EFFECTS OF DIFFERENT EXPLANT TYPES, PLANT GROWTH REGULATORS AND SHOOT DENSITY ON *IN VITRO* REGENERATION OF ASPARAGUS (*Asparagus officinallis* L.)

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The thesis attached here to, entitled **"EFFECTS OF DIFFERENT EXPLANT TYPES, PLANT GROWTH REGULATORS AND SHOOT DENSITY ON *IN VITRO* REGENERATION OF ASPARAGUS (*Asparagus officinallis* L.)"** was prepared under the direction of the chairperson of the candidate supervisory committee and has been approved by all members of that committee as a partial fulfillment of the requirements for the degree of **Master of Agricultural Science (Horticulture).**

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This thesis represents the original works of the author, except where otherwise stated. It has not been submitted previously for a degree at any other University.

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DEDICATED TO MY BELOVED PARENTS
U PYONE CHO AND DAW AYE AYE HNIN

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ABSTRACT

Asparagus are grown throughout the world. The most economically important Asparagus species is garden Asparagus (*Asparagus officinallis* L.). It is a kind of health caring vegetables contributing distinctive taste and an abundant source of various nutrients. The production of Asparagus species is increasing and becoming popular. This experiment was conducted with three objectives; (1) to observe the effects of different explants on *in vitro* shoot induction of asparagus, (2) to determine the optimum concentrations of 6-benzyladeno-purine (BAP) for shoot multiplication of asparagus, and (3) to compare the single shoots and cluster shoots for root induction of asparagus. Three different explant types; apical bud, lateral bud and spear segment were cultured onto MS medium to induce shoots in initial culture. Shoot tips derived from apical and lateral buds of initial culture were sub-cultured onto MS medium with 0.5 mg. L^{-1} 1-naphthaleneacetic acid (NAA) and 0, 2, 3 and 4 mg. L^{-1} 6-benzyladeno-purine (BAP) for shoot multiplication stage. After multiplication, two types of shoots (single and cluster shoots) were cultured onto MS medium with 1.5 and 2.5 mg. L^{-1} indole-3-butyric acid (IBA) for root induction.

Shoot formation of three different explant types was observed approximately 20 days after culture. For initial culture, apical bud gave 63 % of shoot formation followed by lateral bud (44 %) and spear segment (20 %) respectively. The maximum number of shoots (3 shoots per explant) was observed in apical bud. Although the maximum number of nodes (2 nodes per shoot) was found in apical and lateral buds, lateral bud gave longer shoot length (2.6 cm). The medium supplement with 2 mg. L^{-1} BAP gave the highest number of shoots (9 shoots per explant), shoot length (2 cm), and number of nodes (2 nodes per shoot) in multiplication stage. Cluster shoots produced higher root formation percentage in both levels of IBA concentrations than single shoot. Among two different IBA levels, 2.5 mg. L^{-1} IBA gave higher root formation percentage than 1.5 mg. L^{-1} IBA. Based on this research finding, apical bud is a suitable explant type for shoot formation. The medium supplement with 2 mg. L^{-1} BAP could be selected to get maximum number of shoots per explant, nodes per shoot and longest shoot length. It was observed that cluster shoots formed roots well in 2.5 mg. L^{-1} IBA containing medium. Cluster shoots have potential for rooting of *in vitro* developing shoots rather than single shoots.

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CHAPTER I
INTRODUCTION

Asparagus is a large genus with over 150 species of herbaceous perennials crop of high economic value with a chromosome number of 2n=20 that belongs to the Liliaceae family. They are grown throughout the world although they originated mainly from Asia, Africa and Europe (Prohens et al. 2008). The most economically important Asparagus species is garden asparagus (*Asparagus officinallis* L.), which is a highly priced vegetable (Stajner et al. 2002). Young asparagus crowns established a rhizome structure with a large root system and stems. Each rhizome has a few lateral buds that develop into succulent fleshy shoots (spears). *Asparagus officinallis* L. is a dioecious species with male and female plants (sex ratio 1:1). Female plants produce seed and larger-diameter spears, but have lower yields. Male plants have higher yields, longer productive life, and produce spears earlier, however, do not produce seeds.

Asparagus has been identified as having marketable value as a medicinal plant with residential and commercial applications. Asparagus is a kind of health-caring vegetable contributing both to nutrition and fitness and contains manifold functional elements (Shao et al. 1996). The asparagus spears which are crisp and tender with a distinctive taste, is an abundant source of various nutrients such as vitamins (A, C, K and E) amino acids and trace elements. Green asparagus (*Asparagus officinallis* L.) also contains antioxidants, such as rutin, ascorbic acid, tocopherol, ferulic acid and glutathione (Lia et al. 2006). Green asparagus scores higher in micro-nutrient (Fe 1.5 mg, ascorbic acid 48 mg) than white asparagus. The characteristic flavour of Asparagus is due to sugars and bitter components. By means of its valuable uses, tender asparagus shoots are popular in many countries.

