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Factors Affecting *In Vitro* Direct Shoot Regeneration in the Greek Strawberry Tree, *Arbutus andrachne* L.

By

Shahd Aziz Odeh

In Partial Fulfillment of the Requirements for the Degree

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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:

Factors Affecting *In Vitro* Direct Shoot Regeneration in the Greek Strawberry Tree, *Arbutus andrachne* L.

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Factors affecting *in vitro* direct shoot regeneration in the Greek Strawberry Tree, *Arbutus andrachne* L.

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ABSTRACT

Arbutus andrachne L., the Greek Strawberry tree, (Ericaceae) is a small tree distributed naturally throughout many regions in the Mediterranean and southwest Asia. It is also part of the Palestinian flora and known for its medicinal value. Recently populations of *A. andrachne* in Palestine are facing severe genetic erosion due to extensive agricultural and human activities, over-exploitation, overgrazing, habitat fragmentation, and woody cutting by local people. Plant tissue culture offers a powerful alternative for the massive production of many endangered and difficult-to-propagate plants. Direct shoot regeneration aims to produce shoots directly from different explants such as leaves, hypocotyls, cotyledons, and roots. The main objective of this work was to investigate the effect of concentration of different growth regulators and explant type and age on *A. andrachne* direct shoot regeneration. Motherstock plants of *A. andrachne* were initiated from seeds. Seeds were cold stored and were soaked in 5.0 mg/ml GA_3 for seven days followed by culturing on WP media. Old leaves, young leaves, hypocotyls, cotyledons, and roots were cultured on WP media supplemented with different concentrations of TDZ (1.0, 1.5, 2.0 mg/l) and 0.5 mg/l NAA. The cotolydonary explants showed significant differences among media with different TDZ concentrations; media that contains 1.0mg/l TDZ + 0.5 mg/l NAA, or 1.5 mg/l TDZ+ 0.5 mg/l NAA gave the highest shoot regeneration (4.0 shoots/explant,3.9 shoots/explant), while media with 2.0 mg/l TDZ+ 0.5 mg/l NAA gave the least number of shoot regeneration (0.6 shoots/explant). Other explant types showed no significant differences on media with different TDZ concentrations, but old leaves gave the highest regeneration on media with 1.0 mg/l TDZ + 0.5 mg/l NAA (0.4 shoots/explant), young leaves gave the highest regeneration on media with 1.5 mg/l TDZ + 0.5 mg/l NAA (2.3 shoots/explant), and hypocotyls highest response was on media with 1.5 mg/l TDZ + 0.5 mg/l NAA (3.7 shoots/explant), while roots showed no response in all concentrations. Cotyledonary segments and hypocotyl gave the highest number of shoots (4.0 shoots, 3.0 shoots, respectively) on media with 1.0 mg/l TDZ and (3.9 shoots, 3.8 shoots, respectively) on media with 1.5 mg/l TDZ .Young leaves gave highest number of shoots 2.1 shoots on media with 2.0 mg/l while old leaves show very low response and roots did not give any direct shoot regeneration in all PGRs concentrations. At 1.0mg/l TDZ, cotyledons gave the highest proliferation 47.6%, hypocotyls gave 28.6%, then new leaves 42.8%, old leaves and roots showed no response. When TZD level increased to 1.5 mg/l TDZ cotyledons gave 42.9% proliferation, hypocotyls gave 38%, then new leaves 33.3%, old leaves 19% but no response was seen in roots. Whereas in media with 0.5mg/l NAA and 2.0mg/l TDZ, hypocotyls gave responsive by 28.6%, cotyledons gave 9.5% and young leaves gave 9.5%, 4.8% for old leaved and no responsive in roots. The rooting medium of WP supplemented with 15 g/l sucrose and 1.5 mg/l IBA gave 100% responsiveness. This study can be used in genetic transformation of *A. andrachne* tree.

العوامل المؤثرة على تكثير الأشطاء المباشر لنبات القيقب

Arbutus andrachne L. داخل الأنابيب

شهد عزيز عودة

ملخص

شجرة القيقب أو شجرة الفراولة الإغريقية تنتمي إلى عائلة الخلنجيات، تنتشر هذه الشجرة على امتداد منطقة البحر الأبيض المتوسط وجنوبي غربي آسيا. على غرار كثير من نباتات فلسطين الطبية يواجه القيقب خطر الانقراض بسبب تعرضه للعديد من الظروف القاسية والتي تشمل: الممارسات الزراعية والبشرية الخاطئة، الرعي الجائر، الإفراط في استغلال الأراضي الزراعية وقطع الأشجار. يعتبر التكثير الدقيق للنباتات داخل المختبر بديل فعال لتكثير النباتات المهددة بالانقراض. التكثير المباشر داخل المختبر يهدف إلى إنتاج أجيال جديدة وتنميتها مباشرة من خلال استخدام أجزاء مختلفة من نفس النبات. الهدف الأساسي للدراسة الحالية هو اختبار تأثير اختلاف تركيز منظمات النمو المدعمة للوسط الغذائي، واختلاف أجزاء النبات المستخدم في التكثير المباشر للقيقب. لتأسيس النبات داخل المختبر تمت معالجة بذور القيقب بالتبريد لمدة ٧ أيام على درجة حرارة ٤ سيليسيوس وغمسها في GA3 بتركيز ٥.٠ ملغم/مل ثم زراعتها على الوسط الغذائي WP حيث كانت نسبة الإنبات ٩٣%. تمت زراعة أجزاء مختلفة من القيقب المنبت في المختبر على الوسط الغذائي WP المدعم بمنظم النمو (1.0، 1.5، 2.0 ملغم/ لتر) TDZ، بالإضافة إلى ٠.٥ ملغم/ لتر من منظم النمو NAA. الكوتيليدون أعطى اختلاف إحصائي بالنسبة للتراكيز المختلفة من ال TDZ، حيث الوسط الغذائي WP المدعم ب ١.٠ ملغم/لتر TDZ و٠.٥ ملغم/ لتر NAA بالإضافة إلى الوسط الغذائي WP المدعم ب ١.٥ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA أعطوا أكبر عدد من الفروع(٤.٠ و ٣.٩ فرع، بالترتيب) الوسط الغذائي WP المدعم ب ٢.٠ ملغم/ لتر TDZ و ٠.٥ ملغ/لتر NAA أعطى نتائج ضعيفة لم تتجاوز ال ٠.٦ فرع)، بينما الوسط الغذائي الذي لا يحتوي على منظمات النمو لم يعطي أي نتيجة. أجزاء النبات الأخرى لم تظهر اختلافات إحصائية على الوسط الغذائي المدعم بالتراكيز المختلفة، ولكن أعلى عدد فروع نتج من الأوراق القديمة كان على الوسط الغذائي WP المدعم ب ١.٠ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA (٠.٤ فرع)، الأوراق الجديدة أعطت أعلى تفرع (٢.٣ فرع) على الوسط الغذائي WP المدعم ب ١.٥ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA ،أعلى استجابة في الهايبوكوتيل كانت على الوسط الغذائي WP المدعم ب ١.٥ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA (٣.٧فرع)، أما بالنسبة للجذور فكانت النتيجة سلبية. الكوتيليدون والهايبوكوتيل أعطوا أعلى نسبة تفرع (٤.٠فروع، ٣.٠ فروع بالترتيب) على الوسط الغذائي WP المدعم ب ١.٠ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA، و (٣.٩ فرع، ٣.٨ فرع بالترتيب) على الوسط الغذائي WP المدعم ب ١.٥ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA. الأوراق الجديدة أعطت أعلى تفرع (٢.١ فرع) على الوسط الغذائي WP المدعم ب ٢.٠ ملغم/ لتر TDZ و٠.٥ ملغم/ لتر NAA. في حين أن الأوراق القديمة أظهرت استجابة ضعيفة والجذور لم تستجب أبدا على مختلف التراكيز. في الوسط الغذائي WP المدعم ب ١.٠ ملغم/ لتر TDZ أعطت أجزاء الكوتيليدون أعلى نسبة تفرع ٤٧.٦%، الهايبوكوتيل أعطت ٢٨.٦%، الأوراق الجديدة ٤٢.٨%، بينما الأوراق القديمة والجذور لم تعطي نتائج. عند رفع تركيز ال TDZ إلى ١.٥ ملغ/لتر، أعطت الكوتيليدون نسبة تفرع ٤٢.٩%، الهايبوكوتيل أعطت ٣٨%، الأوراق الجديدة ٣٣.٣%، الأوراق القديمة ١٩%، والجذور لم تعطي نتائج. عند تركيز ٢.٠ ملغ/لتر TDZ الكوتيليدون أعطت نسبة تفرع ٩.٥%، الهايبوكوتيل أعطت ٢٨.٦%، الأوراق الجديدة ٩.٥%، الأوراق القديمة ٤.٨%، والجذور لم تعطي نتائج. كما أعطى النبات نسبة تجذير ١٠٠% باستخدام الوسط الغذائي WP بإضافة ١٥ غم سكروز و ١.٥ غم/ لتر من هرمون ال IBA . هذه الدراسة يمكن أن تستخدم في تكثير نبات القيقب في المختبر بأعداد كبيرة وتعديلها جينياً.

DECLARATION

I declare that the Master Thesis entitled "Factors Affecting *in vitro* Direct Shoot Regeneration in *Arbutus andrachne* L." 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 acknowledgment is made in the text.

Shahd Aziz Odeh

Date: January, 2017.

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Shahd Aziz Odeh

Date: January, 2017.

DEDICATION

First and above all, I thank God, the almighty for providing me this opportunity and granting me the capability to proceed successfully.

I would like to dedicate this thesis to my parents for providing me with trustworthy support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis.

And finally, I must express my very profound gratitude to the person who always was there for me, this accomplishment would not have been possible without his supports and encouragements; my lovely husband Alaa', thank you for everything and may Allah give you all the best in return.

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List of abbreviations

Term/Phrase	Abbreviations
And others	*et al.*
Degree Celsius	°C
Gibberellic acid	GA_3
gram	g
Indole-3-butyric acid	IBA
Liter	l
Lloyd & McCown Woody plant medium	WP
Microliter	µl
Micro mole per square meter per second	µmoles/m^2/sec
Milligram	mg
Milliliter	ml
Minutes	min
Molar	M
Naphthalene acetic acid	NAA
percentage	%
Plant growth regulator	PGR
Sterilized Distilled Water	SDW
Thidiazuron	TDZ
Volume per volume	v/v

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Chapter 1

Introduction

Palestine being part of the Eastern Mediterranean region is considered a treasure of natural resources especially plants. Due to varying climate and soil types, there are more than 2600 plant species living in an area of 27000 square km. From this number, there are more than 600 plant species have medicinal or therapeutic uses in traditional medicine (Said *et al.* 2002). Part of this group of wild plants with medicinal value or uses is facing danger of extinction and genetic erosion year after year because of massive collection, grazing pressure and destruction of the natural habitats. A comparison between the floral surveys over the past 20 to 40 years was done by a specialized team from Applied Research Institute of Jerusalem (ARIJ). It was found that up to 636 species growing in Palestine were found endangered of which 90 species are extremely rare (ARIJ, 2014).

Several projects and attempts have focused on conserving the biodiversity and natural resources, mostly at *in situ* scale. On the other hand, *in vitro* culture or (micropropagation) is one of the modern techniques that is highly implemented for conservation and rapid production of plants especially the rare and endangered at very large scale.

1.1 *Arbutus andrachne* L.

