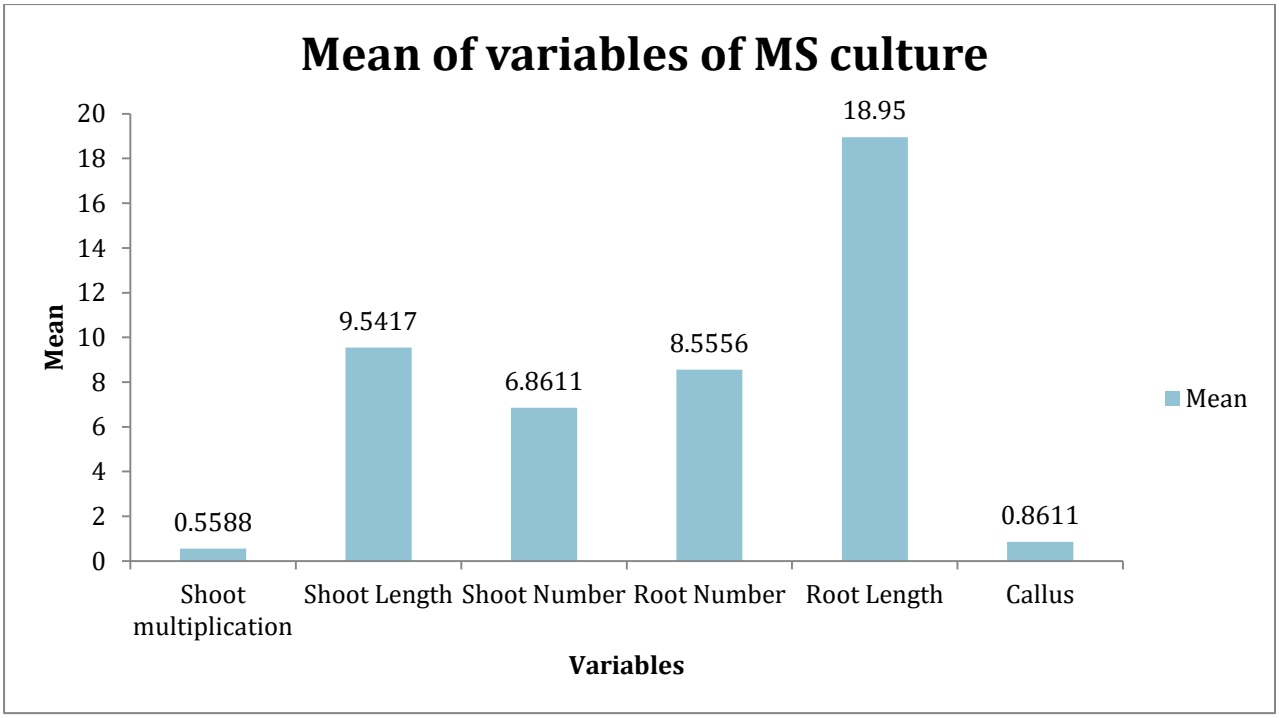


Figure 3.4. Mean of variables of MS culture's experiments.

Faqoua' samples had callogenesis, 20% of samples formed callus with diameter range 1.0 – 3.0 cm. Specifically, some cases formed large fragile friable callus structures with 3 cm diameter and massive rooting (+30) with abnormal shoot regeneration. In fact, 15% of samples had aggressive abnormal callus growth, examples shown in (figure 3.6). Likewise, Davarpanah et al. concluded that full strength MS was good for callogenesis and half-strength MS was used for embryo culture in research for common yew micropropagation (family: Taxaceae) using embryo culture (Davarpanah et al., 2014).

In conclusion, a half-strength MS medium is the optimum media that can be used for iris species for plant regeneration and multiplication. Although plant shoot multiplication mean was considered low (0.555) comparing with other variables but it is high considering low sample size and the absence of growth regulators which will encourage the multiplication of plantlets. On the other hand, the mean of shoot number and shoot length were higher which indicates that half-strength MS medium is convenient for the studied iris species (Figure 3.5).

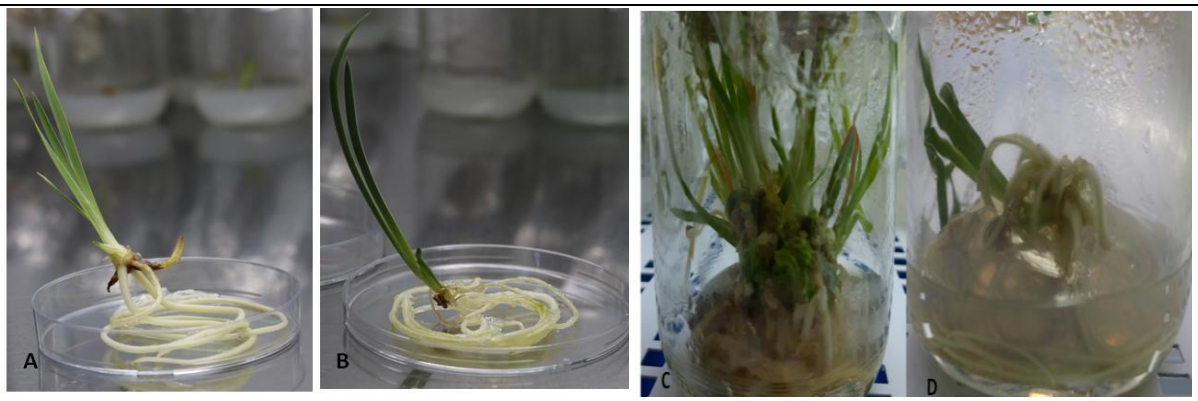


Fig. 3.5. Effect of full-strength and half-strength MS medium on Irises. (A+B) effect of half-strength MS medium on irises. (C+D) Effect of full-strength MS medium on Irises (callus formation and abnormal growth).

Effect of media type on *in vitro* growth of excised embryos

Results show that MS salts gave average number of shoots = 4.6 after six weeks, other tested media gave lower number in which QL= 4.25, NN= 4.20 and B5= 3.20. Furthermore, NN medium resulted in highest average number of roots =9.40 six weeks (Table 3.4)

Table 3.2**.** : Descriptive analysis of the Number of (Shoots and Roots) according to media type.

Variable		After 2 weeks		After 4 weeks		After 6 weeks	
		Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Number of shoots	QL	3.0000a	3.36650	3.5000a	4.35890	4.2500a	5.85235
	MS	1.8000a	1.92354	2.6000a	2.60768	4.6000a	2.70185
	B5	2.6000a	1.14018	3.2000a	1.48324	3.2000a	1.48324
	NN	3.0000a	2.00000	3.6000a	2.96648	4.2000a	3.56371
	Average	2.5789	2.03622	3.2105	2.69936	4.0526	3.30780
Number of roots	QL	0.7500a	0.50000	0.7500a	0.50000	1.2500b	1.25831
	MS	1.8000a	1.30384	2.8000ab	1.64317	2.8000ab	1.64317
	B5	1.2000a	1.30384	1.4000ab	1.51658	1.4000b	1.51658
	NN	6.4000a	7.76531	8.0000a	9.30054	9.4000a	9.76217
	Average	2.6316	4.43735	3.3684	5.38734	3.8421	5.88088

The effect of basal salts on the number of shoots and root number differs significantly as shown in (figures 3.6 and 3.7). The mean shoot number in QL medium was 3.0 which was the highest in the first two weeks and increased by time but MS medium mean was the lowest of 1.8 in the first two weeks and increased by time to reach the highest mean of 4.6 by six weeks of culture (figure 3.6).

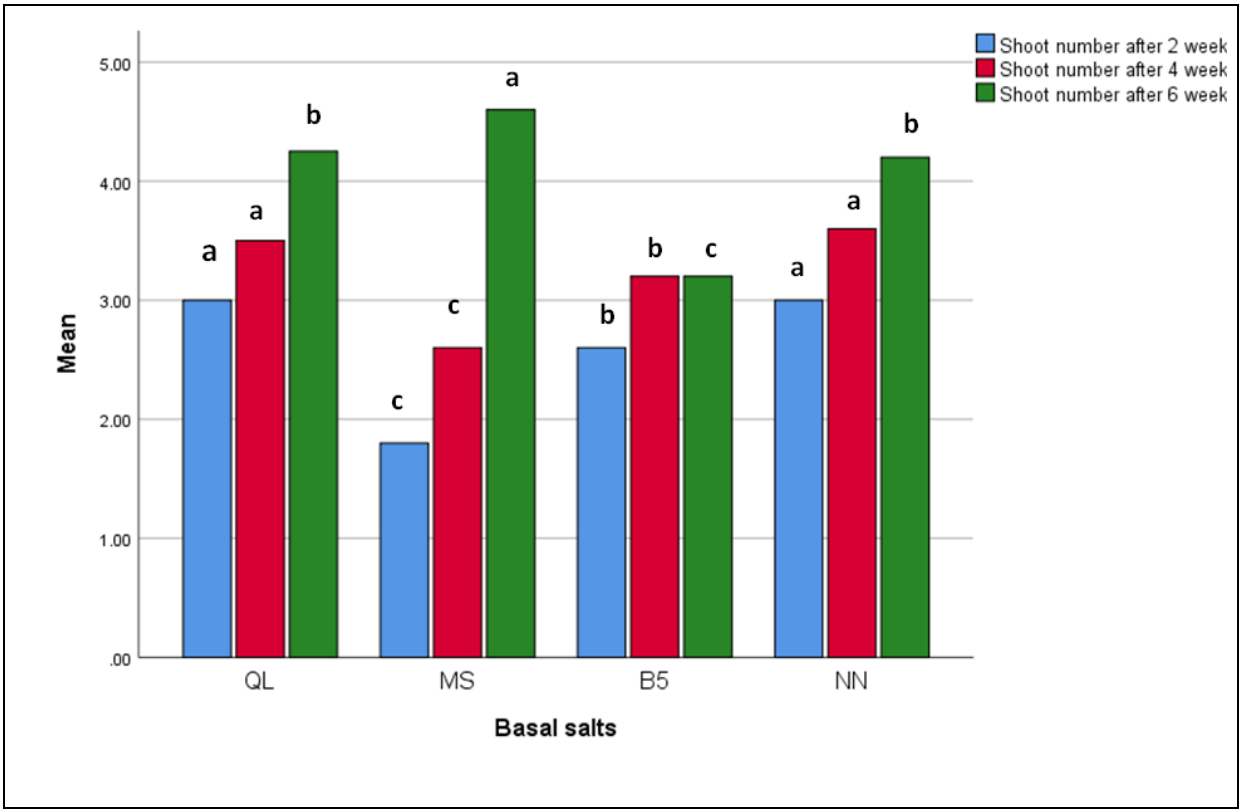


Figure 3.6. The effect of basal salts on shoot number of royal irises after 2 weeks, 4 weeks and 6 weeks.

As for root number, NN medium has a significantly high mean through the first two weeks, four weeks and six weeks of 6.4, 8.0 and 9.4 respectively as shown in figure 3.7. Moreover, other basal salts effect on root number was low considering their mean in two weeks, four weeks and six weeks.

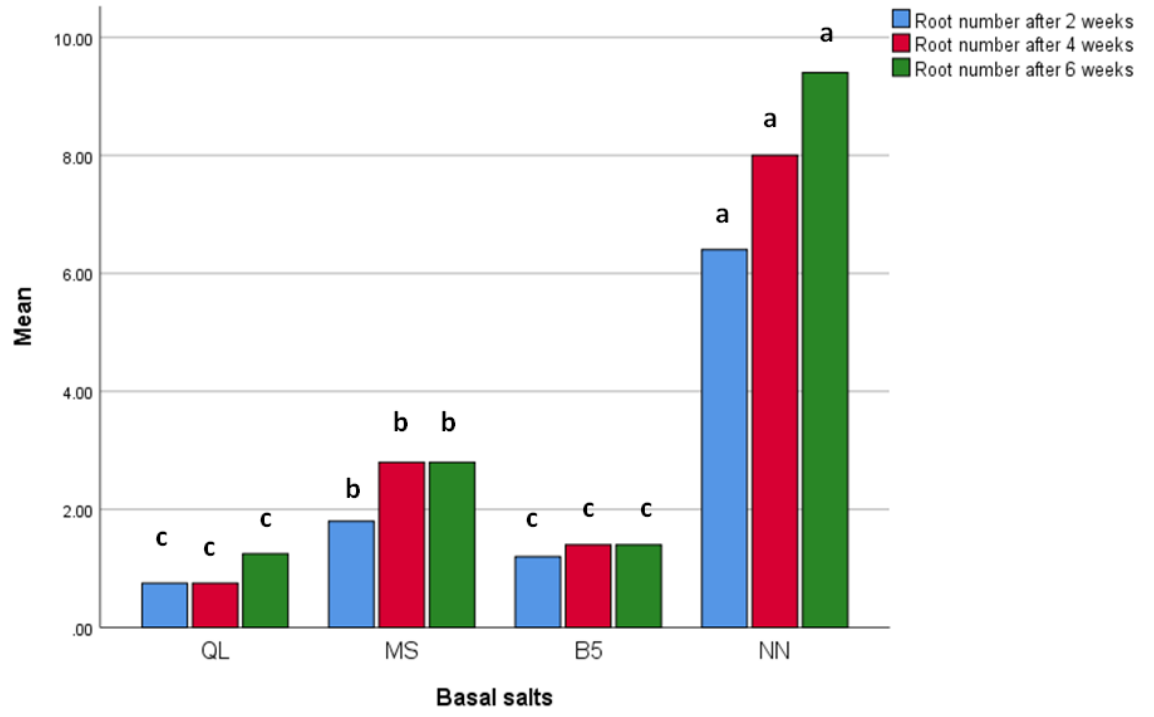


Figure 3.7. The effect of basal salts on root number of royal irises after 2 weeks, 4 weeks and 6 weeks.

The current experiment compared different basal growth media to test the response of irises during in vitro plant growth and development (figure 3.8). According to statistical analysis by one-way ANOVA, a significant differences in the number of shoots in MS media treatment at two, four and six weeks can be observed. On the other hand, NN media gave massive root growth which was in some cases very dense compared to other basal salts. NN media resulted in more root growth than shoots and results showed that roots were significantly higher than other basal salts.