In sixteenth century, asparagus gained popularity in France and England. From there, the early colonists brought it to America. It was first planted in California during the 1850s in the San Joaquin Delta (Baayen et al. 2000). Recently, asparagus (*Asparagus officinallis* L.) growing has become popular in Myanmar and it has been widely cultivated in the eastern mountainous regions of the country. The asparagus plants known as Ka-nyut in Myanmar are commercially cultivated in many places of Upper Myanmar. It has been much grown and successfully developed in Pyin-oo-lwin, Kyauk-me and Thi-paw townships (Hninn 2012). Moreover, it has also been widely cultivated in the central lowlands (Naypyitaw and Mandalay regions). And then, commercial production is also

start in other areas by private growers. In 2015 the crop was grown on approximately 900 ha, more than twice the area grown in 2010 (Zaw et al. 2017). Thus, in near future, it could find its way to international market if the growing areas could be extended for production of quality produces. So, large amount of planting materials are needed for area extension. However, its initial glamour and appeal have pale, simply because of the limited local market and lower than expected yields.

Propagation on asparagus seed or division of individual crowns is commonly used in Myanmar. However, propagation of *Asparagus officinallis* L. by seed results in a low percentage of germination and clonal propagation by division of individual crowns is very slow as one plant gives only 2-4 new plants per year under optimum conditions in the absence of any pest invasion of injured surface (Ornstrup 1997). Seedling transplants have the additional problem that their fern growth can be damaged by machinery during transplanting (Alder et al. 1985). As populations of *Asparagus officinallis* L. seedlings are highly heterozygous, pure inbred homozygous lines are difficult to develop. Therefore, individual seeds harvested from the same plant normally have different genotypes, a variability that can adversely affect the yield of asparagus production fields because the productivity of individual plants may be markedly differ. The problem with commercial production of this crop is availability of plant materials required for conventional vegetative propagation and also to obtain sufficient quantities of uniform products. To avoid yield variation expected from the use of heterozygous seed lots in asparagus fields, micropropagation techniques have been used to multiply individual high producing plants (McCormick and Franklin 1990 and Desjardins and Bajai 1992). Early reports of tissue culture of *Asparagus officinallis* L. dated back to 1945 and since then, this sector has experienced a great deal of development. So, micropropagation could be an alternative solution. Although micropropagation has been applied to asparagus for more than twenty years, weak roots or the lack of root formation has been problematic for some genotypes. The benefit of this study is particularly valuable in the areas of clonal propagation.

In micropropagation techniques, shoot and root induction can be affected by explant source, plant density and plant growth regulators. Explant derivation can influence growth and morphogenetic potential. Moreover, the maximum rate of micropropagation depends on the selection of the most suitable explants. Lateral bud density in solid medium can affect shoot and root growth of asparagus. The number of young shoot can influence on asparagus rooting (Fortes et al. 1997).

Both 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) or 6-benzyladenine (BA), auxin and cytokinin, respectively, are commonly used in culture of asparagus (Yang and Clore 1973) to regulate cell division, internode elongation and shoot differentiation (Razdan 2003). Auxin plays a key role in rooting, and exogenous auxin is required in many species (Diaz-Sala et al. 1996). NAA is preferable to other auxins for asparagus rooting on solid medium. In many commercial propagation systems of other species, however, indole-3-butyric acid (IBA) is most common (Deklerk et al. 1999). Experiments to optimize the kind and concentration of PGRs will be critical in culture of asparagus. Therefore, the present study was carried out;

- to observe the effect of different explants on *in vitro* shoot induction of asparagus,
- to determine the optimum concentration of BAP for shoot multiplication of asparagus, and
- to compare the single shoots and cluster shoots for root induction of asparagus.

CHAPTER II
LITERATURE REVIEW

2.1 Botany and Morphology of Asparagus

Asparagus (*Asparagus officinallis* L.) is a perennial vegetable belonging to the family Liliaceae (Tutin et al. 1980 and Nonnecke 1989) and is grown commercially in a wide range of environments including temperate regions, humid tropics, and arid tropics. Asparagus is a dioecious plant with over 150 species (Prohens et al. 2008). Asparagus species are cultivated for food as well as medicinal and ornamental plants. The genus is categorized into three subgenera; *Asparagus*, *Protasparagus* and *Myrsiphyllum*, based on morphology (Clifford and Conran 1987 and Kubota et al. 2012). Among these species, only *Asparagus officinallis* L. is grown for its edible spears (Nonnecke 1989).

As an herbaceous perennial, asparagus produces an underground crown which consists of a rhizome, and storage and feeder roots. Buds are produced at the ends of the rhizome and develop into shoots (Blasberg 1932). As lateral buds are formed and develop on the rhizome, the crown is extended horizontally in the soil. When a dominant bud completes its development into a shoot, an axillary bud differentiates followed by formation of a spear. The spears are triangular and comprise short internodes and lateral buds. Expansion of individual spear occurs foremost at the base internodes and then in successive internodes, resulting in dynamic growth of the spear into a 4 to 6 feet tall fern. Apical dominance from the presence of developed stems can inhibit spear emergence, however, continual removal of spears prevents apical dominance. The cladophylls, found on the main stem at the base of branches, or as the ‘scales’ at the tip of a young spear are the true leaves (Blasberg 1932). The false leaves (modified stems) are linear or hair-like, called cladodia, which cluster at interval of the branches.

