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Factors Affecting *In Vitro* Culture of *Asphodelus aestivus* L. and Secondary Metabolites Production

By

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Factors Affecting *In Vitro* Culture of *Asphodelus aestivus* L. and Secondary Metabolites Production

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ABSTRACT

Asphodelus aestivus L. (Liliaceae), Arabic name "Gysalan" is a geophytes plant distributed all over the Mediterranean region. It has been used in traditional (folk) medicine to treat skin disease, jaundice, and psoriasis. *Asphodelus aestivus* propagation by conventional methods is problematic, since the seed show low germination percentage, and also show high sensitivity against light, humidity, and salt concentration. Therefore using plant biotechnology techniques offer an excellent alternative to propagate and study *A. aestivus*. In the present study, the main objective was to implement plant *in vitro* culture to propagate *Asphodelus aestivus*, and then assess secondary metabolites content in different parts of the plant. To test *in vitro* seed germination, seeds were cultured on water-agar medium, full strength or half strength MS medium. Seeds show slow and low germination rates (10%). Seeds pretreatment with 1.0 mg/L GA_3 increased germination percentage to 43% on half strength MS medium. More rapid establishment of motherstock plants was achieved using sterilized nodes excised from the plant tuberous roots. Highest number of shoot (8 shoot/flask) was achieved on MS medium with 4.0 mg/L BA, 0.4 mg/L IAA and 1.0 g/L PVP. *In vitro* shoot proliferation was tested by culturing shoots on MS medium with IAA at 0.0 or 0.5 mg/L in combination with BA, and 2-ip at the levels 0.0, 0.5, 1.0, 2.0 or 4.0 mg/L. MS medium supplemented with 0.5 mg/L IAA, 1.0 mg/L BA and 1.0 mg/L 2-ip gave the highest number of shoots (3.5 ± 0.56) and leaves (18.63 ± 2.36). For *in vitro* rooting, shoots were cultured on MS medium supplemented with 1.0, 2.0, and 3.0 mg/L of IAA, NAA, or IBA. Results showed that MS medium with 2.0 mg/L NAA show the highest rooting percentage (93.8%) with a mean root number of (8.75 ± 1.66), and the longest roots was observed on MS medium with 1.0 mg/L NAA with a mean of (3.69 ± 0.76). Callus was induced from immature seed embryos and from newly developed roots under dark condition. The highest percentage of callus induction was obtained from seed embryos cultured on MS medium with 2.0 mg/L BA, and 1.0 mg/L 2,4-D. Thin layer chromatography

(TLC) analysis between *ex vitro* and *in vitro* growing roots revealed several and different biochemical compounds in the root extract. This implies that growing *A. aestivus* under *in vitro* techniques resulted in different biochemical content of root extract.

Key words: *Asphodelus aestivus*, *In vitro* propagation, Callus culture, Secondary metabolites, Traditional medicine.

ملخص بالعربية

العوامل المؤثرة على تكثير نبات الغيصلان "البرواق" داخل الانابيب وانتاج المواد الايضية الثانوية

من قبل

لؤي عبد الهادي الشوامرة

نبات الغيصلان او البرواق نبات عشبي معمر من العائلة الزنبقية ينتشر في مناطق حوض البحر المتوسط. يستخدم البرواق في الطب الشعبي لعلاج العديد من امراض مثل امراض الجلد الطفيلية، الصدفية و اليرقان. ان تكثير نبات *A. aestivus* بالطرق الزراعية التقليدية صعب وذلك لانخفاض نسبة انبات البذور و بطئ نموها. ايضا فان البذور تظهر حساسية عالية ضد الضوء والرطوبة و الضغط الاسموزي لذلك فان استخدام تقنيات التكنولوجيا الحيوية وزراعة الانسجة النباتية يوفر بديلا ممتازا للحصول على عدد كبير من نباتات *A.aestivus*. تهدف الدراسة الحالية الى تكثير نبات البرواق باستخدام تقنية زراعة الانسجة و من ثم دراسة المواد الايضية الثانوية الناتجة من اجزاء مختلفة من النبات . لدراسة نسبة انبات البذور ، تم زراعتها مخبريا على وسط مائي صلب، وبيئة MS بتركيز كامل او تركيز مخفف للنصف. اظهرت البذور معدلات نمو متدنية (10%) و نمو بطيء. ادت معاملة البذور بإضافة 1.0 ملغم/لتر من منظم النمو GA_3 زادت معدل الانبات لتصل الى 43% على الوسط الغذائي MS المخفف للنصف. تم أيضا تجربة بديل آخر وهو تأسيس أمهات داخل الأنابيب باستخدام البراعم المقتطعة من جذور النبات. سجلت أعلى نسبة تفرع (8 فروع/وعاء) على الوسط الغذائي MS مدعما بـ 4.0 ملغم/لتر من منظم النمو BA و 4.0 ملغم/لتر من منظم النمو IAA مع اضافة 1.0 غم/لتر من مضاد الاكسدة PVP. تكثير الفروع المخبري تم دراسته وذلك بزراعة الافرع على وسط MS مدعما بمنظم النمو IAA بتركيز 0.5 و 1.0 ملغم/لتر و منظمي النمو BA و 2-ip بتراكيز 0.0، 0.5، 1.0، 2.0 او 4.0 ملغم/لتر. الوسط الغذائي MS مزوداً بـ 0.5 ملغم/لتر IAA و 1.0 ملغم/لتر BA و 1.0 ملغم/لتر 2-ip اعطى اعلى نسبة تفرع (3.5 ± 0.56) و عدد اوراق (18.63 ± 2.36).اما بالنسبة لتجذير النبات المخبري فقد تم دراسته على الوسط الغذائي MS مدعما بـ 1.0، 2.0، 3.0 ملغم/لتر من منظمات النمو BA او NAA او IBA. اظهرت النتائج ان الوسط الغذائي MS مدعما بـ 2.0 ملغم/لتر من NAA اعطى اعلى نسبة من النباتات التي كونت جذور (93.8) بمتوسط عدد جذور (8.75 ± 1.66)، و الوسط الغذائي MSمدعما بـ 1.0 ملغم/لتر NAA اعطى اطول جذر بمتوسط 3.69 ± 0.76. ايضا تم استحثاث انتاج الكالوس من البذور الغير ناضجة و من الجذور حديثة النمو حيث تم زراعتها في بيئة مظلمة. اعلى نسبة انتاج للكالوس كانت من البذور الغير ناضجة على الوسط الغذائي MS مدعما بـ 2.0 ملغم/لتر BA و 1.0 ملغم/لتر 2,4-D. باستخدام تقنية TLC تم الكشف عن عدة مركبات في خلاصة الجذور النامية في البيئة الخارجية او الجذور النامية في المختبر. هذا يعني ان زراعة نبات *A. aestivus* في المختبر غيرت التركيبة الكيميائية للمكونات الناتجة.

كلمات مفتاحية: الغيصلان " برواق"، تكثير مخبري، الكالوس، المواد الايضية الثانوية، الطب الشعبي.

DECLARATION

I declare that the Master Thesis entitled " **Factors Affecting *In Vitro* Culture of *Asphodelus aestivus* and Secondary Metabolites Production** " 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.

Name and signature:

Loay Abdelhadi Ahmed Alshawamreh

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DEDICATION

I would like to dedicate this work to

The man who I am carrying his name my beloved Father

The source of wisdom, patience, optimism and love my Mother

My strength and support my beloved brothers and sisters

All of my beloved friends……

Loay

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May Allah give you all the best in your life.

ABBREVIATIONS

Abbreviation	Word or Sentence
%	percent
°C	Degree centigrade Celsius
ANOVA	Analysis of variance
LSD	Least Significant Difference
CRD	Completely Randomized Design
et al.	And others
g	gram
mg	Milligram
l	liter
ml	Milliliter
µl	Microliter
rpm	Round per minute
sec.	Second
m^2	Square meter
PPFD	Photosynthetic Photon Flux Density
MS	Murashige and Skoog
1X MS	Full strength MS
½X MS	Half strength MS
2,4-D	2,4-dichlorophenoxyacetatic acid
Kn	Kinetin
2ip	Isopentenyl adenine
BAP	6-Benzylaminopurine
IAA	Indole-3-acetic acid
GA_3	Gibberellic acid
NAA	Naphthalene acetic acid
PVP	Polyvinylpyrrolidone
PGRs	Plant Growth Regulators
pH	Potensial hydrogen
Rf	Retardation factor
SDW	Sterile distilled water
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organization
BERC	Biodiversity and Environmental Research Center
SPSS	Statistical Package for the Social Science

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CHAPTER ONE

General Introduction and Literature Review

Asphodelus aestivus

Near the separation wall – Der Alasal alfoqa - Dora - Hebron - Palestine

(Photo by Alshawamreh, L)

CHAPTER ONE

Introduction:

Since the early civilization, man has depended on plants to obtain food, beverages, shelter and medication. Medicinal herbs represent the basic source of most drugs; many modern drugs are originated or derived from plants. For example, Aspirin is a derivative of a compound isolated from willow bark (Berg J, 2012), Ephedrine is the active compound in *Ephedra sinica* (Elujoba et al., 2005) and Aloe emodin is isolated from different *Aloe* species (Coopoosamy and Magwa, 2006;Birincioglu et al., 2012). Modern drugs are synthesized depending on chemical and biotechnological approaches to assess their activity; these approaches are composed of two steps. Firstly, the effective compounds are extracted and then applied to the target tissue or organ. In the second step the physiological change is assessed and this change represents the activity and affectivity of the drug, and in some cases the molecular target is identified (Berg J, 2012;Talib and Mahasneh, 2010).

Traditional medicine can be defined as the knowledge, skills, and practices based on theories, and experiences that are used in the maintenance of health, prevention, diagnosis and treatment of physical and mental illness (WHO, 2000). Herbal medicine is one of the best examples of traditional medicine, and it can be classified into four systems; Traditional Chinese Herbalism, Ayurvedic Herbalism, Western Herbalism (Europe and America), and Traditional Arabic and Islamic Medicine (Azaizeh et al., 2010). Herbal or green medicine had developed rapidly during the last century. According to the World Health Organization (WHO) three quarters of the population of Asia and Africa depends on traditional medicine, especially herbal remedies to treat many illnesses. In 2005 China's income from herbal remedies reached US$ 14 billion (WHO, 2008). Herbal plants have been used for the treatment of many diseases such as asthma, diabetes, kidney stones, skin disorders, respiratory disorders, urinary system disorders, cardiovascular diseases and cancer (Al-qurain, 2008;Ali-Shtayeh et al., 2000;Jaradat, 2005;Talib and Mahasneh, 2010).

1.1 Biodiversity and floral richness in Palestine (The Holy Land):

Palestine as part of the Eastern Mediterranean region is known for its floral richness. There are more than 47,000 organisms within 27000 Km^2, 2700 of them are plant species, in which more than 600 are known to have medicinal values, and many of these plants are mentioned in the three religions: Islam, Christianity and Judaism (Al-qurain, 2008;Saad et al., 2005;Ali-Shtayeh et al., 2000). This floral richness is a result of its diverse geography where it is loacated in the fertile cresent or as it was called before as "The cradle of civilization". Paleatine has five different ecosystems, i) coastal, ii)central highlands, iii) eastern slopes, iv) desert and v) the Jordan Rift Valley (Ali-Shtayeh et al., 2000;Al-qurain, 2008).

Main threats to biodiversity in Palestine are obvious these days, including urbanization, unplanned agricultural activity, overgrazing, overharvesting, uncontrolled fire setting, and lack of knowledge about plant species due to the death of old people (Ali-Shtayeh et al., 2008). In the last three decades threats have increased due to the Israeli settlements expansion, settlements industrial pollution, and the separation wall (Al Butmah A et al., 2013;Ramahi S, 2012). Many plant species are endangered and listed in the red list of endangered species (Ali-Shtayeh and Jamous, 2006;BERC, 2002).

1.2 Asphodel, *Asphodelus aestivus* (Liliaceae)

One of the known and common medicinal plants in Palestine is *Asphodelus aestivus* (synonyms *A. ramosus*, *A. microcarpus*). It is a perennial, Mediterranean geophyte belongs to the Liliaceae family (Asphodelaceae), subfamily Asphodeloideae (Samocha and Sternberg, 2010;Samocha et al., 2009;Ozturk and Pirdal, 1986). *Asphodelus aestivus* is distributed all over the Mediterranean region, especially in Palestine, Lebanon, Syria, Jordan, Turkey, Portugal, Spain, Greece and Italy. Furthermore, it is also found in regions resembling Mediterranean ecosystem such as West Asia, Canary islands, and California. These regions are usally defined as the Asphodel dessert as in Palestine or the Asphodel semi-desert as in Greece (Ayyad and Hilmy, 1974;Samocha et al., 2009).

The Arabic names of *A.aestivus* is Gysalan غيصلان, Basol باصول , Swai صوي and Berwaq برواق (IUCN, 2005;Ali-Shtayeh and Jamous, 2006;Abu-Rabia, 2012;Rai et al., 2011). The name has a Greek origin in which *Asphodel* means the unsurpassed,

aestivus means of summer, or as in summer. *Asphodelus aestivus* was mentioned in the Odyssey of Homer, according to this epic the souls of the dead when arrive to the underworld it firstly come to the meadows of Asphodel where only Asphodel blooms, and so Asphodel is a symbol of death and it was planted near graveyards (Sawidis et al., 2005).

Asphodelus aestivus is a synanthous geophyte, synanthous geophytes can be defined as geophytes that have leaves and flowers at the same time or in some cases the plant develop the leaves followed by the flowers (Samocha and Sternberg, 2010;Kirmizi et al., 2014;Kamenetsky and Okubo, 2012). *A. aestivus* may reach two meters in high and the plant consists of a long leafless stalk (~70 cm) that ends with an elongated inflorescence, that is composed of clusters of flowers (Samocha and Sternberg, 2010). Flowers are large white with reddish midrib, each day one flower open from the base towards the apex of the inflorescence. After pollination flowers develop to give a large oval fruit that contains 6 seeds (Samocha and Sternberg, 2010). The leaves are bluish-green, simple, sword shaped, slightly thick and leathery, and they are protected against herbivorous by steroid saponins (Ayyad and Hilmy, 1974;Sawidis et al., 2005). The most important part in *Asphodelus aestivus* is the underground storage tuberous roots (Figure 1.1C) that stores water, minerals and many other compounds that help in plant survival by regulating osmotic pressure and reduction of water loss (Sawidis et al., 2005).

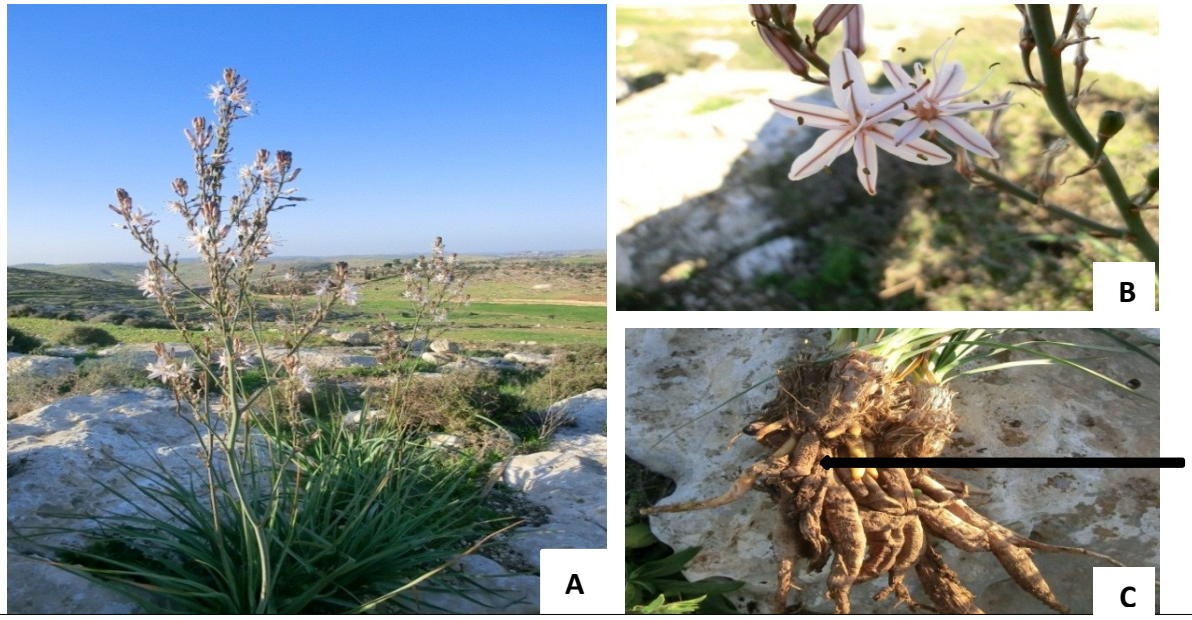

Figure 1.1: *Asphodelus aestivus* plant.
(A) Whole plant structure. (B) Flower of Asphodel. (C) Tuberous roots showing old and new roots "arrow". Photo by Alshawamreh, L.(Der Alasal Alfoqa -Dora – Hebron – Palestine).