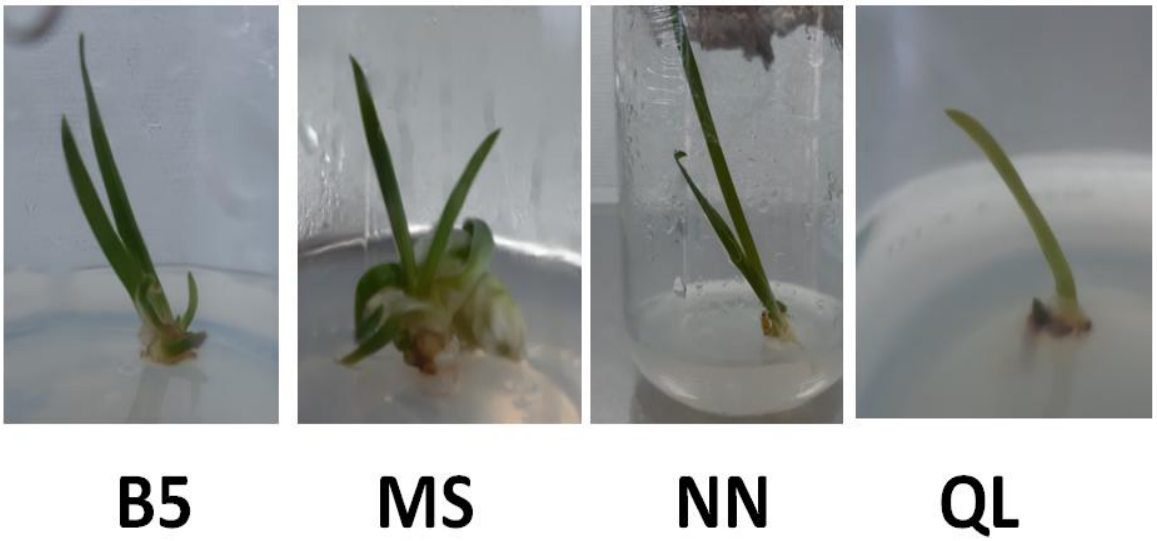


Figure 3.8. Effect of different basal salts on embryo growth after four weeks.

Supporting our findings, De Munk and Schipper recommended using MS medium at full or half-strength, with addition of 2-4% sucrose for irises shoot regeneration (Munk and Schipper, 1993).

As an example of rhizomatous irises micropropagation, *I. germanica* was studied and cultured on modified MS with the addition of casine hydrolysate and adenine sulphate that were recommended to iris tissue culture specifically for the purpose of callus formation with the addition of 2.5 mg/L NAA and 0.5 mg/L kinetin (Kyte, 2013).

Effect of Cytokinin concentrations on shoot regeneration and multiplication.

The results showed that the highest number of shoot multiplication (number of shoots) between the six treatments was in treatment "C", with an average = 1.44 and 5.88 respectively after 4 weeks. Whereas the highest number of roots was observed in treatment "B", with an average = to 1.66. In addition, the mean of the number of roots of treatment "A" and "C" equals 1.5 and 1.1 respectively. Additionally, results showed that the highest number of multiplications of the six treatments was treatment "C" and "B", with means: 2.11 and 2 respectively after 8 weeks. Whereas the highest number of the shoots and roots was for treatment "B", with means equal to 11.5 and 6.6. (Table 3.3)

Table 3.3. Results of cytokinin treatments and mean of variables.

symbol	1/2MS + (Cytokinin Concentration)	Number of multiplication (mean)		Number of leaves (mean)		Number of roots (mean)	
		4 weeks	8 weeks	4 weeks	8weeks	4 weeks	8 weeks
A	BA 0.25 mg/L	0.0000	0.3333	3.0000	4.3333	1.5000	5.0000
B	BA 0.5 mg/L	0.1667	2.0000	2.8333	11.5000	1.6667	6.6667
C	BA 1.0 mg/L	1.4444	2.1111	5.8889	6.4444	1.1111	4.2222
D	KN 0.25 mg/L	0.5000	0.7500	3.3750	5.5000	0.3750	0.7500
E	KN 0.5 mg/L	0.1250	0.2500	2.3750	3.0000	0.6250	0.7500
F	KN 1.0 mg/L	0.5714	1.2857	2.1429	9.5714	0.7143	0.8571

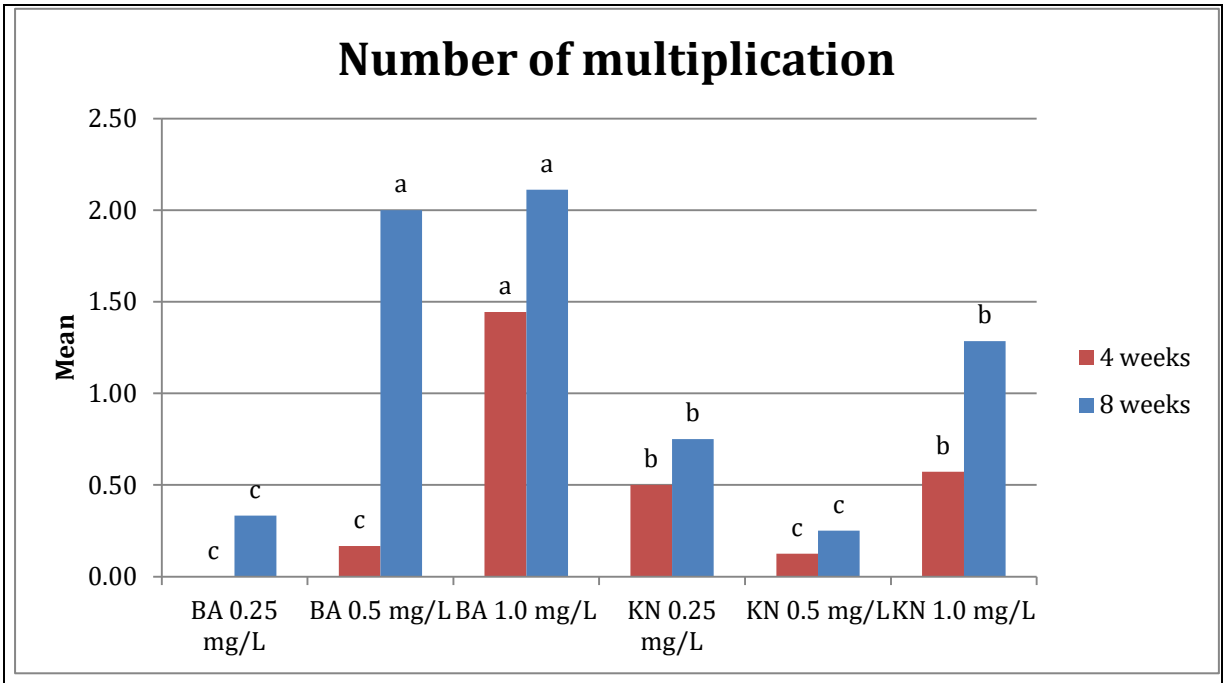


Figure 3.9. Mean comparison between number of shoot multiplication after 4 weeks and 8 weeks.

As shown in figure 3.9, the number of shoot multiplications in all treatments increases significantly from 4 to 8 weeks. Treatment C gave the highest mean value with 1.4 after 4 weeks whereas treatments C and B mean of 2.0 and 2.1 respectively after 8 weeks.

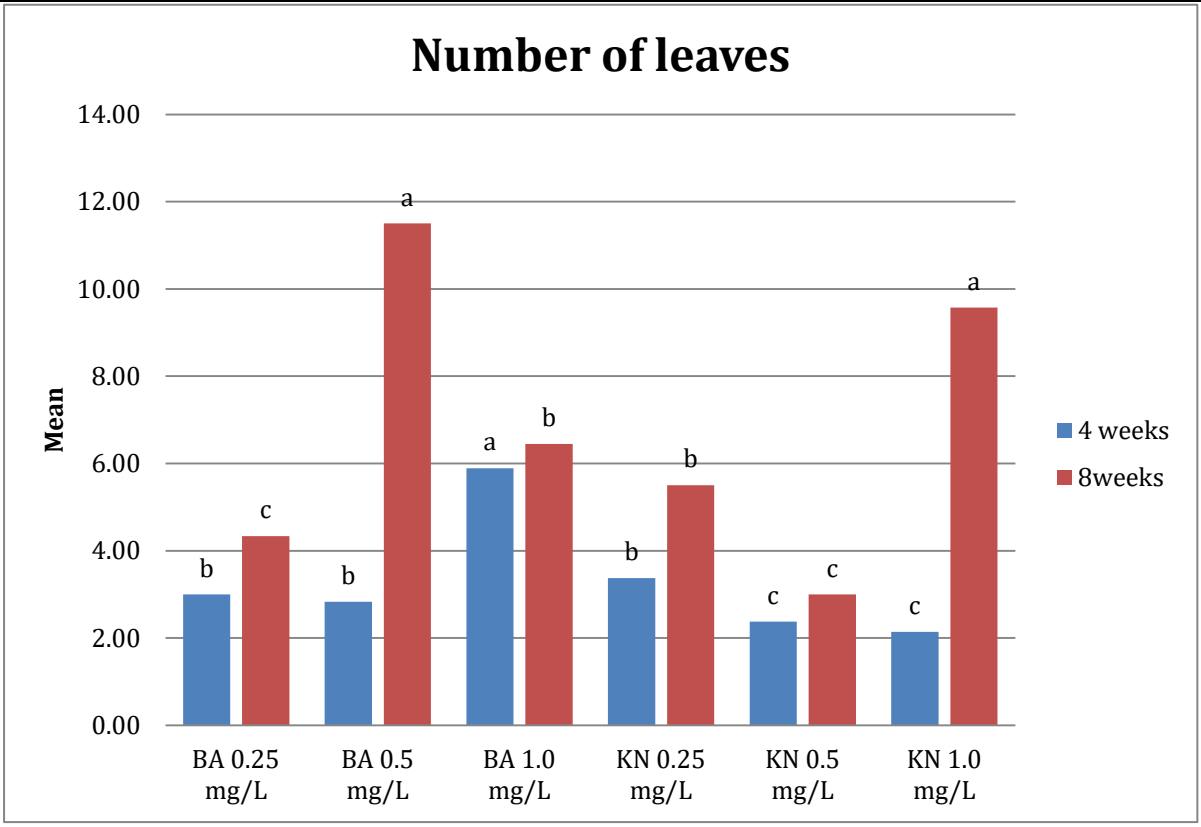


Figure 3.10. Mean comparison between the number of leaves after 4 weeks and 8 weeks.

As shown in figure 3.10, the mean values for the number of leaves vary from 4 to 8 weeks significantly. After 4 weeks, treatment C had the highest mean value with 5.8 whereas after 8 weeks treatment B mean value was the highest with 11.5.

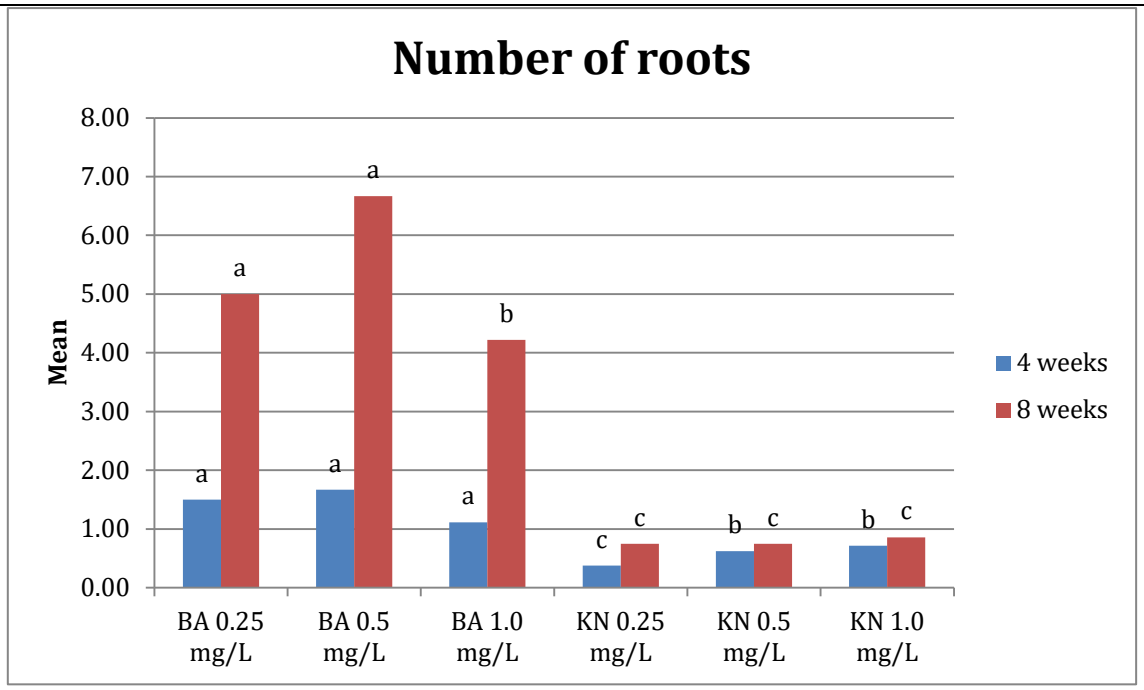


Figure 3.11. Comparison of means between the number of roots after 4 weeks and 8 weeks.

As shown in figure 3.11 mean values of the number of roots varies from 4 to 8 weeks significantly. After 4 weeks, treatments B and A gave the highest average of 1.6 and 1.5 respectively whereas after 8 weeks treatments B mean value was the highest with 6.6.

Figure 3.12 and figure 3.13 show the effect of plant growth regulators on irises after 8 weeks.

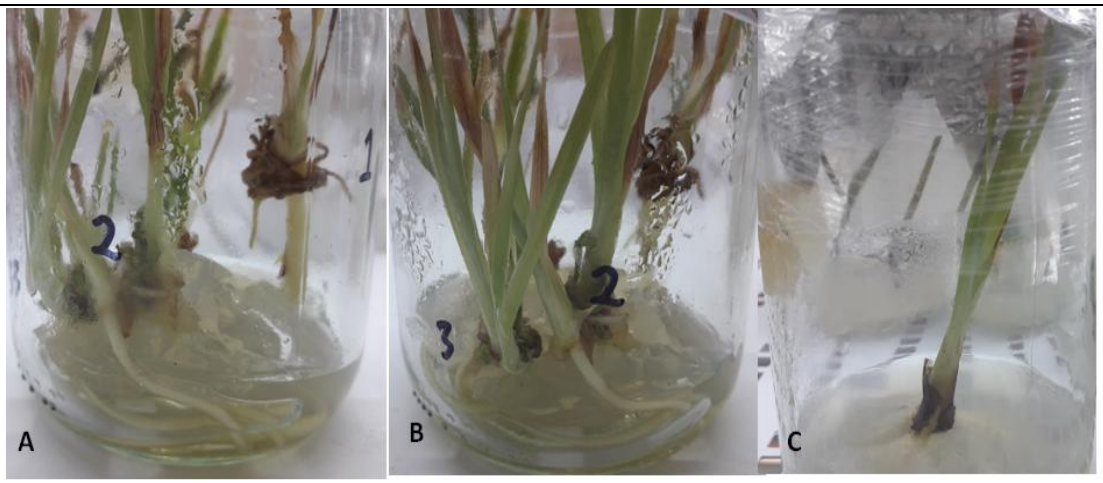


Fig. 3.12. Effect of BA on local Royal Irises. (A)+(B) represents treatment C which contains 1.0 mg/L of BA.(C) represents treatment A which contains 0.25 mg/L of BA.