Male and female flowers are separated on the different plants (Lazarte and Palser 1979). Flowers produced by staminate plants have functional stamens and mostly rudimentary pistils with undeveloped ovules. Pistillate plants have normal stigmas, styles, and ovaries, but degenerate anthers without pollen. Ocassionally, andromonoecious males generate both functional hermaphroditic and male flowers (Sneep 1953). The asparagus fruit is a red berry at maturity and contains up to six small globular seeds. The seed is mostly endosperm tissue surrounding a small embryo. One gene (*M*) controls the gender of asparagus (Jiang and Sink 1997). Female plants are homozygous recessive (*mm*) while male plants are heterozygous (*Mm*). Ocassional hermaphroditic flowers on male plants

result in selfing or a male × male cross, and the recovery of homozygous (*MM*) supermale plants (e.g. *Mm* × *Mm* = 1 *MM* : 2 *Mm*: 1 *mm*). Both MM and Mm genotypes have male phenotypes and supermales can be distinguished by testcrossing to the female (mm). Ultimately, an all-male hybrid is produced by crossing a female (mm) with a supermale (MM) to yield only male (Mm) progeny. When a superior all-male hybrid is identified, tissue culture of the parents is necessary for large scale seed production. The female and supermale parents cannot be propagated by seed, as in the corn inbred-hybrid system, because they have imperfect flowers and cannot be selfed. Genetic identity can only be preserved by vegetative or asexual propagation.

2.2 Taxonomic Classification of *Asparagus officinallis* L.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Liliidae
Order	Liliales
Family	Liliaceae
Genus	*Asparagus* L.
Species	*Aspragus officinallis* L.
Common name	Garden asparagus

(by Prohens et al. 2008)

2.3 Importance of Asparagus

In recent years there has been renewed interest in natural medicines that are obtained from plant parts or plant extracts. Asparagus has been identified as having marketable value as a medicinal plant with residential and commercial applications. It is a popular vegetable consumed in most parts of the world. Asparagus shoots which are frequently used in salads, vegetable dishes and soups are the edible part of the plant. In Chinese traditional medicine, this plant has been used as a tonic, antifebrile, antitussive, hair growth stimulator and diuretic agent. The asparagus extracts have been demonstrated to possess certain biological activities including antifungal, antimutagenic diuretic, cytotoxic, antiviral and molluscicide properties. Moreover, asparagus (*Asparagus officinalis* L.) is a kind of health-caring vegetable contributing both to nutrition and fitness and contains manifold functional elements (Shao et al. 1996).

2.4 Production of Asparagus

Asparagus officinalis production areas in 65 countries are continually changing and are difficult to monitor since asparagus is considered a minor crop in most countries and the production data is usually reported under the broad term of "vegetable production". The production areas in the asparagus industries throughout the world are the result of the worldwide increased consumer demand for this product and the changes in the economic conditions in the producing and consuming nations. International movements of fresh, frozen and canned asparagus are inter-related and dependent upon the consumption and production periods of the various countries. The largest increases in asparagus production in the last ten years has occurred in countries in the Southern Hemisphere and China and in countries with low labor rates where they can produce spears relatively cheaply and/or market their production during a higher priced market window in another country or hemisphere.

When asparagus was introduced into Myanmar, it was deemed to be a very profitable crop. However, its initial glamour and appeal have waned, simply because of the limited local market and lower than expected yields. There are many varieties of Asparagus species. Among the most common in Myanmar is the garden asparagus (*Asparagus officinalis*). Recently, asparagus (*Asparagus officinallis* L.) growing has become popular in Myanmar and it has been widely cultivated in the central lowlands and eastern mountainous regions of the country. In 2015, the crop was grown on approximately 900 ha, more than twice the area grown in 2010 (Zaw et al. 2017).

2.5 Propagation of Asparagus

Traditionally, asparagus fields were established either from dormant one year old crowns, by direct seeding, or by 10-12 week old seedling transplants (Alder et al. 1985). Asparagus crowns used as planting stock for establishing commercial fields are obtained by sowing seeds into nursery stock beds in the spring. Dormant crowns are dug in autumn to early winter and planted out the following winter or early spring (Nichols 1989). Ideally, crowns should be of uniform size, 25-30 mm across the bud cluster, with firm white fleshy roots 200 mm long (McCormick and Franklin 1990). Direct seeding *in situ* is used to establish high density stands, but is no longer a common practice because of the high cost of hybrid seed and difficulties involved in weed control in young seedlings (Nichols 1989). Seedling transplants have a number of advantages over crowns and direct seeding for they extend spring planting; can be planted mechanically; are grown in sterile

media; and, if grown to specification, have unbroken root systems which reduce infection from soil borne fungal diseases. Because seedlings have live fern growth and are smaller than crowns, they require more care when transplanted. Normally, asparagus beds established by seedling transplants require two full growing seasons before harvest. In contrast, crowns require only one full growing season (McCormick and Franklin 1990).