Kirmizi etal. (2014) have suggested that the geophyte tubers have two main roles; firstly, they store carbon and nitrogen in forms that can be easily mobilized when the plant needs it. Secondly, these tubers help in the production of new plants by the development of new propagules. Every year at the beginning of autumn *A. aestivus* develops new tubers that store water in order to be used during the hot and dry summer (Samocha et al., 2009;Polycarpou, 2009;Post, 1980).

A. aestivus is well adapted to the Mediterranean climate, where there is a huge fluctuation in the humidity and rain percentage. *A. aestivus* has two life phases, the active phase starts by the emergence of the first leaves in autumn until the end of spring, then the dormant phase in the hot summer until the next autumn (Polycarpou, 2009;Kirmizi et al., 2014). *A. aestivus* morphology helps in the adaptation to semi-arid climate. Ozturk and Pirdal (1986) have addressed that Asphodel seed has hard seed coat and mucilage layer when freshly harvested and so it possesses deep dormancy which delays the germination. Also, they have suggested that dormancy is due to some physiological factors, in which the seed fail to germinate; this is approved by seed stratification and GA_3 pretreatment. Results revealed that GA_3 pretreatment increased germination up to 68% (Ozturk and Pirdal, 1986).

Asphodelus aestivus is a self-compatible plant, however, for successful pollination insects are needed to transfer the pollen grains. Insects especially honey bees and the female mirid bug *Capsodes infuscatus* is the main pollinator for Asphodel and the female mirid bug *C. infuscatus* is the main enemy for it (Figure 1.2) (Izhaki et al., 1996). The bug nymphs hide insides the old leaves and tubers of *A. aestivus* during summer and when the plant starts to develop the leaves they immerge out and feed on it causing leaves discoloration. If *C. infuscatus* nymphs reached Asphodel capsule so they well feed on it leading to the reduction of the number of viable seeds. For this reason the germination rate of *A. aestivus* is very low and the plant depends on the tuberous root to produce new plants (Samocha and Sternberg, 2010). Another reason for low seed production depends on the quality and quantity of pollen grains reaching the plant. It has been reported that if the pollen grains are from the same colony so the number of viable seeds will be low and this indicates a sort of self-sterility on this plant due to polyploidy since *A. aestivus* is hexaploid. Cross fertilization between different colonies increased the number of mature capsules and seeds (Schuster et al., 1993).

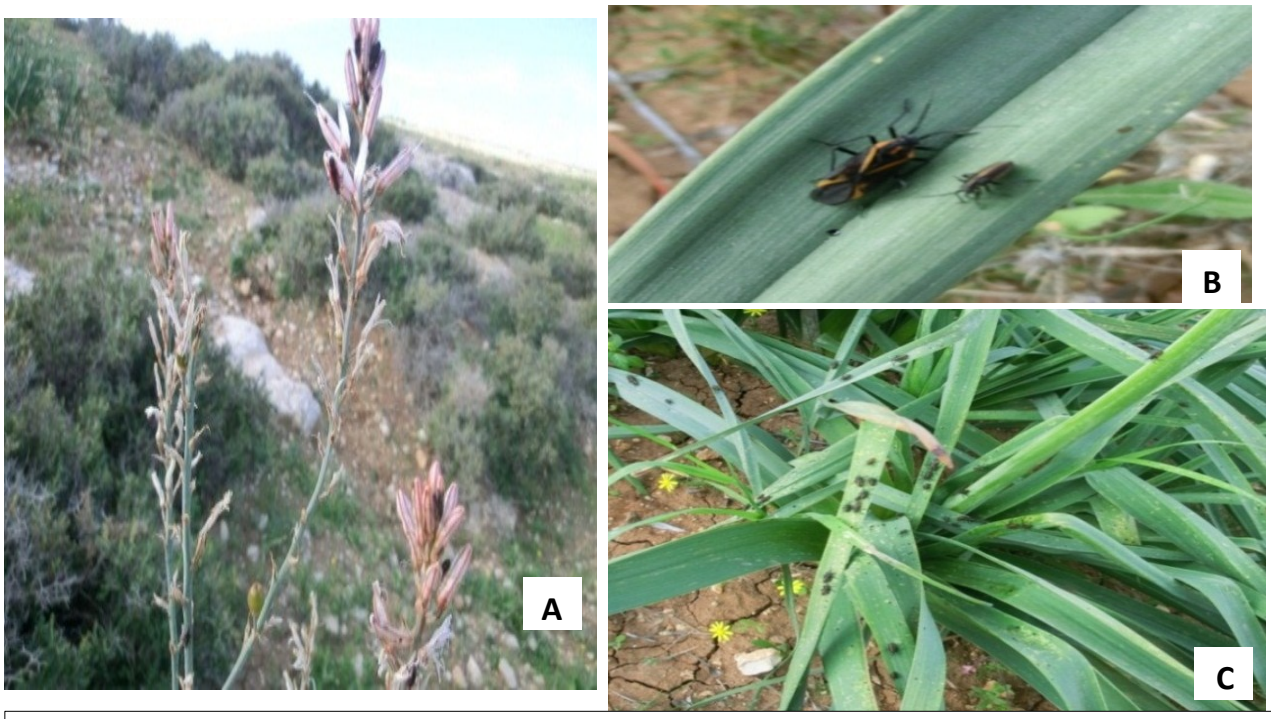

Figure 1.2: Infection of *Asphodelus aestivus* with mirid bug *Capsodes infuscatus*.
(A) Asphodelus inflorescence infected with the bug. (B) The mirid bug *C. infuscatus* and a newly emerged small nymph. (C) Asphodelus leaves covered with mirid bug *C.infuscatus.*
Photo by Alshawamreh, L. (Der Alasal alfoqa -Dora –Hebron – Palestine).

1.2.1 *Asphodelus aestivus* in Palestine

A. aestivus is distributed all over the mountains and valleys of Palestine from the upper Galilee mountains (height 1208 meters, mean annual rain fall 780 mm) in the north to the Negev desert (height 175 meters, mean annual rainfall 90 mm) in the south (Ayyad and Hilmy, 1974;Samocha et al., 2009;Izhaki et al., 1996).

1.2.2 Uses of *Asphodelus aestivus* in Traditional Medicine

For centuries *Asphodelus aestivus* has been used in traditional medicine to treat many illnesses, and all plant parts are believed to have medicinal values. The green leaves of *A. aestivus* are poisonous. Ingestion of green leaves by cattle can cause neurological disorders with intense neuronal pigmentation (Calisa et al., 2006;Birincioglu et al., 2012). But, when leaves are dry they can be used as animal fodder (Tastad et al., 2010;Lev yadun and Ne'eman, 2004). *Asphodelus aestivus* has been used in most Mediterranean countries to treat ectodermal parasites, jaundice, and psoriasis (Ghoneim et al., 2014;Abuhamdah et al., 2013;El-Seedi, 2007;Lev yadun and Ne'eman, 2004). In Palestine *Asphodelus aestivus* have been used for the treatment of hair and skin diseases and wounds (Alkowni and Sawalha, 2012;Ali-

Shtayeh and Jamous, 2006). Abu–Rabia (2012), has reported that Bedouins in Negev desert have used *A. aestivus* for the treatment of paralysis, treatment applied externally by rubbing the body with roasted tubers as an ointment and drinking decoction from leaves (Said et al., 2002). Additionally, in northern Palestine a herbal tea is prepared by cutting the tuberous roots of *A.aestivus*, the roots is grinded and mixed with hot water in a tea pot then it can be drank, sugar or honey may be added, this tea is very beneficial for digestive system disorders such as stomach ulcer (Lev, 2002;Einav, 2011).

In Turkey the tubers are used for the treatment of hemorrhoids, nephritis, burns and wounds (Aslantürk and Çelik, 2013;Kirmizi et al., 2014;Peksel et al., 2012;Ilker et al., 2009), also it is used for the treatment of mesentrual obstraction (Leyel, 1984), and as a diuretic agent (Abu-Rabia, 2012;Kirmizi et al., 2014;Reynaud et al., 1997). In north African countries such as Tunisia, Algeria and Morroco they use *A. aestivus* root extracts for the treatment of skin diseases including eczema, vitiligo and paronchia (Lardos, 2006;IUCN, 2005;Ouarghidi et al., 2013;Zellagui et al., 2013). Moreover, *A. aestivus* is used to treat ear problems, where treatment is prepared by grinding the tubers and mixing with warm olive oil, and then three drops are applied daily (IUCN, 2005). In Algeria, root decoction is used to treat digestive system problems such as stomach ulcer (González-Tejero et al., 2008). In central Spain, a decoction from the roots of *A. aestivus* is made for the treatment of muscular spasm (Rai et al., 2011). Zellagui et al (2013) have reported the use of *A. aestivus* for the treatment of rheumatism, colds and to gain weight. Lardose (2006) reported the use of *A. aestivus* roots and tubers for the treatment of migraine, toothache and as a cosmetic agent to promote growth of the hair. Additionally, many Muslim and Arab scholars such as Ibn al-Baytar, Daud al-Antaki and Abu'l- Abbas an-Nabati has reported the use of *A. aestivus* for the treatment of internal, and sexual diseases (Lev, 2002). *Asphodelus aestivus* is also used in veterinary medicine, crushed roots are used as a curative agent to treat skin problems and wounds in horses (Sarker et al., 2006).

1.2.3 Phytochemical Composition of *Asphodelus aestivus*

Asphodelus aestivus contains several compounds such as glycosides, alkaloids, anthraquinones, flavonoids, anthranoids, and triterpenes (Aslantürk and Çelik, 2013;Abu-Rabia, 2012;Calisa et al., 2006). These compounds are distributed in all

plant parts (Table 1.1). First attempts for the isolation of secondary metabolites from *A.aestivus* was conducted by Hammouda et al (1971), they have isolated two alkaloid compounds, Stachydrine and Choline. Stachydrine is a potent anticancer agent, it has been reported that stachydrine have inhibited the metastasis of prostate caner cell line (Rathee et al., 2012). Choline is a very important compound that has been used in the treatment of liver disorders, hepatitis, glucoma, atherosclorosis, and alzheimer (Chan et al., 2009;Van Beek and Claassen, 2011). Recently, El-Seedi (2007), have isolated two arylcoumarins from the tubers of *A.aestivus*, the active form is called asphodelin-A that works as antibacterial and antifungal agent.

Ghoneim, Elokely et al. (2014) have isolated five compound, two of them show high potent activities against bacterial cells and leshmania. The first compound was methyl-1,4,5-trihydroxy-7-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylate show potent activity against Methicillin-resistant *Staphylococcus aureus* bacteria (MRSA) and *Staphylococcus aureus* with a half maximal inhibition concentration (IC_{50}) value of 1.5 mg/ml and 1.2 mg/ml respectively. The second compound, 3,10-dimethoxy-5-methyl-1*H*-1,4-epoxybenzo[*h*] isochromene show antileshmanial activity with IC_{50} value of 33.2 mg/ml. The tubers also contain starch, inulin, fatty acid, and high percentage of sugars such as sucrose, glucose, and fructose (Polycarpou, 2009;Ozturk and Pirdal, 1986). Most of the isolated compounds possess anti-oxidant, anti-cancer, antimicrobial, anti-malarial and anti-leshmanial activity.

Table 1.1: Main phytochemical compounds present in different parts of *A.aestivus*

Phytochemical Compound	**Part used**	**Reference**
Methyl Syringate	Flowers	(Tuberoso et al., 2009)
Germacrene - D	Flowers	(Zellagui et al., 2013)
Germacrene- B	Flowers	(Zellagui et al., 2013)
Luteolin	leaves	(Reynaud et al., 1997)
Chlorogenic acid	Leaves	(Calisa et al., 2006)
7-O- glucosyl apigenin	Leaves	(Reynaud et al., 1997)

Isoorientin	Leaves	(Reynaud et al., 1997)
Adenosin	Leaves	(Calisa et al., 2006)
Aloe – emodin acetate	Leaves	(Calisa et al., 2006)
Chrysophanol -O- gentiobiosides	Leaves	(Calisa et al., 2006)
Isovitexin	Leaves	(Calisa et al., 2006)
Asphodelin–A	Tubers	(El-Seedi, 2007;Ghoneim et al., 2013)
Aloe – emodin	Tubers and leaves	(Rizk et al., 1972;Calisa et al., 2006)
7-O- glucosyl luteolin	Tubers	(Reynaud et al., 1997)
Chrysophanol	Tubers	(Rizk et al., 1972;Ghoneim et al., 2013)
Emodin	Tubers	(Ghoneim et al., 2013)
Ramosin	Tubers	(Ghoneim et al., 2013)
Aestivin	Tubers	(Ghoneim et al., 2013)
Stachydrin	Tubers	(Hammouda et al., 1971)
Choline	Tubers	(Hammouda et al., 1971)
Bichrysophanol	Tubers	(Rizk et al., 1972)
Chrysophanol glycoside	Tubers	(Rizk et al., 1972)
1,8 – dihydroxy - anthraquinine	Tubers	(Rizk et al., 1972)
methyl-1,4,5-trihydroxy-7-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylate	Tubers	(Ghoneim et al., 2014)
3,10-dimethoxy-5-methyl-1*H*-1,4-epoxybenzo[*h*]isochromene	Tubers	(Ghoneim et al., 2014)

1.2.4 *In vitro* Culture of *Asphodelus aestivus*

According to a recent literature review *in vitro* culture of *A. aestivus* has not been addressed. Traditional propagation methods mainly depend on the propagation either by seeds or by division of the tubers. Seeds are sown in the soil for three months at 15°C, and then when the seedling grows enough they are transferred and cultivated in pots or in soil (Sawidis et al., 2008). Ozturk and Pirdal (1986) have reported that the seeds of *A. aestivus* have deep coat dormancy, this coat is impermeable to moisture and so they do not germinate under normal conditions, also *A. aestivus* seeds show high sensitivity to light and salt concentration. Division of tubers is applied in early spring or autumn when there is enough humidity and the plant is in its active phase (La Mantia et al., 2012).

1.3 *In vitro* Culture of plants

Plant tissue culture has several applications such as micropropagation, production of disease free plants especially from viruses, conservation of endangered plant species, and the production of secondary metabolites. Plant *in vitro* culture is rapid, season independent, not limited to species or genus, independent from political interferences and it ensures the continuous production of novel secondary metabolites. However, plant tissue culture has various complications like instability of cell lines, low yield, slow growth and expensive method (Murthy et al., 2008;Ramachandra Rao and Ravishankar, 2002).Various factors influence plant tissue cultures as plant growth regulators (PGRs), nutrient medium, pH of the media, oxygen, and temperature levels. Plant growth regulators are crucial factors in plant tissue culture development pathway (Saad and Elshahed, 2012). These PGRs are mainly classified into three families; auxins, cytokinins and Gibberellins, and concentration of each cause different developmental changes. Auxins such as (IAA, IBA, 2.4-D and NAA) in high concentrations induce cell division and root formation, and high cytokinins such as (BA, kinetin, TDZ, Zeatin and 2-ip) concentrations induce cell division and shoot proliferation. If the ratio between the auxin and cytokinin level is equal, this will lead to the formation of the undifferentiated cells that known as callus tissue. The third family is Gibberellins, it is composed of about twenty compounds, and GA_3 is the most commonly used one. Gibberellins enhance growth, help in plant development and in many studies they have been used to overcome seed dormancy and increase

germination rates (Haque and Ghosh, 2013;Hussain et al., 2012;Ramachandra Rao and Ravishankar, 2002;Zakia et al., 2013).

1.3.1 Micropropagation of medicinal plants and the production of secondary metabolites

In the last years, several approaches have been developed for the propagation of medicinal plants in order to produce and isolate secondary metabolites. McCaratan and Van Studen (2003) have developed a protocol for the propagation of *Kniphofia leucocephala*, a medicinally important plant belongs to Asphodelaceae family (Afolayan and Adebola, 2005). Lee et al. (2011) have established a propagation protocol for the production of aloe emodin from *Aloe* roots, this protocol depend on the induction of adventitious roots from leaf tissue. Plant tissue culture is an excellent approach to study and produce secondary metabolites. There are several advantages for production of secondary metabolites by *in vitro* techniques. They include: a) secondary metabolites production and isolation are simpler and more reliable compared to production from *ex vitro* sources, b) compounds production can be enriched by elicitation, c) the quality and quantity of the produced compounds are higher in many cases than conventional methods, and d) isolated secondary metabolites can be labelled and used in food for laboratory animals that can be traced (Karuppusamy, 2009;Ramachandra Rao and Ravishankar, 2002).

Advances in plant tissue culture technology have led to the production of many pharmaceutical compounds such as, Taxol (plaxitaxol) an anticancer agent isolated from Taxus tree. Morphine and codeine, an important compounds used to relive pain in many illness, isolated from opium poppy. Another example is L-Dopa (L-3,4-dihydroxyphenylalanine), L-Dopa is a potent drug for Parkinson's disease, and it is isolated from several plants as Fava bean , Mucuna and Baptsia (Vanisree et al., 2004)

1.4 Isolation and qualitative determination of secondary metabolites

1.4.1 Extraction of secondary metabolites

Secondary metabolites are essential compounds for plant growth and development; they play an important role in cell structure maintenance, support and protection against biotic and a biotic agents (Kennedy and Wightman, 2011). Furthermore, plant secondary metabolites play an important role in human health by working as antioxidant, free radical-scavenging, and antiproliferative agents, also it can be used as nutritional additives (Kennedy and Wightman, 2011;Menghani et al., 2012). In

order to investigate the importance of these compounds several techniques has been developed, extraction is one of the best techniques.