1.1.1 Description of the study species.

Arbutus andrachne, also known as "Greek Strawberry Tree", Arabic name “Qyqab or Qatlab”, is a member of the Ericaceae family, distributed along the Mediterranean region and southwestern Asia (Ergun *et al*. 2014). It grows on rocky hills and alkaline soil with low aeration. *A. andrachne* has a smooth and red trunk that peels off in curly strips during summer. It has thick evergreen leaves, white flowers in a bell shape that bloom in spring. Mature fruits are red in color, round, sweet and edible, full of small seeds, and the fruit peel is also edible with a rough surface that softens after ripening in Autumn (Ergun *et al*. 2014)(Figure 1.1). The flowers are hermaphrodite and pollinated by bees, and the plant is self-fertile (Danin, 2005).

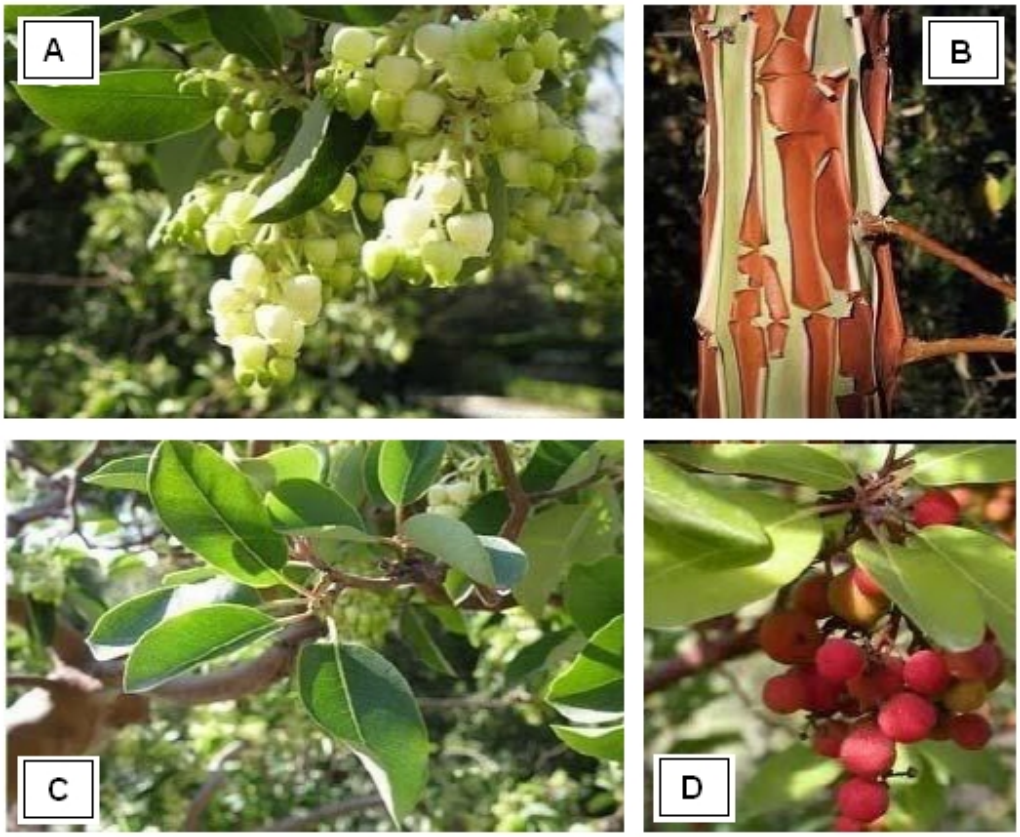

Figure 1.1. *Arbutus andrachne* L. plant parts: (a) flower (b) trunk's peeling bark (c) leaves (d) ripe fruits.

1.1.2 *Arbutus andrachne* in Palestine.

Arbutus andrachne as many other wild plants in Palestine is facing genetic erosion and extinction danger due to several factors including habitat fragmentation, over-exploitation, extensive agricultural and human activities, overgrazing, and wood cutting by local people for heating. (Aljabary *et al.* 2011).

Difficult and low seed germination rates under natural conditions, poor rooting of stem cuttings and slow plant growth are the main propagation problems in *A. Andrachne* (Karam and Al-Salem, 2001). In order to satisfy the demand for *A. andrachne*, more productive propagation methods are needed.

1.1.3 Importance of *Arbutus andrachne* in traditional medicine.

In traditional (folk) medicine *A. andrachne* is being known to have many benefits in curing wounds, treating joints ache, and urinary system ailments. It is also used as an astringent, urinary antiseptic, anticancer agent, and as blood tonic. (Ergun *et al.* 2014)

In study on 51 medicinal plant species in Jordan, *A. andrachne* was found to have the highest level of antioxidant activity (Tawaha *et al.* 2007). Several classes of naturally occurring antioxidants such as phenolic compounds like anthocyanins; gallic acid derivatives, tannins and flavonoids, organic acids; vitamin C, vitamin E, and carotenoids were found in the fruits and leaves (Serçea *et al*. 2010). Moreover, arbutin, monotropeins, unedoside, and catechin were isolated from bark and leaves. Bark, leaves, and fruits also contain triterpenoides, sterols, and lipids (Sakar *et al*. 1991).

1.2 *In Vitro* plant culture and micropropagation.

Many tree species are propagated by traditional vegetative methods like cutting, grafting, and budding. These propagation techniques are sometimes difficult, expensive, and successful rates are very low. The use of plant tissue culture or (micropropagation), which deals with *in vitro* plant propagation, offers a powerful alternative method mainly for massive production of difficult- to- propagate plants (Pierek, 1997).

Plant tissue culture is the aseptic technique used for organs and tissues (explants) which are cultured on nutrient medium under aseptic growing conditions to induce rapid production of new shoots, roots, or even large number of new plantlets (Thrope, 2007).

1.2.1 Micropropagation advantages and disadvantages

Micropropagation provides continuous source of high numbers of plantlets from material of plants (explants) in a reduced time period with low cost. Moreover, cultivated tissues are free of contamination and produce pathogen-free plants. It is not affected by external conditions besides the flexibility in adjusting the influencing factors that affect the regeneration process such as media components, growth regulators' levels, media pH, light, and temperature. Although micropropagation aims to produce genetically identical and uniform plants, genetic changes in plant cells could take place (Srivastava *et al*. 2005; George *et al*. 2008; Saini *et al*. 2010).

On the other hand, the main limitations of the micropropagation methods are that they are labor intensive and advanced skills and specialized mechanization are needed for the implementation (George *et al*. 2008; Chawla, 2009).

1.2.2 Micropropagation phases

In 1974, Murashige was the first to describe micropropagation stages for *in vitro* plant multiplication. He described the process in the following five phases:

- Phase 0: A preparative stage before *in vitro* culture that aims to precondition the starting material (motherstock plants) in order to reduce microbial contamination.
- Phase I: The establishment phase, which involves sterilization of the explant for clean culture and growth.
- Phase II: The multiplication of shoots. This phase is considered the most important step in micropropagation.
- Phase III: Rooting phase, by culturing the proliferated shoots from phase II in specific media with a rooting PGR.
- Phase IV: After roots have become well established, *in vitro* plantlets have to be acclimatized to a normal growing conditions in the greenhouse.

1.2.3 Micropropagation strategies:

In vitro propagation of plants can be achieved by using three different strategies; the simplest and most common is propagation from axillary shoots or buds. This technique is based on the repeated formation of axillary shoots which are then used to initiate new cycles of propagation. Axillary buds are usually present on the axil of leaves and have the potential to differentiate into shoots. In the axillary bud culture, cytokinins added to the growth medium break bud dormancy and stimulate new shoots growth from pre-existing meristems in large numbers. This option is considered the safest and it gives *in vitro* plant shoots with high genetic stability. (George *et al*. 2008; Chawla, 2009; Ngezahayo *et al*. 2014). This method was used for propagation different species of the Ericaceae family such as *Arbutus unedo* (Mereti *et al*. 2002; Gomes *et al*. 2010), *Arbutus xalapensis* (Mackay, 1996), *Elliottia racemosa* (Radcliffe *et al*. 2011), *Gaultheria fragantissima* (Bantawa *et al*. 2011), and *Calluna vulgaris* (Gebhardt and Friedrich 1987).

Unlike axillary shoot proliferation, the other two strategies of plant regeneration are achieved by the induction of adventitious shoots from non meristematic tissues or organs. They are important means of propagation, and have higher chances to get mutant plants. Shoots are directly induced from explants by direct organogenesis, or may involve a callus intermediate formation via indirect organogenesis and somatic embryogenesis (Phillips *et al*. 1995; George *et al*. 2008; Chawla, 2009).

Propagation by organogenesis include indirect adventitious shoot regeneration and indirect somatic embryogenesis. While indirect adventitious shoot organogenesis includes shoot or root formation, somatic embryogenesis indicate the formation of a bipolar structure that contains shoots and roots together which can form a complete plant (Phillips *et al*. 1995; George *et al*. 2008).

The first step in the indirect micropropagation is the formation of actively dividing and unorganized calluses from any tissue explants. Callus induction is highly affected by the exogenous growth regulators present in the culturing medium. Pierik (1997) reported that auxin and cytokinin concentration adjustment was important for callus formation. Generally, inducing callus requires a high auxin level with low or no cytokinin concentrations in the nutrient medium, then at the shoot development stages the auxin level in the medium should be reduced or the concentration of cytokinin might be increased. Explant size, age, and physiological state are additional factors that can affect callus formation (Aljabary, 2011; Tan *et al*. 2011).

Indirect plantlet production from callus has been achieved for numerous ornamental plants such as freesia and pelargonium (George *et al*. 2008), *Portulaca grandiflora* L. (Safdari *et al*. 2010), in addition to a wide variety of woody plants. Somatic embryogenesis for regeneration is a tool widely used in monocotyledon plants such as cereals, date palm, banana, and oil palm (George *et al*. 2008).

The other strategy is direct shoot regeneration. It is an agriculturally important process due to its benefits in clonal plantlet production for large scale propagation, and in genetic transformation studies which introduce a transgene into the genome of various plant species (Kulkarni *et al*. 2000). Moreover, it is useful in the production of polyploid plants (Safdari *et al.* 2010). In direct shoot regeneration, shoots are directly induced from different explants such as leaves, shoot buds, hypocotyls, cotyledons, and roots. The first step in direct shoot regeneration is the appropriate establishment of contamination free explants which will later be cultured on nutrient

medium supplemented with specific exogenous growth regulators. Usually, high cytokinin concentrations in combination with low auxin are critical in shoot induction viability (Pierik, 1997). Literature described that direct shoot regeneration is affected by numerous factors that widely affect the reproducibility of shoot inductions. These factors will be discussed later in this thesis.

Kalanchoe (Khan *et al*. 2006) and African violet (Sunpui *et al*. 2002) are two examples of ornamental plant species that are propagated by direct shoot regeneration. Some woody plants are widely propagated by direct shoot regeneration, such as *Fraxinus americana* (Palla *et al.* 2011), pear (Yousefiara *et al*. 2014), and *Millettia pinnata* (Nagar *et al.* 2015).

In summary, direct shoot regeneration deals directly with organized explant tissue. In comparison, indirect regeneration occurs from an unorganized callus tissue. The plant source and the research aims will determine the micropropagation procedure to be used, as many plant species are easily regenerated from callus, while others are exclusively regenerated by a direct method. Commercial micropropagation prefers to use axillary bud proliferation strategy which produces more genetically stable plants.