2.6 *In Vitro* Cloning of Asparagus

Utilisation of asparagus clones offers many advantages including: the potential for earlier harvest after field establishment; improved uniformity in field establishment, as well as improvement in spear colour, shape, length and diameter at harvest; and increased yields (Wilson 1991, Abernethy and Conner 1992).Plant tissue culture is widely used technique and carries immense potential in abroad spectrum of plant biotechnology and plant genetics applications. *In vitro* propagation is a promising method of rapidly producing numerous, uniform plants that are free of microbial contamination. The development of an *in vitro* protocol is advantageous not only for maintainable utilization of a species, but also for germplasm conservation and genetic improvement (Chen et al. 2006). Tissue culture involves the use of small pieces of plant tissue (explants) which are cultured in a nutrient medium under sterile condition. Using the appropriate growing conditions with the addition of suitable plant growth regulators (PGRs) for each explant type, plant can be induced to rapidly produce new shoots and new roots.

In vitro plant propagation or micropropagation is a useful technique for medicinal plants. The rapid growth and uniformity of the planting material are some major benefits of the micropropagation techniques. Futhermore, tissue culture techniques can be used to commercially produce and can save rare plants of medicinal value. Such propagation techniques could be necessary as a result of species physiological factors such as low productivity due to low seed germination rates (Chen et al. 2006). In addition, micropropagation of medicinal plants is a means of producing disease free superior quality planting material. It is a viable alternative for species where elites have been identified based on their potential for yielding higher amount of active principle; which are difficult to regenerate by conventional methods, and, where conventional methods are inadequate to meet the demand of quality planting material.

Within the Asparagus genus, micropropagation protocols have been extensively studied in *Asparagus officinallis* (Murashige et al. 1972) and other species used mainly as ornamental or medicinal plants, using media supplemented with MS (Murashige and

Skoog 1962) medium and various concentrations of auxins and cytokinins. Several methods of *in vitro* regeneration of asparagus have been established namely: direct organogenesis (Murashige et al. 1972), indirect organogenesis (Reuther 1977, 1984) and somatic embryogenesis (Reuther 1977). Micropropagation using shoot tip culture promotes the growth and multiplication of terminal and axillary shoots. Asparagus shoot tip culture was first reported by (Loo 1945) who demonstrates that it is possible to grow asparagus stem tips continuously on a basal medium supplemented with sucrose, and inorganic and organic constituents. To improve asparagus micropropagation, several factors should be concerned, including explant types, density and concentration of growth regulators, basal medium, carbohydrates.

2.6.1 Explant materials

Selection of explant material is a crucial aspect of micropropagation that requires three important condierations: 1) genetic and epigenetic characteristics of the source plant 2) pathogen control 3) physiological conditions of the plant prior to explant excision in order to optimize its abilty to establish in a culture (Hartmann et al. 1990). Different parts of mother plants can be chosen to establish an asparagus clone through *in vitro* culture, e.g. anthers (Doré 1977, Tsay et al. 1980, Inagaki et al. 1981 and Reuther 1983), shoot segments (Harada and Yakuwa 1983), bud clusters (Kohmura et al. 1994). Recent studies on asparagus *in vitro* culture conducted in China proved the importance of explant material selection. Shoot basal segments were proved to be better explants compared to shoot middle or apical segments after some studies by Cai et al. (2005), Lin et al. (2006), Chen (2007). Nevertheless, Lin et al. (2008) clarified shoot tips could be ideal explant materials. Explants derived from different parts of the mother plants, after initiate growth on the callus induction medium, can be developed into plantlets. However, not all types of explants are favorable for root stimulation. Fortes et al. (1997) studied the influence of young shoot number on asparagus rooting. Gebler (2005) stated that none of the single shoot explant could develop storage roots. Furthermore, very small explants, whether they are shoot or meristem tips, fragments of whole plant tissues, or pieces of callus, do not survive well in culture, but large explants may be difficult to decontaminate effectively or are less easily manipulated (Roberta 2013).

2.6.2 Plant growth regulators

Hormones also known as plant growth regulators are chemicals used to alter the growth of a plant or plant parts. Good development of tissues and organs are influenced by the present of hormones in medium though a few monocotyledonous species find it optional. In tissue culture, five major classes of growth regulators are important: auxins, cyokinins, gibberellins, abscisic acid and ethylene (Razdan 2003 and Gaba 2005). Among these classes, auxins and cytokinins are the most important for regulating growth and morphogenesis in plant tissue and organ cultures. 6-Benzylaminpurine (BAP) from cykokinins and Indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) from auxins were used for this study. BAP with the formula $C_{12}H_{11}N_5$ from cytokinins and IBA with the molecular formula $C_{12}H_{13}NO_2$ and NAA with molecular formula $C_{12}H_{10}O_2$ are among the auxins are the most favored and common hormones employed in various experiments for tissue culture and micropropagation (George 1993). Cykonins can stimulate protein synthesis. The cytokinins are used to stimulate cell division in the culture medium and induce shoot formation or auxillary shoot proliferation (George 1993). Auxins are generally used to stimulate callus production and cell growth in a culture medium or to initiate shoots, particularly roots, and to induce somatic embryogenesis and stimulate growth from shoot apices and shoot tip cultures (George 1993).