Extraction is a very old technique back to the time of Egyptians and Sumerians where it was used to separate and isolate several substances including perfumes, oils and waxes. Extraction is a process, in which secondary metabolites are separated from other plant tissues by using special techniques and solvents (Jörg Bart, 2011). After plant collection, the whole plant or the desired part of the plant is grinded to powder using either mortar and pestle or blender. Then, the powder is dissolved in the appropriate solvent for 24, 48 hours or more depending on the solvent components and the secondary metabolites themselves. Extraction methods includes maceration, decoction, reflux, microwave-assisted extraction (MAE), hot continuous extraction (Soxhlet), and ultrasonic assisted extraction (Jörg Bart, 2011;Margeretha et al., 2012). Moreover, there are several parameters affecting extraction method and their yield like the solvent type and concentration, time, temperature, and liquid to solid ratio (Margeretha et al., 2012;Liu et al., 2010). Since solvent is the most important factor affecting any extraction method there is several criteria for the selection of solvents. They include, solubility of compounds, polarity, stability, selectivity, toxicity, flammability, surface tension, recoverability of solvent, environmental impact, cost and availability of the solvent (Jörg Bart, 2011).

1.4.2 Thin layer chromatography (TLC)

Chromatography is a method in which a compound is isolated, separated, purified and identified from a mixture of compounds. There are several chromatography techniques such as adsorption chromatography, partition chromatography, gel chromatography and bio-affinity chromatography (Jörg Bart, 2011). Thin layer chromatography (TLC) is a classical chromatography types, simple, easy, rapid, and non-expensive; it is used as an analytical method for the qualitative and semi-quantitative detection of natural and synthetic products (Sarker et al., 2006;Wagner, 1996). TLC consists of two phases, the stationary phase that is usually made of a polar solid substance, e.g. silica or alumina plates. The second phase is the mobile phase and it is composed of a solvent or a mixture of solvents, which differ in their polarity (Sarker et al., 2006;Jörg Bart, 2011). The mobile phase solvents move through the stationary phase by capillary action, and through its movement it separates the secondary metabolites. Separation of secondary metabolites depends

mainly on the solute polarity, non-polar compounds move faster than polar compound. Migration or movement of any compound through TLC plates is known as development, and it is assessed by retardation factor (R_f) value. R_f value is between 0 and 1 and always written as rations, it is defined by the equation as cited in (Jörg Bart, 2011).

$$R_f = \frac{\text{Compound distance from origin (midpoint)}}{\text{Solvent front distance from origin}}$$

2. Problems Statement and Objectives

2.1 Problems Statement

Asphodelus aestivus is highly adapted to hot and dry Mediterranean climate where a vast fluctuations in mean annual rainfall. *A. aestivus* propagation by conventional methods is problematic, since the seed show low germination percentage and high sensitivity against some factors like, light, humidity, salt concentration and osmotic pressure. Therefore, using plant biotechnology techniques offers an excellent alternative to propagate and study *A. aestivus*. Moreover, it is known that plants growing in harsh environments such as deserts and semi-deserts produce different secondary metabolites that play roles in defense against abiotic factors such as drought, salinity and biotic factors as insects, herbivorous, and pathogens. Therefore, using plant *In vitro* propagation techniques can be used to increase secondary metabolites production.

2.2 Objectives:

The main objective of the present study is.

To implement biotechnological methods mainly plant *in vitro* culture to propagate *Asphodelus aestivus*, and then to study the secondary metabolites production.

Specific objectives:

1. To develop micropropagation protocol for *Asphodelus aestivus.*

2. Optimizing a suitable growth medium with optimal PGRS for culture induction, shooting, and rooting

3. Optimizing a suitable growth medium for callus induction and maintenance from different parts.

4. To assess secondary metabolites produced by different *in vitro* and *ex vitro* sources of *A. aestivus*.

CHAPTER TWO

Materials and Methods

This study was conducted in the Plant Tissue Culture Laboratory, Biotechnology Research Center at Palestine Polytechnic University, Hebron, Palestine.

2.1 Collection of plant Material

Seeds, leaves and growing shoot tips of *Asphodelus aestivus* were collected from Der-Alasal Alfoqa village (latitude: 31.466, longitude: 34.939, altitude: 481 meters), Dora, Hebron. Seeds were collected twice; in September, and May. Asphodel tuberous roots collected during October – December 2013. All plant parts were collected and stored in plant tissue culture laboratory for further analysis.

2.2 Chemicals and Reagents

The basal growth media; Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) were purchased from Duchefa biochemie, Netherlands. The plant growth regulators (PGRs); 6-Benzyleaminopurine (BA), Gibberellic acid (GA_3), Indole-3-acetic acid (IAA), 1-Naphtalene acetic acid (NAA), and Isopentenyl adenine (2-ip) were purchased from Duchefa biochemie, whereas kinetin (Kn) 2,4-Dichlorophenolxyacetic acid (2,4-D), and Indole-3-butyric acid (IBA) were purchased from Sigma-Aldrich. Polyvinylpyrrolidone (PVP) was purchased from Duchefa. Media was gelled by agar purchased from HyLabs Company for laboratory chemicals. Cat number of every chemical are listed in appendix Table 9.

2.3 Media Preparation and Sterilization

Growth media was prepared using MS basal salts including vitamins. Different concentrations of the MS basal salt have been used. MS medium was prepared by dissolving 4.4 g/L of the MS salt, and the addition of 30 g/L of sucrose. Suitable plant growth regulators (PGR) were added, after that the mixture was completed to its final volume. The pH of the medium was adjusted to 5.8 by using 1.0 M KOH or 1.0 M HCl. All media were autoclaved at 121°C and 121 pa for 20 min.

2.4 Sterilization of Plant Material

Seeds, roots and growing shoots were first washed with running tap water for 10 min. In the laminar flow cabinet, and for starting *in vitro* seed culture, the seeds were removed from its capsule; seeds were dipped in 100 ml of 20 % v/v Chlorex (5.0 % NaOCl) with continuous shaking for 25 min. then Chlorex was removed by rinsing seeds with sterile distilled water (SDW) three times two min each, then, seeds were dipped in 70% ethanol for 30 sec. Finally, seeds were washed by sterile distilled water three times for 2 min each. After sterilization, seeds were cultured on gelled-water agar (8 g/l) at pH 5.8, and MS media. Plates were incubated in growth room at 25°C under photoperiod of 16 hours light duration with white fluorescent tubes 45 µmol/m²/sec. With the same steps tuberous roots is sterilized, after that they were cultured on different MS media with different concentrations supplemented with different plant growth regulators.

2.5 Growth Conditions

All cultures were incubated in the growth room at 24 ± 1ºC with 16:8 light: dark photoperiod under cool-white fluorescent illumination of 40-45 µmol/m^2/sec photosynthetic photon flux density (PPFD). Seeds then incubated at room temperature, in order to break seed dormancy.

2.6 Culture Types

2.6.1 *In Vitro* Seed Germination

Before the *in vitro* culture of seeds, the seeds were divided in to three lots; one lot was exposed to pretreatment by soaking in: i) water, ii) 1.0 mg/l of GA_3, iii) 5.0 mg/l of GA_3. Seeds were soaked for one week at room temperature. In the second lot, seeds were pretreated by soaking for 48 hours in either distilled water, 1.0 mg/l of GA_3, and 5.0 mg/l of GA_3. Then they were cultured on two growth media MS and water agar medium and stored at 4°C for 30 days. After that seeds were transferred to growth room and stored in dark cabinet. The third lot was sterilized and directly cultured.

The sterilized seeds were cultured on full strength MS, half strength MS, and gelled-water agar medium.

2.6.2 Subculture of *In Vitro* Germinated Seeds

The germinated *A. aestivus* seeds were transferred to glass jars filled with MS medium or gelled-water agar medium. MS medium supplemented with different combination of PGRs as listed in Table 2.1. Germinated seedlings were monitored for two weeks to evaluate their growth and then were subcultured to new media. These media combinations were used as a starting media to design the next experiments and 1.0 g/l of PVP was added to all media combination.

Table 2.1: Treatments that were used for sub-culturing of *In Vitro* Germinated Seeds

Treatment	IAA (mg/L)	BA (mg/L)	NAA (mg/L)
1	1.0	2.0	0
2	1.0	4.0	0
3	1.0	6.0	0
4	2.0	2.0	0
5	2.0	4.0	0
6	2.0	6.0	0
7	0	1.0	0.25
8	0	4.0	0.25
9	0	1.0	0.5
10	0	4.0	0.5
11	0	1.0	1.0
12	0	4.0	1.0
13	1.0	4.0	1.0

2.6.3 Micropropagation of *Asphodelus aestivus*

2.6.3.1 Establishment of Motherstock Culture

Asphodels growing shoot tips were excised from wild mother plant. They were cleaned by washing for 5 min, then peeled off and cut into 2.0 cm x 1.0 cm (basal diameter x length) segments. They were surface sterilized with 5% Chlorex and 70% ethanol, followed by three times washing with sterile distilled water (SDW). Then they were cultured on MS medium supplemented with PGR as the following:

a. 4.0 mg/L BA, 0.4 mg/L IAA, and 1.0 g/L PVP.
b. 2.0 mg/L Kn.
c. 4.0 mg/L 2.4-D and 1.0 g/L PVP.

These media was designed depending on the results obtained in section 2.6.2

2.6.3.2 Shoot Proliferation and Regeneration

Growing shoots were excised from the *in vitro* mother plant and cultured in 250 ml flasks filled with 50.0 ml MS medium supplemented with IAA, BA, and 2-ip as the following:

Table 2.2: Treatments used for testing shoot proliferation.

Treatment	IAA (mg/L)	BA (mg/L)	2-ip (mg/L)
1	**0**	**0**	**0**
2	**0.5**	**0.5**	**0.5**
3	**0.5**	**1.0**	**1.0**
4	**0.5**	**2.0**	**2.0**
5	**0.5**	**4.0**	**4.0**

Treatments were arranged in a CRD with four replicates per treatment with four shoots per replicate. Cultures were transferred to growth room at light regime of 16:8 hours (light: dark) and photoperiod (photosynthetic photon flux density (PPFD) = 40-45 µmoles/m^2/sec) at 25 ± 1°C. Data were recorded after five weeks of culture to evaluate the percentage of shoot proliferation, shoot height, number of proliferated leaves/shoot, and tissue browning.

2.6.3.3 *In vitro* Rooting

Shoots about 2.0 cm length that grown on media mentioned in section (2.6.3.2) was used. Shoots were cultured on full MS supplemented with 1.0, 2.0, and 3.0 mg/L of IAA, NAA, or IBA. Each treatment was arranged in a CRD design with four microshoots per flask and four flasks per treatment. Cultures were transferred to growth room at light regime of 16:8 hours (light: dark) and photoperiod (photosynthetic photon flux density (PPFD) = 40-45 μmoles/m^2/sec), at 25 ± 1°C. Data were recorded after 4 weeks of culturing to evaluate the percentage of rooting, mean number of roots, mean of root length, mean of shoot height, mean number of proliferated leaves/shoots, and callus formation.

2.7 Callus Induction and Culture

2.7.1 Callus Induction

Callus was induced from both *A. aestivus in vitro* growing roots and immature seeds "embryos" from *ex vitro* plant. The immature seeds were collected from the wild. Unripe capsules containing the immature seeds were sterilized and then opened by blade and carefully the seeds were removed from it. Roots and seeds were cultured on 5.0 cm Petri-dishes filled with full strength MS with three combinations: (i) 2.0 mg/L BA, and 1.0 mg/L 2,4-D. (ii) 2.0 mg/L KN, and 0.5 mg/L NAA. (iii) 4.0 mg/L BA, and 2.5 mg/L NAA. All plant tissue was stored in dark condition.

2.7.2 Callus Subculture

Calli clumps were transferred again on the media which gave highest growth results. Callus tissue developed from immature seeds, *ex vitro* and *in vitro* roots were subcultured on the same induction media and monitored for two weeks to evaluate general growth.

2.8 Extraction and determination of chemical constituents of *Asphodelus aestivus* using Thin Layer Chromatography (TLC)

2.8.1 Plant material

For *ex vitro* sources roots of *A. aestivus* were collected from Der-Alasal Alfoqa village, south Hebron (latitude: 31.466, longitude: 34.939, altitude: 481 meters) in the beginning of summer 2013. The roots were then washed with tap water, cleaned with tissue paper to remove any soil particles, and

dried at room temperature away from direct light. After four weeks the dried roots were powdered by grinder and stored in glass container until the beginning of the extraction. For *in vitro* grown roots of *A. aestivus* were collected from seedlings grown on MS medium as described in section (2.6.3.3).

2.8.2 Extraction Procedure

One gram of the dry powder of *ex vitro* roots and 0.5 gram of *in vitro* roots were used for extraction. Each sample was mixed with 50.0 ml of either water, ethyl acetate or 99% ethanol in a 50.0 ml falcon tubes with continuous stirring for 48 hours. After that, the extract was centrifuged for 10 min at 4000 rpm to separate the supernatant. The supernatant was air dried under suction hood. The yield was calculated by measuring the weight for each sample divided by the initial weight.

2.8.4 Thin Layer Chromatography (TLC) analysis

For the detection of secondary metabolites, 10.0 mg of each sample was dissolved in 1.5 ml distilled water. The analyses of these samples were conducted using pre-coated silica gel plates (Merck, Germany Silica gel 60 F254, 0.25 mm) as a stationary phase. The solvent system used for TLC analysis were: chloroform: water (15:1), butanol: acetic acid: water (4:1:5), ethyl acetate: n-butanol: water (10:10:4), ethyl acetate: n-butanol: water: formic acid (10:10:4:2), and methanol: water: formic acid (13.3: 19: 1) was used. All plates were examined under ultraviolet (UV) light.

2.9 Experimental Design and Statistical Analysis

All experiments were arranged in a Completely Randomized Design (CRD) and significant difference between means was tested by the analysis of variance ANOVA with Statistical Package for the Social Science SPSS version 13. In the case of Significant at $p = 0.05$, means were separated by Fisher's least significant difference (LSD) test.

CHAPTER THREE

Results and Discussion

Based on recent literature review, this is the first study to address the *in vitro* culture of *Asphodelus aestivus*, and so all the *in vitro* experiments conducted on *A. aestivus* and listed below are conducted for the first time to our knowledge.

3.1 *In Vitro* Culture of *Asphodelus aestivus*

3.1.1 *In Vitro* Seed Germination

The aim of this experiment was to investigate the factors influencing *in vitro* seed germination of *A. aestivus*. Ozturk and Pirdal (1986) have studied *Asphodelus aestivus* seed dormancy. They have mentioned that *A. aestivus* seeds have deep mechanical dormancy. Dormancy is caused because *A. aestivus* has hard seed coat and mucilage layer, results in a very low germination percentages. Also they have mentioned that *A. aestivus* seeds are very sensitive to temperature, light period, salt concentration and substrate osmotic potential. Furthermore, this unique type of germination is known as intermittent seed germination, in which the seed store inhibitory chemicals and in order to germinate it must remove them (Keeley, 1995). The newly harvested seed were soaked in water or 1.0 mg/L or 5.0 mg/L GA_3 for one week and then they were cultured on gelled-water agar, MS and half strength MS medium, all cultures were incubated in dark and light regimes, germinated seeds in dark were transferred to the light for further growth. Germination percentages for the treatments are presented in Table 3.1.

Table 3.1: Germination percentage of *Asphodelus aestivus* cultured onto different media after five months of culture. Sample size (n) = 30.

Treatment	Media type		
	MS	½MS	Agar
Water	0	0	0
1.0 mg/L GA_3	7	43	10
5.0 mg/L GA_3	6	7	4
Control	0	7	7

Germination percentages were very low in all treatments, and seeds cultured in dark incubation gave the highest result. Some germinated seeds under light have failed to maintain their growth. In nature viable *A. aestivus* seeds germinate well when sown in 1.0 cm depth from the soil surface.

Results show that seeds pretreated by soaking in 1.0 mg/L GA_3 for 30 days and cultured on half strength MS gave the highest results (43%). Also, it is clear that water stratification result in no germination and this may be due to osmotic pressure alteration between the seeds and the media. Moreover, it has been observed that seeds pre-soaked in water show excessive watery exudates on the medium surface, and this may causes reduction in germination. Several studies have addressed the relation between water loss and the reduction of germination percentage such as in the case of the wild onion *Asphodelus tenuifolius* (Tanveer et al., 2014;Hassan et al., 2006). Basal salts play a critical role in the enhancement of germination and growth of seedlings. MS salt has huge effect on germination since no germination was observed on full strength medium in all the three treatments. The previous results are in agreement with the results of Ozturk and Pirdal (1986). However, germination time differs in this study since seeds start to germinate after nine days of culture especially on ½MS with 1.0 mg/L GA_3 treatment. It must be mentioned that the procedures to overcome seed dormancy between Ozturk and Pirdal (1986) and this study differs in the preparation procedure and the used media. Ozturk and Pirdal (1986) have scarified the seeds and then cultured them in petri dishes containing two layers of filter paper and 5.0 ml of distilled water under 5, 10, 15, 20, 25 and 30°C temperatures and 3, 6, 9, 12, 18 and 24 h light regimes. Results show that the highest germination was observed on 15°C and 6/18 hour photoperiod and the seeds show sensitivity toward light and high salt concentrations.