In comparison with regeneration through callus, direct shoot regeneration has a higher success rate in shoot production in addition to producing regenerants with somaclonal variations at lower rates than callus mediated regeneration pathways. This could be due to the explants that are used, as meristems are subject to somaclonal variation at lower frequencies compared to nonmeristematic explants used in direct and indirect regeneration (Phillips *et al*. 1995; George *et al*. 2008; Saini *et al.* 2010; Safdari *et al.* 2010).

There are several studies addressed the *in vitro* propagation of plants from the Ericacea family, for example, Mereti *et al.* (2002), Gomes and Conhoto (2009), and Gomes *et al*. (2010) studied different aspects of *in vitro* culture in *Arbutus unedo*, a sister species and very close to *A. andrachne*. Mackay (1996) studied *Arbutus xalapensis* micropropagation, and *Arbutus andrachne* was successfully *in vitro* propagated by Mostafa *et al*. (2010) and Bertsouklis and Papafotiou (2009).

1.3 Factors affecting *in vitro* direct shoot regeneration.

The *in vitro* direct shoot regeneration process is affected by different components of the culture medium such as basal salt composition, gelling agent, carbon source, media pH, and plant

growth regulator combination and concentration, in addition to other factors connected to explant's status and condition like age, organ, and cutting position. For a better understanding of the process, it is important to evaluate the effect of all of these factors on *in vitro* direct shoot regeneration (Ishag *et al*,2009). In addition, studies revealed that plant regeneration strategies are genotype specific, and genetic potential also control shoot regeneration (George *et al*. 2008).

1.3.1 The effect of plant growth regulators on*in vitro* direct shoot regeneration.

Throughout the various stages of plant development PGRs are used to alter the shape and size of plants, and they also play an important role in shoot induction and growth (Van Staden *et al.* 2008). PGRs like auxins, cytokinins, and gibberellins, are reported to play important factor that affects the *in vitro* direct shoot regeneration.

During the multiplication phase, in order to induce shoot formation one or more cytokinin are added to the nutrient medium (Van Staden *et al.* 2008; Chawla, 2009). Although zeatin, benzyladenine (BA), kinetin, and N6-(2-isopentenyl) adenine (2-iP) are the most common cytokinins used in micropropagation (Chawla, 2009), thidiazuron (TDZ) is widely used for promoting shoot initiation for the micropropagation of some woody plants including species from the Ericaceae family such as lingonberry and blueberry, (Van Staden *et al.* 2008; Ranyaphia *et al*. 2011; SEFASI *et al.* 2013).

Generally, the ratio of cytokinin to auxin is a key in controlling plant regeneration, and it was found that shoot induction requires low auxin and high cytokinin concentrations in the medium (Pierik, 1997; Van Staden *et al.* 2008). The optimal PGR concentration to be used differs among plant species (Singh *et al*, 2002). In a study on *Psidium guajava*, the maximum percentage of shoot regeneration (44.6%) was obtained by a treatment consisting of 1.0 µm TDZ and 0.54 µm NAA (Singh *et al*. 2002). The highest percent of shoot regeneration of pear (56%) was achieved by combination of 7.5 µm TDZ and 1 µm NAA (Yousefiara *et al*. 2014).

A number of species in the Ericaceae family including *A. andrachne* (Bertsouklis and Papafotiou, 2009), *A. unedo* (Mereti *et al.* 2002), *A. xalapensis* (Mackay, 1996), *Rhododendron* sp. (Almeida *et al.* 2005) have been micropropagated by axillary shoot strategy. The most common factor was the requirement of a combination of a cytokinin and an auxin in the growth medium in order to promote shoot induction, although it was observed that the regeneration rate varied according to the concentration and type of the PGRs used.

1.3.2 The effect of explant type on *in vitro* direct shoot regeneration.

Widely successful *in vitro* direct shoot regeneration processes have been achieved for woody plants, but the majority of these studies used leaf segments to initiate shoot regeneration (Gomes *et al*, 2009, Woo and Wetzstein, 2008, Al-Wasel, 2000). On the other hand, few studies propose that explant type to be used for shoot regeneration is an interesting feature to be concerned (Singh *et al*, 2002, Du *et al*,2008, Palla *et al*, 2011). Studies about shoot regeneration are associated with the induction of shoots from different explant types including leaves, cotyledons, hypocotyls, and roots. For examle; shoots of *Brassica oleracea* were directly regenerated from hypocotyl, cotyledon, leaf, and petiole explants (Kumar *et al*. 2015), whereas in *Psidium guajava* (Singh *et al*. 2002), and *Milletia pinnata* (Nagar *et al*. 2015) shoots were regenerated from hypocotyl explants. Lombardo *et al*. (2011) used cotyledonary explants for *Citrus clementina* shoot proliferation. Apricot leaves were suitable source for direct shoot regeneration (Pe´rez-Tornero, 2000). *In vitro* direct shoots have been regenerated from root explants in very small number of species like in *Passiflora edulis* Sims. Rocha *et al*. (2012).

1.3.3 The effect of explant age on *in vitro* direct shoot regeneration.

One of the essential factors that affect regeneration capacity is explant age. Many reports revealed that old explants lose their regeneration ability and show lower shoot proliferation rates than juvenile explants. This is represented by the results of Tornero *et al*. (2000) on apricot leaves, and the hypocotyls and cotyledons of *Fraxinus pennsylvanica* by Du *et al*. (2008).

Chapter 2

Problem statement and study objectives

2.1. Problem statement

A. andrachne L. is a rare medicinal tree in Palestine and sometimes the demand on it is increasing for uses in traditional medicine and for different therapeutic uses. This wild species is facing severe genetic erosion and extinction danger, slow growth and difficult seeds germination under natural conditions make *A. andrachne* recovery in its natural habitat very low. Plant tissue culture is a powerful alternative for propagating rare and difficult-to-propagate plants like *A. andrachne* and this takes place without being affected by season or climatic or other conditions. *In vitro* direct shoot regeneration is one of the efficient propagation strategies that could provide reliable amounts of *A. andrachne* plants. Consequently, an efficient, rapid and reproducible shoot regeneration system is required to propagate this plant species through adjusting *in vitro* growing factors.

2.2. Objectives

The main objective of this study is to outline efficient protocol for *in vitro* direct shoot regeneration in *A. andrachne* under different conditions including:

1. Finding optimum cytokinin concentration (TDZ) that results in highest direct shoot regeneration.
2. The effect of different explant type like root, hypocotyle, cotyledon, young and old leaves on direct shoot regeneration

Chapter 3

Methodology

This part of the experimental work was conducted in the Plant Tissue Culture Laboratory at the Biotechnology Training and Research Center, Palestine Polytechnic University, Hebron- Palestine.

3.1 Chemicals and reagents for *in vitro* culture

All basal plant growth media, plant growth regulators (PGRs), and chemical reagents were obtained from Duchefa Biochemie and Sigma-Aldrich chemical companies.

3.2 Plant growth Regulators (PGRs) and media preparation

The PGRs (TDZ and NAA) were prepared as concentrated stock solutions at 1.0 mg/ml concentration. Lloyd and McCown Woody Plant basal media (WP) with vitamins (Prod No. L449) was used for the *in vitro* growth experiments. For one liter of final volume, (3.0% w/v) or 30 g/l of sucrose and 2.41g/l of WP media were dissolved in 900 ml of deionized water. According to each experiment's purpose, the required concentrations (mg/l) of the PGRs were added. After that, the media was brought to the final volume, the pH was adjusted to 5.8 by using 1.0M NaOH or 1.0M HCl. Finally, 7.0 g/l of agar were used to solidify the media. The prepared media was poured into autoclavable bottles closed with autoclavable screw caps. Media was autoclaved at 121°C and 15psi (= 104 kPs) for 20min then was transferred to the media storage cabinet. Detailed media components are listed in Appendix 1 table 1.

3.3. Plant material

Seeds of *A. andrachne* were collected from wild trees in Dora, West of Hebron city in 2014. The ripe fruits were soaked in tap water for 72 hours before seeds were separated by hand and washed from the fruit pulp, then dried at room temperature and preserved for the experiment.

3.4. Breaking seed dormancy, surface sterilization of explants, and *in vitro* germination

Seeds were soaked in a solution containing 5.0 mg/ml GA_3 and cold stored at 4°C for seven days to break seed dormancy and induce germination. Surface sterilization was carried out by gentle shaking of seeds in soap and water for 2 min. Then, under the laminar flow hood, seeds were dipped in the antiseptic solution of 20% (v⁄v) commercial sodium hypochlorite (Chlorox®) with shaking for 15 min followed by two rinses with autoclaved distilled water. The sterilized seeds were washed with 70% ethanol for 30 seconds. Finally, seeds were washed twice with autoclaved distilled water for 3 min each.

Surface sterilized seeds were cultured aseptically under the laminar flow hood on PGRs free WP media to obtain sterile seedlings. Germination percentage was recorded after six weeks of culture.

3.5. *In vitro* direct shoot regeneration.

3.5.1. Effect of PGR concentration on direct shoot regeneration:

WP medium was supplemented with different concentrations of TDZ (1.0, 1.5 or 2.0 mg/l), each in combination with 0.5 mg/l NAA (Table 3.1). Media were prepared and 25 ml were poured into 9.0cm sterile Petri dishes (three replicates for each treatment) (Figure 3.1).

Explants were obtained from the sterile seedlings that have been previously germinated. Cultures were maintained in the growth room at light regime of 16-h light: 8-h dark, and light intensity of 40-45 μmoles.m^{-2}.sec^{-1} photosynthetic photon flux density (PPFD). Temperature in the growth room was kept at 24 ± 1°C. After 4 weeks, data were recorded for the appearance of callus, callus color and texture, occurrence and percentage of shoot regeneration, and number of regenerated shoots per explants.

Table 3.1. The concentrations of PGRS in WP media

WP media	TDZ (mg/l)	NAA (mg/l)
M1	0	0
M2	1.0	0.5
M3	1.5	0.5
M4	2.0	0.5

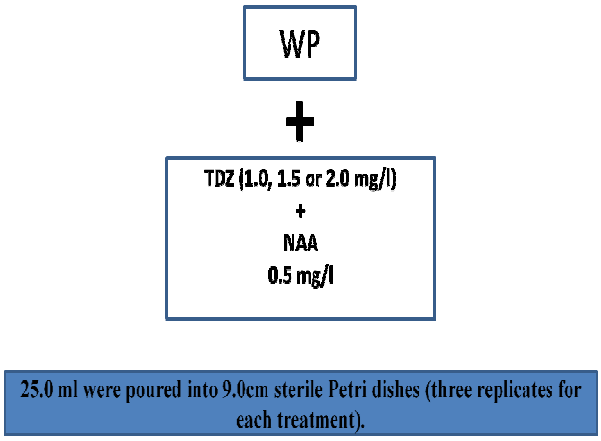

Figure 3.1. Media prepared for PGRs treatments

3.5.2. Effect of explant type on direct shoot regeneration:

The media that is used in this experiment was prepared as mentioned in section 3.5.1. Hypocotyl segments, cotyledonary segments, roots, young and old leaves obtained from *in vitro* germinated seedlings (Figure 3.2) were cut and inoculated on the surface of media with seven explants in each petri dish. Cultures were maintained in the growth room at light region of 16-h light/8 dark and photoperiod (photosynthetic photon flux density (PPFD) = 40-45 µmoles.m^{-2}.sec^{-1}), at (24 ± 1°C). Results were recorded after four weeks for callus color and texture, percentage of responsive explants for direct shoot regeneration, and number of regenerated shoots appeared on each responsive explant.