In the culture of asparagus shoots with lateral buds, varying concentrations of auxins and cytokinins affected both shoot and root growth (Yang and Clore 1973). Pant and Joshi (2009) reported that while 1-naphthaleneacetic acid (NAA) promoted bud initiation, 6-benzylaminopurine (BAP) promoted both shoot and bud initiation and their combination promoted shoot, bud, callus and root initiation *in vitro* propagation of *Asparagus racemosus*. Similarly, Afroz et al. (2010) used 6-benzylaminopurine (BAP) as a cytokine source and 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA as auxin source for the successful *in vitro* multiplication of *Aasparagus racemosus* as the best shoot multiplication was obtained from MS medium supplemented with BAP (0.1 mg. L^{-1}) and NAA (0.05 mg. L^{-1}). According to Kamile et al. (2014), apical buds were the best explants, MS medium containing 2.0 mg. L^{-1} BAP and 0.5 mg. L^{-1} NAA was the best propagation medium in *Asparagus stipularis* Forssk. Kumar (2009) reported that nodal segements were suitable for *in vitro* shoot development and bud induction in *Asparagus racemosus*. The best results were achieved on MS medium supplemented with 3.0 mg. L^{-1}

BAP and 0.5 mg. L^{-1} NAA, and 40-45 shoot bud of explants were obtained on the medium. It is possible that a high BAP concentration positively affects shoot development. Damiano et al. (2004) tested IAA, NAA and IBA in hazelnut culture, and the highest rooting rate and root number per rooted plants was achieved with media containing supplement with 1.8 mg. L^{-1} IBA. Benmoussa et al. (1997) and Mehta and Subramanian (2005) induced roots on media containing IBA in *Asparagus densiflorous*, *Asparagus adscendens* and *Asparagus racemosus* shoots respectively whereas Gosh and Sen (1996) reported good rooting on MS mdia containing IAA in *Asparagus verticillatus*. Better root induction was observed on MS medium supplement with 1.5 mg. L^{-1} IBA in nodal segments of *Asparagus racemosus*.

2.6.3 Plant density

Lateral bud density affected shoot and root growth of asparagus (Matsubara and Clore 1974). Shen et al. (1995) found that when both clustered shoots (with 2–5 shoots) and a single shoot tip were used as explants to be subcultured on 1/2 MS medium with 0.1 ml. L^{-1} NAA, a higher rooting percentage (78.9 %) was received from clustered shoots in comparison to that from a single shoot tip, which only gave 25.7 % rooting. Fortes et al. (1997) and Gebler (2005) illustrated similar results. Fortes et al. (1997) studied the influence of young shoot number on asparagus rooting. It was reported that four-shoot explants rooted better than those with one, two or three shoots. Longer roots were also observed from the explant with four shoots. Gebler (2005) stated that none of the single shoot explant could develop storage roots.

2.6.4 *In vitro* rooting of microshoots

Rooting is an important step in micropropagation studies (Moncousin 1991). Although, a number of plant root spontaneously in culture (some monocotyledons and other herbaceous species), shoots of most species multiplied *in vitro* lack a root system (Yeoman 1987). Rooting can be achieved either by transferring the microshoots to medium lacking cytokinins with or without a rooting hormone or by treating the shoots as conventional cutting after removal from sterile culture medium. There is a great variation between species in the ease with which cultured shoots can be rooted and systematic trials are often needed to find the most effective conditions for rooting. All cytokinins inhibit rooting and BA (which is widely used for shoot multiplication) does so strongly, even after transfer to cytokinins-free medium (George and Sherrington 1984).

Many species require the stronger auxin i.e., IBA or NAA to stimulate root formation. Among the auxins tested, IBA was found to be the most suitable for *in vitro* rooting (Sreekumar et al. 2000, Faisal and Anis 2002, Liu et al. 2003, Sivaram and Mukundan 2003, Anis and Shahzad 2005, Feyissa et al. 2005, Faisal et al. 2005 and Faisal et al. 2007).

2.6.5 Acclimatization

Acclimatization is the adaptation of plantlets developed *in vitro* to new external uncontrolled environment during which normal photosynthesis activity and water relations have to be developed (Desjardins 1995). However, the commercial utilization of the micropropagation technology is often limited due to poor survival rates of many plant species during acclimatization period (Kozai 1991 and Preece and Sutter 1991). The main problem of *ex vitro* transfer is high rates of water loss from the shoots of plantlets taken out from the culture vessel. Even if the water potential of the substrate (sand or soil with nutrient solution) is usually higher than the water potential of the media with sucrose, the plantlets may quickly wilt. The abnormalities in morphology, anatomy and physiology of plantlets cultivated *in vitro* can be repaired after transfer to *ex vitro* conditions. However, many plant species need gradual changes in environmental conditions to avoid desiccation losses and photo inhibition.