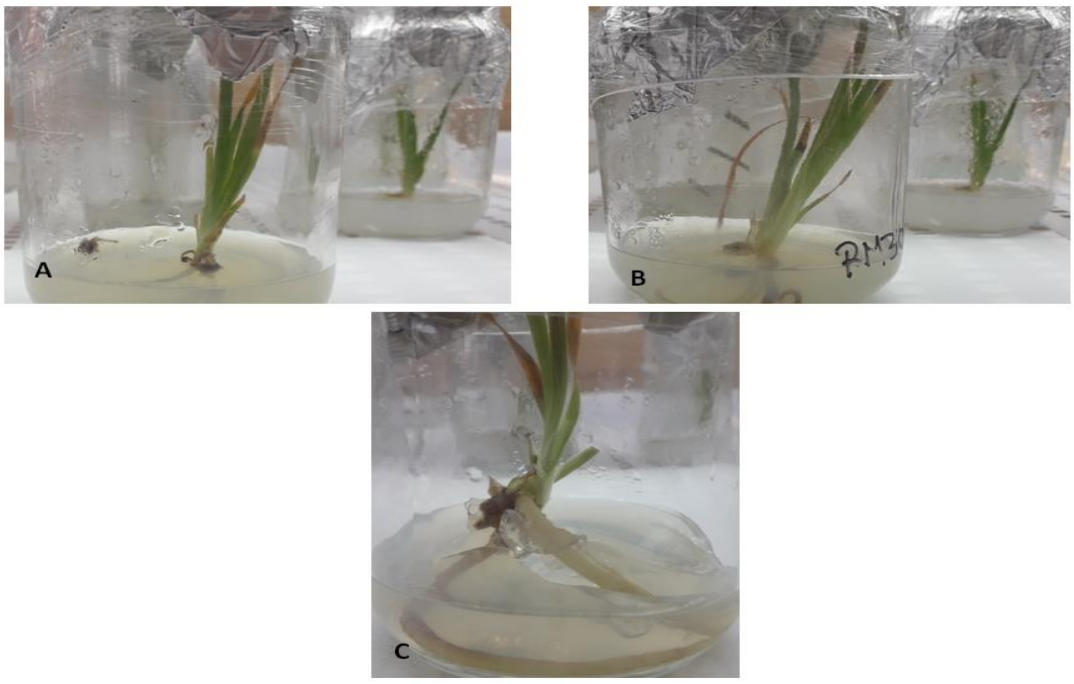


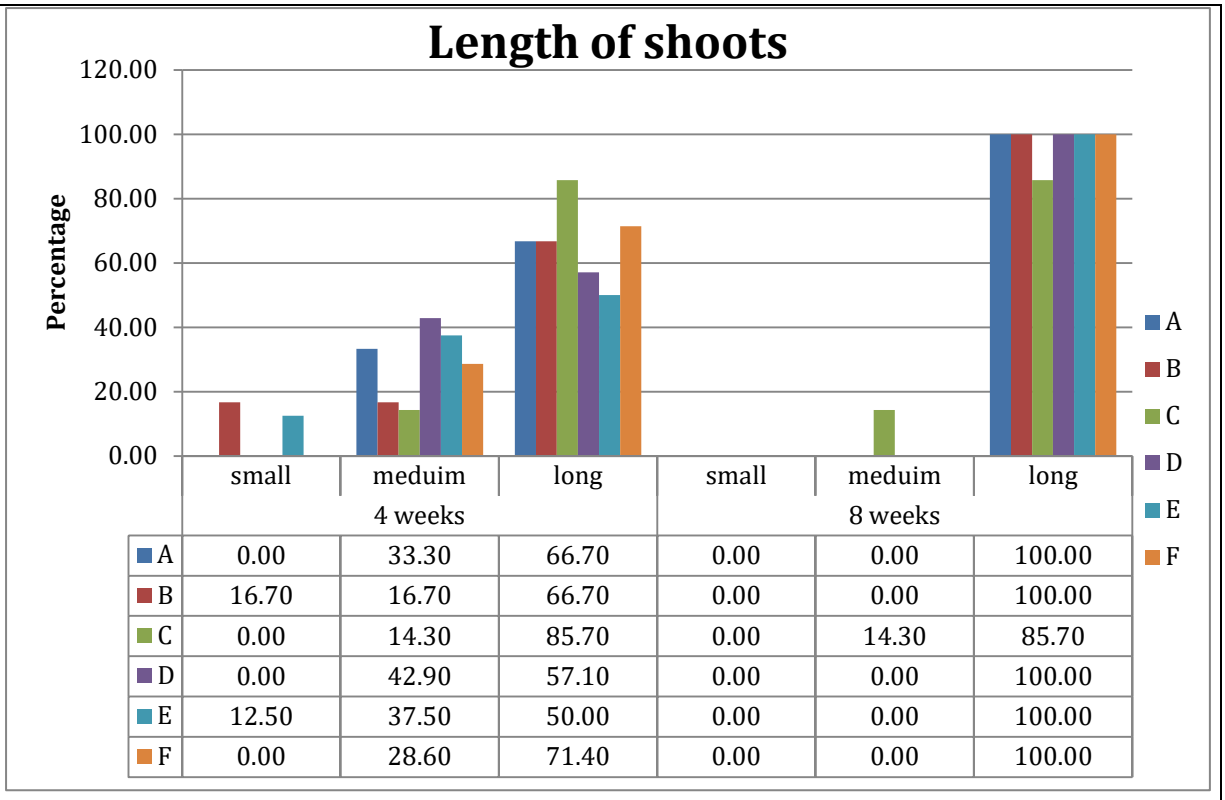
Figure 3.13. Effect of KN on local Royal Irises. (A) Represents treatment E which contains 0.5 mg/L of KN. (B)+(C) represents treatment F which contains 1.0mg/L of KN.

Comparison between shoot length and root diameter after four weeks and eight weeks:

The below table shows the results of Pearson chi-square's test comparison of the shoot length and root diameter after 4 and 8 weeks, Pearson chi-square values for after 8 weeks records were significant compared to 4 weeks records.

Table 3.4. Person chi-square values of shoot length and root after 4 and 8 weeks.

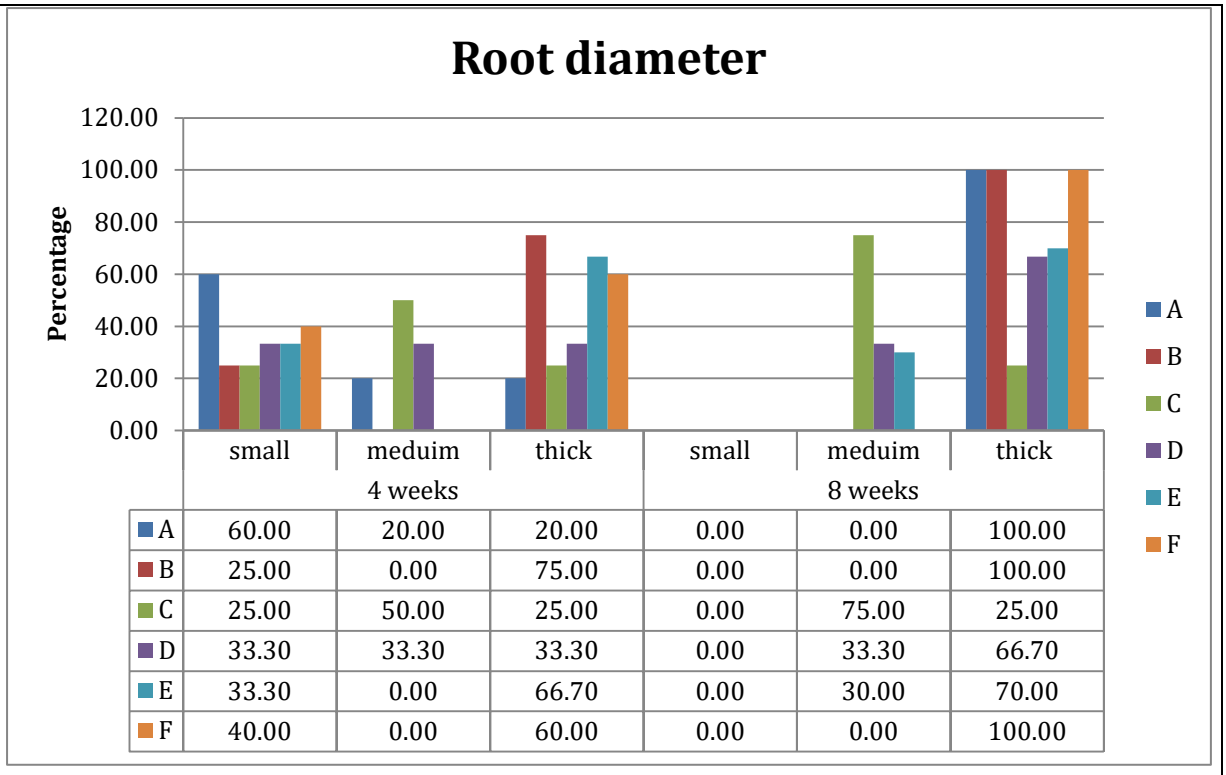
Variable	Pearson chi-square value	Significance (p-value)
Shoot length (4 weeks)	12.125	0.670
Shoot length (8 weeks)	30.528	0.000
Root diameter (4 weeks)	16.750	0.334
Root diameter (8 weeks)	30.494	0.010



	small	meduim	long	small	meduim	long
	4 weeks			8 weeks		
A	0.00	33.30	66.70	0.00	0.00	100.00
B	16.70	16.70	66.70	0.00	0.00	100.00
C	0.00	14.30	85.70	0.00	14.30	85.70
D	0.00	42.90	57.10	0.00	0.00	100.00
E	12.50	37.50	50.00	0.00	0.00	100.00
F	0.00	28.60	71.40	0.00	0.00	100.00

Figure 3.14 Comparison in shoot length and cytokinin treatments after 4 weeks and 8 weeks.

The results showed that after 4 weeks, all treatments had a high percentage of large shoots but treatment C percentage was significant with 85.70%, while after 8 weeks, treatments B, D, E and F percentage was 100% which is significantly high compared to other treatments. (Figure 3.14)



	small	meduim	thick	small	meduim	thick
	4 weeks			8 weeks		
A	60.00	20.00	20.00	0.00	0.00	100.00
B	25.00	0.00	75.00	0.00	0.00	100.00
C	25.00	50.00	25.00	0.00	75.00	25.00
D	33.30	33.30	33.30	0.00	33.30	66.70
E	33.30	0.00	66.70	0.00	30.00	70.00
F	40.00	0.00	60.00	0.00	0.00	100.00

Figure 3.15. : Comparison of root diameter between cytokinin treatments after 4 weeks and 8 weeks.

The results showed that after 4 weeks, treatments B and E gave thick root diameter with 75% and 66.7% respectively, whereas percentages of treatments A, B and F after 8 weeks were 100% thick which is significantly higher than other treatments. (Figure 3.15)

To encourage plant multiplication, cytokinin experiments were conducted to find a suitable cytokinin concentration that triggers iris multiplication without forming callus. Some studies on iris species suggested medium formulas for shoot multiplication contains growth regulators such as adenine sulphate, BA, NAA and tyrosine with modifications on MS medium by adding myo-inositol to the mixture. This method was conducted on callus as an explants source which was induced to form somatic embryogenesis as a method to iris micropropagation (Jevremović et al., 2013).

On the contrary, the present study offers a promising method of embryo culture followed by shoot multiplication with one cytokinin that could multiplicate plantlets without callogenesis. To illustrate, the results of treatment C (half-strength MS with 1.0 mg/L of BA) after 4 weeks shows the highest number of shoot multiplications and the highest number of shoots between 6 treatments with means: 1.44 and 5.88 respectively. In addition, the percentage of shoot length was 85.7% large and 14.30% medium which was high compared to other treatments as

shown in figure 3.16 and table 3.6. Comparatively, the results after 8 weeks show that this treatment gave the highest multiplication with an average of 2.11, whereas treatment (half-strength MS with 0.5 mg/L BA) had the highest number of shoots with a mean of 11.5. (Table 3.3)

According to data analysis, treatment C is suitable for local irises multiplication. Despite that, treatment B has a relatively high number of multiplications mean of 2.00 and the highest mean number of shoots after 8 weeks. To summarize, half-strength MS with 0.5 or 1.0 mg/L of BA is recommended for shoot multiplication of royal irises in Palestine. Higher concentrations of BA will encourage callogenesis and can be used for callus induction; this result is also similar to the result found by Shibli and Ajlouni (2000). Other studies indicate that BA can induce shoot regeneration at the rate of 100% when cultured on medium with 1.0 or 3.0 mg/L of BA, although the highest BA concentration at 3.0 mg/L appeared to show a suppressive effect on shoot differentiation, in other species like *I. germanica* no effect on shoot development was found (Bae et al., 2012). As for KN, it was generally used in the induction of embryogenic callus in irises but it was reported that KN has an inhibitory effect on somatic embryogenesis when supplemented at a high concentration of 2-4 mg/L (Shimizu et al., 1996; Wang et al., 1999).

In vitro rooting

As for in vitro rooting experiments, auxin treatment “E” (1/2 MS with 0.5 mg/L of IBA) gave the highest number of roots after four weeks with an average of 1.62, whereas treatment B’ (1/2 MS with 0.5 mg/L of NAA) and E’ (1/2 MS with 0.5 mg/L of IBA) gave the highest number of roots with means of 3.5 and 3.6 respectively after eight weeks. Results also showed that the total number of roots was (111 roots) after 8 weeks which was higher than the total number after 4 weeks (43 roots). Also, the results showed that the highest number of roots between the six treatments is for the treatment “E’”, with a mean: 1.62 after 4 weeks whereas the highest number of roots for treatment “B’ and E’ ”, with means equal to 3.5, 3.6 respectively after 8 weeks (table 3.5).