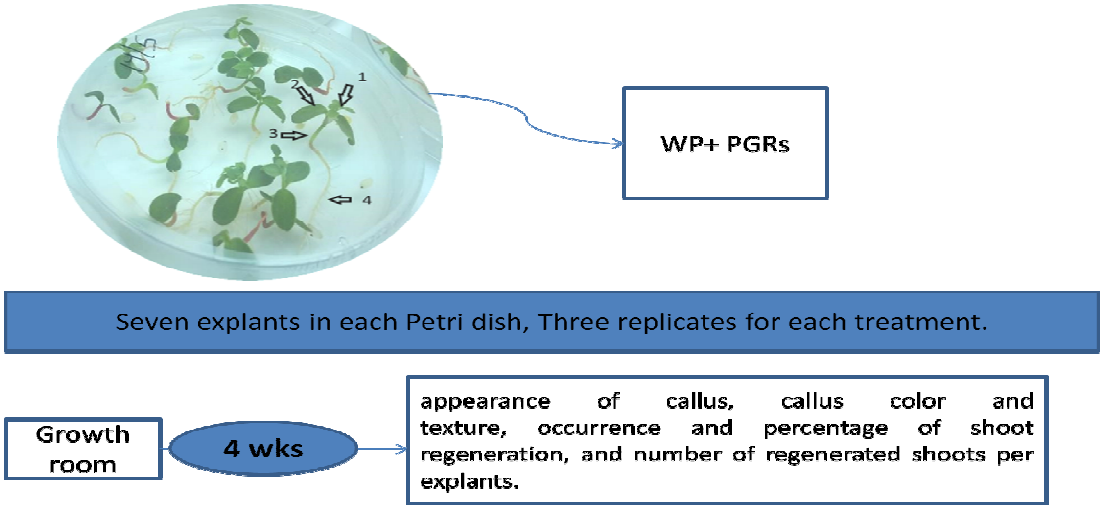

Figure 3.2. Five weeks old*in vitro* growing *A. andrachne* seedlings on WP media. Arrows are pointed at (1) true leaves, (2) cotyledons, (3) hypocotyl, (4) primary roots.

3.5.3. Effect of explant age: young vs. old growing leaf explants

To evaluate the effect of tissue age on direct shoot regeneration, leaves from 6 and 15 weeks old plants were cultured on WP medium supplemented with 1.0, 1.5, or 2.0 mg/l TDZ, each in combination with 0.5mg/l NAA. Three replicates for each treatment were made. Cultures were maintained in the growth room at light region of 16-h light/8 dark and photoperiod (photosynthetic photon flux density (PPFD) = 40-45 μmoles.m^{-2}.sec^{-1}), at (24 ± 1°C). Results were e recorded after four weeks for callus color and texture, percentage of responsive explants for direct shoot regeneration, and number of regenerated shoots in explants.

3.6. *In vitro* rooting

Two cm long microshoots were cultured in test tube contains 7.0 ml of WP medium contains 15 g/l sucrose and 1.5 mg/l IBA. Test tubes were maintained in the growth room until rooting took place. Data were reported after 6 weeks for the percentage of rooting (figure 3.3).

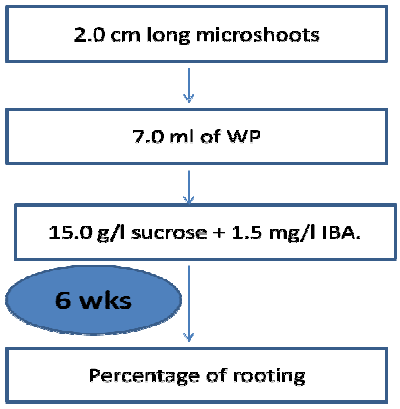

Figure 3.3. In vitro rooting

3.7. Data collection and statistical analysis

Each treatment was represented in three replicates (25.0ml plates) with seven explants were cultured in each. Experimental units were organized at completely randomized design (CRD). Percentage of the responsive explants that showed shoot regeneration, and the average number of shoots in each treatment were analyzed using Minitab ver. 16. Analysis of variance (ANOVA) was used to test the statistical significance between treatment means, and the significance of differences among means was carried out using Fisher test for mean separation at p=0.05.

Chapter 4

Results

The main objective of this study is to investigate the effect of PGRs particularly TDZ and NAA, in addition to explant type and age on *in vitro* direct shoot regeneration ability of the endangered *A. andrachne*.

4.1. Breaking seed dormancy, surface sterilization of explants, and germination

A. andrachne seeds were successfully germinated with 92%. The germination percentage was achieved after cold storage and soaking in a solution contains 5.0 mg/ml GA_3 for seven days.

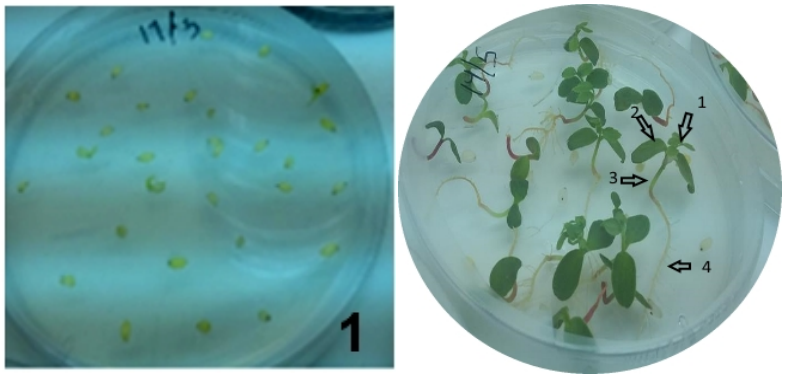

Figure 4.1: *A. andrachne* seeds cultured on WP growth media: Left: immediately after inoculation, right: after 5 weeks of germination.

4.2 The effect of PGRs and explant type on *in vitro* direct shoot regeneration ability in *A. andrachne*.

Direct shoot regeneration on different explants was noted after four weeks of growth. Different explants varied in their regeneration percentage on different medium that was used. Figure 4.2 shows variation and percentage of responsiveness on different media composition. As noticed, the maximum percentage of responsive explants was observed in cotyledons that were cultured on WP media with 0.5mg/l NAA and 1.0mg/l TDZ.

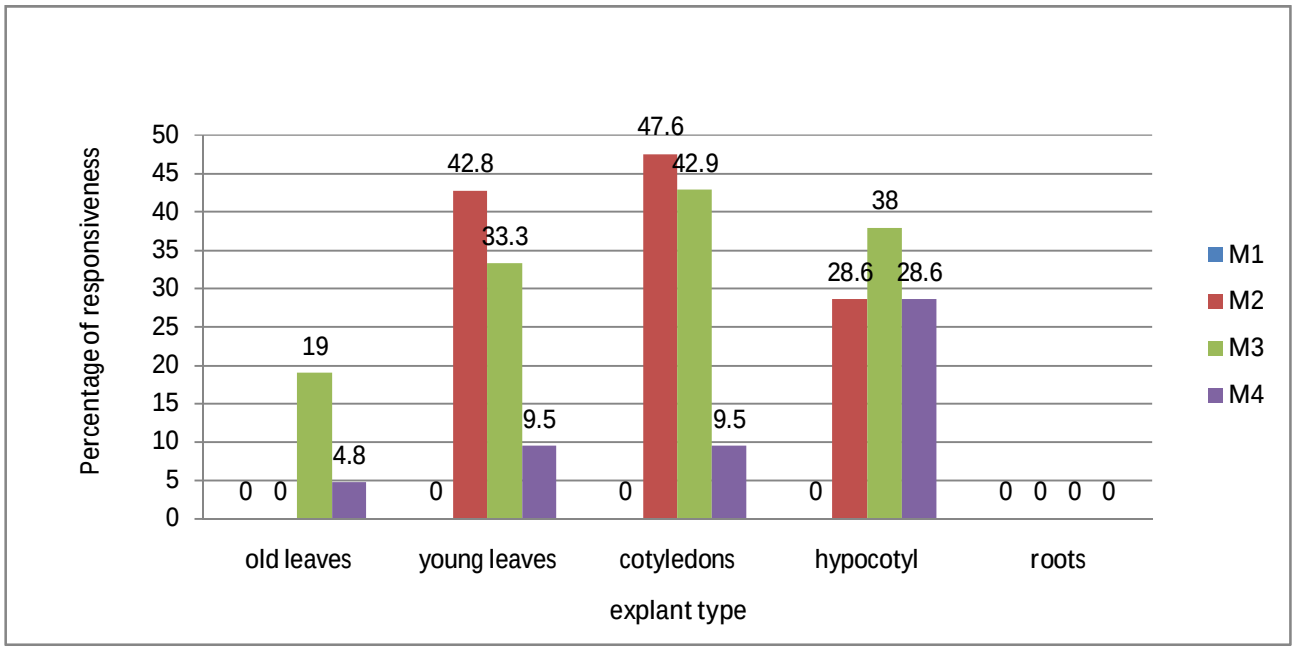

Figure 4.2: Percentage of responsive explants for direct shoot regeneration on M1 (control), M2 (0.5mg/l NAA + 1.0mg/l TDZ, M3 (0.5mg/l NAA + 1.5mg/l TDZ, M3 (0.5mg/l NAA + 2.0mg/l TDZ).

4.2.1 The effect of PGR concentrations on *in vitro* direct shoot regeneration

PGRs concentration effect on the number of regenerated shoots is shown in figure 4.3. The cotolydonary explants showed significant differences among media with different TDZ concentrations (p = 0.05); media that contains 1.0mg/l TDZ + 0.5 mg/l NAA, or 1.5 mg/l TDZ+ 0.5 mg/l NAA gave the highest shoot regeneration, while the media with 2.0 mg/l TDZ+ 0.5 mg/l NAA gave the lowest number of regenerated shoots. Although hypocotyls showed no significant differences on media with different TDZ concentrations (p = 0.153), media with 1.5 mg/l TDZ gave low number of regenerated shoots, followed by media with 1.0 mg/l TDZ then media with 2.0 mg/l TDZ. Young leaves showed no significant differences on media with different TDZ concentrations (p = 0.185), but media with 1.0 mg/l TDZ gave low number of regenerated shoots, then media with 1.5 mg/l TDZ and media with 2.0 mg/l TDZ. Also old leaves showed no significant differences on media with different TDZ concentrations (p = 0.074), but media with 1.5 mg/l TDZ gave the highest number of shoot regeneration, followed by media with 2.0 mg/l TDZ, and media with 1.0 mg/l TDZ gave no shoots. The control media with no PGRs showed no responsiveness in all explant types.