CHAPTER III
MATERIALS AND METHODS

3.1 Mother Plant Selection and Preparation

Mother plant selection and preparation were conducted during September and November, 2016 in asparagus hybrid farm located from Kohywar Thapyay Kone Village, Pobbathiri Township and Plant Tissue Culture Laboratory of Department of Horticulture, Yezin Agricultural Uinversity, firstly. Secondly, shoot induction was carried out at Plant Tissue Culture Laboratory to observe the effect of different explants on *in vitro* shoot induction of asparagus. Thirdly, the optimum concentration of BAP for asparagus shoot multiplication was investigated in multiplication stage and finally, root induction stage was carried out to compare the single shoots and cluster shoots for asparagus rooting.

3.1.1 Preparation of plant materials

Fifteen to twenty-centimeter long healthy young spears emerging in the early morning from two year old asparagus hybrid farm were harvested (Plate 3.1). The basal (10 cm) region of these spears was discarded and the remaining portions were collected and carried with the ice-bag as explant types.

3.1.2 Surface sterilization

After collecting the spears, they were surface sterilized before placing them on the culture medium. Firstly, the collected spears were washed thoroughly with running water. The surface of explants were sterilized with commercial bleach concentration (15 %) for 15 minutes and then rinsed 3 times with sterilized water. After sterilization, spears were cut into about 5 mm pieces and apical buds (AB), lateral buds (LB) and spear segments (SS) under *in vitro* condition. Leaf scales from lateral buds were discarded (Plate 3.1).

3.1.3 Preparation of shoot induction medium

MS basal medium was used for all culture in this study. The stock solutions for MS medium (macronutrients, micronutrients, iron and organic) were prepared using distilled water. Appropriate amounts of major, minor, iron and organic were taken from the stock solutions and the volume made up to at least half that required with distilled water, 3 % (w/v) sucrose was added for this experiment unless otherwise stated to the media as the carbon source. Prior to autoclaving pH of the media was adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCL with a pH meter. Six g/L agar, used for gelling, was

added to the medium and heated to melt it completely. The molten media were dispensed into 150 ml cornical flask (30 ml). Aluminium foil and parafilm were used to plug the culture flasks. The medium culture flask was sterilized by autoclaving at 121 °C, 15 Psi (1.06 kg. m^{-2}) for 15 min. after sterilization the flasks were taken out in culture flask racks and allowed to gel.

3.2 Effects of Different Explant Types on *In Vitro* Shoot Induction of Asparagus

3.2.1 Experimental procedure

Three different explants: apical buds, lateral buds and spear segments (approximately 0.5 cm in length) were excised from sterilized spears and cultured onto MS medium. The culture vessels were incubated at 25 ± 2 °C with 16 hr photoperiod under light intensity (approximately 30 μ. mol. m^{-2}. s^{-1}). In this experiment, each of explant was inoculated in each culture vessel and three culture vessels were cultured in each treatment. The experiment was set up in Completely Randomized Design (CRD) with twelve replications.

3.2.2 Data collection

The following data were collected during six weeks culture for *in vitro* shoot induction of asparagus.

(1) Survival percent

Survival rate of *in vitro* shoots was recorded after initial culture for six weeks.

(2) Number of days to induce first shoots

Day to induce first shoot was counted first day to form *in vitro* shoot from three different explant types after inoculation.

(3) Shoot formation percent

$$\text{Shoot formation (\%)} = \frac{\text{Total number of shoot produced}}{\text{Total number of shoot cultured}} \times 100$$

(4) Number of shoots per explant

The number of shoots was recorded by counting the shoot producing form each treatment.

(5) Shoot length (cm)

Shoot length was measured the longest shoot length from each treatment after six weeks culture.

(6) Number of nodes per shoot

The number of nodes was recorded by counting the node forming from the longest shoot length after six weeks culture.

3.3 Effects of Different Explant Types and Different Concentrations of 6-Benzylaminopurine (BAP) on *In Vitro* Shoot Multiplication of Asparagus

3.3.1 Experimental procedure

Two different explant types (approximately 0.5 cm in length, shoot tips derived from apical and lateral bud cultures) formed at six weeks after initial culture were used as explant in shoot multiplication stage. Two different explant types developed from initial culture were transfered onto PGRs free medium for two weeks and then cultured onto fresh medium for shoot multiplication. Shoot multiplication medium was composed of MS medium (Murashige and Skoog 1962) supplemented with different concentrations of PGRs, BAP (0.0, 2.0, 3.0, 4.0 mg. L^{-1}) and NAA (0.5 mg. L^{-1}) (Table 3.1). The culture vessels were incubated at 25 ± 2 °C with 16 hr photoperiod under light intensity (approximately 30 μ. mol. m^{-2}. s^{-1}) for eight weeks. In this experiment, each of explant was inoculated in each culture vessel and three culture vessels were cultured in each treatment. The experiment was set up in factorial Randomized Complete Block Design (RCB) with twelve replications.

3.3.2 Data collection

The following data were collected during the *in vitro* shoot proliferation of asparagus.

(1) Shoot formation percent

$$\text{Shoot formation (\%)} = \frac{\text{Total number of shoot produced}}{\text{Total number of shoot cultured}} \times 100$$

(2) Number of shoots per explant

The number of shoots was recorded by counting the shoot producing from each treatment.