Table 3.5: mean of variables and number of roots for auxin treatments after 4 weeks and 8 weeks.

Symbol	1/2 MS+ (Auxin Concentration)	4 weeks		8 weeks	
		Number of roots	Mean	Number of roots	Mean
A'	NAA 0.25 mg/L	3	0.3000	11	1.1000
B'	NAA 0.5 mg/L	8	0.8000	35	3.5000
C'	NAA 1.0 mg/L	9	1.0000	17	1.8889
D'	IBA 0.25 mg/L	7	1.0000	12	1.7143
E'	IBA 0.5 mg/L	13	1.6250	29	3.6250
F'	IBA 1.0 mg/L	3	0.5000	7	1.1667
Total		**43**		**111**	

The in vitro rooting phase in irises was mentioned in other studies with various recommendations depending on the cultivars and species. For example, the rooting of iris shoots was done successfully on BM medium which is composed of MS mineral salts , organics and 20-50 g/l sucrose, supplemented with adenine sulphate and tyrosine for rooting of *I. germanica*, whereas other cultivars response to half-strength MS only or BM medium supplemented with 0.1mg/L IAA or NAA to improve rooting (Jevremović et al., 2013). Moreover, the root formation occurred in 0.5 mg/L of NAA media with no shoot formation in the *Iris kirkwoodiea* plant (Doğan and Çağlar, 2018). Furthermore, Uzun et al, (2014) demonstrated that MS basal medium supplemented with 1.0 mg/L IBA gave better impact on root development in *I.sari* and *I. schachtii* and it was found that IBA- containing medium allowed rapid root development in *Iris* species (Laublin et al., 1991; Uzun et al., 2014). Additionally, high NAA concentrations increased root formation and suppressed callus growth under light conditions in these species (Uzun et al., 2014). Despite that, it has been determined that increasing NAA concentrations tend to form callus in *Iris kirkwoodiea* (Doğan and Çağlar, 2018).

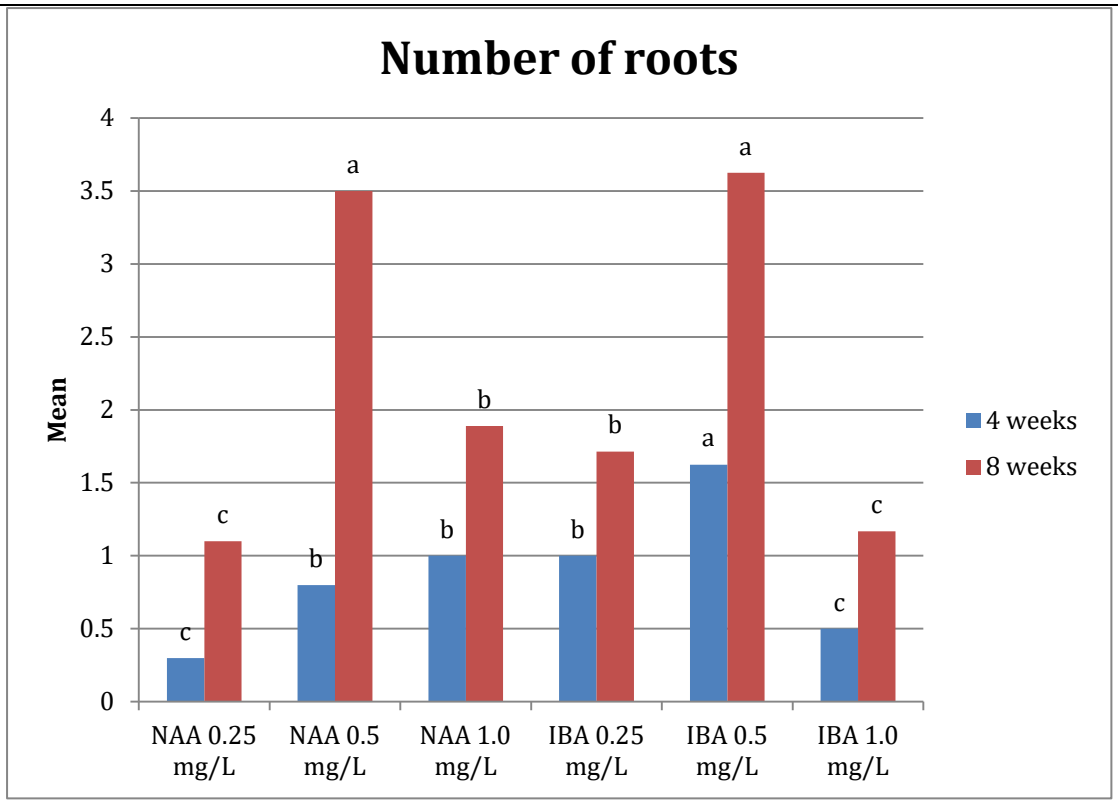


Figure 3.16. Mean for the number of roots in after 4 weeks and 8 weeks.

Similarly, the best rooting results were obtained on medium with half-strength MS and 0.5 mg/L IBA or 0.5mg/L NAA where the mean was 3.5 and 3.6 respectively. Despite that, rooting results of half strength MS were satisfactory (figure 3.16). Figure 3.17 and figure 3.18 show the effect of plant growth regulators on irises after 8 weeks; this is a sample of results.

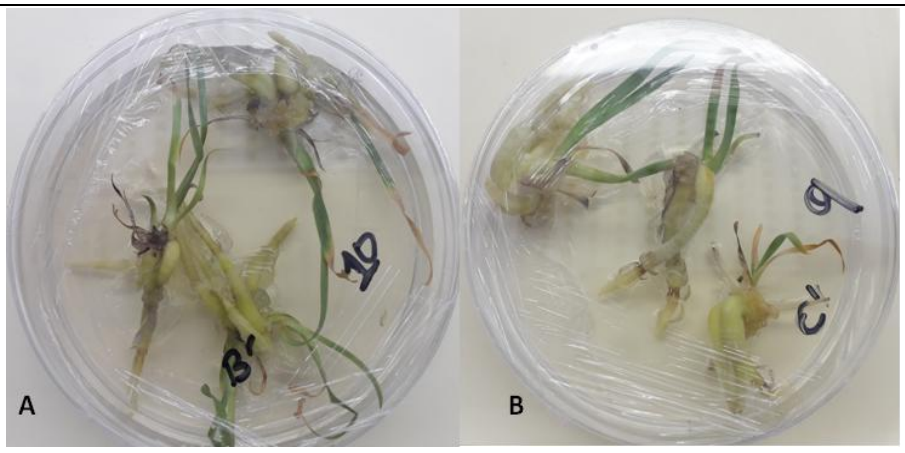


Figure 3.17. Effect of NAA on local royal irises. (A) represents treatment B' which contains 0.5 mg/L of NAA.. (B) represents treatment C' which contains 1.0 mg/L of NAA.

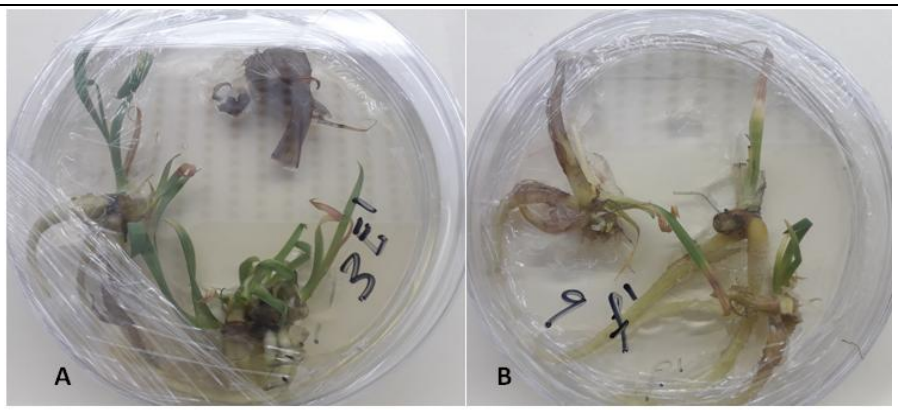


Figure 3.18. Effect of IBA on iris rooting. (A) Treatment E' which contains 0.5 mg/L of IBA. (B) Treatment F' which contains 1.0 mg/L of IBA.

Acclimatization and hardening of royal irises.

Three groups of *in vitro* grown plantlets with roots were transferred to the greenhouse successfully from Faqoua population. Survival percentage was = 100% for plants planted in December and February and 75.00% for the ones planted in November (Figure 3.19). Despite that, all groups survival rate was considered high. Notably, November and December groups were all cultured on half-strength MS whereas the February group was cultured on half-strength MS with 0.5 and 1.0 BA and all were not cultured on rooting mediums before transferring to the greenhouse.

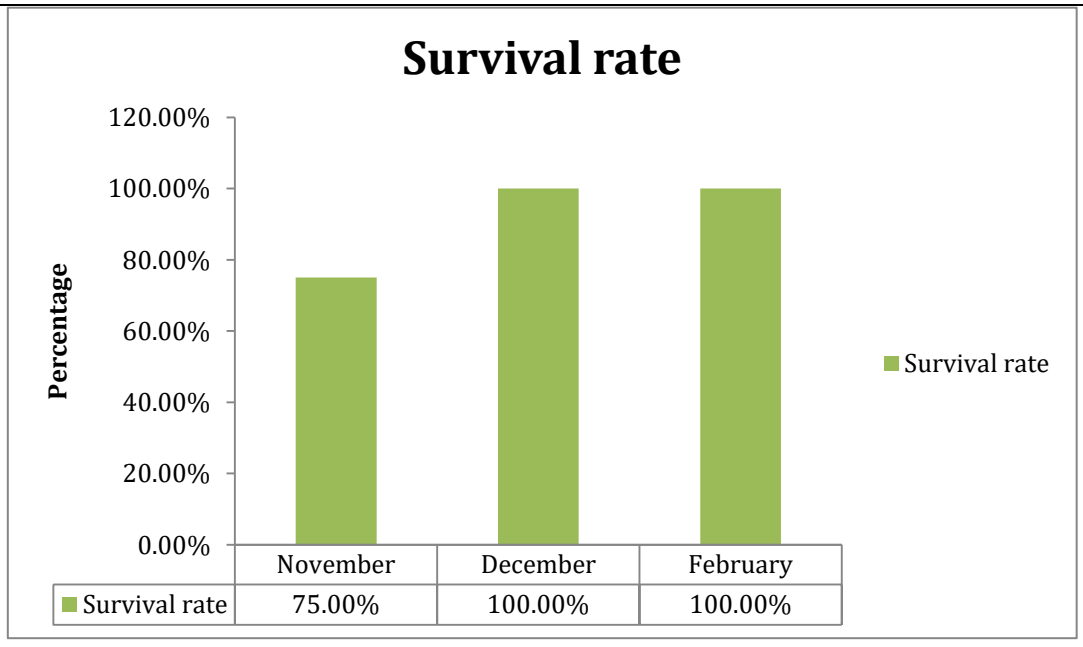


Figure 3.19. Survival rate of transplanted iris plantlets in the greenhouse

Acclimatization is a critical stage and it requires gradual transfer from *in vitro* to *ex vitro* conditions because this stage is associated with slow growth and significant plant loss (Uzun et al., 2014). In this research, acclimatized plantlets were obtained successfully from plantlets cultured on half-strength MS media only or supplemented with (0.5-1.0 mg/L) of IBA. Similarly, Uzun et al, (2014), observed root initiation in shoots cultured on medium without PGRs with 52.5% in *Iris sari* and *Iris schachtii* (Uzun et al., 2014). Other species like *Iris ensata required* PGRs for root initiation (Boltenkov et al., 2007), the effect of PGRs differ from species to species .

In this experiment, the combination of (cytokinin – auxin) was discarded due to its encouragement of callogenesis; this would be great for other micropropagation purpose if callus was needed but in this study, callus was discarded from micropropagation phases to obtain healthy plantlets without callogenesis.

Transferred plantlets were successfully acclimatized and hardened in the greenhouse. After one season, iris seedlings had dry-out and new shoots were germinated and 1- year juvenile rhizomes were formed which indicates that seedlings are established in the greenhouse and ready for the next season (figure 3.20).

Figure 3.20. Representative images of hardened plantlets. (A) Iris seedlings in December. (B) Iris seedlings after one week. (C) Iris seedlings after 3 months. (D) Iris seedlings after one season from Faqoua' and Rammoun populations.

Notably, root formation was not affected by the lack of added auxins. To clarify, roots were initiated on half-strength MS medium. In fact; the mean of number of root and the length of roots of half-strength MS medium were 8.5 and 18.9 respectively. On half-strength MS only, the diameter of obtained roots was thin and hair-like roots but these plantlets can be transferred to greenhouse after one month of incubation and survival rate was 100%. Moreover, half-strength MS medium supplemented with 0.5 mg/l of BA had also initiated roots and plantlets were transferred after one month on incubation to green house, the survival rate was 100%. The reason behind root initiation despite the lack of added auxins is the presence of endogenous auxin in sufficient amounts that enable the plantlets to form roots. The difference in roots between cytokinin treatments, auxin treatments and half-strength MS only is that auxin treatments have initiated thicker roots and root initiation time was less than mediums that are not supplemented with auxin. Supporting these observations, a study performed on black iris in Jordan (*I. nigricans*) in 2009 concluded that immature seeds had concentrations of ABA, cytokinin and IAA of 0.88, 17.8 and 36.9 μg/g dw (dry-weight), respectively, whereas mature seeds had 31.5, 36.2 and 9.7 μg/g dw, respectively (Samarah et al., 2009). To conclude, immature seeds had lower ABA and cytokinin, but higher IAA than mature seeds. Moreover, ABA is the only plant hormone that maintains plant dormancy (Liu et al., 2013). High amount of endogenous IAA in immature seeds could alter the root initiation despite the lack of exogenous auxins from nutrient media.