Many studies have reported the effect of cold stratification on breaking seed dormancy. In this experiment, seeds were soaked either in water, 1.0 mg/L GA_3, or 5.0 mg/L GA_3 for one week then were cultured on gelled-water agar medium, MS and ½MS medium and stored in cold condition at 4°C for one month. Seeds were then transferred to growth room, and monitored for two months. Data is shown in Table 3.2.

Table 3.2: Germination percentage of *Asphodelus aestivus* seeds stored in cold for one month, germination percentages is recorded in different media after two months of culture. Sample size (n) = 10.

Treatment	Media type		
	MS	**½ MS**	**Agar**
Water	0	0	20
1.0 mg/L GA_3	10	0	10
5.0 mg/L GA_3	0	0	0

Results indicate that low temperature treatment has decreased germination rates, the highest germination rates was observed on gelled water agar medium and the seeds was soaked in water for one week (Figure 3.1). This experiment also shows the little effect of GA_3 on seed germination, where only the treatment with 1.0 mg/L GA_3 on both gelled water agar and MS medium result in 10% germination. In conclusion, the results obtained from this experiment may be in contradiction with previous studies conducted to overcome seed dormancy by cold treatment; one explanation is that the time of treatment was not enough since some seeds require long periods of cold to release inhibitors and overcome dormancy.

3.1.2 Subculture of *In Vitro* Germinated Seeds

The germinated *A. aestivus* seeds were subcultured to glass jars containing free MS, gelled-water agar or MS medium supplemented with different concentrations of PGRs. This is done to evaluate the best growth medium for *A. aestivus* motherstock plant establishment. Some seedlings failed to maintain their growth, other seedlings developed low number of shoots without rooting. In other cases seedlings has developed long and thick root with one shoot composed of two or three leaves, the plantlets show low growth and proliferation rates compared to other plantlets (Figure 3.2). MS medium supplemented with 4.0 mg/L BA, 1.0 mg/L IAA and 1.0 mg/L NAA show the highest percentage of proliferation. This is the first *in vitro* study conducted to assess germination rates in *Asphodelus aestivus* using plant *in vitro* culture.

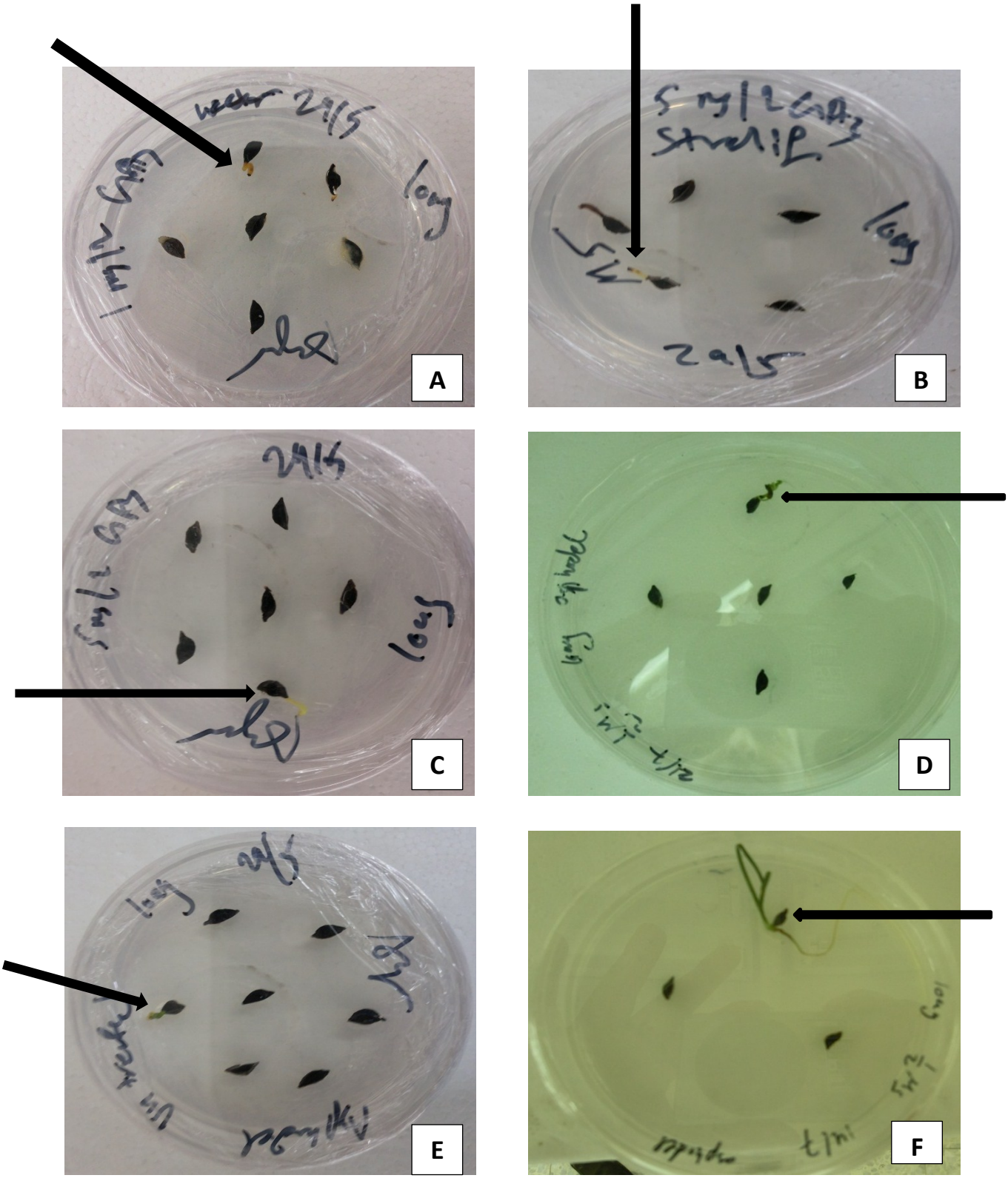

Figure 3.1: Seed germination of *Asphodelus aestivus* on different media.

(A) Seed germination on water agar medium after 1.0 mg/L GA_3 stratification. (B) Seed germination on MS medium after 5.0 mg/L GA_3 stratification. (C) Seed germination on water agar medium after 5.0 mg/L GA_3 stratification. (D) Untreated seeds germinated on ½ MS medium. (E) Untreated seeds germinated on gelled water agar medium. (F) Seed germination on ½MS medium after 1.0 mg/L GA_3 stratification. (Arrows indicates germinated seeds)

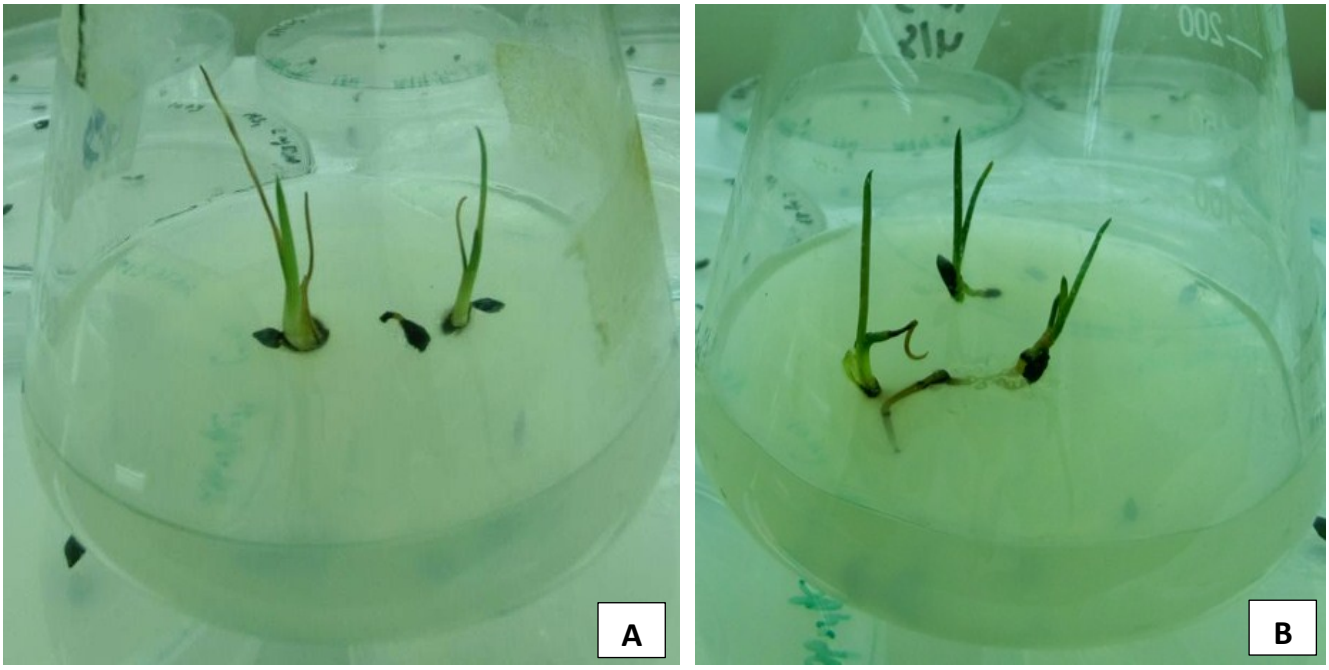

Figure 3.2: *Asphodelus aestivus* seedlings germination, Seeds subcultured into growth media (A) MS with 2.0 mg/L Kinetin. (B) MS free media.

3.2 *In Vitro* Multiplication of Motherstock Plant

Since seeds are not a best source for *A. aestivus* propagation, we have depended on the other second approach, were wild plants are used as a source for the establishment of motherstock material. As mentioned in section (2.6.3.1) the shoot tips "suckers" from wild plant were excised and cut into pieces 2.0 cm x 1.0 cm (basal diameter x length) and then cultured on three MS medium supplemented with different hormones (Figure 3.3). Results show that MS medium supplemented with 4.0 mg/L BA, 0.4 mg/L IAA and 1.0 g/L PVP show the highest plant proliferation rates compared to the other two mediums, the mean shoot number per treatment was 8.

BA enhanced plant proliferation and number of leaves and shoots have increased, in some cases plantlets have developed small roots. The addition of PVP have decreased tissue browning and played a major role in plant growth enhancement. MS medium supplemented with BA and IAA was used as the starting medium for the multiplication of the *in vitro* mother plant.

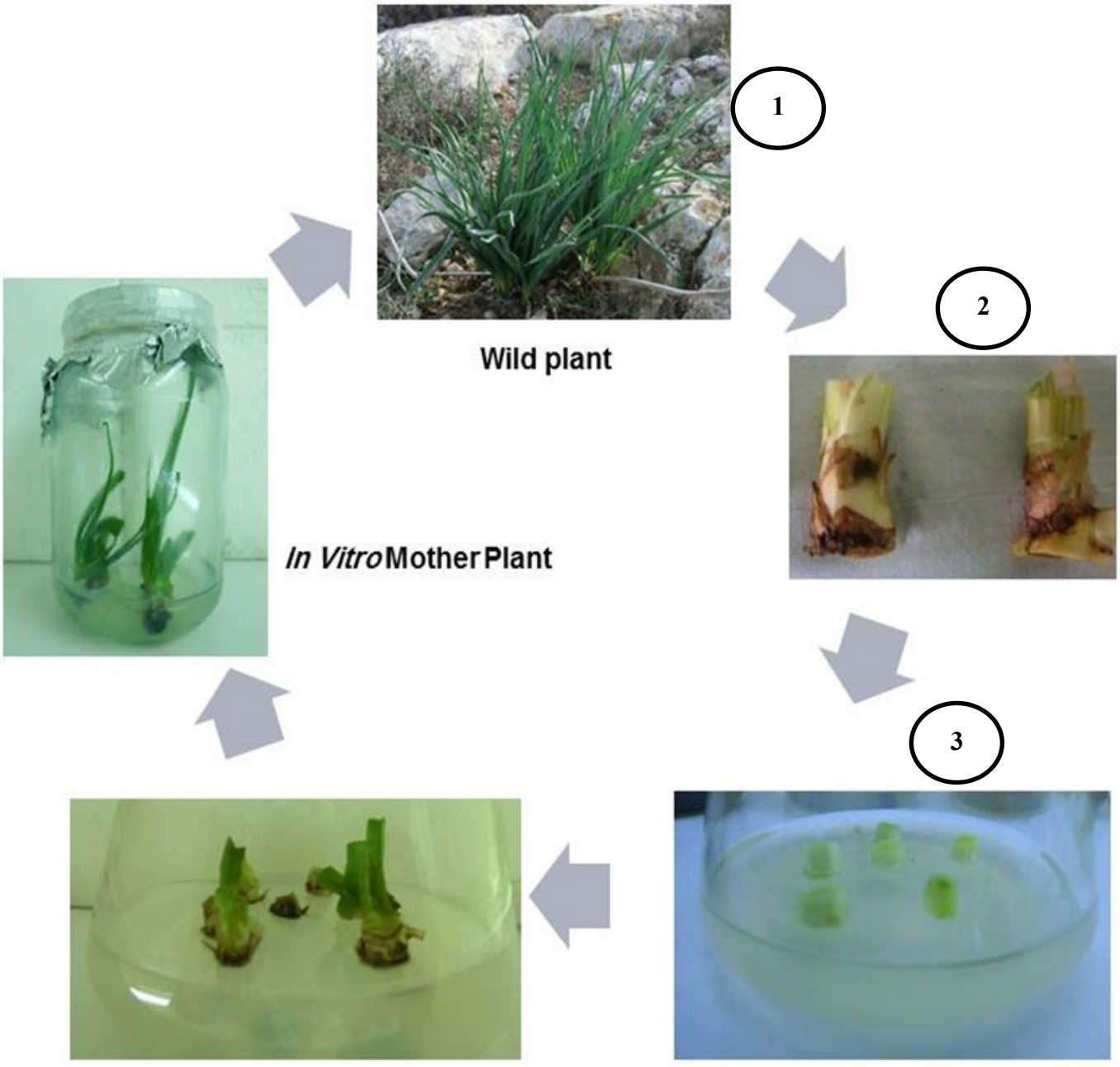

Figure 3.3: Schematic illustration of the *in vitro* culture process.

(1) Motherstock plants were established from shoot tips grown from the tuberous roots. (2) The tissue was peeled off and cut into 2.0 cm x 1.0 cm segments and cultured on free MS medium, and MS medium supplemented with different hormones. (3) MS medium was supplemented with BA at three levels 2.0, 4.0 and 6.0 mg/l, with IAA at two levels 1.0 and 2.0 mg/l, 1 g/l of PVP was added to all media combination. Moreover, in the second combination BA was used at two levels 1.0 and 4.0 mg/l with NAA at three levels 0.25, 0.5 and 1.0 mg/l. in the third combinations, BA at 4.0 mg/l, IAA at 1.0 mg/l and NAA at 1.0 mg/l.

Based on all the previous results it can be concluded that seeds are not preferable starting materials for the *in vitro* establishment of motherstock plant due to low germination percentage caused by dormancy of seeds. Moreover, using of shoot tips was a better method since it produces higher number of seedlings compared to seeds, also it takes short time. However, there is little contamination due to the use of wild plants material (Figure 3.4).