(3) Shoot length (cm)

Shoot length was measured the longest shoot length from each treatment after eight weeks culture.

(4) Number of nodes per shoot

The number of nodes was recorded by counting the node forming from the longest shoot length after eight weeks culture.

3.4 Effects of Shoot Density and Different Concentrations of Idole-3-butyric Acid (IBA) on *In Vitro* Root Induction of Asparagus

3.4.1 Experimental procedure

At eight weeks after shoot multiplication, shoots produced from shoot multiplication stage (approximately 4-5 cm in length) were separated as clustered shoots type and single shoot type (Plate 3.2). And then, two different shoot types were transferred onto MS medium with IBA (1.5 mg. L^{-1} and 2.5 mg. L^{-1}) for root induction under light intensity (approximately 30 μ. mol. m^{-2}. s^{-1}) (Table 3.2). In this experiment, each of explant was inoculated in each culture vessel and three culture vessels were cultured in each treatment. The experiment was set up in factorial Randomized Complete Block Design (RCB) with twelve replications.

3.4.2 Data collection

The following data was collected during eight weeks culture for *in vitro* root induction of asparagus.

(1) Root formation percent

$$\text{Root formation (\%)} = \frac{\text{Total number of root produced}}{\text{Total number of shoot cultured}} \times 100$$

10 cm

Washing with detergent under running tap water

AB LB SS

Separating explants from spear

Surface sterilization with 20 %
Sodium hypochloride for 20 mins

Rinsing 3 times with sterile water

Plate 3.1 Explant preparation from asparagus hybrid farm and surface sterilization procedure under *in vitro* condition

AB - Apical buds

LB - Lateral buds

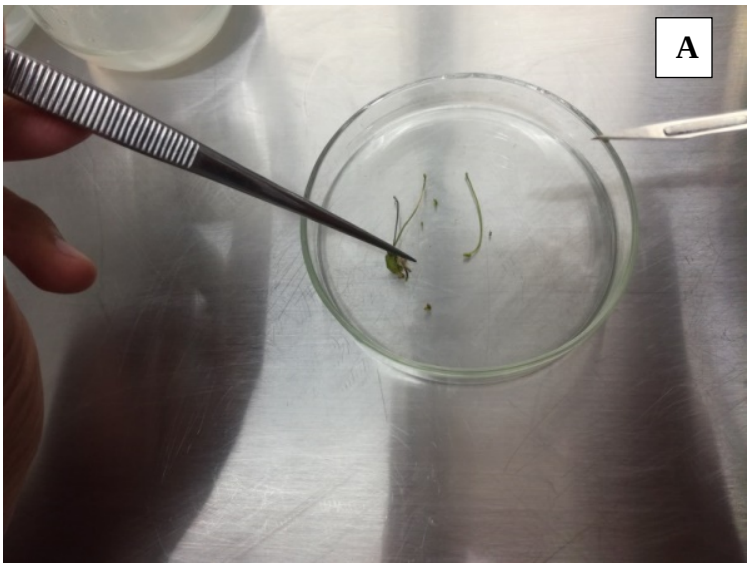
SS - Spear segments

Table 3.1 Different combinations and concentrations of PGRs used to culture of two different explant types for shoot multiplication

Explant types	PGRs (mg. L^{-1})	
	NAA	BAP
Shoot tips derived from apical bud cultures	0.50	0.00
	0.50	2.00
	0.50	3.00
	0.50	4.00
Shoot tips derived from lateral bud cultures	0.50	0.00
	0.50	2.00
	0.50	3.00
	0.50	4.00

Table 3.2 Different concentrations of IBA used to culture of two different shoot types for root induction

Shoot types	IBA concentrations (mg. L^{-1})
Cluster shoots (3-5 shoots) in length of 4-5 cm	1.5
	2.5
Single shoots in length of 4-5 cm	1.5
	2.5



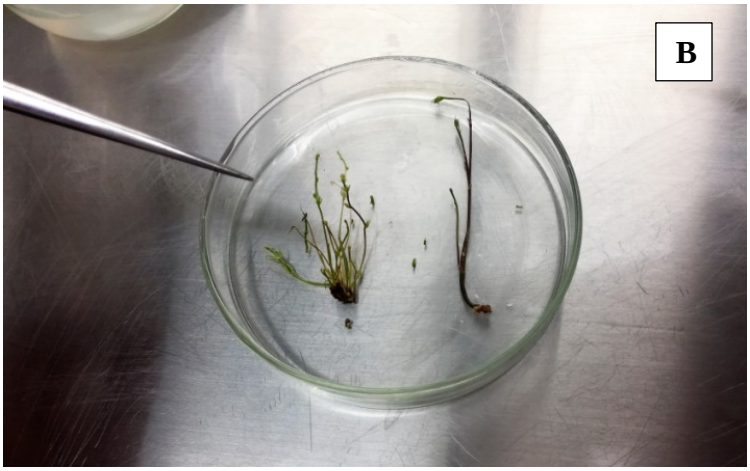


Plate 3.2 Different types of shoot; (A) single and (B) cluster shoot, excised from multiplication stage for root induction

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Effects of Different Explant Types on *In Vitro* Shoot Induction of Asparagus

4.1.1 Survival percent

Explant types were found to influence on the survival percent of explants. However, in this experiment lateral buds and spear segments produced the 100 % survival rate while the apical bud explants has 91 %.