Chapter five

Conclusions and future work

Oncocyclus iris seeds possess two types of dormancy; morphological dormancy and morph-physiological dormancy. To overcome seed dormancy, an in vitro embryo germination experiments were conducted with 100% germination rate, embryo culture is a successful method because it removes the thick seed coat that prevents water from penetrating the seed. A protocol was developed for micropropagtion of irises as an important step in the conservation of endangered irises and solving seed dormancy problem. This micropropagation protocol contains four stages: first, the selection of the explants depending on the purpose of micropropagtion, here embryo culture was efficient for conservation of irises and overcoming seed dormancy with 100% germination rate of embryo culture. Moreover, shoot regeneration of irises using half-strength MS supplemented with 0.5 mg/L of BA with the highest mean of number of shoot multiplications of all cytokinin treatment with mean of 2.11 , and finally for rooting using half-strength MS supplemented with 0.5 mg/L of IBA with the highest mean of all auxin treatments with 3.6 for number of roots .as a final stage; hardening of plantlets in the greenhouse in peatmoss: sand mixture (2:1) had survival rate from 75%-100% , seedlings were dry-out and obtained 1-year juvenile rhizomes and were ready for the next season after one year in the greenhouse. This protocol would be considered as a conservation method of irises in Palestine.

Future work on irises in Palestine should focus on increasing the population of these plants by introducing these micropropagated seedlings into botanical gardens, increase the population in their natural habitat (*in situ* conservation) or relocating these endangered plants to protected area (*ex situ* conservation). Spreading these plants can help in minimizing the effect of their annual loss in their natural habitat. Furthermore, the government must increase their effort to protect irises in Palestine.

References:

1. Abobkar, I.M.S., and Ahmed, M.E. (2012). Plant Tissue Culture Media.
2. Agboola, D.A., Ogunyale, O.G., Fawibe, O.O., and Ajiboye, A.A. (2014). A Review of Plant Growth Substances: Their Forms, Structures, Synthesis and Functions. Journal of Advanced Laboratory Research in Biology *5*, 152-168.
3. Al-Gabbiesh, A., Hassawi, D., and Afifi, F. (2006). In vitro Propagation of Endangered Iris Species. Journal of Biological Sciences *6*.
4. Ali-shtayeh, M.s., and Jamous, R. (2018). Updating the plant "Red List" of Palestine (West Bank and Gaza Strip): conservation assessments and recommendations.
5. Alkowni, R., and Sawalha, K. (2012). Biotechnology for conservation of palestinian medicinal plants. Journal of Agricultural Technology *8*, 1285-1299.
6. Altaf Hussain, I.A.Q., Hummera Nazir and Ikram Ullah (2012). Plant Tissue Culture: Current Status and Opportunities,. IntechOpen.
7. Arafeh, R.M., Sapir, Y., Shmida, A., Iraki, N., Fragman, O., and Comes, H.P. (2002). Patterns of genetic and phenotypic variation in Iris haynei and I. atrofusca (Iris sect. Oncocyclus = the royal irises) along an ecogeographical gradient in Israel and the West Bank. Mol Ecol *11*, 39-53.
8. ARIJ (2007). The Status of the Environment in the West Bank. Bethlehem, Palestine.
9. Atrash, I. (2021). Iris haynei (observation.org: Imad Atrash), pp. 1-6.
10. Avishai, M. (1977). Species relationships and cytogenetic affinities in section Oncocyclus of the genus Iris. Ph.D. Thesis. The Hebrew University of Jerusalem.
11. Avishai, M., and Zohary, D. (1980). Genetic Affinities among the Oncocyclus irises. Botanical Gazette *141*, 107-115.
12. Bae, K.-H., Yoo, K.-H., Lee, H.-B., and Yoon, E.-S. (2012). Callus induction and plant regeneration of Iris dichotoma Pall. in endangered species. Journal of Plant Biotechnology *39*.
13. Barazani, O., Mayzlish Gati, E., Lifshitz, D., Hadas, R., Keren, A., Golan, S., Faraj, T., Singer, A., Beerman, A., and Perevolotsky, A. (2016). Strategies and priorities in field collections for ex situ conservation: the case of the Israel Plant Gene Bank. Genetic Resources and Crop Evolution.
14. Baskin, C., and Baskin, J. (1998). Seeds: Ecology, Biogeography, and, Evolution of Dormancy and Germination.

15. Baskin, C., and Baskin, J.M. (2014). Seed Ecology, Biogeography, and Evolution of Dormancy and Germination.
16. Baskin, J., and Baskin, C. (2004). A classification system for seed dormancy. Seed Science Research *14*, 1 - 16.
17. Baskin, J.M., Baskin, C.C., and Li, X. (2000). Taxonomy, anatomy and evolution of physical dormancy in seeds. Plant Species Biology *15*, 139-152.
18. Berger, F., Waites, W.M., and Leifert, C. (1994). An improved surface disinfection method for shoot expiants from Iris rhizomes infected with bacterial soft rot (Erwinia carotovora subsp, carotovora). Journal of Horticultural Science *69*, 491-494.
19. Blumenthal, A., Lerner, H.R., Werker, E., and Poljakoff-Mayber, A. (1986). Germination Preventing Mechanisms in Iris Seeds. Annals of Botany *58*, 551-561.
20. Boltenkov, E.V., Mironova, L.N., and Zarembo, E.V. (2007). Effect of phytohormones on plant regeneration in callus culture of Iris ensata Thunb. Biology Bulletin *34*, 446.
21. Bridgen, M. (1994). A review of plant embryo culture. Hortscience *29*, 1243-1246.
22. Bridgen, M., Houtven, W., and Eeckhaut, T. (2018). Plant Tissue Culture Techniques for Breeding. In, pp. 127-144.
23. Carta, A., Hanson, S., and Müller, J.V. (2016). Plant regeneration from seeds responds to phylogenetic relatedness and local adaptation in Mediterranean Romulea (Iridaceae) species. Ecology and Evolution *6*, 4166-4178.
24. Çöçü, S., Uranbey, S., İpek, A., Khawar, K.M., Sarihan, E.O., Kaya, M.D., Parmaksiz, İ., and Özcan, S. (2004). Adventitious Shoot Regeneration and Micropropagation in Calendula officinalis L. Biologia Plantarum *48*, 449-451.
25. Coelho, N., Gonçalves, S., and Romano, A. (2020). Endemic Plant Species Conservation: Biotechnological Approaches. Plants *9*, 345.
26. Dahl, A.O., R. Schreiner, and G. S. Joachim. (1949). Culture of Iris in vitro. Bull Am Iris Soc, 99-101.
27. Danin, A. (2004). Distribution atlas of plants in the Flora Palaestina area.
28. Davarpanah, S.J., Lahouti, M., and Karimian, R. (2014). Micropropagation of Common Yew Using Embryo Culture. Journal of Applied Biotechnology Reports *1*, 77-80.
29. Doğan, S., and Çağlar, G. (2018). In Vitro Shoot Proliferation via Immature Embryos of Iris kirkwoodiae Chaudhary.

30. Donohue, K., Casas, R.R.d., Burghardt, L., Kovach, K., and Willis, C.G. (2010). Germination, Postgermination Adaptation, and Species Ecological Ranges. Annual Review of Ecology, Evolution, and Systematics *41*, 293-319.
31. Dorman, M., Melnikov, P., Sapir, Y., and Volisa, S. (2009). Factors affecting dormancy of Oncocyclus iris seeds. Israel Journal of Botany *57*, 329-333.
32. Fenner, M., and Thompson, K. (2005). The ecology of seeds.
33. Finch-Savage, W., and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. The New phytologist *171 3*, 501-523.
34. Fu, Z., Tan, D.-Y., Baskin, J., and Baskin, C. (2013). Seed dormancy and germination of the subalpine geophyte Crocus alatavicus (Iridaceae). Australian Journal of Botany *61*, 376.
35. Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research *50*, 151-158.
36. George, E., Hall, M., and De Klerk, G.-J. (2008). Plant Propagation by Tissue Culture. In, pp. 283-333.
37. Gilmour, D. (2006). Micropropagation and horticultural potential of native Tasmanian Liliaceae and Iridaceae. PhD thesis, University of Tasmania.
38. Hilhorst, H., Finch-Savage, W., Buitink, J., Bolingue, W., and Leubner, G. (2010). Dormancy in Plant Seeds. In, pp. 43-67.
39. Hussey, G. (1975). Totipotency in Tissue Explants and Callus of Some Members of the Liliaceae, Iridaceae, and Amaryllidaceae. Journal of Experimental Botany *26*, 253-262.
40. IUCN (2016). The IUCN Red List of Threatened Species. Version 2016-1. Available at: www.iucnredlist.org.(Accessed: 30 June 2016).
41. Jéhan, H., Courtois, D., Ehret, C., Lerch, K., and Pétiard, V. (1994). Plant regeneration of Iris pallida Lam. and Iris germanica L. via somatic embryogenesis from leaves, apices and young flowers. Plant Cell Reports *13*, 671-675.
42. Jevremović, S., Jeknić, Z., and Subotić, A. (2013). Micropropagation of Iris sp. Methods Mol Biol *11013*, 291-303.
43. Jevremovic, S., and Radojevic, L. (2002). Plant regeneration from suspension cultures of Iris pumila L. Acta Horticulturae *572*.

44. Kildisheva, O.A., Dixon, K.W., Silveira, F.A.O., Chapman, T., Di Sacco, A., Mondoni, A., Turner, S.R., and Cross, A.T. (2020). Dormancy and germination: making every seed count in restoration. Restoration Ecology *28*, S256-S265.
45. Kyte, L. (2013). Plants from test tubes : an introduction to micropropagation.
46. Laublin, G., Saini, H.S., and Cappadocia, M. (1991). In vitro plant regeneration via somatic embryogenesis from root culture of some rhizomatous irises. Plant Cell, Tissue and Organ Culture *27*, 15-21.
47. Lenz, L.W. (1954). The Endosperm as a Barrier to Intersectional Hybridization in Iris. Aliso: A Journal of Systematic and Floristic Botany: *3*.
48. Lenz, L.W. (1955). Studies in Iris Embryo Culture: I. Germination of Embryos of the Subsection Hexapogon Benth (sect. Regelia sensu Dykes),. Aliso: A Journal of Systematic and Floristic Botany: *3*, 10.
49. Liu, X., Zhang, H., Zhao, Y., Feng, Z., Li, Q., Yang, H.-Q., Luan, S., Li, J., and He, Z.-H. (2013). Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated activation in Arabidopsis. Proceedings of the National Academy of Sciences *110*, 15485-15490.
50. Martin, A.C. (1946). The Comparative Internal Morphology of Seeds. The American Midland Naturalist *36*, 513-660.
51. Meng, L., Xiao, K., Zhao, M.L., and Zhang, G.F. (2009). Technological system of tissue culture and rapid propagation of Iris lactea Pall. var. chinensis (Fisch.) Koidz. B Bot Res *29*, 193-197.
52. Meyer, M.M.J. (1984). In vitro propagation of Siberian iris from flower stalks. HortScience 19(13), 575.
53. Millam, S. (2005). Plant Development and Biotechnology. Edited by R. N. Trigiano and D. J. Gray. Boca Raton, USA: CRC Press (2005), pp. 358, £49.99. ISBN 0-8493-1614-6. Experimental Agriculture *41*, 505-505.
54. Munk, W.J.D., and Schipper, J. (1993). The physiology of flower bulbs: Iris - bulbous and rhizomatous. Elsevier Science Publishers,, 349- 379.
55. Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum *15*, 473-497.
56. Nitsch, J.P., and Nitsch, C. (1969). Haploid Plants from Pollen Grains. Science *163*, 85-87.

57. Okba, M., Baki, P., Khaleel, A., El-Sherei, M., and Salem, M. (2020). Discrimination of common Iris species from Egypt based on their genetic and metabolic profiling. Phytochemical Analysis *32*.
58. Pierik, R.L.M. (1987). In vitro culture of higher plants (Dordrecht; Boston; Hingham, MA: M. Nijhoff ; Distributors for the U.S. and Canada, Kluwer).
59. Randolph, L.F. (1945). Embryo culture of Iris seeds. Plants and Gardens *1*, 241-246.
60. Samarah, N., Qurashi, S.A., Karam, N.S., and Shibli, R.A. (2009). In vivo and in vitro seed germination in black iris: A potential new floricultural crop from Jordan. Acta Horticulturae *813*, 113-120.
61. Sapir, Y. (2016a). Iris atrofusca. . The IUCN Red List of Threatened Species 2016: eT13161435A18610636.
62. Sapir, Y. (2016b). Iris haynei. . The IUCN Red List of Threatened Species 2016: eT13161709A18612375.
63. Sapir, Y., Shmida, A., and Ne'eman, G. (2005). Pollination of Oncocyclus irises (Iris: Iridaceae) by Night-Sheltering Male Bees. Plant biology (Stuttgart, Germany) *7*, 417-424.
64. Shibli, R.A., and Ajlouni, M.M. (2000). Somatic embryogenesis in the endemic black iris. Plant Cell, Tissue and Organ Culture *61*, 15-21.
65. Shimizu, K., Yabuya, T., and Adachi, T. (1996). Plant regeneration from protoplasts of Iris germanica L. Euphytica *89*, 223-227.
66. Shimshi, D. (1967). Raising irises from embryo culture. Teva Va ' Arez, 4.
67. Skoog, F., and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol *11*, 118-130.
68. Tikhomirova, L. (2020). Scientific framework for selecting explants of Iris L. genus for direct shoot regeneration. IOP Conference Series: Earth and Environmental Science *421*, 052014.
69. Uranbey, S. (2011). In vitro bulblet regeneration from immature embryos of Muscari azureum. Archives of Biological Sciences *63*, 209-215.
70. Uzun, S., Ilbas, A., Ipek, A., Arslan, N., and Barpete, S. (2014). Efficient in vitro plant regeneration from immature embryos of endemic Iris sari and I. schachtii. TURKISH JOURNAL OF AGRICULTURE AND FORESTRY *38*, 348-353.
71. Volis, S. (2019). Plant Conservation: The Role of Habitat Restoration.