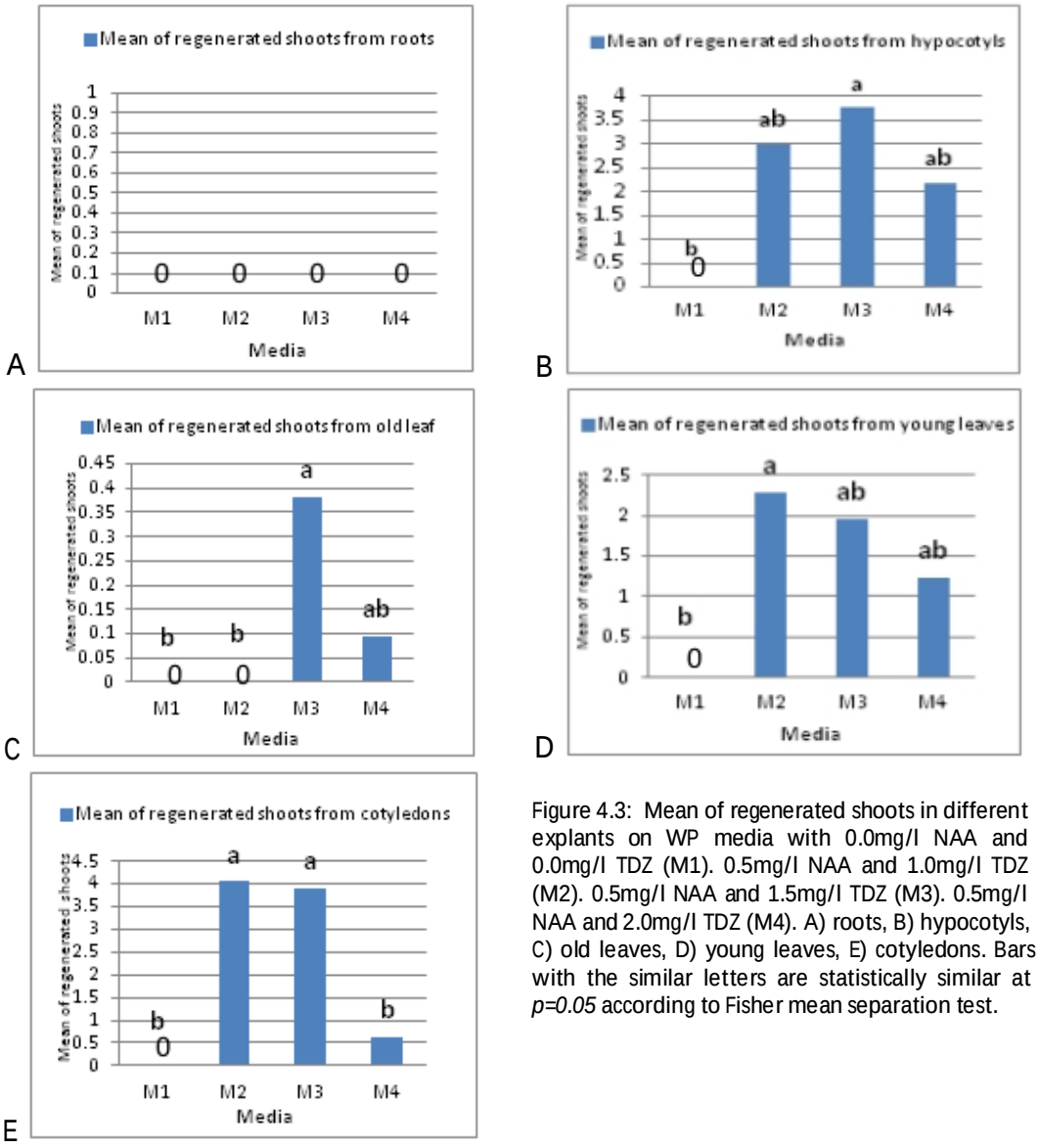

Figure 4.3: Mean of regenerated shoots in different explants on WP media with 0.0mg/l NAA and 0.0mg/l TDZ (M1). 0.5mg/l NAA and 1.0mg/l TDZ (M2). 0.5mg/l NAA and 1.5mg/l TDZ (M3). 0.5mg/l NAA and 2.0mg/l TDZ (M4). A) roots, B) hypocotyls, C) old leaves, D) young leaves, E) cotyledons. Bars with the similar letters are statistically similar at *p=0.05* according to Fisher mean separation test.

4.2.2 The effect of explant type on *in vitro* direct shoot regeneration

Figure 4.4 illustrates the response of different explants with the number of regenerated shoots from each. Significant differences between the explants have been observed. All explants on media without PGRs failed to produce any shoots. On media with 0.5 mg/l NAA and 1.0 mg/l TDZ (p = 0.026) hypocotyle and cotolydonary explants gave the highest shoot regeneration ability, followed by young leaves, while old leaves and root explants failed to produce any

shoots. On media with 0.5 mg/l NAA and 1.5 mg/l TDZ ($p = 0.005$) hypocotyl and cotolydonary explants gave the highest shoot regeneration ability, followed by young leaves, old leaves gave the least number of shoot regeneration and the root explants failed to produce any shoots. Media with 0.5 mg/l NAA and 2.0 mg/l TDZ ($p = 0.154$) showed no significant differences between the different explants, but hypocotyls gave the highest number of regenerated shoots, followed by cotyledons and young leaves, and old leaves gave the least number of shoot regeneration and the root explants failed to produce any shoots.

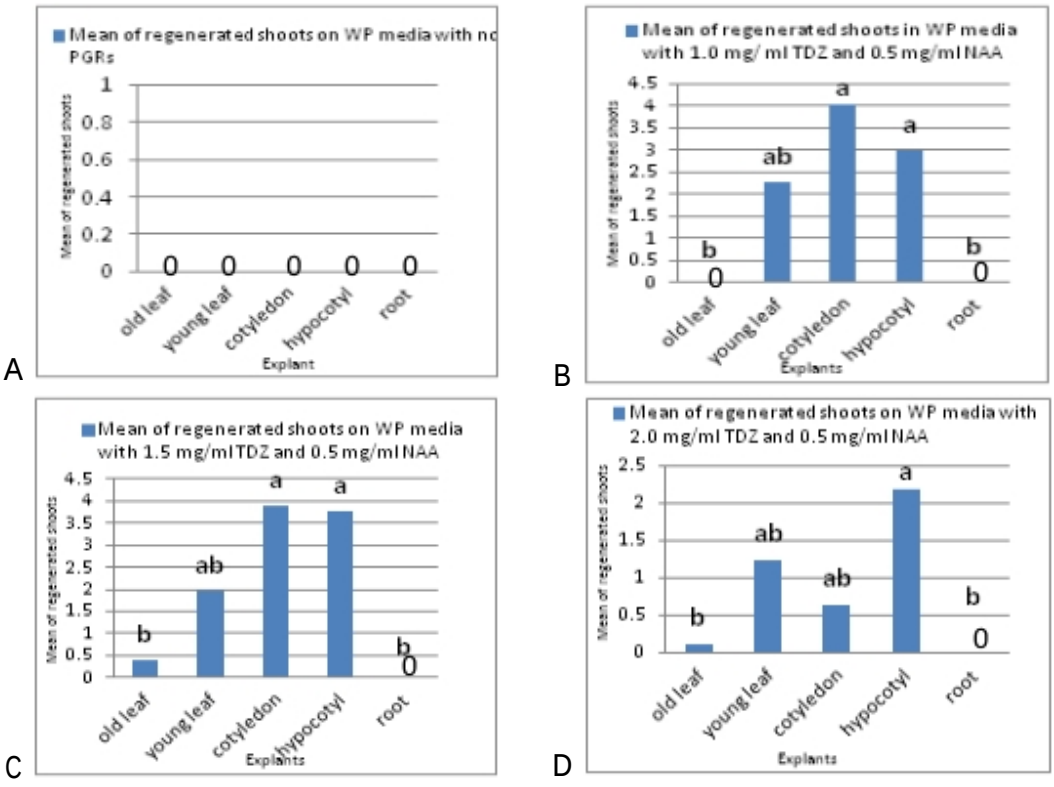

Figure 4.4: Mean of regenerated shoots in different explants on WP media with different PGRs concentration. A) 0.0mg/l NAA and 0.0mg/l TDZ, B) 5.0mg/l NAA and 1.0mg/l TDZ, C) 5.0mg/l NAA and 1.5mg/l TDZ, D) 5.0mg/l NAA and 2.0mg/l TDZ. Bars with the similar letters are statistically similar at *p=0.05* according to Fisher mean separation test.

Shoot regeneration was clearly seen under dissecting microscope at 10X magnification (Figure 4.5). Additionally, all explants (except control treatment) gave growth to a compact, dark red callus beside shoots.

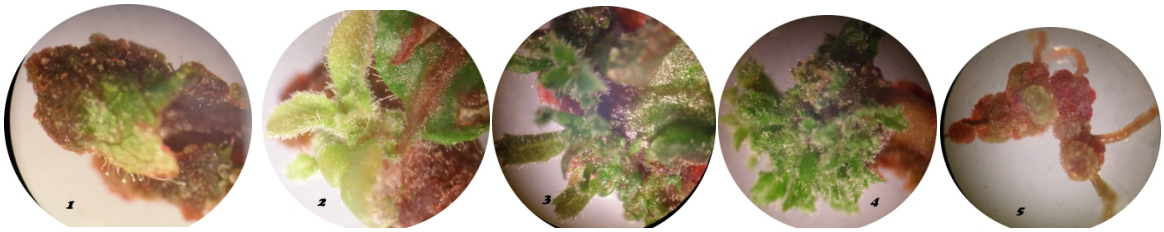

Figure 4.5: Growth in different explants of*A. andrachne* were cultured in WP media supplemented with 0.5mg/l NAA and 1.0mg/l TDZ. 1-old leaf, 2- young leaf, 3- cotyledon, 4- hypocotyl, 5- root.

WP media supplied with 0.5mg/l NAA and 1.5mg/l TDZ induced shoots regeneration in young leaves, cotyledons and hypocotyls explants. Old leaves showed low number of regenerated shoots and roots showed no response (Figure 4.6).

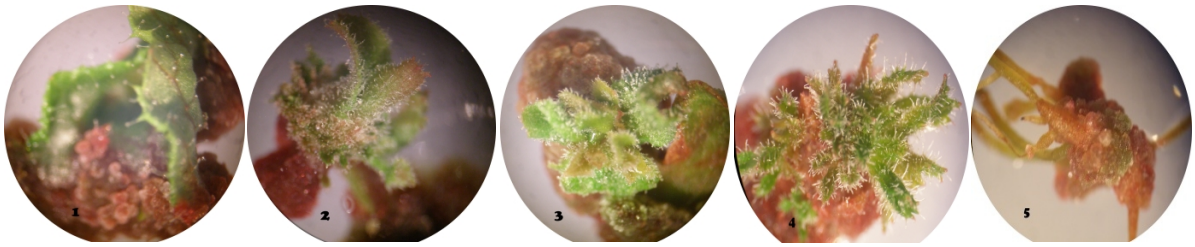

Figure 4.6: Regenerated shoots in different explants types of*A. andrachne* were cultured in WP media supplied with 0.5mg/l NAA and 1.5mg/l TDZ. 1-old leaf, 2- young leaf, 3-cotyledon, 4- hypocotyl, 5- root.

When explants are cultured on WP media supplemented with 0.5mg/l NAA and 2.0mg/l TDZ, dark red compact callus was observed and direct shoots formation was clearly seen in all types except roots. (Figure 4.7)

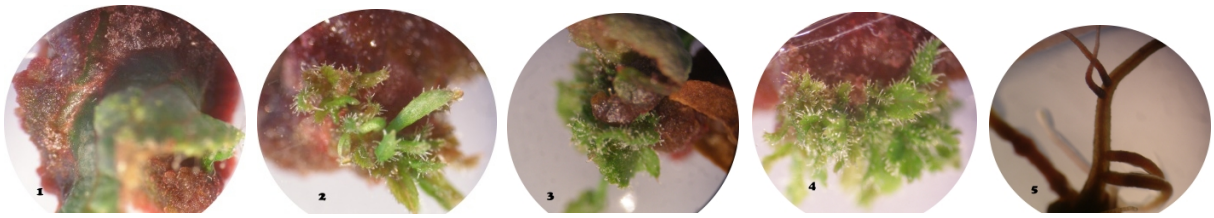

Figure 4.7: Regenerated shoots in different explants types of *A. andrachne* were cultured in WP media supplimented with 0.5mg/l NAA and 2.0mg/l TDZ. 1-old leaf, 2- young leaf, 3-cotyledon, 4- hypocotyl, 5- root.