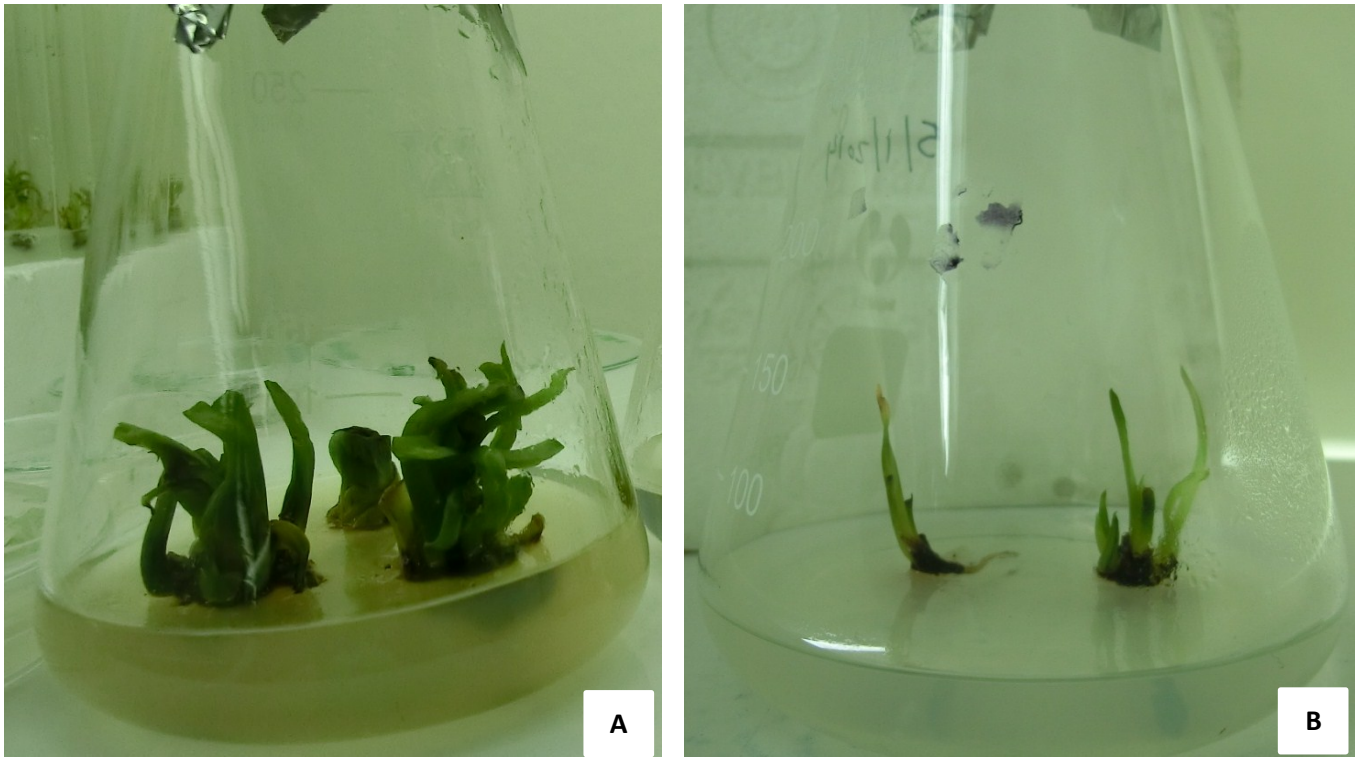

Figure 3.4: *Asphodel aestivus* seedlings from different sources.
(A) Produced from culturing shoot tips on MS with 4.0 mg/L BA and 0.4 mg/L IAA. (B) Produced from culturing of seeds on MS media.

3.3 Micropropagation of *Asphodelus aestivus*

3.3.1 Shoot Proliferation

In order to induce shoot proliferation, shoots were obtained from the established motherstock plant; 2.0 cm height shoots were excised from mother plant and cultured on MS medium supplemented with the auxin IAA and 2-ip. Results of shoot height, number of shoots, number of leaves and browning of media after five weeks of culturing is listed in Table 3.3.

Table 3.3: Effect of PGRs on shoot proliferation, number of leaves, number of shoots, shoots height of *Asphodelus aestivus.* Also shown the browning of the media, (browning was at three levels, thick and donated 3, moderate and donated 2 and slight and donated 1). Data were recorded after five weeks of culturing.

Medium type	Number of Leaves ± SE	Number of Shoots ± SE	Shoot Height (cm) ± SE	Browning of the media ± SE
Free MS (control)	4.44 ± 0.56^{c}	1.3 ± 0.12^{b}	9.34 ± 1.22^{a}	1.0 ± 0.0^{e}
MS + 0.5 mg/L IAA , 0.5 mg/L BA , 0.5 mg/L 2-ip	9.00 ± 0.92^{b}	1.94 ± 0.25^{ab}	7.72 ± 0.94^{a}	2.5 ± 0.13^{c}
MS + 0.5 mg/L IAA ,1.0mg/L BA,1.0 mg/L 2-ip	18.63 ± 2.36^{a}	3.5 ± 0.56^{a}	8.16 ± 0.73^{a}	3.0 ± 0.0^{a}
MS + 0.5 mg/L IAA ,2.0 mg/L BA, 2.0 mg/L 2-ip	15.69 ± 1.96^{a}	3.5 ± 0.39^{a}	5.19 ± 0.73^{b}	2.0 ± 0.0^{d}
MS + 0.5 mg/L IAA ,4.0 mg/L BA, 4.0 mg/L 2-ip	12.50 ± 1.49^{ab}	3.13 ± 0.40^{a}	6.97 ± 0.68^{ab}	3.0 ± 0.0^{b}

Letters within columns showed significant difference at $p < 0.05$ as determined by Fisher's LSD. Sample size (n) = 16.

The significance between treatments was tested by ANOVA and means were separated with Fisher's LSD test. There was a statistical difference at ($p \leq 0.05$) between the five media types in the number of leaves, number of shoots and shoot height. Also it has been observed that there is significance in the media browning between the five different media types.

Results indicate that MS medium supplemented with 0.5 mg/L IAA, 1.0 mg/L BA, 1.0 mg/L 2-ip was the most efficient medium for shoot proliferation. The mean number of leaves was 18.63 ± 2.36 and the mean number of shoots was 3.5 ± 0.56 per explant, and the mean shoot height was 8.16 ± 0.73. Moreover, results indicate that the addition of BA and 2-ip increases the number of leaves in *A. aestivus*, high concentration of these PGRs more than 2.0 mg/L decreased the number of leaves and shoot height. Free MS medium as a control showed the lowest result for the mean number of leaves and the mean number of shoots 4.44 ± 0.56, and 1.3 ± 0.12, respectively. However, it show higher results for mean of shoot height and it was 9.34 ± 1.22, followed by MS supplemented with 0.5 mg/L IAA, 1.0 mg/L BA, 1.0 mg/L 2-ip and it was 8.16 ± 0.73 (Figure 3.5). In conclusion MS medium supplemented with 0.5 mg/L IAA, 1.0 mg/L BA, and 1.0 mg/L 2-ip show the highest results for number

of leaves, number of shoots and shoot height, and so it can be used for the micropropagation of *Asphodelus aestivus*.

Results reported from this experiment are the first that address the development of protocol for the micropropagation of *A. aestivus*. There are few plants in the genus Asphodelus, (18 species) distributed over all the Mediterranean region (Naderi Safar et al., 2014). The closest group to *Asphodelus* is *Aloe*, *Aloe* species differ from *Asphodelus* species in morphology, structure and many characteristics (Daru, 2012). In contrast with *Asphodelus* species there are extensive studies on the micropropagation of *Aloe* species, and in the last years several protocols have been developed for the micropropagation of *Aloe* and for the production of several secondary metabolites that can be used as drugs and supplements such as aloe-emodin (Lee et al., 2011). Zakia et al (2013) has optimized a protocol for the micropropagation of *Aloe vera*, they have found that MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA gave the highest number of shoots and shoot height. In addition, Haque and Ghosh (2013) have optimized a protocol for the massive production of true–to-type *Aloe* plantlets. They have used the rhizomatous stem as a source of explant then they were cultured on MS medium supplemented with two cytokinins either BAP or kinetin at 1.0, 2.5, and 4.0 mg/L, in addition to *Aloe vera* leaf gel. Results revealed that the highest shoot number was observed on MS medium supplemented with 2.5 mg/L BAP, and MS supplemented with 4.0 mg/L kinetin. On the first generation results were 14.5 ± 0.31 for MS with 2.5 mg/L BAP and 9.7 ± 0.29 for MS with 4.0 mg/L kinetin, and on the third generation results were 27.6 ± 0.53 MS with 2.5 mg/L BAP and 20.3 ± 0.33 MS with 4.0 mg/L kinetin.

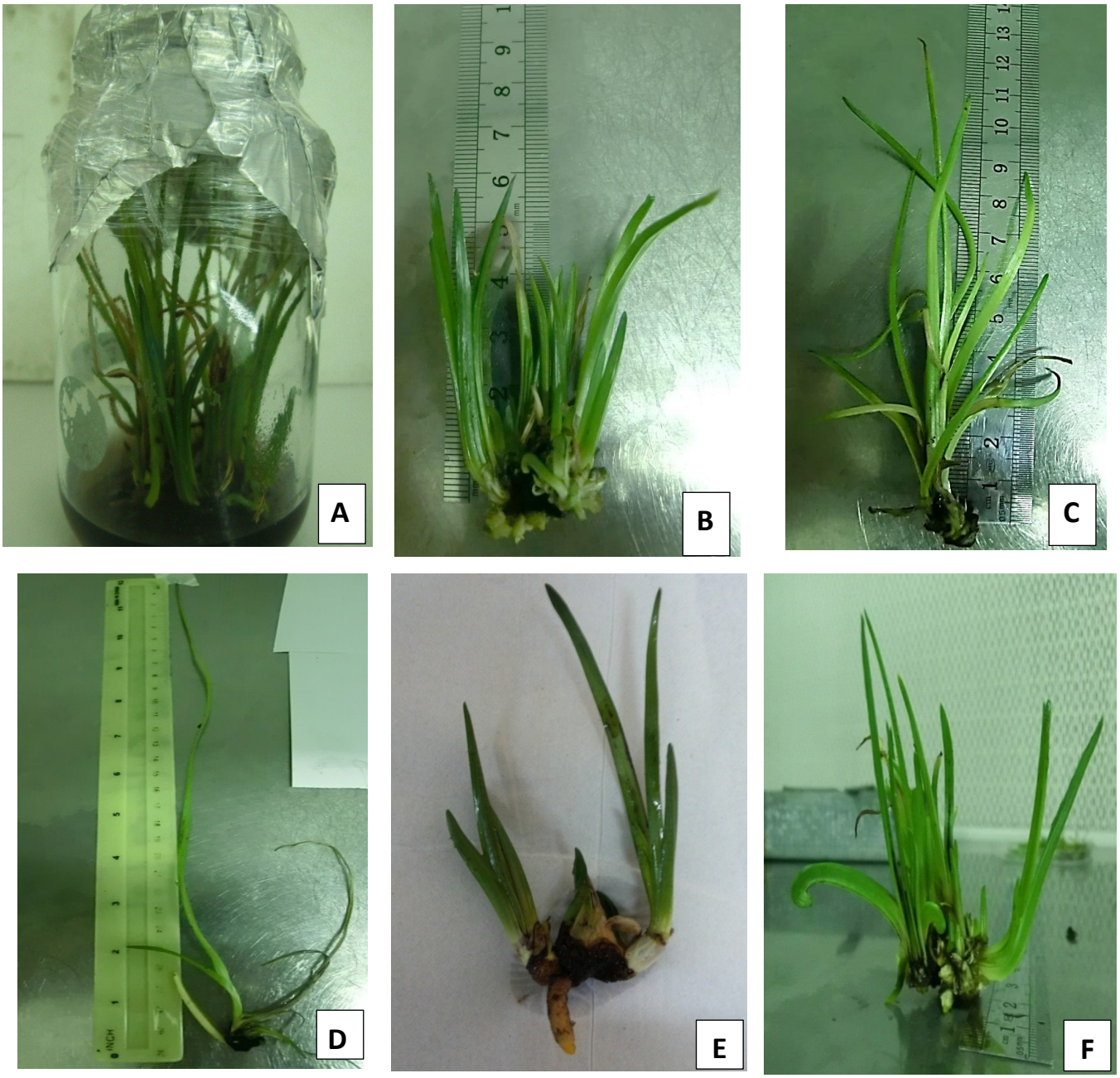

Figure 3.5: Shoot proliferation of *Asphodelus aestivus* after five weeks of culturing. Shoots were cultured on MS medium supplemented with different hormones. (A) 0.5 mg/L IAA, 1.0 mg/L BA and 1.0 mg/L 2-ip. (B) 0.5 mg/L IAA, 4.0 mg/L BA and 4.0 mg/L 2-ip. (C) 0.5 mg/L IAA, 1.0 mg/L BA and 1.0 mg/L 2-ip. (D) Free MS media, it show the highest shooting "leaf length". (E) MS supplemented with 0.5 mg/L IAA, 0.5 mg/L BA and 0.5 mg/L 2-ip, in this case plantlets develop small root. (F) *A. aestivus* proliferated shoots on MS supplemented with 0.5 mg/L IAA, 1.0 mg/L BA and 1.0 mg/L 2-ip.

3.3.2 *In vitro* Rooting

Results for the number of roots, root length, shoot height, number of proliferated leaves/shoots, and callus formation percentage was recorded after 4 weeks of culturing and data is listed in Table 3.4.

Results revealed that they are statistically significant differences ($p \leq 0.05$) in the number of roots; root length, shoot height and number of shoots between the nine media types, and no callusing were observed. The highest percentage of rooting was observed on MS medium supplemented with 2.0 mg/L NAA and it was 93.8%, where it was 87.5% on both 1.0 and 3.0 mg/L NAA. Moreover, the highest number of roots was on MS medium supplemented with NAA, and the mean root number from MS medium with 2.0 mg/L NAA was (8.75 ± 1.66) followed by 3.0 mg/L NAA with a mean (7.75 ± 2.09), and 1.0 mg/L NAA with a mean number (5.44 ± 1.48). MS medium supplemented with IAA or IBA show poor results. From the previous results we can conclude that MS medium supplemented with 2.0 mg/L NAA is the most optimum media for the induction of roots in *Asphodelus aestivus* since it showed the highest results between the nine treatments.

The same result was reported for root length and MS medium supplemented with NAA showed the highest results. The highest root length was observed on MS medium supplemented with 1.0 mg/L NAA with a mean length (3.69 ± 0.76), followed by 2.0 mg/L NAA with a mean root length (3.03 ± 0.43), and 3.0 mg/L NAA mean root length was (1.88 ± 0.45). These results are in agreement with the previous results obtained for the number of roots, and so we can conclude that MS medium supplemented with NAA gives the most efficient root induction. Figure 3.6 shows the results of root induction.

Table 3.4: Effect of different auxins on rooting. The number of roots, root length, shoot height, number of proliferated leaves/shoots, and callus formation percentage was recorded after four weeks of culturing of *Asphodelus aestivus* microshoots.

Growth regulator	Level mg/L	Rooting %	Number of roots ± SE	Root length ± SE	Shoot Height ± SE	Number of shoots ± SE	Callusing percentage
IAA	1	18.8	0.25 ± 0.11^{c}	0.72 ± 0.34^{d}	16.66 ± 2.12^{a}	2.00 ± 0.27^{def}	0
	2	6.3	0.19 ± 0.19^{c}	0.19 ± 0.19^{d}	7.90 ± 0.69^{b}	4.63 ± 0.51^{a}	0
	3	25	0.33 ± 0.19^{c}	0.79 ± 0.43^{d}	9.33 ± 0.80^{b}	1.33 ± 0.19^{f}	0
IBA	1	25	0.42 ± 0.23^{c}	1.17 ± 0.64^{c}	7.75 ± 0.91^{b}	3.83 ± 0.47^{ab}	0
	2	6.3	0.06 ± 0.06^{c}	0.06 ± 0.06^{d}	7.63 ± 0.67^{b}	3.25 ± 0.46^{bc}	0
	3	25	0.38 ± 0.18^{c}	0.53 ± 0.30^{d}	8.03 ± 0.84^{b}	2.63 ± 0.38^{cd}	0
NAA	1	**87.5**	$\mathbf{5.44 \pm 1.48^{b}}$	3.69 ± 0.76^{a}	6.66 ± 0.67^{b}	2.50 ± 0.50^{cd}	0
	2	**93.8**	$\mathbf{8.75 \pm 1.66^{a}}$	3.03 ± 0.43^{ab}	$4.31 \pm 0.62b^{c}$	2.06 ± 0.29^{de}	0
	3	**87.5**	$\mathbf{7.75 \pm 2.09^{ab}}$	1.88 ± 0.45^{bc}	3.44 ± 0.29^{c}	1.94 ± 0.23^{ef}	0

Letters within columns showed significant difference at $p < 0.05$ as determined by Fisher's LSD. Sample size (n) = 16.

Results for shoot number and height after four weeks of culture indicate that MS medium supplemented with IAA and IBA shows higher result than MS with NAA. MS medium supplemented with 1.0 mg/L IAA gave the highest results with a mean shoot number (16.66 ± 2.12) followed by 3.0 mg/L IAA with a mean shoot number (9.33 ± 0.80) and 3.0 mg/L IBA with a mean shoot number (8.03 ± 0.84). On the other hand, the highest shoot number was observed on MS medium supplemented with 2.0 mg/L IAA with a mean (4.63 ± 0.51), followed by MS medium supplemented with 1.0 and 2.0 mg/L IBA with a mean shoot height of (3.83 ± 0.47), and (3.25 ± 0.46), respectively. The lowest rooting was observed on MS medium supplemented with 3 mg/L IAA with a mean shoot number (1.33 ± 0.19).