72. Volis, S., Blecher, M., and Sapir, Y. (2008). Complex ex situ-in situ approach for conservation of endangered plant species and its application to Iris atrofusca of the Northern Negev. BioRisk *3*, 20-23.
73. Volis, S., Blecher, M., and Sapir, Y. (2010). Application of complex conservation strategy to Iris atrofusca of the Northern Negev, Israel. Biodiversity and Conservation *19*, 3157-3169.
74. Volis, S., and Dorman, M. (2019). Effects of soil type, period of burial and moisture levels on the germination of Oncocyclus iris seeds. Plant Ecology *220*, 1021 - 1028.
75. Volis, S., Zhang, Y.-H., Dorman, M., and Blecher, M. (2015). Iris atrofusca genetic and phenotypic variation, the role of habitat-specific selection in this variation structuring, and conservation implications using quasi in situ guidelines. Israel Journal of Plant Sciences.
76. Wang, Y., Jeknj , Z., Ernst, R.C., and Chen, T.H.H. (1999). Efficient Plant Regeneration from Suspension-cultured Cells of Tall Bearded Iris. Hortscience *34*, 730-735.
77. Werckmeister, P. (1936). Über Herstellung und kunstliche Aufzucht von Bastarden der Gattung Iris. . Gartenbauwiss, 500-520.
78. Wilson, C., Padiernos, j., and Sapir, Y. (2016). The royal irises (Iris subg. Iris sect. Oncocyclus): Plastid and low-copy nuclear data contribute to an understanding of their phylogenetic relationships. Taxon *65*, 35.
79. Yildiz, M. (2012). The Prerequisite of the Success in Plant Tissue Culture: High Frequency Shoot Regeneration, Vol Recent Advances in Plant in vitro Culture.

APPENDICES

Appendix 1

Appendix 1 table 1. Basal salts used concentration and Inc.

Basal salts	Concentration	Inc.
Murashige and Skoog (MS)	4,408 g/l	Duchefa Biochemie
Gamborg B5 medium	2.203 g/l	Duchefa Biochemie
Nitsch & Nitsch Basic Medium (NN)	2.18 g/l	Duchefa Biochemie
Quoirin & Lepoivre medium (QL)	3.378 g/l	Duchefa Biochemie

Appendix 1 table 2. PGR used and their solvents

PGR	Solvent	Diluents	Working Conc. (mg/L)	Inc.
Kinetin(KN)	KOH	Water	50 g KN dissolved in 1 L. 0.1-5.0	Sigma-Aldrich
6-Benzylaminopurine (BA)	NaOH	Water	0.1-5.0	Sigma-Aldrich
Indole-3-butyric acid (IBA)	EtOH/NaOH	Water	0.1-10.0	Sigma-Aldrich
1-Naphthaleneacetic acid (NAA)	NaOH	Water	0.1-10.0	Sigma-Aldrich

Appendix 1 table 3. Composition of Randolph's nutrient medium (a mix of solution A and solution B)

	Composition	**g/L**
Solution A	Calcium Nitrate — $Ca(NO_2)_2 = -4H_2O$	23.6 gms.
	Potassium Nitrate — KNO_2	8.5 gms.
	Potassium Chloride — KCl	6.5 gms.
	Distilled Water	500 ml.
Solution B	Ferrous Sulfate — $FeSO_4 \cdot 7H_2O$	0.2 gms.
	Calgon — $(NaPO_3)_n$	1.0 gms.
	Magnesium Sulfate — $MgSO_4 \cdot 7H_2O$	3.6 gms.
	Distilled Water	500 ml.

Appendix 1 table 4. Tissue culture laboratory instruments and equipment

Laboratory instruments and equipment	
Beakers	pH meter
Test tubes	Laminar flow hood
Pippets	Balance scale
Jars	Magnetic stirrer
Petri dish	Microwave
Graduated cylinder	Different size forceps
Refrigerator	Scalpel blade scalpel holders
Containers	Freezer
Flasks	Bunsen burner

Appendix 2

Appendix 2 table 1. Analysis of variance (ANOVA) for Cytokinin experiments on Ramon (RM) and Faqoua' (Fq) performed via SPSS software:

One way ANOVA, Data from December:

Descriptives									
						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Number of Multiplication(4weeks)	A	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	B	6	.1667	.40825	.16667	-.2618-	.5951	.00	1.00
	C	9	1.4444	2.35112	.78371	-.3628-	3.2517	.00	6.00
	D	8	.5000	1.06904	.37796	-.3937-	1.3937	.00	3.00
	E	8	.1250	.35355	.12500	-.1706-	.4206	.00	1.00
	F	7	.5714	1.13389	.42857	-.4772-	1.6201	.00	3.00
	Total	44	.5227	1.30275	.19640	.1267	.9188	.00	6.00
Number of shoots(4weeks)	A	6	3.0000	2.52982	1.03280	.3451	5.6549	1.00	8.00
	B	6	2.8333	.75277	.30732	2.0433	3.6233	2.00	4.00
	C	9	5.8889	8.32833	2.77611	-.5128-	12.2906	.00	24.00
	D	8	3.3750	2.66927	.94373	1.1434	5.6066	.00	9.00
	E	8	2.3750	1.30247	.46049	1.2861	3.4639	1.00	5.00
	F	7	2.1429	.89974	.34007	1.3107	2.9750	1.00	3.00
	T	44	3.38	4.13277	.6230	2.1299	4.6428	.00	24.00

	otal		64		4				
Number of Roots(4weeks)	A	6	1.5000	2.25832	.92195	-.8700-	3.8700	.00	6.00
	B	6	1.6667	1.36626	.55777	.2329	3.1005	.00	3.00
	C	9	1.1111	1.36423	.45474	.0625	2.1597	.00	3.00
	D	8	.3750	.51755	.18298	-.0577-	.8077	.00	1.00
	E	8	.6250	.74402	.26305	.0030	1.2470	.00	2.00
	F	7	.7143	1.11270	.42056	-.3148-	1.7434	.00	3.00
	Total	44	.9545	1.29318	.19495	.5614	1.3477	.00	6.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Number of Multiplication(4weeks)	Between Groups	11.332	5	2.266	1.397	.247
	Within Groups	61.645	38	1.622		
	Total	72.977	43			
Number of shoots(4weeks)	Between Groups	78.102	5	15.620	.904	.488
	Within Groups	656.329	38	17.272		
	Total	734.432	43			
Number of Roots(4weeks)	Between Groups	9.008	5	1.802	1.088	.382
	Within Groups	62.901	38	1.655		
	Total	71.909	43			

Multiple Comparisons

LSD

Dependent Variable	(I) Trial	(J) Trial	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Number of	A	B	-.16667-	.73535	.822	-1.6553-	1.3220

Multiplication(4weeks)		**C**	**-1.44444-***	**.67128**	**.038**	-2.8034-	-.0855-
		D	-.50000-	.68786	.472	-1.8925-	.8925
		E	-.12500-	.68786	.857	-1.5175-	1.2675
		F	-.57143-	.70860	.425	-2.0059-	.8631
	B	A	.16667	.73535	.822	-1.3220-	1.6553
		C	-1.27778-	.67128	.065	-2.6367-	.0812
		D	-.33333-	.68786	.631	-1.7258-	1.0592
		E	.04167	.68786	.952	-1.3508-	1.4342
		F	-.40476-	.70860	.571	-1.8393-	1.0297
	C	A	1.44444*	.67128	.038	.0855	2.8034
		B	1.27778	.67128	.065	-.0812-	2.6367
		D	.94444	.61889	.135	-.3084-	2.1973
		E	1.31944*	.61889	.040	.0666	2.5723
		F	.87302	.64187	.182	-.4264-	2.1724
	D	A	.50000	.68786	.472	-.8925-	1.8925
		B	.33333	.68786	.631	-1.0592-	1.7258
		C	-.94444-	.61889	.135	-2.1973-	.3084
		E	.37500	.63683	.559	-.9142-	1.6642
		F	-.07143-	.65919	.914	-1.4059-	1.2630
	E	A	.12500	.68786	.857	-1.2675-	1.5175
		B	-.04167-	.68786	.952	-1.4342-	1.3508
		C	-1.31944-*	.61889	.040	-2.5723-	-.0666-
		D	-.37500-	.63683	.559	-1.6642-	.9142
		F	-.44643-	.65919	.502	-1.7809-	.8880
	F	A	.57143	.70860	.425	-.8631-	2.0059
		B	.40476	.70860	.571	-1.0297-	1.8393
		C	-.87302-	.64187	.182	-2.1724-	.4264
		D	.07143	.65919	.914	-1.2630-	1.4059
		E	.44643	.65919	.502	-.8880-	1.7809
Number of shoots(4weeks)	A	B	.16667	2.39943	.945	-4.6907-	5.0241
		C	-2.88889-	2.19037	.195	-7.3231-	1.5453
		D	-.37500-	2.24446	.868	-4.9187-	4.1687
		E	.62500	2.24446	.782	-3.9187-	5.1687
		F	.85714	2.31215	.713	-3.8236-	5.5378

	B	A	-.16667-	2.39943	.945	-5.0241-	4.6907
		C	-3.05556-	2.19037	.171	-7.4897-	1.3786
		D	-.54167-	2.24446	.811	-5.0853-	4.0020
		E	.45833	2.24446	.839	-4.0853-	5.0020
		F	.69048	2.31215	.767	-3.9902-	5.3712
	C	A	2.88889	2.19037	.195	-1.5453-	7.3231
		B	3.05556	2.19037	.171	-1.3786-	7.4897
		D	2.51389	2.01942	.221	-1.5742-	6.6020
		E	3.51389	2.01942	.090	-.5742-	7.6020
		F	3.74603	2.09440	.082	-.4939-	7.9859
	D	A	.37500	2.24446	.868	-4.1687-	4.9187
		B	.54167	2.24446	.811	-4.0020-	5.0853
		C	-2.51389-	2.01942	.221	-6.6020-	1.5742
		E	1.00000	2.07797	.633	-3.2066-	5.2066
		F	1.23214	2.15090	.570	-3.1221-	5.5864
	E	A	-.62500-	2.24446	.782	-5.1687-	3.9187
		B	-.45833-	2.24446	.839	-5.0020-	4.0853
		C	-3.51389-	2.01942	.090	-7.6020-	.5742
		D	-1.00000-	2.07797	.633	-5.2066-	3.2066
		F	.23214	2.1509	.915	-4.1221-	4.5864

				0			
	F	A	-.85714-	2.3121 5	.713	-5.5378-	3.8236
		B	-.69048-	2.3121 5	.767	-5.3712-	3.9902
		C	-3.74603-	2.0944 0	.082	-7.9859-	.4939
		D	-1.23214-	2.1509 0	.570	-5.5864-	3.1221
		E	-.23214-	2.1509 0	.915	-4.5864-	4.1221
Number of Roots(4weeks)	A	B	-.16667-	.74281	.824	-1.6704-	1.3371
		C	.38889	.67809	.570	-.9838-	1.7616
		D	1.12500	.69483	.114	-.2816-	2.5316
		E	.87500	.69483	.216	-.5316-	2.2816
		F	.78571	.71579	.279	-.6633-	2.2347
	B	A	.16667	.74281	.824	-1.3371-	1.6704
		C	.55556	.67809	.418	-.8172-	1.9283
		D	1.29167	.69483	.071	-.1149-	2.6983
		E	1.04167	.69483	.142	-.3649-	2.4483
		F	.95238	.71579	.191	-.4967-	2.4014
	C	A	-.38889-	.67809	.570	-1.7616-	.9838
		B	-.55556-	.67809	.418	-1.9283-	.8172
		D	.73611	.62516	.246	-.5295-	2.0017
		E	.48611	.62516	.442	-.7795-	1.7517
		F	.39683	.64837	.544	-.9157-	1.7094
	D	A	-1.12500-	.69483	.114	-2.5316-	.2816
		B	-1.29167-	.69483	.071	-2.6983-	.1149
		C	-.73611-	.62516	.246	-2.0017-	.5295
		E	-.25000-	.64329	.700	-1.5523-	1.0523
		F	-.33929-	.66587	.613	-1.6873-	1.0087
	E	A	-.87500-	.69483	.216	-2.2816-	.5316
		B	-1.04167-	.69483	.142	-2.4483-	.3649
		C	-.48611-	.62516	.442	-1.7517-	.7795
		D	.25000	.64329	.700	-1.0523-	1.5523
		F	-.08929-	.66587	.894	-1.4373-	1.2587
	F	A	-.78571-	.71579	.279	-2.2347-	.6633
		B	-.95238-	.71579	.191	-2.4014-	.4967
		C	-.39683-	.64837	.544	-1.7094-	.9157

		D	.33929	.66587	.613	-1.0087-	1.6873
		E	.08929	.66587	.894	-1.2587-	1.4373
*. The mean difference is significant at the 0.05 level.							

Appendix 2 table2. Analysis of variance (ANOVA) for Cytokinin experiments on Ramon (RM) and Faqoua' (Fq) performed via SPSS software:

One way ANOVA, Data from February:

Descriptives									
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Number of Multiplication(8weeks)	A	6	.3333	.81650	.33333	-.5235-	1.1902	.00	2.00
	B	6	2.0000	4.89898	2.00000	-3.1412-	7.1412	.00	12.00
	C	9	2.1111	3.88730	1.29577	-.8769-	5.0992	.00	11.00
	D	8	.7500	1.38873	.49099	-.4110-	1.9110	.00	3.00
	E	8	.2500	.70711	.25000	-.3412-	.8412	.00	2.00

	F	7	1.2857	3.40168	1.28571	-1.8603-	4.4317	.00	9.00
	Total	44	1.1364	2.87403	.43328	.2626	2.0101	.00	12.00
Number of shoots(8 weeks)	A	6	4.3333	1.96638	.80277	2.2697	6.3969	3.00	8.00
	B	6	11.5000	16.92040	6.90773	-6.2569-	29.2569	4.00	46.00
	C	9	6.4444	10.51322	3.50441	-1.6367-	14.5256	.00	33.00
	D	8	5.5000	7.63451	2.69921	-.8826-	11.8826	.00	22.00
	E	8	3.0000	3.70328	1.30931	-.0960-	6.0960	.00	10.00
	F	7	9.5714	16.21581	6.12900	-5.4257-	24.5685	.00	46.00
	Total	44	6.5455	10.52893	1.58730	3.3444	9.7465	.00	46.00
Number of Roots(8weeks)	A	6	5.0000	3.89872	1.59164	.9085	9.0915	2.00	10.00
	B	6	6.6667	9.64711	3.93841	-3.4574-	16.7907	1.00	26.00
	C	9	4.2222	7.10243	2.36748	-1.2372-	9.6816	.00	22.00
	D	8	.7500	1.03510	.36596	-.1154-	1.6154	.00	3.00
	E	8	.7500	1.16496	.41188	-.2239-	1.7239	.00	3.00
	F	7	.8571	1.46385	.55328	-.4967-	2.2110	.00	3.00
	Total	44	2.8636	5.29410	.79812	1.2541	4.4732	.00	26.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Number of Multiplication(8weeks)	Between Groups	24.531	5	4.906	.564	.727
	Within	330.651	38	8.701		