4.3. *In vitro* rooting

After 6 weeks, microshoots that were cultured in WP medium supplemented with 15.0 g/l sucrose and 1.5 mg/l IBA showed 100% rooting. Figure 4.8 shows rooting where arrows are pointed at the roots.

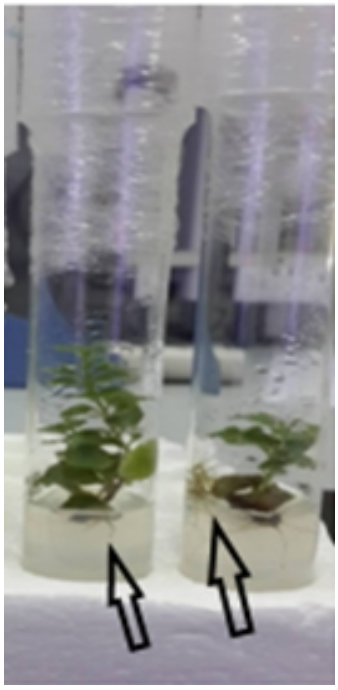

Figure 4.8: Six weeks aged microshoots cultured in test tube contains 7.0 ml of WP medium supplemented with 15 g/l sucrose and 1.5 mg/l IBA.

Chapter 5

Discussion

5.1. Breaking seed dormancy and germination

Seed dormancy is a major problem that encounters propagation by seeds. Dormant seeds do not germinate easily even if placed in germination favorable conditions. Many studies have taken place in order to examine methods of breaking seed dormancy and inducing germination (Tilki, 2004; Karam and Al-Salem, 2001; Aljabari, 2011). A combination of cold storing and gibberlic acid GA_3, a plant growth regulator, was the most effective treatment for breaking seed dormancy in *A. andrachne*. Seeds were successfully germinated with 92% success. According to Aljabary (2011), seeds showed the highest germination percentage of 84% by storing at 4°C for 24 hours and treatment with 5.0 mg/l GA_3. Compared to this study, the germination percentage was improved to 92% after seeds were treated for seven days in GA_3.

5.2. The effect of PGRs on *in vitro* direct shoot regeneration

According to many studies, PGRs were found to be an essential factor for efficient *in vitro* direct shoot regeneration. In many studies focused on woody plants, TDZ in combination with low concentration of auxin was more effective than other cytokinines for inducing shoot proliferation (Guo *et al*. 2011; Aggarwal *et al*. 2012; Yousefiara *et al*. 2014).On the other hand, Ranyaphia (2011) observed that a combination of TDZ with auxins reduced the percentage of regeneration of *Gaultheria fragrantissima*. TDZ is considered to be more stable than other cytokinines, as other cytokinines are more prone than TDZ to become biologically inert by degradation or conjugation with other media components (Ranyaphia *et al*. 2011). NAA also helps in reducing the period needed for shooting (Singh *et al*. 2002).

Many studies showed that TDZ stimulates shoot regeneration at low concentrations. In the present study, TDZ concentrations of (1.0, 1.5, or 2.0 mg/l) were combined with 0.5 mg/l NAA to investigate the ability to induce direct shoot regeneration. Both combinations of 1.0 mg/l TDZ

and 0.5 mg/l NAA and 1.5 mg/l TDZ together with 0.5 mg/l NAA showed the highest number of shoots (4.0 shoots/explant, 3.9 shoot/explant, respectively) in the cotolydonary explants, with no significant differences between them. For hypocotyles, media with 1.5 mg/l TDZ gave the highest number of regeneration (3.8 shoot/explant) and in young leaves media with 1.0 mg/l TDZ gave the highest number of regeneration (2.3 shoots/explant) The TDZ at 1.0 mg/l gave the highest regeneration percentage (47.6%) as compared to other combinations. Similar results have been reported by other studies, but the optimum level of TDZ varies among different species. Singh, in his study on *Psidium guajava*, found that the maximum percentage of shoot regeneration and maximum number of regenerated shoots were obtained by a treatment consisting of 1.0 µm TDZ and 0.54 µm NAA (Singh *et al*. 2002). Also, in a study of factors affecting direct shoot regeneration of pear, Yousefiara et al. (2014) found that the highest percent of shoot regeneration was achieved by a combination of 7.5 µm TDZ together with 1.0 µm NAA. Furthermore, Hamidoghli *et al*. (2011) found that direct shoot regeneration of *Primula heterochroma* was stimulated by a high TDZ concentration (2.0mg/l) more efficiently than lower concentrations (0.2-1.0 mg/l).

TDZ, when used in high concentrations, will produce undesirable morphogenic developments. Singh (2002) noticed that high concentrations of TDZ induce explant browning and necrosis, leading to a decrease in the efficiency of shoot induction. Similar observation in *A. andrachne* has been reported in this study (Figure 5.1). Ishag (2009) observed that production of excessive callus at high levels of TDZ decreases percentage and shoot regeneration frequency. Other disadvantages that can be overcome by reducing TDZ concentration include the production of stunt and compact shoots, beside the difficulties in regenerated shoot elongation and rooting. (Lu, 1993).

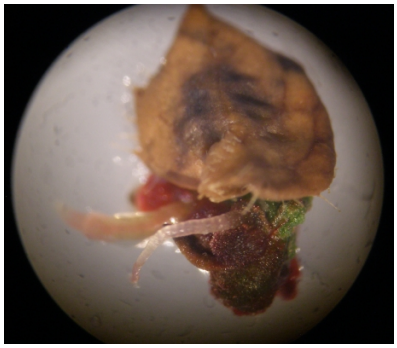

Figure 5.1: Browning and necrosis of leaves at 2.0 mg/l TDZ

5.3. The effect of explant age and type on *in vitro* direct shoot regeneration

Many studies about shoot regeneration are associated with the induction of shoots from different explant types including hypocotyls, cotyledons, and leaves. This is the first study that was applied on *A. andrachne* using hypocotyls, cotyledons, leaves, and roots to investigate the effect of explant type and age on direct shoot regeneration.

A. andrachne was successfully proliferated by *in vitro* direct shoot regeneration from different explant types on WP medium fortified with different concentrations of TDZ (1.0- 1.5 - 2.0 mg/L) in combination with (0.5 mg/L) NAA. Shoots were induced in all the concentrations of TDZ from cotyledons, hypocotyles, and fresh leaves, but old leaves showed very little response and roots had no response at all. The maximum percentage of responsive explants (47.6%) was recorded by cotyledons, and the highest number of shoots was recorded by cotyledonary and hypocotyl explants (4.0 and 3.0 shoots/explants, respectively) and young leaves gave (2.3 shoots/explant) on media with 1.0 mg/l TDZ. On media with 1.5 mg/l cotyledonary and hypocotyl explants (3.9 and 3.8 shoots/explants, respectively) and young leaves gave (2.0 shoots/explant). While on media with 2.0 mg/l TDZ young leaves gave the highest number of shoots (2.2 shoots/explant).

In a comparative study on *Brassica oleracea* organogenesis, Kumar *et al*. (2015) described the regeneration ability of hypocotyl, cotyledon, leaf, and petiole explants. He report that the best explant for regeneration was cotyledonary segments, followed by hypocotyl, and leaves gave the least shoot regeneration percentage. By contrast, other scientists reported hypocotyls being more regenerative than cotyledons (Du *et al*. 2008; Palla *et al*. 2011). Singh *(2002)* regenerated *Psidium guajava* from hypocotyl explants with a maximum shoot number of (3.2 shoot/explant) . Also, multiple shoots of *Milletia pinnata* were induced from hypocotyl segments by direct shoot regeneration (Nagar *et al*. 2015). Apricot was successfully regenerated from leaves with 24.3% (Pe´rez-Tornero, 2000). Lombardo *et al*. declared that *Citrus clementina* can be proliferated through direct shoot regeneration using cotyledon explants (Lombardo *et al*. 2011). Few studies showed that the use of roots as explants for *in vitro* direct shoot regeneration is limited to a small number of species (Rocha *et al*. 2012).

The differences in regeneration response between explants excised from different areas of the mother plant could be due to the presence of different levels of endogenous hormones in the

different parts of the plant, or the interaction between endogenous hormones and exogenous hormones (Yousefiara *et al*. 2014; Nagar *et al*. 2015). George *et al*.(2008), indicated that shoot proliferation is controlled by the genetic potential of the propagated plant. Plants that effectively regenerated by using their roots as an regenerated explant were found to have adventitious shoot buds on their roots (George *et al*. 2008).

Explant age has also been found to be a factor in the success of shoot regeneration, with young explants displaying a greater regeneration ability and more rapid rates of proliferation than aged explants. In the present study on *A. andrachne*, aged leaves showed a very low regeneration response. This result corroborates the results of Pe´rez-Tornero (2000) which showed that the regeneration response of juvenile apricot leaves was greater than aged leaves. Younger explants of most *Brassica* species were also shown to have superior regeneration ability compared to aged plants (Kumar *et al*. 2015).

The results could possibly be explained by the fact that young explants are physiologically and biochemically more active and that they have a less rigid cell wall which allows them to be easily affected by some factors like exogenous plant growth regulators.

Chapter 6

Conclusion

Culture media components and type of explant are considered to have wide effect on the *in vitro* direct shoot regeneration of *A. andrachne* . Seeds pretreated with 5.0 mg/ml GA_3 at 4°C for seven days are a good starting material and give a high germination rate. Culturing of cotyledons or hypocotyl explants on WP media supplemented with 0.5 mg/l NAA and up to 1.5 mg/l TDZ is recommended to get high shoot regeneration with maximum number of regenerated shoots.

This is among few studies that addressed the effects of some plant parts in trees which cannot be obtained easily in nature like hypocotyls and cotyledons. In conclusion, the present study reports efficient direct shoot regeneration protocol for *A. andrachne* which could be combined with future biotechnological programs like genetic manipulation and *Agrobacterium* mediated transformation.

Additionally, a closer look at other factors related to the explants can be studied in the future like the excision position and the inoculation surface, position or direction.