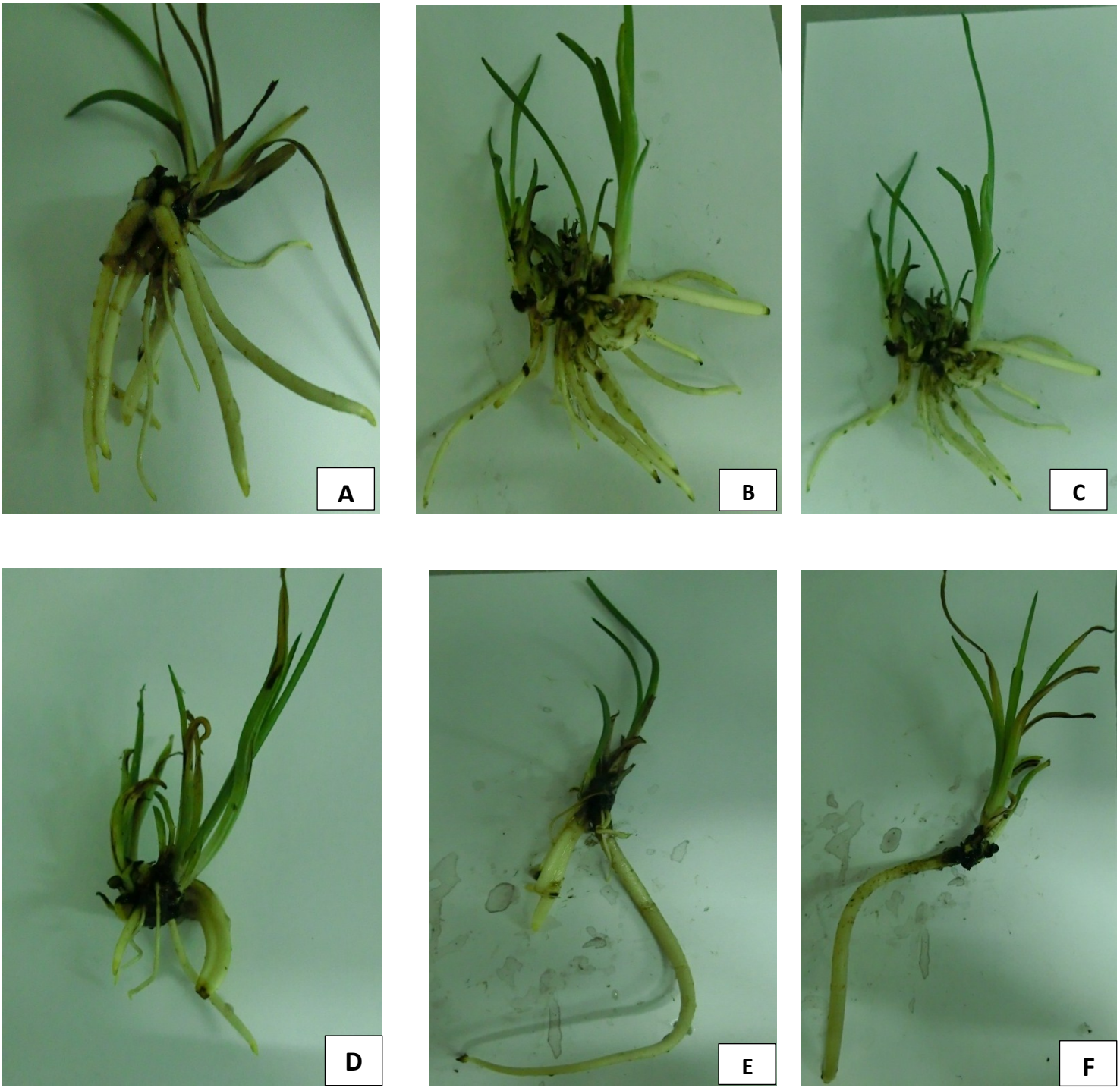

Figure 3.6: Root induction of *Asphodelus aestivus* after four weeks of culturing at MS medium supplemented with different concentrations of IAA, IBA and NAA. (A) 2.0 mg/L NAA. (B) 1.0 mg/L NAA. (C) 3.0 mg/L NAA. (D) 2.0 mg/L IBA. (E) 2.0 mg/L IAA. (F) I.0 mg/L IAA.

From the previous results we can conclude that MS medium supplemented with NAA show the best results from the number of roots and root length, and MS medium supplemented with IAA or IBA show the lowest results. The number and length of the induced roots on either IAA or IBA was very poor in number and in morphology. However, the highest number of shoot and shoot height was observed on MS medium supplemented with IAA, and IBA. Media supplemented with NAA shows poor results, and it is clear that high concentrations of NAA induce root formation but it is have an inhibitory effect on number and height of shoots. In conclusion, results indicate that shoots cultured on MS medium supplemented with NAA gave the highest percentage of root formation, and high concentrations of NAA show poor shooting.

In several studies IAA, IBA and NAA have been used for root induction in several plants. However, IBA and NAA are mostly used rather than IAA and they have helped in the micropropagation of several plants from different families (Ahmed et al., 2007). Results reported in this experiment are the first that address the *in vitro* rooting of *Asphodelus aestivus*, and to our knowledge there is no previous work on rooting experiments in the genus *Asphodelus*. In the Asphodelaceae family, most studies have been conducted on the micropropagation of *Aloe* species, and there were extensive researches on root induction. Ahmed et al (2007) have reported that *Aloe vera* have induced roots on MS medium supplemented with 0.2 and 0.5 mg/L NAA. Moreover, the study of Yun Sun Lee et al (2011) showed that best results for root induction of *Aloe vera* was observed on MS medium supplemented with 0.5 mg/L NAA and 0.2 mg/L BAP. Zakia et al (2013) has reported that the microshoots of *Aloe vera* showed high percentage of rooting in MS medium supplemented with 1.5 mg/l IBA and it was 84.67%. However, lower percentage of rooting was observed in MS medium supplemented with 1.5 mg/l NAA (86.67%).These results are in agreement with the results in this study were low concentrations of NAA yielded better results than high concentrations.

3.4 Callus Induction and Culture

3.4.1 Callus Induction

The highest percentage of callus induction was obtained from seed embryos cultured on MS supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D, and MS medium supplemented with 2.0 mg/L KN, 0.5 mg/L NAA. Callus tissue inducted from embryos was dark-pink in color, compact in texture, and starts to grow after two weeks of culture. Callus show excessive production of secondary metabolites that gives pinkish color in the induction medium. Immature seeds cultured on MS medium with 4.0 mg/L BA, 2.5 mg/L NAA show low callus tissue production, and no callus was produced when MS free medium was used. Callus tissue also induced from the roots. Highest callus percentage was achieved from roots cultured on MS supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D, and MS supplemented with 2.0 mg/L KN, 0.5 mg/L NAA. Small percentage of callus was induced from roots cultured on MS with 4.0 mg/L BA, 2.5 mg/L NAA, and no callus was initiated on free MS medium. Callus tissue induced from roots was dark in color, compact in texture and showed slow growth rate. However, callus tissue size and a mount from roots was higher than callus tissue from embryos, and the average size was 2.0 cm in diameter. However, callus growth from root tissue took a long time. No callus induction or growth was obtained from Asphodel leave explants. From these results we can conclude that MS medium supplemented with 2.0 mg/L KN, 0.5 mg/L NAA and MS medium supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D is the best for callus induction in *Asphodelus aestivus*. This is the first report on trials for the production of callus tissue from the genus *Asphodelus L*. Callus tissue production is shown in Figure 3.7.

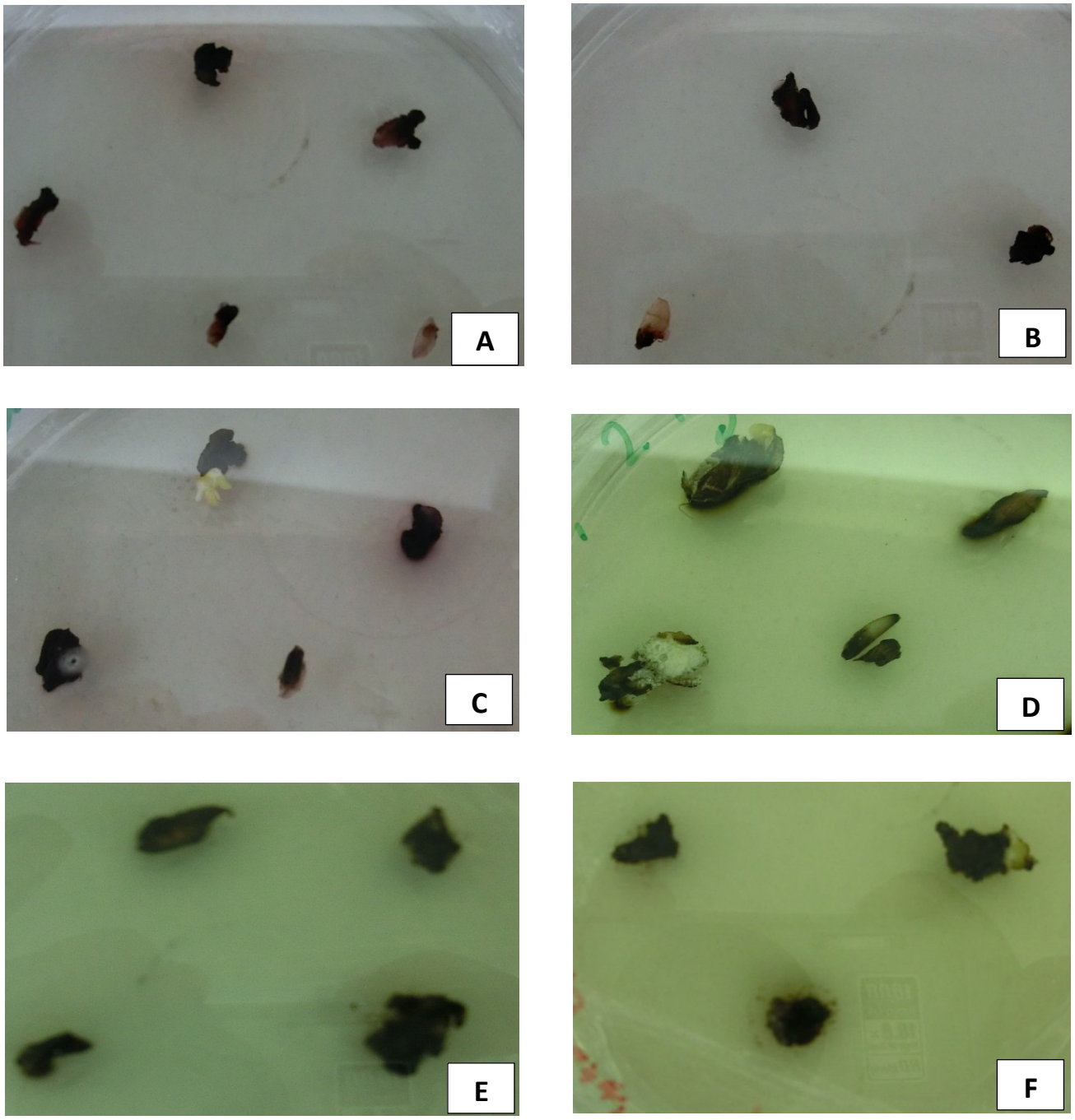

Figure 3.7: Callus induction of *Asphodelus aestivus* after one month of culture.

A, B, C, and F callus tissue induced from embryos and cultured on (A) 2.0 mg/L BA and 1.0 mg/L 2,4-D. (B) 2.0 mg/L KN and 0.5 mg/L NAA. (C) 4.0 mg/L BA and 2.5 mg/L NAA. D and E Callus tissue induced from roots cultured on (D) 2.0 mg/L BA and 1.0 mg/L 2,4-D. (E) 2.0 mg/L KN and 0.5 mg/L NAA. (F) 2.0 mg/L BA and 2.5 mg/L 2,4-D.

3.4.2 Callus Proliferation

In this experiment the developed callus tissue from immature seeds "embryo" and roots was subcultured to the most optimum media that show the highest yield and the fastest proliferation in the induction experiment. Callus tissue was subcultured on MS medium supplemented with 2.0 mg/L KN, 0.5 mg/L NAA and MS media supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D. Callus tissue was monitored for two weeks to evaluate their growth and data were recorded after five weeks of culture, results are shown in Table 3.5.

Table 3.5: Root and immature seed "embryos" callus tissue color, average diameter size, and texture after five weeks of culturing. Callus tissue was maintained under dark condition. Sample size (n) = 20.

Medium type	Callus source	Callus Color	Average callus diameter (cm)	Callus texture
2.0 mg/L KN, 0.5 mg/L NAA	Embryo	Different colors; yellow, pink, and dark	1.5^{a}	compact
2.0 mg/L BA, 1 mg/L 2,4-D	Embryo	Different colors; Pink, dark pink. and Brown-pink	0.5^{c}	compact
2.0 mg/L KN, 0.5 mg/L NAA	Roots	dark	1.0^{b}	compact
2.0 mg/L BA, 1 mg/L 2,4-D	Roots	dark	1.0^{b}	compact

Callus from MS medium supplemented with 2.0 mg/L KN, 0.5 mg/L NAA show better results. Callus tissue produced from immature seeds callus subcultured on 2.0 mg/L KN, 0.5 mg/L NAA show different colors (yellow, pink, and dark). Callus texture was compact and has average diameter higher than the other treatments with an average diameter size of 1.5 cm. Callus tissue produced from root callus subcultured on 2.0 mg/L KN, 0.5 mg/L NAA show higher proliferation than MS supplemented with 2 mg/L BA, 1 mg/L 2,4-D, and callus maintain more compact texture compared to other callus with other treatments. Results are shown in Figure 3.8.

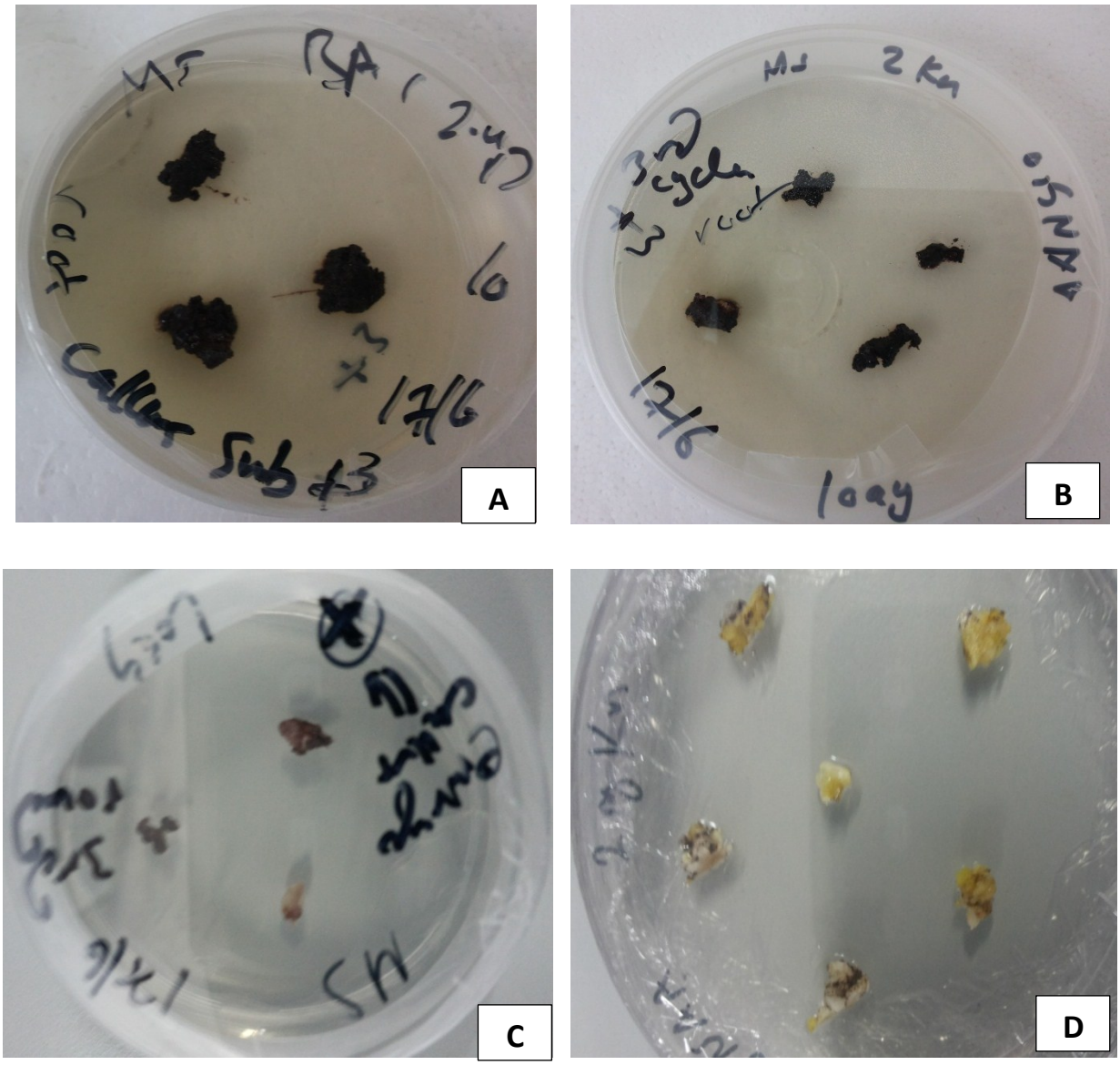

Figure 3.8: *Asphodelus aestivus* Callus tissue growth after two months of sub-culturing for three cycles. (A) and (B) Root callus tissue subcultured on MS medium with (A) 2.0 mg/L BA and 1.0 mg/L 2,4-D. (B) 2.0 mg/L KN and 0.5 mg/L NAA. (C) and (D) Immature seeds Callus tissue subcultured on MS with (C) 2.0 mg/L BA and 1.0 mg/L 2,4-D. (D) 2.0 mg/L KN and 0.5 mg/L NAA.