	Groups					
	Total	355.182	43			
Number of shoots(8 weeks)	Between Groups	350.139	5	70.028	.602	.698
	Within Groups	4416.770	38	116.231		
	Total	4766.909	43			
Number of Roots(8 weeks)	Between Groups	230.436	5	46.087	1.797	.137
	Within Groups	974.746	38	25.651		
	Total	1205.182	43			

Multiple Comparisons

LSD

Dependent Variable	(I) treatement	(J) treatement	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Number of Multiplication(8 weeks)	A	B	-1.66667-	1.70307	.334	-5.1144-	1.7810
		C	-1.77778-	1.55468	.260	-4.9251-	1.3695
		D	-.41667-	1.59308	.795	-3.6417-	2.8083
		E	.08333	1.59308	.959	-3.1417-	3.3083
		F	-.95238-	1.64112	.565	-4.2747-	2.3699
	B	A	1.66667	1.70307	.334	-1.7810-	5.1144
		C	-.11111-	1.55468	.943	-3.2584-	3.0362
		D	1.25000	1.59308	.438	-1.9750-	4.4750
		E	1.75000	1.59308	.279	-1.4750-	4.9750
		F	.71429	1.64112	.666	-2.6080-	4.0366

	C	A	1.77778	1.55468	.260	-1.3695-	4.9251
		B	.11111	1.55468	.943	-3.0362-	3.2584
		D	1.36111	1.43335	.348	-1.5405-	4.2628
		E	1.86111	1.43335	.202	-1.0405-	4.7628
		F	.82540	1.48656	.582	-2.1840-	3.8348
	D	A	.41667	1.59308	.795	-2.8083-	3.6417
		B	-1.25000-	1.59308	.438	-4.4750-	1.9750
		C	-1.36111-	1.43335	.348	-4.2628-	1.5405
		E	.50000	1.47490	.736	-2.4858-	3.4858
		F	-.53571-	1.52667	.728	-3.6263-	2.5549
	E	A	-.08333-	1.59308	.959	-3.3083-	3.1417
		B	-1.75000-	1.59308	.279	-4.9750-	1.4750
		C	-1.86111-	1.43335	.202	-4.7628-	1.0405
		D	-.50000-	1.47490	.736	-3.4858-	2.4858
		F	-1.03571-	1.52667	.502	-4.1263-	2.0549
	F	A	.95238	1.64112	.565	-2.3699-	4.2747
		B	-.71429-	1.64112	.666	-4.0366-	2.6080
		C	-.82540-	1.48656	.582	-3.8348-	2.1840
		D	.53571	1.52667	.728	-2.5549-	3.6263
		E	1.03571	1.526	.502	-2.0549-	4.1263

				67			
Number of shoots(8 weeks)	A	B	-7.16667-	6.22444	.257	-19.7674-	5.4340
		C	-2.11111-	5.68211	.712	-13.6139-	9.3917
		D	-1.16667-	5.82243	.842	-12.9536-	10.6202
		E	1.33333	5.82243	.820	-10.4536-	13.1202
		F	-5.23810-	5.99802	.388	-17.3804-	6.9043
	B	A	7.16667	6.22444	.257	-5.4340-	19.7674
		C	5.05556	5.68211	.379	-6.4473-	16.5584
		D	6.00000	5.82243	.309	-5.7869-	17.7869
		E	8.50000	5.82243	.153	-3.2869-	20.2869
		F	1.92857	5.99802	.750	-10.2138-	14.0709
	C	A	2.11111	5.68211	.712	-9.3917-	13.6139
		B	-5.05556-	5.68211	.379	-16.5584-	6.4473
		D	.94444	5.23864	.858	-9.6606-	11.5495
		E	3.44444	5.23864	.515	-7.1606-	14.0495
		F	-3.12698-	5.43313	.568	-14.1258-	7.8718
	D	A	1.16667	5.82243	.842	-10.6202-	12.9536
		B	-6.00000-	5.82243	.309	-17.7869-	5.7869
		C	-.94444-	5.23864	.858	-11.5495-	9.6606
		E	2.50000	5.39052	.645	-8.4125-	13.4125

		F	-4.07143-	5.579 72	.470	-15.3670-	7.2241
	E	A	-1.33333-	5.822 43	.820	-13.1202-	10.4536
		B	-8.50000-	5.822 43	.153	-20.2869-	3.2869
		C	-3.44444-	5.238 64	.515	-14.0495-	7.1606
		D	-2.50000-	5.390 52	.645	-13.4125-	8.4125
		F	-6.57143-	5.579 72	.246	-17.8670-	4.7241
	F	A	5.23810	5.998 02	.388	-6.9043-	17.3804
		B	-1.92857-	5.998 02	.750	-14.0709-	10.2138
		C	3.12698	5.433 13	.568	-7.8718-	14.1258
		D	4.07143	5.579 72	.470	-7.2241-	15.3670
		E	6.57143	5.579 72	.246	-4.7241-	17.8670
Number of Roots(8weeks)	A	B	-1.66667-	2.924 11	.572	-7.5862-	4.2529
		C	.77778	2.669 33	.772	-4.6260-	6.1816
		D	4.25000	2.735 25	.129	-1.2872-	9.7872
		E	4.25000	2.735 25	.129	-1.2872-	9.7872
		F	4.14286	2.817 74	.150	-1.5614-	9.8471
	B	A	1.66667	2.924 11	.572	-4.2529-	7.5862
		C	2.44444	2.669 33	.366	-2.9593-	7.8482
		D	5.91667*	2.735 25	.037	.3794	11.4539
		E	5.91667*	2.735	.037	.3794	11.4539

				25			
		F	5.80952*	2.817 74	.046	.1053	11.5137
	C	A	-.77778-	2.669 33	.772	-6.1816-	4.6260
		B	-2.44444-	2.669 33	.366	-7.8482-	2.9593
		D	3.47222	2.461 00	.166	-1.5098-	8.4543
		E	3.47222	2.461 00	.166	-1.5098-	8.4543
		F	3.36508	2.552 37	.195	-1.8019-	8.5321
	D	A	-4.25000-	2.735 25	.129	-9.7872-	1.2872
		B	-5.91667-*	2.735 25	.037	-11.4539-	-.3794-
		C	-3.47222-	2.461 00	.166	-8.4543-	1.5098
		E	.00000	2.532 35	1.00 0	-5.1265-	5.1265
		F	-.10714-	2.621 23	.968	-5.4136-	5.1993
	E	A	-4.25000-	2.735 25	.129	-9.7872-	1.2872
		B	-5.91667-*	2.735 25	.037	-11.4539-	-.3794-
		C	-3.47222-	2.461 00	.166	-8.4543-	1.5098
		D	.00000	2.532 35	1.00 0	-5.1265-	5.1265
		F	-.10714-	2.621 23	.968	-5.4136-	5.1993
	F	A	-4.14286-	2.817 74	.150	-9.8471-	1.5614
		B	-5.80952-*	2.817 74	.046	-11.5137-	-.1053-
		C	-3.36508-	2.552 37	.195	-8.5321-	1.8019

		D	.10714	2.621 23	.968	-5.1993-	5.4136
		E	.10714	2.621 23	.968	-5.1993-	5.4136
*. The mean difference is significant at the 0.05 level.							

Appendix 2 table 3. Comparison of shoot length of RM population treated with different cytokinin concentrations (Chi-square of shoot lengths, 4 weeks):

Treatment * Length of Shoot(4weeks) Crosstabulation							
			Length of Shoot(4 weeks)				Total
			.00	Small	Medium	Large	
Treatment	A	Count	0	0	2	4	6
		% within Treatment	0.0%	0.0%	33.3%	66.7%	100.0%
	B	Count	0	1	1	4	6
		% within Treatment	0.0%	16.7%	16.7%	66.7%	100.0%
	C	Count	2	0	1	6	9
		% within Treatment	22.2%	0.0%	11.1%	66.7%	100.0%
	D	Count	1	0	3	4	8
		% within Treatment	12.5%	0.0%	37.5%	50.0%	100.0%
	E	Count	0	1	3	4	8
		% within Treatment	0.0%	12.5%	37.5%	50.0%	100.0%
	F	Count	0	0	2	5	7
		% within Treatment	0.0%	0.0%	28.6%	71.4%	100.0%

Total	Count	3	2	12	27	44
	% within Treatment	6.8%	4.5%	27.3%	61.4%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	12.125[a]	15	.670
Likelihood Ratio	13.152	15	.591
Linear-by-Linear Association	.001	1	.971
N of Valid Cases	44		

a. 23 cells (95.8%) have expected count less than 5. The minimum expected count is .27.

Appendix 2 table 4. Comparison of shoot length of RM population treated with different cytokinin concentrations (Chi-square of shoot lengths, 8 weeks):

Trial * Length of Shoot(February) Crosstabulation

			Length of Shoot(8 weeks)		
			Medium	Large	Total
Trial	A	Count	6	0	6
		% within Trial	100.0%	0.0%	100.0%
	B	Count	0	6	6
		% within Trial	0.0%	100.0%	100.0%
	C	Count	1	6	7
		% within Trial	14.3%	85.7%	100.0%
	D	Count	0	6	6
		% within Trial	0.0%	100.0%	100.0%
	E	Count	0	5	5
		% within Trial	0.0%	100.0%	100.0%
	F	Count	0	6	6
		% within Trial	0.0%	100.0%	100.0%
Total		Count	7	29	36
		% within	19.4%	80.6%	100.0%

	Trial			

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	30.528[a]	5	.000
Likelihood Ratio	29.726	5	.000
Linear-by-Linear Association	13.775	1	.000
N of Valid Cases	36		

a. 11 cells (91.7%) have expected count less than 5. The minimum expected count is .97.

Appendix 2 table 5. Comparison of root diameter of RM population treated with different cytokinin concentrations (Chi-square of root diameter, 8 weeks):

Crosstab

			Root Diameter(December)				
			small	medium	Thick	Very Thick	Total
Trial	A	Count	0	0	3	1	4
		% within Trial	0.0%	0.0%	75.0%	25.0%	100.0%
	B	Count	0	0	4	0	4
		% within Trial	0.0%	0.0%	100.0%	0.0%	100.0%
	C	Count	1	2	1	0	4
		% within Trial	25.0%	50.0%	25.0%	0.0%	100.0%
	D	Count	1	1	1	0	3
		% within Trial	33.3%	33.3%	33.3%	0.0%	100.0%
	E	Count	1	0	2	0	3
		% within Trial	33.3%	0.0%	66.7%	0.0%	100.0%
	F	Count	0	0	3	0	3
		% within Trial	0.0%	0.0%	100.0%	0.0%	100.0%
Total		Count	3	3	14	1	21
		% within Trial	14.3%	14.3%	66.7%	4.8%	100.0%

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	16.750[a]	15	.334
Likelihood Ratio	17.566	15	.286
Linear-by-Linear Association	1.155	1	.283
N of Valid Cases	21		
a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .14.			

Appendix 2 table 6. Comparison of root diameter of RM population treated with different cytokinin concentrations (Chi-square of root diameter, 8 weeks):

Trial * Root Diameter(February) Crosstabulation							
			Root Diameter(February)				
			small	meduim	Thick	Very Thick	Total
Trial	A	Count	3	0	1	1	5
		% within Trial	60.0%	0.0%	20.0%	20.0%	100.0%
	B	Count	1	0	3	0	4
		% within Trial	25.0%	0.0%	75.0%	0.0%	100.0%
	C	Count	6	0	0	1	7
		% within Trial	85.7%	0.0%	0.0%	14.3%	100.0%
	D	Count	1	2	0	0	3
		% within Trial	33.3%	66.7%	0.0%	0.0%	100.0%
	E	Count	2	0	3	0	5
		% within Trial	40.0%	0.0%	60.0%	0.0%	100.0%
	F	Count	2	0	3	0	5
		% within Trial	40.0%	0.0%	60.0%	0.0%	100.0%
Total		Count	15	2	10	2	29
		% within	51.7%	6.9%	34.5%	6.9%	100.0%

	Trial					

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	30.494[a]	15	.010
Likelihood Ratio	25.442	15	.044
Linear-by-Linear Association	.061	1	.804
N of Valid Cases	29		
a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .21.			

Appendix 2 table 7. Analysis of variance (ANOVA) for auxin experiments on Ramon (RM) and Faqoua' (Fq) performed via SPSS software: (One way ANOVA, Data from December):

The below table describes the Descriptive analysis of the Number of Roots, according to the trial, on December 2020 and February 2021.