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APPENDICES

Appendix 1 Table 1:

Composition of growth media used in this study; Lloyd & McCown WP

All components expressed in mg/L	**Lloyd & McCown Woody Medium**
Ammonium Nitrate (NH4NO3)	**400**
Boric Acid(H2BO3)	**6.2**
Calcium Chloride (CaCl2.2H2O)	**96**
Calcium Nitrate (Ca(NO3)2.4H2O)	**556**
Cupric Sulfate (CuSO4.5H2O)	**0.025**
Magnesium Sulfate (MgSO4)	**370**
Manganese Sulfate (MnSO4.H2O)	**22.3**
Potassium Phosphate (KH2PO4)	**170**
Sodium Molybdate (Na2MoO4.2H2O)	**0.025**
Zinc Sulfate (ZnSO4.7H2O)	**8.6**
Ferrous Sulfate (FeSO4·7H2O)	**27.8**

Na2-EDTA	**37.3**
Inositol	**100**
Nicotinic Acid	**0.3**
Pyridoxine-HCl	**0.3**
Thiamine-HCl	**1.0**
Sucrose	**30.00**
Agar	**8.000**

Appendix 2

Analysis of variance (ANOVA) for number of regenerated shoots at different types of explants

M2- 1.0 TDZ

One-way ANOVA: old leaf, young leaf, cotyledon, hypocotyl, root

```
Source   DF      SS    MS     F      P
Factor    4   276.9  69.2  2.90  0.026
Error   100  2385.2  23.9
Total   104  2662.1

S = 4.884   R-Sq = 10.40%   R-Sq(adj) = 6.82%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level        N   Mean  StDev  --------+---------+---------+---------+-
old leaf    21  0.000  0.000  (-------*-------)
young leaf  21  2.286  4.617           (-------*--------)
cotyledon   21  4.048  7.074                   (-------*--------)
hypocotyl   21  3.000  6.921              (-------*-------)
root        21  0.000  0.000  (-------*-------)
                            --------+---------+---------+---------+-
                                  0.0       2.5       5.0       7.5

Pooled StDev = 4.884
```

```
Grouping Information Using Fisher Method

             N   Mean  Grouping
cotyledon   21  4.048  A
hypocotyl   21  3.000  A
young leaf  21  2.286  A B
root        21  0.000    B
old leaf    21  0.000    B

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 71.86%


old leaf subtracted from:

             Lower  Center  Upper  --------+---------+---------+---------+-
young leaf  -0.705   2.286  5.276                  (-------*------)
cotyledon    1.057   4.048  7.038                       (------*-------)
hypocotyl    0.010   3.000  5.990                    (-------*------)
root        -2.990   0.000  2.990             (------*------)
                                  --------+---------+---------+---------+-
                                       -4.0       0.0       4.0       8.0


young leaf subtracted from:

             Lower  Center  Upper  --------+---------+---------+---------+-
cotyledon   -1.228   1.762  4.752                (------*-------)
hypocotyl   -2.276   0.714  3.705             (-------*------)
root        -5.276  -2.286  0.705       (------*-------)
                                 --------+---------+---------+---------+-
                                      -4.0       0.0       4.0       8.0


cotyledon subtracted from:

             Lower  Center   Upper  --------+---------+---------+---------+-
hypocotyl   -4.038  -1.048   1.943          (------*-------)
root        -7.038  -4.048  -1.057  (-------*------)
                                    --------+---------+---------+---------+-
                                         -4.0       0.0       4.0       8.0


hypocotyl subtracted from:

       Lower  Center   Upper  --------+---------+---------+---------+-
root  -5.990  -3.000  -0.010     (-------*------)
                               --------+---------+---------+---------+-
                                    -4.0       0.0       4.0       8.0
```

M3- 1.5 TDZ

One-way ANOVA: old leaf, young leaf, cotyledon, hypocotyl, root

```
Source   DF      SS    MS     F      P
Factor    4   280.5  70.1  3.95  0.005
```

```
Error   100  1775.5  17.8
Total   104  2056.0

S = 4.214   R-Sq = 13.64%   R-Sq(adj) = 10.19%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level        N   Mean  StDev  ---------+---------+---------+---------+
old leaf    21  0.381  0.973    (--------*--------)
young leaf  21  1.952  3.263             (--------*--------)
cotyledon   21  3.905  5.224                      (---------*--------)
hypocotyl   21  3.762  7.063                      (--------*--------)
root        21  0.000  0.000  (--------*--------)
                              ---------+---------+---------+---------+
                                     0.0       2.0       4.0       6.0

Pooled StDev = 4.214


Grouping Information Using Fisher Method

             N   Mean  Grouping
cotyledon   21  3.905  A
hypocotyl   21  3.762  A
young leaf  21  1.952  A B
old leaf    21  0.381    B
root        21  0.000    B

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 71.86%


old leaf subtracted from:

             Lower  Center  Upper  ---------+---------+---------+---------+
young leaf  -1.008   1.571  4.151                  (------*-------)
cotyledon    0.944   3.524  6.104                       (------*------)
hypocotyl    0.801   3.381  5.961                      (-------*------)
root        -2.961  -0.381  2.199            (------*------)
                             ---------+---------+---------+---------+
                                   -3.5       0.0       3.5       7.0


young leaf subtracted from:

             Lower  Center  Upper  ---------+---------+---------+---------+
cotyledon   -0.628   1.952  4.532                  (-------*------)
hypocotyl   -0.770   1.810  4.389                  (------*-------)
root        -4.532  -1.952  0.628        (------*-------)
                             ---------+---------+---------+---------+
                                   -3.5       0.0       3.5       7.0


cotyledon subtracted from:

             Lower  Center   Upper  ---------+---------+---------+---------+
hypocotyl   -2.723  -0.143   2.437            (-------*------)
```

```
root       -6.485  -3.905  -1.325  (-------*------)
                                   ---------+---------+---------+---------+
                                         -3.5       0.0       3.5       7.0


hypocotyl subtracted from:

       Lower  Center   Upper  ---------+---------+---------+---------+
root  -6.342  -3.762  -1.182   (------*-------)
                              ---------+---------+---------+---------+
                                    -3.5       0.0       3.5       7.0
```

M4- 2.0 TDZ

One-way ANOVA: old leaf, young leaf, cotyledon, hypocotyl, root

```
Source   DF      SS    MS     F      P
Factor    4    69.1  17.3  1.71  0.154
Error   100  1011.8  10.1
Total   104  1080.9

S = 3.181   R-Sq = 6.39%   R-Sq(adj) = 2.65%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level        N   Mean  StDev  ---------+---------+---------+---------+
old leaf    21  0.095  0.436  (---------*--------)
young leaf  21  1.238  4.493          (--------*--------)
cotyledon   21  0.619  2.247      (--------*--------)
hypocotyl   21  2.190  5.016                (---------*--------)
root        21  0.000  0.000  (--------*--------)
                              ---------+---------+---------+---------+
                                     0.0       1.5       3.0       4.5

Pooled StDev = 3.181


Grouping Information Using Fisher Method

             N   Mean  Grouping
hypocotyl   21  2.190  A
young leaf  21  1.238  A B
cotyledon   21  0.619  A B
old leaf    21  0.095    B
root        21  0.000    B

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 71.86%


old leaf subtracted from:

             Lower  Center  Upper  -------+---------+---------+---------+--
young leaf  -0.805   1.143  3.090                (-------*------)
cotyledon   -1.424   0.524  2.471             (-------*-------)
hypocotyl    0.148   2.095  4.043                    (------*-------)
root        -2.043  -0.095  1.852           (-------*------)
```

```
                                  -------+---------+---------+---------+--
                                      -2.5       0.0       2.5       5.0


young leaf subtracted from:

            Lower  Center  Upper  -------+---------+---------+---------+--
cotyledon  -2.567  -0.619  1.329         (-------*------)
hypocotyl  -0.995   0.952  2.900                 (-------*-------)
root       -3.186  -1.238  0.709      (-------*-------)
                                  -------+---------+---------+---------+--
                                      -2.5       0.0       2.5       5.0


cotyledon subtracted from:

            Lower  Center  Upper  -------+---------+---------+---------+--
hypocotyl  -0.376   1.571  3.519                 (-------*-------)
root       -2.567  -0.619  1.329         (-------*------)
                                  -------+---------+---------+---------+--
                                      -2.5       0.0       2.5       5.0


hypocotyl subtracted from:

       Lower  Center   Upper  -------+---------+---------+---------+--
root  -4.138  -2.190  -0.243  (-------*-------)
                               -------+---------+---------+---------+--
                                   -2.5       0.0       2.5       5.0
```

M1 - 0TDZ

One-way ANOVA: old leaf, young leaf, cotyledon, hypocotyl, root

```
Source   DF         SS         MS  F  P
Factor    4  0.0000000  0.0000000  *  *
Error   100  0.0000000  0.0000000
Total   104  0.0000000

S = 0   R-Sq = *%   R-Sq(adj) = *%


Level        N         Mean        StDev
old leaf    21  0.000000000  0.000000000
young leaf  21  0.000000000  0.000000000
cotyledon   21  0.000000000  0.000000000
hypocotyl   21  0.000000000  0.000000000
root        21  0.000000000  0.000000000

            Individual 95% CIs For Mean Based on Pooled StDev
Level         +---------+---------+---------+---------
old leaf      *
young leaf    *
cotyledon     *
hypocotyl     *
root          *
              +---------+---------+---------+---------
            0.000000  0.000010  0.000020  0.000030

Pooled StDev = 0.000000000
```

Grouping Information Using Fisher Method

	N	Mean	Grouping
root	21	0.000000000	A
hypocotyl	21	0.000000000	B
cotyledon	21	0.000000000	C
young leaf	21	0.000000000	D
old leaf	21	0.000000000	E

Means that do not share a letter are significantly different.

Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 71.86%

old leaf subtracted from:

	Lower	Center	Upper
young leaf	0.000000000	0.000000000	0.000000000
cotyledon	0.000000000	0.000000000	0.000000000
hypocotyl	0.000000000	0.000000000	0.000000000
root	0.000000000	0.000000000	0.000000000

```
             +---------+---------+---------+---------
young leaf    *
cotyledon     *
hypocotyl     *
root          *
             +---------+---------+---------+---------
           0.000000  0.000010  0.000020  0.000030
```

young leaf subtracted from:

	Lower	Center	Upper
cotyledon	0.000000000	0.000000000	0.000000000
hypocotyl	0.000000000	0.000000000	0.000000000
root	0.000000000	0.000000000	0.000000000

```
             +---------+---------+---------+---------
cotyledon     *
hypocotyl     *
root          *
             +---------+---------+---------+---------
           0.000000  0.000010  0.000020  0.000030
```

cotyledon subtracted from:

	Lower	Center	Upper
hypocotyl	0.000000000	0.000000000	0.000000000
root	0.000000000	0.000000000	0.000000000

```
             +---------+---------+---------+---------
hypocotyl     *
root          *
             +---------+---------+---------+---------
           0.000000  0.000010  0.000020  0.000030
```

```
hypocotyl subtracted from:

            Lower       Center        Upper
root  0.000000000  0.000000000  0.000000000

        +---------+---------+---------+---------
root    *
        +---------+---------+---------+---------
      0.000000  0.000010  0.000020  0.000030
```

Appendix 3

Analysis of variance (ANOVA) for number of regenerated shoots at different PGRs concentrations.