Callus production is one of the major techniques in plant tissue culture that depends on factors that may alter callus production growth, amount and many other characteristics. Factors include internal factors such as the genetic and chemical composition of explants, and external factors such as the culture condition, media type, and hormone concentration that affect the color, texture and growth of callus tissue (Xiaohong et al., 2011;Alkowni and Sawalha, 2012). Callus induction in the genus Asphodelus was not addressed before, and the recent results indicate this genus

needs to be studied since it contains different secondary metabolites that play an essential role in medicine.

Different explants of *Asphodelus aestivus* was used to produce callus tissue, callus produced from these explants was different in its characteristics such as color, texture and growth rate. All explants of *A. aestivus* were incubated in dark condition to increase callus amount and growth rate. Immature seeds have produced callus tissue that has different color that range from pink, dark pink, to brown-pink. The texture of all callus tissue on all media types was compact and show slow growth rate. MS medium supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D gave the highest percentage of callus and callus maintain its color and texture. The other two media types show slow or no callus formation. Moreover, the main character of callus produced from immature seeds was that the browning of the media have increased when the callus become older, and this indicate that the callus tissue to secrete or synthesize secondary metabolites that may have medicinal values. In the other hand, the a mount of callus tissue produced from *in vitro* roots was lower and show slower growth rate compared to immature seeds. The color in the majority of callus tissue was dark and maintained a compact texture. MS medium supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D gave the highest percentage of callus. Free MS medium did not produce any callus from the two explants. Asphodel leaves did not produce any callus in any media type, and this is may be related to the structure and composition of the leaves. Moreover, the leaves show fast browning; the leaf explants show dark color within one week. To the best of my knowledge, there is no available data or publications on callus induction of *Asphodelus aestivus*.

3.5 Extraction and Determination of Chemical Constituents of *Asphodelus aestivus* Using Thin Layer Chromatography (TLC)

3.5.1 Extraction Yields

The aim of this experiment was to compare the chemical constituents between *ex vitro* and *in vitro* roots of *A. aestivus*. Results show that there are differences between the extract of the *ex vitro* and the *in vitro* sources in the extract yield percentage. Ethyl acetate gave the highest yield for *ex vitro* and *in vitro* roots, and it was 31.1% for *ex vitro* root extract and 27.08% for *in vitro* root extract. The percentages of extraction yield was calculated as a result of dividing the weight of the extracted powder by

the weight of the extracted plant material, the yield and percentages of extract for the three solvents is shown in Table 3.6.

Table 3.6: Extraction yield and percentages of 1.0 gram of *ex vitro* and 0.5 g *in vitro* *A. aestivus* roots dissolved in different solvents.

Extract source	**Water**		**Ethyl acetate**		**99 % Ethanol**	
	Yield weight	Yield percent	Yield weight	Yield percent	Yield weight	Yield percent
ex vitro roots	0.1 g	10 %	0.311 g	31.1 %	25.8 g	12.4 %
in vitro roots	0.0197 g	3.93 %	0.135 g	27.08 %	0.023 g	4.61 %

Ethyl acetate gave the highest yield percentage for *ex vitro* and *in vitro* roots, also the extract texture and solubility was better than when water was used. Moreover, 99% ethanol extracts, and ethyl acetate extracts dissolved rapidly without the formation of any sedimentation. Water gave the lowest percentage of yield for both *ex vitro* and *in vitro* roots, and this may be due the high polarity of water and the chemical composition of *A. aestivus* roots.

Currently, the information on the extraction of *A. aestivus* based on solvents polarities is rare, and this experiment will provide good information for future work. El-seedi (2007) has conducted a similar experiment were he extracted *A.aestivus* (synonym: *A .microcarpus*) *ex vitro* roots with ethyl acetate and this leads him to the isolation of a new aryl coumarin glucoside, asphodelin A 4′-O-â-D-glucoside, and its aglycon, asphodelin A. These two compounds possess a potent antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Botrytis cinerea*. To my knowledge this is the first report for the extraction of *in vitro* roots of *A. aestivus*.

3.5.2 Thin Layer Chromatography (TLC) analysis

The extracts derived from the six treatments were subjected to TLC analysis; where 10.0 mg of each extract was dissolved in 1.5 ml D.H_2O. Ten µL were loaded on the TLC plate and were run with a mobile phase composed of a mixture of methanol: water: formic acid (13.3: 19: 1). The TLC plate was examined under ultraviolet (UV)

light. Results show that *ex vitro* root extract has separated to several chemical profiles (bands), while *in vitro* root extract especially ethyl acetate extract show two bands at the mid and top of the TLC plate (Figure 3.9). These bands differ from the other bands separated from the *ex vitro* roots in there color and *Rf* value. The first chemical profile was in the middle of the TLC plate and has a florescent blue color, and *Rf* value of (≈0.6). The second chemical profile was at the top of the TLC plate and has a pale green color and *Rf* value of (≈ 0.85). This indicates that the *in vitro* culturing of *Asphodelus aestivus* may leaded to the expression of high amount of these secondary metabolites, or the expression of these compounds depends on the root type that developed. Water extract of both *ex vitro* and *in vitro* show minor residues of chemical profiles, and this indicate that water is not a suitable solvent for extracting secondary metabolites from *A. aestivus*.

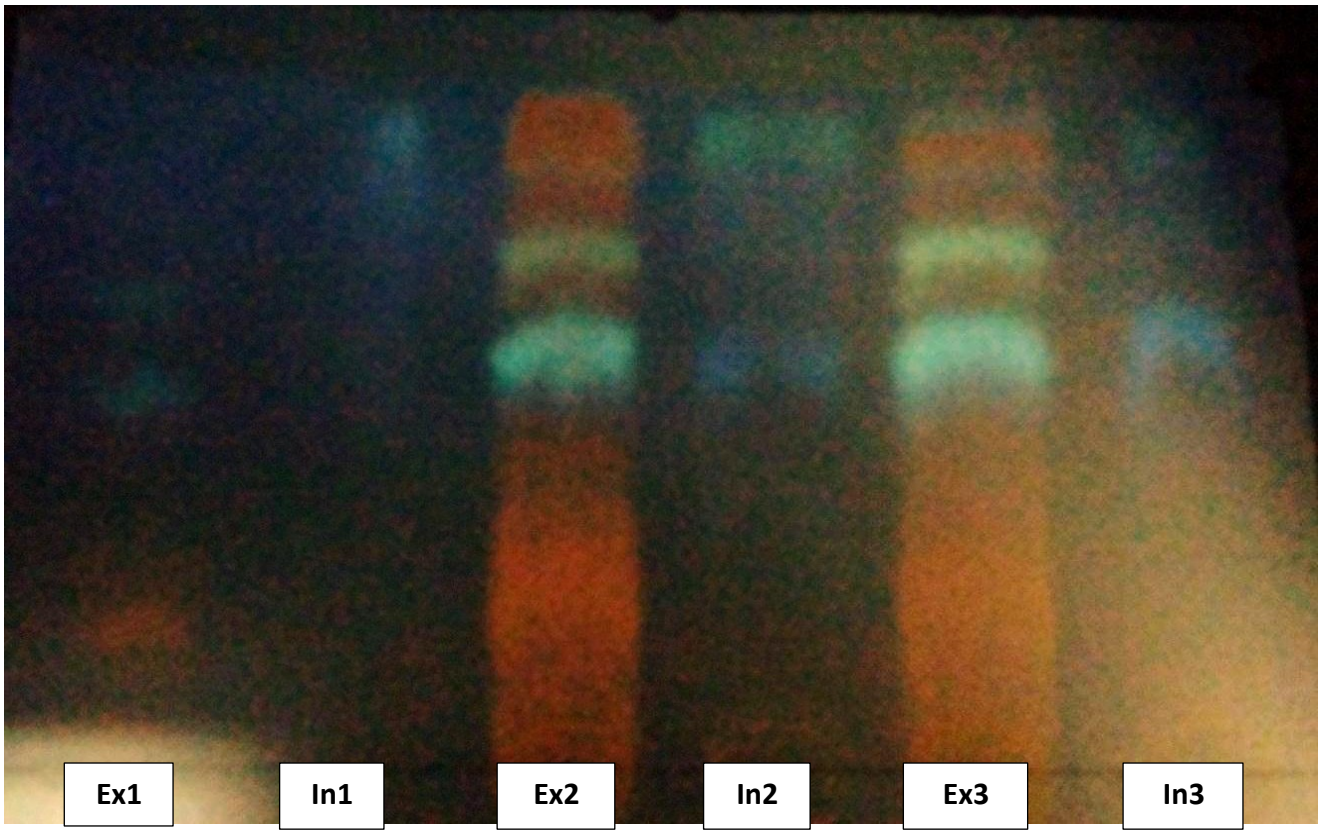

Figure 3.9: TLC plate viewed under UV light for *ex vitro* and *in vitro* root extract dissolved in 1.5 ml D.H_2O and run on a mixture of methanol: water: formic acid (13.3: 19: 1).
(Ex1) *Ex vitro* root water extract. (In1) *In vitro* root water extract. (Ex2) *Ex vitro* root ethyl acetate extract. (In2) *In vitro* root ethyl acetate extract. (Ex3) *Ex vitro* root 99% ethanol extract. (In3) *In vitro* root 99% ethanol extract

The biochemical composition of *ex vitro* sources of *Asphodelus aestivus* has been extensively investigated, and several compounds were isolated from tubers, leaves, and flowers (Rizk et al., 1972;Reynaud et al., 1997;Calisa et al., 2006;Tuberoso et al., 2009;Ghoneim et al., 2013;Ghoneim et al., 2014). However, to our knowledge there are no previous reports on the investigation of the *in vitro* nature of the biochemical composition of *A. aestivus* and so this is the first report on TLC analysis of in vitro root extract. According to the TLC image and depending on the *Rf* values the first chemical compounds can be assumed to be chrysophanol, or Aloin A, B and the second compound can be assumed to be aloe-emodin, or emodin.

CHAPTER FOUR

Conclusion and Future Work

4.1 Conclusions

From the results reported in this study it can be concluded that: Firstly, there is a necessity to apply *in vitro* propagation methods because conventional methods are not satisfactory. Using seeds as a starting material for the propagation of *Asphodelus aestivus* show low percentage of germination and some plants fail to maintain their growth, and in order to germinate they requires special treatment. Secondly, the produced plants from seeds grow slowly and consume a lot of time during their growth compared to *in vitro* propagated materials. The percentage of shoots proliferated and rooted were very low. Treating seeds by soaking with 1.0 mg/L GA_3 gave the highest results and so it can be used to increase the germination rate, germination percentage after pretreatment with 1.0 mg/L GA_3 was 43 %.

Thirdly, results suggest that using shoot tips is a very good method for the *in vitro* propagation of *Asphodelus aestivus* compared to seed propagation, since it shows higher proliferation rates and the consumed time is lower than seeds. A full protocol for the *in vitro* propagation of *Asphodelus aestivus* has been developed were the shoot tips of wild plant have been excised, sterilized then cultured on MS medium supplemented with different PGRs, MS medium supplemented with 4.0 mg/L BA, 0.4 mg/L IAA and 1.0 g/L PVP show the highest proliferation rates. Additionally, this is the first study conducted to develop a propagation protocol for *Asphodelus aestivus*, and there is no previous studies regarding this plant or its genus.

Fourthly, for shoot proliferation MS medium supplemented with 0.5 mg/L IAA, 1.0 mg/L BA, 1.0 mg/L 2-ip was the most efficient medium for shoot proliferation, since it gives the highest number of shoots and leaves. The mean number of leaves of this medium was 18.63 ± 2.36 and the mean number of shoots was 3.5 ± 0.56. The combination of BA and 2-ip has increased shoot proliferation rates compared to using BA a lone. Fifthly, for root induction MS medium supplemented with 2.0 mg/L NAA show the best results with a mean root number of 8.75 ± 1.66, and the longest roots was observed on MS medium supplemented with 1.0 mg/L NAA with a mean length of 3.69 ± 0.76.

In this study, callus was induced from immature seed embryos and from newly developed roots. The highest percentage of callus induction was obtained from seed embryos cultured on MS supplemented with 2.0 mg/L BA, 1.0 mg/L 2,4-D, and MS medium supplemented with 2.0 mg/L KN, 0.5 mg/L NAA, callus tissue was compact and have a dark pink color. Callus induced from roots on the same previous media and it was compact and dark, it shows slow proliferation rates compared to callus produced from seed embryos.

Crude extraction of *in vitro* and *ex vitro* roots shows the highest percentage when ethyl acetate was used. In TLC analysis, several solvent systems were used and the mixture composed of methanol: water: formic acid at (13.3: 19: 1) detected most compounds. TLC results for *ex vitro* roots show different chemical profile and they were separated completely. For *in vitro* roots only two chemical profiles (bands) has appeared, and these band differs from the bands detected in the *ex vitro* roots and this indicates the production of new compounds. Depending on the retention factor of these compounds it can be assumed they are emodin, aloe-emodin, Aloin A, B and chrysophanol.

4.2 Future Work

Since this is the first study conducted to develop a micropropagation protocol for *Asphodelus aestivus* more studies is required to improve shoot proliferation, and rooting percentages. Also more studies are required to enhance callus production for different sources rather than immature seeds or roots. In the future, further chemical analysis should be conducted to qualitate and quantitate the produced compounds in the *ex vitro* and *in vitro* extracts. Moreover, the produced extracts should be subjected to bioassay analysis to evaluate the activity of extracts especially the *in vitro* root extract that has the medicinal properties against cancer cells, fungi, bacteria and viruses.

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APPENDICES

Appendix Table 1: Analysis of variance (ANOVA) for number of leaves at different media type after five weeks of the *in vitro* multiplication of the mother stock plant.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
Free MS	16	71	4.44	5.1
MS + 0.5 mg/L IAA , 0.5 mg/L BA , 0.5 mg/L 2-ip	16	144	9.00	13.5
MS + 0.5 mg/L IAA ,1.0 mg/L BA, 1.0 mg/L 2-ip	16	298	18.63	89.3
MS + 0.5 mg/L IAA ,2.0 mg/L BA, 2.0 mg/L 2-ip	16	251	15.69	61.2
MS + 0.5 mg/L IAA ,4.0 mg/L BA, 4.0 mg/L 2-ip	16	200	12.50	35.5
Total	80	964	12.05	63.9

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	1982.68	4	495.67	12.121	0.000
Within Groups	3067.13	75	40.90		
Total	5049.80	79			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	-4.563	0.047	accepted
1 vs 3	-14.188	0.000	accepted
1 vs 4	-11.250	0.000	accepted
1 vs 5	-8.063	0.001	accepted
2 vs 3	-9.625	0.000	accepted
2 vs 4	-6.688	0.004	accepted
2 vs 5	-3.500	0.126	rejected
3 vs 4	2.938	0.198	rejected
3 vs 5	6.125	0.008	accepted
4 vs 5	3.188	0.163	rejected

Appendix Table 2: Analysis of variance (ANOVA) for number of shoots at different media type after five weeks of the *in vitro* multiplication of the mother stock plant.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
Free MS	16	21.01	1.313	0.23
MS + 0.5 mg/L IAA , 0.5 mg/L BA , 0.5 mg/L 2-ip	16	31.01	1.938	1.00
MS + 0.5 mg/L IAA ,1.0 mg/L BA, 1.0 mg/L 2-ip	16	56.00	3.500	4.93
MS + 0.5 mg/L IAA ,2.0 mg/L BA, 2.0 mg/L 2-ip	16	56.00	3.500	2.40
MS + 0.5 mg/L IAA ,4.0 mg/L BA, 4.0 mg/L 2-ip	16	50.00	3.125	2.52
Total	80	214.00	2.675	2.91

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	63.425	4	15.856	7.159	0.000
Within Groups	166.125	75	2.215		
Total	229.550	79			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	-0.6250	0.239	rejected
1 vs 3	-2.1875	0.000	accepted
1 vs 4	-2.1875	0.000	accepted
1 vs 5	-1.8125	0.001	accepted
2 vs 3	-1.5625	0.004	accepted
2 vs 4	-1.5625	0.004	accepted
2 vs 5	-1.1875	0.027	accepted
3 vs 4	0.0000	1.000	rejected
3 vs 5	0.3750	0.478	rejected
4 vs 5	0.3750	0.478	rejected