Descriptives									
						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Root Number in December	A'	10	.3000	.67495	.21344	-.1828-	.7828	.00	2.00
	B'	10	.8000	1.31656	.41633	-.1418-	1.7418	.00	4.00
	C'	9	1.0000	1.73205	.57735	-.3314-	2.3314	.00	5.00
	D'	7	1.0000	1.29099	.48795	-.1940-	2.1940	.00	3.00
	E'	8	1.6250	1.40789	.49776	.4480	2.8020	.00	4.00
	F'	6	.5000	1.22474	.50000	-.7853-	1.7853	.00	3.00
	Total	50	.8600	1.30946	.18519	.4879	1.2321	.00	5.00
Root Number in	A'	10	1.10	1.79196	.566	-.1819-	2.3819	.00	4.00

February			00		67				
	B'	10	3.50 00	4.47834	1.41 618	.2964	6.7036	.00	11.00
	C'	9	1.88 89	3.21887	1.07 296	-.5854-	4.3631	.00	9.00
	D'	7	1.71 43	2.36039	.892 14	-.4687-	3.8973	.00	6.00
	E'	8	3.62 50	3.58319	1.26 685	.6294	6.6206	.00	10.00
	F'	6	1.16 67	2.85774	1.16 667	-1.8323-	4.1657	.00	7.00
	T ot al	50	2.22 00	3.22800	.456 51	1.3026	3.1374	.00	11.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Root Number in December	Between Groups	8.945	5	1.789	1.048	.402
	Within Groups	75.075	44	1.706		
	Total	84.020	49			
Root Number in February	Between Groups	54.154	5	10.831	1.044	.404
	Within Groups	456.426	44	10.373		
	Total	510.580	49			

Multiple Comparisons

LSD

Dependent Variable	(I) trial	(J) trial	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Root Number in December	A'	B'	-.50000-	.58417	.397	-1.6773-	.6773
		C'	-.70000-	.60017	.250	-1.9096-	.5096
		D'	-.70000-	.64372	.283	-1.9973-	.5973
		E'	-1.32500-*	.61960	.038	-2.5737-	-.0763-

		F'	-.20000-	.67454	.768	-1.5594-	1.1594
	B'	A'	.50000	.58417	.397	-.6773-	1.6773
		C'	-.20000-	.60017	.741	-1.4096-	1.0096
		D'	-.20000-	.64372	.758	-1.4973-	1.0973
		E'	-.82500-	.61960	.190	-2.0737-	.4237
		F'	.30000	.67454	.659	-1.0594-	1.6594
	C'	A'	.70000	.60017	.250	-.5096-	1.9096
		B'	.20000	.60017	.741	-1.0096-	1.4096
		D'	.00000	.65828	1.000	-1.3267-	1.3267
		E'	-.62500-	.63472	.330	-1.9042-	.6542
		F'	.50000	.68845	.472	-.8875-	1.8875
	D'	A'	.70000	.64372	.283	-.5973-	1.9973
		B'	.20000	.64372	.758	-1.0973-	1.4973
		C'	.00000	.65828	1.000	-1.3267-	1.3267
		E'	-.62500-	.67604	.360	-1.9875-	.7375
		F'	.50000	.72672	.495	-.9646-	1.9646
	E'	A'	1.32500*	.61960	.038	.0763	2.5737
		B'	.82500	.61960	.190	-.4237-	2.0737
		C'	.62500	.63472	.330	-.6542-	1.9042
		D'	.62500	.67604	.360	-.7375-	1.9875
		F'	1.12500	.70545	.118	-.2967-	2.5467
	F'	A'	.20000	.67454	.768	-1.1594-	1.5594
		B'	-.30000-	.67454	.659	-1.6594-	1.0594
		C'	-.50000-	.68845	.472	-1.8875-	.8875
		D'	-.50000-	.72672	.495	-1.9646-	.9646
		E'	-1.12500-	.70545	.118	-2.5467-	.2967
Root Number in February	A'	B'	-2.40000-	1.44037	.103	-5.3029-	.5029
		C'	-.78889-	1.47984	.597	-3.7713-	2.1935
		D'	-.61429-	1.58721	.701	-3.8131-	2.5845
		E'	-2.52500-	1.52774	.105	-5.6040-	.5540
		F'	-.06667-	1.66319	.968	-3.4186-	3.2853
	B'	A'	2.40000	1.44037	.103	-.5029-	5.3029
		C'	1.61111	1.4798	.282	-1.3713-	4.5935

				4			
		D'	1.78571	1.5872 1	.267	-1.4131-	4.9845
		E'	-.12500-	1.5277 4	.935	-3.2040-	2.9540
		F'	2.33333	1.6631 9	.168	-1.0186-	5.6853
	C'	A'	.78889	1.4798 4	.597	-2.1935-	3.7713
		B'	-1.61111-	1.4798 4	.282	-4.5935-	1.3713
		D'	.17460	1.6231 1	.915	-3.0966-	3.4458
		E'	-1.73611-	1.5650 1	.273	-4.8902-	1.4180
		F'	.72222	1.6974 9	.673	-2.6988-	4.1433
	D'	A'	.61429	1.5872 1	.701	-2.5845-	3.8131
		B'	-1.78571-	1.5872 1	.267	-4.9845-	1.4131
		C'	-.17460-	1.6231 1	.915	-3.4458-	3.0966
		E'	-1.91071-	1.6669 0	.258	-5.2701-	1.4487
		F'	.54762	1.7918 7	.761	-3.0637-	4.1589
	E'	A'	2.52500	1.5277 4	.105	-.5540-	5.6040
		B'	.12500	1.5277 4	.935	-2.9540-	3.2040
		C'	1.73611	1.5650 1	.273	-1.4180-	4.8902
		D'	1.91071	1.6669 0	.258	-1.4487-	5.2701
		F'	2.45833	1.7394 1	.165	-1.0472-	5.9639
	F'	A'	.06667	1.6631 9	.968	-3.2853-	3.4186

		B'	-2.33333-	1.66319	.168	-5.6853-	1.0186
		C'	-.72222-	1.69749	.673	-4.1433-	2.6988
		D'	-.54762-	1.79187	.761	-4.1589-	3.0637
		E'	-2.45833-	1.73941	.165	-5.9639-	1.0472
*. The mean difference is significant at the 0.05 level.							

Appendix 2 table 8. The basal salt's effect on Faqoua' (Fq) population without plant growth regulators: (one-way ANOVA)

Descriptives									
						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Shoot number after 2 weeks	QL	4	3.0000	3.36650	1.68325	-2.3569-	8.3569	1.00	8.00
	MS	5	1.8000	1.92354	.86023	-.5884-	4.1884	.00	5.00
	B5	5	2.6000	1.14018	.50990	1.1843	4.0157	1.00	4.00
	NN	5	3.0000	2.00000	.89443	.5167	5.4833	1.00	5.00
	Total	19	2.5789	2.03622	.46714	1.5975	3.5604	.00	8.00
Shoot number after 4 weeks	QL	4	3.5000	4.35890	2.17945	-3.4360-	10.4360	1.00	10.00
	MS	5	2.6000	2.60768	1.16619	-.6379-	5.8379	1.00	7.00
	B5	5	3.2000	1.48324	.66332	1.3583	5.0417	1.00	5.00
	NN	5	3.6000	2.96648	1.32665	-.0834-	7.2834	1.00	8.00
	Total	19	3.2105	2.69936	.61928	1.9095	4.5116	1.00	10.00
Shoot number after 6 weeks	QL	4	4.2500	5.85235	2.92617	-5.0624-	13.5624	1.00	13.00

	MS	5	4.6000	2.70185	1.20830	1.2452	7.9548	2.00	8.00
	B5	5	3.2000	1.48324	.66332	1.3583	5.0417	1.00	5.00
	NN	5	4.2000	3.56371	1.59374	-.2249-	8.6249	1.00	8.00
	Total	19	4.0526	3.30780	.75886	2.4583	5.6469	1.00	13.00
Root number after 2 weeks	QL	4	.7500	.50000	.25000	-.0456-	1.5456	.00	1.00
	MS	5	1.8000	1.30384	.58310	.1811	3.4189	.00	3.00
	B5	5	1.2000	1.30384	.58310	-.4189-	2.8189	.00	3.00
	NN	5	6.4000	7.76531	3.47275	-3.2419-	16.0419	1.00	20.00
	Total	19	2.6316	4.43735	1.01800	.4928	4.7703	.00	20.00
Root number after 4 weeks	QL	4	.7500	.50000	.25000	-.0456-	1.5456	.00	1.00
	MS	5	2.8000	1.64317	.73485	.7597	4.8403	1.00	5.00
	B5	5	1.4000	1.51658	.67823	-.4831-	3.2831	.00	3.00
	NN	5	8.0000	9.30054	4.15933	-3.5481-	19.5481	1.00	24.00
	Total	19	3.3684	5.38734	1.23594	.7718	5.9650	.00	24.00
Root number after 6 weeks	QL	4	1.2500	1.25831	.62915	-.7522-	3.2522	.00	3.00
	MS	5	2.8000	1.64317	.73485	.7597	4.8403	1.00	5.00
	B5	5	1.4000	1.51658	.67823	-.4831-	3.2831	.00	3.00
	NN	5	9.4000	9.76217	4.36578	-2.7213-	21.5213	1.00	26.00
	Total	19	3.8421	5.88088	1.34917	1.0076	6.6766	.00	26.00

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
Shoot number after 2 weeks	Between Groups	4.632	3	1.544	.331	.803
	Within Groups	70.000	15	4.667		
	Total	74.632	18			
Shoot number after 4 weeks	Between Groups	2.958	3	.986	.115	.950
	Within Groups	128.200	15	8.547		
	Total	131.158	18			
Shoot number after 6 weeks	Between Groups	5.397	3	1.799	.141	.934
	Within Groups	191.550	15	12.770		
	Total	196.947	18			
Root number after 2 weeks	Between Groups	98.871	3	32.957	1.934	.167
	Within Groups	255.550	15	17.037		
	Total	354.421	18			
Root number after 4 weeks	Between Groups	155.671	3	51.890	2.122	.140
	Within Groups	366.750	15	24.450		
	Total	522.421	18			
Root number after 6 weeks	Between Groups	216.576	3	72.192	2.668	.085
	Within Groups	405.950	15	27.063		
	Total	622.526	18			

Multiple Comparisons							
LSD							
			Mean			95% Confidence Interval	
Dependent Variable	(I) symbol	(J) symbol	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Shoot number after 2 weeks	QL	MS	1.20000	1.44914	.421	-1.8888-	4.2888
		B5	.40000	1.44914	.786	-2.6888-	3.4888
		NN	.00000	1.44914	1.000	-3.0888-	3.0888
	MS	QL	-1.20000-	1.44914	.421	-4.2888-	1.8888
		B5	-.80000-	1.36626	.567	-3.7121-	2.1121
		NN	-1.20000-	1.36626	.394	-4.1121-	1.7121
	B5	QL	-.40000-	1.44914	.786	-3.4888-	2.6888

		MS	.80000	1.36626	.567	-2.1121-	3.7121
		NN	-.40000-	1.36626	.774	-3.3121-	2.5121
	NN	QL	.00000	1.44914	1.000	-3.0888-	3.0888
		MS	1.20000	1.36626	.394	-1.7121-	4.1121
		B5	.40000	1.36626	.774	-2.5121-	3.3121
Shoot number after 4 weeks	QL	MS	.90000	1.96112	.653	-3.2800-	5.0800
		B5	.30000	1.96112	.880	-3.8800-	4.4800
		NN	-.10000-	1.96112	.960	-4.2800-	4.0800
	MS	QL	-.90000-	1.96112	.653	-5.0800-	3.2800
		B5	-.60000-	1.84896	.750	-4.5410-	3.3410
		NN	-1.00000-	1.84896	.597	-4.9410-	2.9410
	B5	QL	-.30000-	1.96112	.880	-4.4800-	3.8800
		MS	.60000	1.84896	.750	-3.3410-	4.5410
		NN	-.40000-	1.84896	.832	-4.3410-	3.5410
	NN	QL	.10000	1.96112	.960	-4.0800-	4.2800
		MS	1.00000	1.84896	.597	-2.9410-	4.9410
		B5	.40000	1.84896	.832	-3.5410-	4.3410
Shoot number after 6 weeks	QL	MS	-.35000-	2.39719	.886	-5.4595-	4.7595
		B5	1.05000	2.39719	.668	-4.0595-	6.1595
		NN	.05000	2.39719	.984	-5.0595-	5.1595
	MS	QL	.35000	2.39719	.886	-4.7595-	5.4595
		B5	1.40000	2.26009	.545	-3.4173-	6.2173
		NN	.40000	2.26009	.862	-4.4173-	5.2173
	B5	QL	-1.05000-	2.39719	.668	-6.1595-	4.0595
		MS	-1.40000-	2.26009	.545	-6.2173-	3.4173
		NN	-1.00000-	2.26009	.664	-5.8173-	3.8173
	NN	QL	-.05000-	2.39719	.984	-5.1595-	5.0595
		MS	-.40000-	2.26009	.862	-5.2173-	4.4173
		B5	1.00000	2.26009	.664	-3.8173-	5.8173
Root number after 2 weeks	QL	MS	-1.05000-	2.76884	.710	-6.9517-	4.8517
		B5	-.45000-	2.76884	.873	-6.3517-	5.4517
		NN	-5.65000-	2.76884	.059	-11.5517-	.2517
	MS	QL	1.05000	2.76884	.710	-4.8517-	6.9517
		B5	.60000	2.61049	.821	-4.9641-	6.1641
		NN	-4.60000-	2.61049	.098	-10.1641-	.9641
	B5	QL	.45000	2.76884	.873	-5.4517-	6.3517
		MS	-.60000-	2.61049	.821	-6.1641-	4.9641
		NN	-5.20000-	2.61049	.065	-10.7641-	.3641
	NN	QL	5.65000	2.76884	.059	-.2517-	11.5517
		MS	4.60000	2.61049	.098	-.9641-	10.1641
		B5	5.20000	2.61049	.065	-.3641-	10.7641
Root number after 4 weeks	QL	MS	-2.05000-	3.31700	.546	-9.1200-	5.0200
		B5	-.65000-	3.31700	.847	-7.7200-	6.4200

		NN	-7.25000-*	3.31700	.045	-14.3200-	-.1800-
	MS	QL	2.05000	3.31700	.546	-5.0200-	9.1200
		B5	1.40000	3.12730	.661	-5.2657-	8.0657
		NN	-5.20000-	3.12730	.117	-11.8657-	1.4657
	B5	QL	.65000	3.31700	.847	-6.4200-	7.7200
		MS	-1.40000-	3.12730	.661	-8.0657-	5.2657
		NN	-6.60000-	3.12730	.052	-13.2657-	.0657
	NN	QL	7.25000*	3.31700	.045	.1800	14.3200
		MS	5.20000	3.12730	.117	-1.4657-	11.8657
		B5	6.60000	3.12730	.052	-.0657-	13.2657
Root number after 6 weeks	QL	MS	-1.55000-	3.48977	.663	-8.9883-	5.8883
		B5	-.15000-	3.48977	.966	-7.5883-	7.2883
		NN	-8.15000-*	3.48977	.034	-15.5883-	-.7117-
	MS	QL	1.55000	3.48977	.663	-5.8883-	8.9883
		B5	1.40000	3.29019	.677	-5.6129-	8.4129
		NN	-6.60000-	3.29019	.063	-13.6129-	.4129
	B5	QL	.15000	3.48977	.966	-7.2883-	7.5883
		MS	-1.40000-	3.29019	.677	-8.4129-	5.6129
		NN	-8.00000-*	3.29019	.028	-15.0129-	-.9871-
	NN	QL	8.15000*	3.48977	.034	.7117	15.5883
		MS	6.60000	3.29019	.063	-.4129-	13.6129
		B5	8.00000*	3.29019	.028	.9871	15.0129
*. The mean difference is significant at the 0.05 level.							

Appendix 2 table 9. Comparison between variables from MS culture, (one-way ANOVA):

Descriptive Statistics					
	N	Minimum	Maximum	Mean	Std. Deviation
Multiplication	34	.00	4.00	.5588	1.15971
Shoot Length	36	.00	35.00	9.5417	7.74816
Shoot Number	36	.00	37.00	6.8611	7.69101
Root Number	36	1.00	50.00	8.5556	10.21608
Root Length	36	5.00	41.00	18.9500	10.05179
Callus	36	.00	4.00	.8611	1.22247
Valid N (listwise)	34				