cotyledon
One-way ANOVA: M1- 0 TDZ, M2- 1.0 TDZ, M3- 1.5 TDZ, M4- 2.0 TDZ

```
Source  DF      SS    MS     F      P
Factor   3   286.6  95.5  4.64  0.005
Error   80  1647.7  20.6
Total   83  1934.3

S = 4.538   R-Sq = 14.82%   R-Sq(adj) = 11.62%



Level         N   Mean  StDev
M1- 0 TDZ    21  0.000  0.000
M2- 1.0 TDZ  21  4.048  7.074
M3- 1.5 TDZ  21  3.905  5.224
M4- 2.0 TDZ  21  0.619  2.247

             Individual 95% CIs For Mean Based on Pooled StDev
Level           +---------+---------+---------+---------
M1- 0 TDZ       (---------*---------)
M2- 1.0 TDZ                         (---------*---------)
M3- 1.5 TDZ                         (---------*--------)
M4- 2.0 TDZ        (---------*---------)
                +---------+---------+---------+---------
              -2.0       0.0       2.0       4.0

Pooled StDev = 4.538


Grouping Information Using Fisher Method

              N   Mean  Grouping
M2- 1.0 TDZ  21  4.048  A
M3- 1.5 TDZ  21  3.905  A
M4- 2.0 TDZ  21  0.619    B
M1- 0 TDZ    21  0.000    B

Means that do not share a letter are significantly different.
```

```
Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 79.96%


M1- 0 TDZ subtracted from:

              Lower  Center  Upper  --------+---------+---------+---------+-
M2- 1.0 TDZ   1.260   4.048  6.835                      (-------*-------)
M3- 1.5 TDZ   1.118   3.905  6.692                    (-------*-------)
M4- 2.0 TDZ  -2.168   0.619  3.406             (-------*-------)
                                   --------+---------+---------+---------+-
                                        -3.5       0.0       3.5       7.0


M2- 1.0 TDZ subtracted from:

              Lower  Center   Upper  --------+---------+---------+---------+-
M3- 1.5 TDZ  -2.930  -0.143   2.644           (-------*-------)
M4- 2.0 TDZ  -6.216  -3.429  -0.641  (-------*-------)
                                    --------+---------+---------+---------+-
                                         -3.5       0.0       3.5       7.0


M3- 1.5 TDZ subtracted from:

              Lower  Center   Upper  --------+---------+---------+---------+-
M4- 2.0 TDZ  -6.073  -3.286  -0.499   (-------*-------)
                                    --------+---------+---------+---------+-
                                         -3.5       0.0       3.5       7.0
```

hypocotyl

One-way ANOVA: M1- 0 TDZ, M2- 1.0 TDZ, M3- 1.5 TDZ, M4- 2.0 TDZ

```
Source  DF      SS    MS     F      P
Factor   3   166.2  55.4  1.80  0.153
Error   80  2459.0  30.7
Total   83  2625.2

S = 5.544   R-Sq = 6.33%   R-Sq(adj) = 2.82%


Level         N   Mean  StDev
M1- 0 TDZ    21  0.000  0.000
M2- 1.0 TDZ  21  3.000  6.921
M3- 1.5 TDZ  21  3.762  7.063
M4- 2.0 TDZ  21  2.190  5.016

             Individual 95% CIs For Mean Based on Pooled StDev
Level          +---------+---------+---------+---------
M1- 0 TDZ      (---------*---------)
M2- 1.0 TDZ                (---------*---------)
M3- 1.5 TDZ                   (---------*---------)
M4- 2.0 TDZ             (---------*---------)
               +---------+---------+---------+---------
             -2.5       0.0       2.5       5.0
```

```
Pooled StDev = 5.544


Grouping Information Using Fisher Method

              N   Mean  Grouping
M3- 1.5 TDZ  21  3.762  A
M2- 1.0 TDZ  21  3.000  A B
M4- 2.0 TDZ  21  2.190  A B
M1- 0 TDZ    21  0.000    B

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 79.96%


M1- 0 TDZ subtracted from:

              Lower  Center  Upper  ----+---------+---------+---------+-----
M2- 1.0 TDZ  -0.405   3.000  6.405              (---------*--------)
M3- 1.5 TDZ   0.357   3.762  7.167                (---------*--------)
M4- 2.0 TDZ  -1.214   2.190  5.595            (--------*---------)
                                   ----+---------+---------+---------+-----
                                    -3.5       0.0       3.5       7.0


M2- 1.0 TDZ subtracted from:

              Lower  Center  Upper  ----+---------+---------+---------+-----
M3- 1.5 TDZ  -2.643   0.762  4.167       (---------*---------)
M4- 2.0 TDZ  -4.214  -0.810  2.595    (---------*--------)
                                   ----+---------+---------+---------+-----
                                    -3.5       0.0       3.5       7.0


M3- 1.5 TDZ subtracted from:

              Lower  Center  Upper  ----+---------+---------+---------+-----
M4- 2.0 TDZ  -4.976  -1.571  1.834  (---------*--------)
                                   ----+---------+---------+---------+-----
                                    -3.5       0.0       3.5       7.0
```

young leaf

One-way ANOVA: M1- 0 TDZ, M2-1.0 TDZ, M3- 1.5 TDZ, M4- 2.0 TDZ

```
Source  DF      SS    MS     F      P
Factor   3    64.5  21.5  1.65  0.185
Error   80  1043.0  13.0
Total   83  1107.6

S = 3.611   R-Sq = 5.82%   R-Sq(adj) = 2.29%


Level         N   Mean  StDev
M1- 0 TDZ    21  0.000  0.000
M2-1.0 TDZ   21  2.286  4.617
M3- 1.5 TDZ  21  1.952  3.263
```

```
M4- 2.0 TDZ  21  1.238  4.493

             Individual 95% CIs For Mean Based on Pooled StDev
Level           +---------+---------+---------+---------
M1- 0 TDZ       (---------*---------)
M2-1.0 TDZ                   (---------*----------)
M3- 1.5 TDZ                (---------*---------)
M4- 2.0 TDZ             (---------*----------)
                +---------+---------+---------+---------
              -1.5       0.0       1.5       3.0

Pooled StDev = 3.611



Grouping Information Using Fisher Method

              N   Mean  Grouping
M2-1.0 TDZ   21  2.286  A
M3- 1.5 TDZ  21  1.952  A B
M4- 2.0 TDZ  21  1.238  A B
M1- 0 TDZ    21  0.000    B

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 79.96%


M1- 0 TDZ subtracted from:

              Lower  Center  Upper  ------+---------+---------+---------+---
M2-1.0 TDZ    0.068   2.286  4.503                  (----------*----------)
M3- 1.5 TDZ  -0.265   1.952  4.170                (----------*----------)
M4- 2.0 TDZ  -0.979   1.238  3.456            (----------*----------)
                                   ------+---------+---------+---------+---
                                      -2.0       0.0       2.0       4.0


M2-1.0 TDZ subtracted from:

              Lower  Center  Upper  ------+---------+---------+---------+---
M3- 1.5 TDZ  -2.551  -0.333  1.884     (----------*----------)
M4- 2.0 TDZ  -3.265  -1.048  1.170  (----------*----------)
                                   ------+---------+---------+---------+---
                                      -2.0       0.0       2.0       4.0


M3- 1.5 TDZ subtracted from:

              Lower  Center  Upper  ------+---------+---------+---------+---
M4- 2.0 TDZ  -2.932  -0.714  1.503   (----------*----------)
                                   ------+---------+---------+---------+---
                                      -2.0       0.0       2.0       4.0
```

old leaf

One-way ANOVA: M1- 0 TDZ, M2- 1.0 TDZ, M3- 1.5 TDZ, M4- 2.0 TDZ

```
Source  DF      SS     MS     F      P
```

```
Factor   3   2.048  0.683  2.40  0.074
Error   80  22.762  0.285
Total   83  24.810

S = 0.5334   R-Sq = 8.25%   R-Sq(adj) = 4.81%


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level         N    Mean   StDev  ---------+---------+---------+---------+
M1- 0 TDZ    21  0.0000  0.0000  (--------*--------)
M2- 1.0 TDZ  21  0.0000  0.0000  (--------*--------)
M3- 1.5 TDZ  21  0.3810  0.9735               (--------*---------)
M4- 2.0 TDZ  21  0.0952  0.4364      (--------*--------)
                                 ---------+---------+---------+---------+
                                        0.00      0.25      0.50      0.75

Pooled StDev = 0.5334


Grouping Information Using Fisher Method

              N    Mean  Grouping
M3- 1.5 TDZ  21  0.3810  A
M4- 2.0 TDZ  21  0.0952  A B
M2- 1.0 TDZ  21  0.0000    B
M1- 0 TDZ    21  0.0000    B

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 79.96%


M1- 0 TDZ subtracted from:

               Lower  Center   Upper  --------+---------+---------+---------+-
M2- 1.0 TDZ  -0.3276  0.0000  0.3276            (-------*-------)
M3- 1.5 TDZ   0.0534  0.3810  0.7085                     (--------*-------)
M4- 2.0 TDZ  -0.2324  0.0952  0.4228              (-------*--------)
                                   --------+---------+---------+---------+-
                                        -0.40      0.00      0.40      0.80


M2- 1.0 TDZ subtracted from:

               Lower  Center   Upper  --------+---------+---------+---------+-
M3- 1.5 TDZ   0.0534  0.3810  0.7085                     (--------*-------)
M4- 2.0 TDZ  -0.2324  0.0952  0.4228              (-------*--------)
                                   --------+---------+---------+---------+-
                                        -0.40      0.00      0.40      0.80


M3- 1.5 TDZ subtracted from:

               Lower   Center   Upper  --------+---------+---------+---------+-
M4- 2.0 TDZ  -0.6133  -0.2857  0.0419     (-------*-------)
                                   --------+---------+---------+---------+-
                                        -0.40      0.00      0.40      0.80
```

root

One-way ANOVA: M1- 0 TDZ, M2- 1.0 TDZ, M3- 1.5 TDZ, M4- 2.0 TDZ

```
Source  DF         SS         MS  F  P
Factor   3  0.0000000  0.0000000  *  *
Error   80  0.0000000  0.0000000
Total   83  0.0000000

S = 0   R-Sq = *%   R-Sq(adj) = *%



Level          N         Mean        StDev
M1- 0 TDZ     21  0.000000000  0.000000000
M2- 1.0 TDZ   21  0.000000000  0.000000000
M3- 1.5 TDZ   21  0.000000000  0.000000000
M4- 2.0 TDZ   21  0.000000000  0.000000000

              Individual 95% CIs For Mean Based on Pooled StDev
Level           +---------+---------+---------+---------
M1- 0 TDZ       *
M2- 1.0 TDZ     *
M3- 1.5 TDZ     *
M4- 2.0 TDZ     *
                +---------+---------+---------+---------
              0.000000  0.000010  0.000020  0.000030

Pooled StDev = 0.000000000



Grouping Information Using Fisher Method

               N         Mean  Grouping
M4- 2.0 TDZ   21  0.000000000  A
M3- 1.5 TDZ   21  0.000000000    B
M2- 1.0 TDZ   21  0.000000000      C
M1- 0 TDZ     21  0.000000000        D

Means that do not share a letter are significantly different.


Fisher 95% Individual Confidence Intervals
All Pairwise Comparisons

Simultaneous confidence level = 79.96%


M1- 0 TDZ subtracted from:

                    Lower       Center        Upper
M2- 1.0 TDZ  0.000000000  0.000000000  0.000000000
M3- 1.5 TDZ  0.000000000  0.000000000  0.000000000
M4- 2.0 TDZ  0.000000000  0.000000000  0.000000000

                +---------+---------+---------+---------
M2- 1.0 TDZ     *
M3- 1.5 TDZ     *
M4- 2.0 TDZ     *
                +---------+---------+---------+---------
              0.000000  0.000010  0.000020  0.000030
```

```
M2- 1.0 TDZ subtracted from:

                   Lower       Center        Upper
M3- 1.5 TDZ  0.000000000  0.000000000  0.000000000
M4- 2.0 TDZ  0.000000000  0.000000000  0.000000000

                +---------+---------+---------+---------
M3- 1.5 TDZ     *
M4- 2.0 TDZ     *
                +---------+---------+---------+---------
              0.000000  0.000010  0.000020  0.000030


M3- 1.5 TDZ subtracted from:

                   Lower       Center        Upper
M4- 2.0 TDZ  0.000000000  0.000000000  0.000000000

                +---------+---------+---------+---------
M4- 2.0 TDZ     *
                +---------+---------+---------+---------
              0.000000  0.000010  0.000020  0.000030
```