Appendix Table 3: Analysis of variance (ANOVA) for *A.aestivus* shoots height at different media type after five weeks of the *in vitro* multiplication of the mother stock plant.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
Free MS	16	149.5	9.344	23.69
MS + 0.5 mg/L IAA , 0.5 mg/L BA , 0.5 mg/L 2-ip	16	123.5	7.719	14.06
MS + 0.5 mg/L IAA ,1.0 mg/L BA, 1.0 mg/L 2-ip	16	130.5	8.156	8.52
MS + 0.5 mg/L IAA ,2.0 mg/L BA, 2.0 mg/L 2-ip	16	83	5.188	3.89
MS + 0.5 mg/L IAA ,4.0 mg/L BA, 4.0 mg/L 2-ip	16	111.5	6.969	7.35
Total	80	598	7.475	12.85

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	152.07	4	38.02	3.305	0.015
Within Groups	862.87	75	11.51		
Total	1014.95	79			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	1.6250	0.179	rejected
1 vs 3	1.1875	0.325	rejected
1 vs 4	4.1563	0.001	accepted
1 vs 5	2.3750	0.051	rejected
2 vs 3	-0.4375	0.716	rejected
2 vs 4	2.5313	0.038	accepted
2 vs 5	0.7500	0.534	rejected
3 vs 4	2.9688	0.016	accepted
3 vs 5	1.1875	0.325	accepted
4 vs 5	-1.7813	0.142	rejected

Appendix Table 4: Analysis of variance (ANOVA) for browning of the used media types after five weeks of the *in vitro* multiplication of the mother stock plant

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
Free MS	16	16	1	0.00
MS + 0.5 mg/L IAA , 0.5 mg/L BA , 0.5 mg/L 2-ip	16	40	2.5	0.27
MS + 0.5 mg/L IAA ,1.0 mg/L BA, 1.0 mg/L 2-ip	16	48	3	0.00
MS + 0.5 mg/L IAA ,2.0 mg/L BA, 2.0 mg/L 2-ip	16	32	2	0.00
MS + 0.5 mg/L IAA ,4.0 mg/L BA, 4.0 mg/L 2-ip	16	48	3	0.00
Total	80	184	2.3	0.62

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	44.8	4	11.2	210	0.000
Within Groups	4	75	0.053		
Total	48.8	79			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	-1.50	0.000	accepted
1 vs 3	-2.00	0.000	accepted
1 vs 4	-2.00	0.000	accepted
1 vs 5	-1.00	0.000	accepted
2 vs 3	-0.50	0.000	accepted
2 vs 4	-0.50	0.000	accepted
2 vs 5	0.50	0.000	accepted
3 vs 4	0.00	1.000	rejected
3 vs 5	1.00	0.000	accepted
4 vs 5	1.00	0.000	accepted

Appendix Table 5: Analysis of variance (ANOVA) for percentage of rooting at MS medium supplemented with IAA, IBA or NAA at different concentrations after four weeks of *in vitro* culturing of *A.aestivus* shoots.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
1.0 mg/L IAA	16	28	2	0.2
2.0 mg/L IAA	16	31	2	0.063
3.0 mg/L IAA	12	21	2	0.205
1.0 mg/L IBA	12	21	2	0.205
2.0 mg/L IBA	16	31	2	0.063
3.0 mg/L IBA	16	28	2	0.2
1.0 mg/L NAA	16	18	1	0.117
2.0 mg/L NAA	16	17	1	0.063
3.0 mg/L NAA	16	18	1	0.117
Total	136	213	2	0.247

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	16.592	8	2.074	15.667	0.000
Within Groups	16.813	127	0.132		
Total	33.404	135			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	-0.188	0.147	rejected
1 vs 3	0.000	1.000	rejected
1 vs 4	0.000	1.000	rejected
1 vs 5	-0.188	0.147	rejected
1 vs 6	0.000	1.000	rejected
1 vs 7	0.625	0.000	accepted
1 vs 8	0.688	0.000	accepted
1 vs 9	0.625	0.000	accepted
2 vs 3	0.188	0.180	rejected
2 vs 4	0.188	0.180	rejected

2 vs 5	0.000	1.000	rejected
2 vs 6	0.188	0.147	rejected
2 vs 7	0.813	0.000	accepted
2 vs 8	0.875	0.000	accepted
2 vs 9	0.813	0.000	accepted
3 vs 4	0.000	1.000	rejected
3 vs 5	-0.188	0.180	rejected
3 vs 6	0.000	1.000	rejected
3 vs 7	0.625	0.000	accepted
3 vs 8	0.688	0.000	accepted
3 vs 9	0.625	0.000	accepted
4 vs 5	-0.188	0.180	rejected
4 vs 6	0.000	1.000	rejected
4 vs 7	0.625	0.000	accepted
4 vs 8	0.688	0.000	accepted
4 vs 9	0.625	0.000	accepted
5 vs 6	0.188	0.147	rejected
5 vs 7	0.813	0.000	accepted
5 vs 8	0.875	0.000	accepted
5 vs 9	0.813	0.000	accepted
6 vs 7	0.625	0.000	accepted
6 vs 8	0.688	0.000	accepted
6 vs 9	0.625	0.000	accepted
7 vs 8	0.063	0.628	rejected
7 vs 9	0.000	1.000	rejected
8 vs 9	-0.063	0.628	rejected

Appendix Table 6: Analysis of variance (ANOVA) for number of roots at MS medium supplemented with IAA, IBA or NAA at different concentrations after four weeks of *in vitro* culturing of *A.aestivus* shoots.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
1.0 mg/L IAA	16	4	0.25	0.200
2.0 mg/L IAA	16	3.04	0.19	0.563
3.0 mg/L IAA	12	3.96	0.33	0.424
1.0 mg/L IBA	12	5.04	0.42	0.629
2.0 mg/L IBA	16	0.96	0.06	0.063
3.0 mg/L IBA	16	6.08	0.38	0.517
1.0 mg/L NAA	16	87.04	5.44	35.036
2.0 mg/L NAA	16	140	8.75	44.067
3.0 mg/L NAA	16	124	7.75	69.8
Total	136	374	2.75	29.91

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	1637.854	8	204.732	11.476	0.000
Within Groups	2265.646	127	17.84		
Total	3903.50	135			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	0.063	0.967	rejected
1 vs 3	- 0.083	0.959	rejected
1 vs 4	- 0.167	0.918	rejected
1 vs 5	0.188	0.900	rejected
1 vs 6	- 0.125	0.933	rejected
1 vs 7	- 5.188	0.001	accepted
1 vs 8	- 8.500	0.000	accepted
1 vs 9	- 7.500	0.000	accepted
2 vs 3	- 0.146	0.928	rejected
2 vs 4	- 0.229	0.887	rejected

2 vs 5	0.125	0.933	rejected
2 vs 6	- 0.188	0.900	rejected
2 vs 7	- 5.250	0.001	accepted
2 vs 8	- 8.563	0.000	accepted
2 vs 9	- 7.563	0.000	accepted
3 vs 4	-0.083	0.962	rejected
3 vs 5	0.271	0.867	rejected
3 vs 6	-0.042	0.979	rejected
3 vs 7	- 5.104	0.002	accepted
3 vs 8	- 8.417	0.000	accepted
3 vs 9	- 7.417	0.000	accepted
4 vs 5	0.354	0.827	rejected
4 vs 6	0.042	0.979	rejected
4 vs 7	- 5.021	0.002	accepted
4 vs 8	- 8.333	0.000	accepted
4 vs 9	- 7.333	0.000	accepted
5 vs 6	- 0.313	0.835	rejected
5 vs 7	- 5.375	0.000	accepted
5 vs 8	- 8.688	0.000	accepted
5 vs 9	- 7.688	0.000	accepted
6 vs 7	- 5.063	0.001	accepted
6 vs 8	- 8.375	0.000	accepted
6 vs 9	- 7.375	0.000	accepted
7 vs 8	- 3.313	0.028	accepted
7 vs 9	- 2.313	0.124	rejected
8 vs 9	1.000	0.504	rejected

Appendix Table 7: Analysis of variance (ANOVA) for root length at MS medium supplemented with IAA, IBA or NAA at different concentrations after four weeks of *in vitro* culturing of *A.aestivus* microshoots.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
1.0 mg/L IAA	16	11.5	0.719	1.799
2.0 mg/L IAA	16	3.0	0.188	0.563
3.0 mg/L IAA	12	9.5	0.792	2.248
1.0 mg/L IBA	12	14.0	1.167	4.879
2.0 mg/L IBA	16	1.0	0.063	0.063
3.0 mg/L IBA	16	8.5	0.531	1.449
1.0 mg/L NAA	16	59.0	3.688	9.239
2.0 mg/L NAA	16	48.5	3.031	2.882
3.0 mg/L NAA	16	30.0	1.875	3.250
Total	136	185	1.360	4.258

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	206.434	8	25.804	8.895	0.000
Within Groups	368.411	127	2.901		
Total	574.846	135			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	0.5313	0.379	rejected
1 vs 3	- 0.0729	0.911	rejected
1 vs 4	-0.4479	0.492	rejected
1 vs 5	0.6563	0.278	rejected
1 vs 6	0.1875	0.756	rejected
1 vs 7	-2.9688	0.000	accepted
1 vs 8	-2.3125	0.000	accepted
1 vs 9	-1.1563	0.057	accepted

2 vs 3	-0.6042	0.355	rejected
2 vs 4	-0.9792	0.135	rejected
2 vs 5	0.1250	0.836	rejected
2 vs 6	-0.3438	0.569	rejected
2 vs 7	-3.500	0.000	accepted
2 vs 8	-2.8438	0.000	accepted
2 vs 9	-1.6875	0.006	accepted
3 vs 4	-0.3750	0.591	rejected
3 vs 5	0.7292	0.264	rejected
3 vs 6	0.2604	0.690	rejected
3 vs 7	-2.8958	0.000	accepted
3 vs 8	-2.2396	0.001	accepted
3 vs 9	-1.0833	0.098	accepted
4 vs 5	1.1042	0.092	rejected
4 vs 6	0.6354	0.330	rejected
4 vs 7	-2.5208	0.000	accepted
4 vs 8	-1.8646	0.005	accepted
4 vs 9	-0.7083	0.278	accepted
5 vs 6	-0.4688	0.438	rejected
5 vs 7	-3.6250	0.000	accepted
5 vs 8	-2.9688	0.000	accepted
5 vs 9	-1.8125	0.003	accepted
6 vs 7	-3.1563	0.000	accepted
6 vs 8	-2.5000	0.000	accepted
6 vs 9	-1.3438	0.027	accepted
7 vs 8	0.6563	0.278	accepted
7 vs 9	1.8125	0.003	rejected
8 vs 9	1.1563	0.057	rejected

Appendix Table 8: Analysis of variance (ANOVA) for shoot height at MS medium supplemented with IAA, IBA or NAA at different concentrations after four weeks of *in vitro* culturing of *A.aestivus* microshoots.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
1.0 mg/L IAA	16	266.6	16.66	71.557
2.0 mg/L IAA	16	126.5	7.906	7.674
3.0 mg/L IAA	12	112.0	9.333	7.697
1.0 mg/L IBA	12	93.0	7.750	9.841
2.0 mg/L IBA	16	122.0	7.625	7.183
3.0 mg/L IBA	16	128.5	8.031	11.149
1.0 mg/L NAA	16	106.5	6.656	7.224
2.0 mg/L NAA	16	69.0	4.313	6.096
3.0 mg/L NAA	16	55.0	3.438	1.329
Total	136	1079	7.934	27.247

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	1802.300	8	225.288	15.250	0.000
Within Groups	1876.104	127	14.772		
Total	3678.404	135			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	8.7500	0.000	accepted
1 vs 3	7.3229	0.000	accepted
1 vs 4	8.9063	0.000	accepted
1 vs 5	9.0313	0.000	accepted
1 vs 6	8.6250	0.000	accepted
1 vs 7	10.0000	0.000	accepted
1 vs 8	12.3438	0.000	accepted
1 vs 9	13.2188	0.000	accepted
2 vs 3	-1.4271	0.333	rejected

2 vs 4	0.1563	0.915	rejected
2 vs 5	0.2813	0.836	rejected
2 vs 6	-0.1250	0.927	rejected
2 vs 7	1.2500	0.359	rejected
2 vs 8	3.5938	0.009	accepted
2 vs 9	4.4688	0.001	accepted
3 vs 4	1.5833	0.315	rejected
3 vs 5	1.7083	0.247	rejected
3 vs 6	1.3021	0.377	rejected
3 vs 7	2.6771	0.071	rejected
3 vs 8	5.0208	0.001	accepted
3 vs 9	5.8958	0.000	accepted
4 vs 5	0.1250	0.932	rejected
4 vs 6	-0.2813	0.848	rejected
4 vs 7	1.0938	0.458	rejected
4 vs 8	3.4375	0.021	accepted
4 vs 9	4.3125	0.004	accepted
5 vs 6	-0.4063	0.765	rejected
5 vs 7	0.9688	0.477	rejected
5 vs 8	3.3125	0.016	accepted
5 vs 9	4.1875	0.003	accepted
6 vs 7	1.3750	0.314	rejected
6 vs 8	3.7188	0.007	accepted
6 vs 9	4.5938	0.001	accepted
7 vs 8	2.3438	0.087	rejected
7 vs 9	3.2188	0.019	accepted
8 vs 9	0.8750	0.521	rejected

Appendix Table 9: Analysis of variance (ANOVA) for number of shoots at MS medium supplemented with IAA, IBA or NAA at different concentrations after four weeks of *in vitro* culturing of *A.aestivus* microshoots.

Summary				
Treatments	**Sample size**	**Sum**	**Mean**	**Variance**
1.0 mg/L IAA	16	32	2	1.2
2.0 mg/L IAA	16	74	4.63	4.12
3.0 mg/L IAA	12	16	1.33	0.424
1.0 mg/L IBA	12	46	3.83	2.69
2.0 mg/L IBA	16	52	3.25	3.40
3.0 mg/L IBA	16	42	2.63	2.25
1.0 mg/L NAA	16	40	2.50	4.0
2.0 mg/L NAA	16	33	2.06	1.40
3.0 mg/L NAA	16	31	1.94	0.863
Total	136	366	2.69	3.104

ANOVA					
Source of variation	**SS**	**df**	**MS**	**F value**	**Sig.**
Between Groups	126.321	8	15.790	6.851	0.000
Within Groups	292.708	127	2.305		
Total		135			

Fisher LSD			
Group vs Group	**Difference**	**p-level**	**Accepted?**
1 vs 2	-2.625	0.252	rejected
1 vs 3	0.667	0.002	accepted
1 vs 4	-1.833	0.021	accepted
1 vs 5	-1.250	0.246	rejected
1 vs 6	-0.625	0.353	rejected
1 vs 7	-0.500	0.907	rejected
1 vs 8	-0.063	0.907	rejected
1 vs 9	0.063	0.252	rejected
2 vs 3	3.292	0.000	rejected

2 vs 4	0.792	0.175	rejected
2 vs 5	1.375	0.012	accepted
2 vs 6	2.000	0.000	accepted
2 vs 7	2.125	0.000	accepted
2 vs 8	2.563	0.000	accepted
2 vs 9	2.688	0.000	accepted
3 vs 4	-2.500	0.000	accepted
3 vs 5	-1.917	0.001	accepted
3 vs 6	-1.292	0.028	accepted
3 vs 7	-1.167	0.046	accepted
3 vs 8	-0.729	0.211	rejected
3 vs 9	-0.604	0.299	rejected
4 vs 5	0.583	0.316	rejected
4 vs 6	1.208	0.039	accepted
4 vs 7	1.333	0.023	accepted
4 vs 8	1.771	0.003	accepted
4 vs 9	1.896	0.001	accepted
5 vs 6	0.625	0.246	rejected
5 vs 7	0.750	0.165	rejected
5 vs 8	1.188	0.029	accepted
5 vs 9	1.313	0.016	accepted
6 vs 7	0.125	0.816	rejected
6 vs 8	0.563	0.297	rejected
6 vs 9	0.688	0.203	rejected
7 vs 8	0.438	0.417	rejected
7 vs 9	0.563	0.297	rejected
8 vs 9	0.125	0.816	rejected

Appendix Table 10: Chemicals and hormones used for media preparation in this study.

Chemical Name	Company	Cat. No.
MS media	Duchefa	M0222.0050
Gibberellic acid (GA_3)	Duchefa	G0907.0005
European Bacteriological Agar	Hy-labs	705437
2,4-dichlorophenoxyacetic acid (2,4-D)	Sigma	D7299
6-benzylaminopurine (BA)	Duchefa	B-0904.0005
Indole-3-acetic acid (IAA)	Duchefa	10901.0025
1-Naphtalene acetic acid (NAA)	Duchefa	N0903.0025
Isopentenyl adenine (2-ip)	Duchefa	2365-40-4
kinetin (Kn)	Sigma	K0905.005
Indole-3-butyric acid (IBA)	Sigma	I5386
Polyvinylpyrrolidone (PVP)	Duchefa	P1368.0500

Appendix Table 11: Chemicals and Reagents used in the extraction and determination of chemical constituents of *Asphodelus aestivus* using Thin Layer Chromatography (TLC)

Chemical Name	Company	Cat. No.
Absolute ethyl alcohol	Sigma-Aldrich	34870
Ethyl acetate	CS	141-78-6 / lot: 5398
Formic acid	Sigma-Aldrich	F050-100 ml
Methanol	Frutarom	34860