It was found that some apical bud explants showed browning and death. It may be linked to the excision of primary explants and explant quality. The excision of primary explants often promotes the release of polyphenols and stimulates polyphenol oxidase activity within the damaged tissues. The polyphenol oxidation products often blacken the explant tissue and medium. Murashige (1974) also reported that explants exhibit different capacities for establishment *in vitro* depending on their location of the donor plant.

4.1.2 Number of days to induce first shoots

Most different explant types formed shoot induction within 3 weeks (Table 4.1). The earliest shoot induction, 17 days, was found in apical buds among different explants. The latest shoot induction was observed at 19 days in spear segments.

It was observed that the days to induce first shoot was varied with the different explants. Murashige (1974) also suggested that juvenile explants typically are often more responsive than those obtained from the often-nonresponsive mature tissues of the same plant. In this experiment, the most juvenile one was apical buds.

4.1.3 Shoot formation percent

Shoot formation started within 2 weeks of culture in all explants. The shoot formation percent of different explants are shown in figure 4.1. The maximum shoot formation percent (63 %) was found in apical bud explants then followed by lateral bud explants (44 %) and spear segment explants (20 %).

It was observed that apical bud explants have higher shoot formation frequency than the lateral bud explants and spear segment explants. Brown and Sommer (1992) also found that the apical meristem is normally associated with shoot morphogenesis.

Table 4.1 Effects of different explant types on survival percent and days to induce first shoots of asparagus in initial culture

Explants	Survival percent	Days to induce first shoot
Apical bud	91	17
Lateral bud	100	16
Spear segment	100	19

Table 4.2 Effects of different explant types on number of shoots per explant, shoot length and number of nodes per shoot in shoot induction of asparagus

Explants	Number of shoots per explant	Shoot length (cm)	Numbe of nodes per shoot
Apical bud	**2.71 a**	1.79 b	**2.07 a**
Lateral bud	2.02 b	**2.64 a**	**2.39 a**
Spear segment	1.34 c	1.28 b	1.10 b
LSD (0.05)	0.62	0.73	0.69
CV %	69.67	88.04	85.03
Pr>F	**	**	**

Means within a column followed by the same letter are not significantly difference at 5 % LSD.

***** Significant at 5 % level, ****** Significant at 1 % level

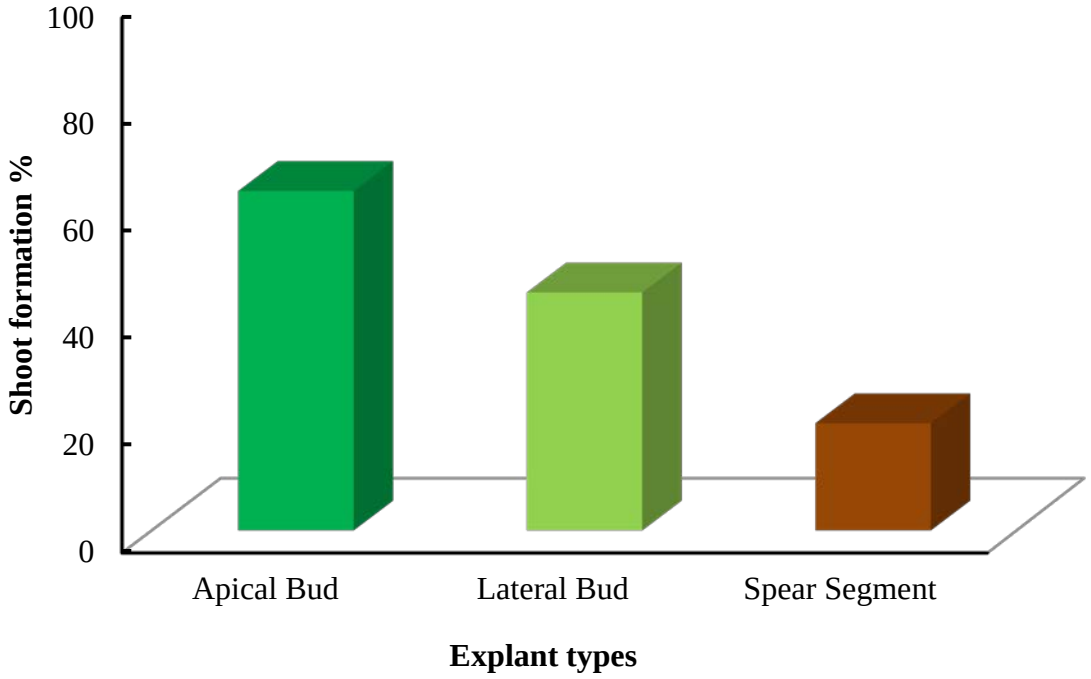


Figure 4.1 Shoot formation % of different explant types of asparagus in initial culture

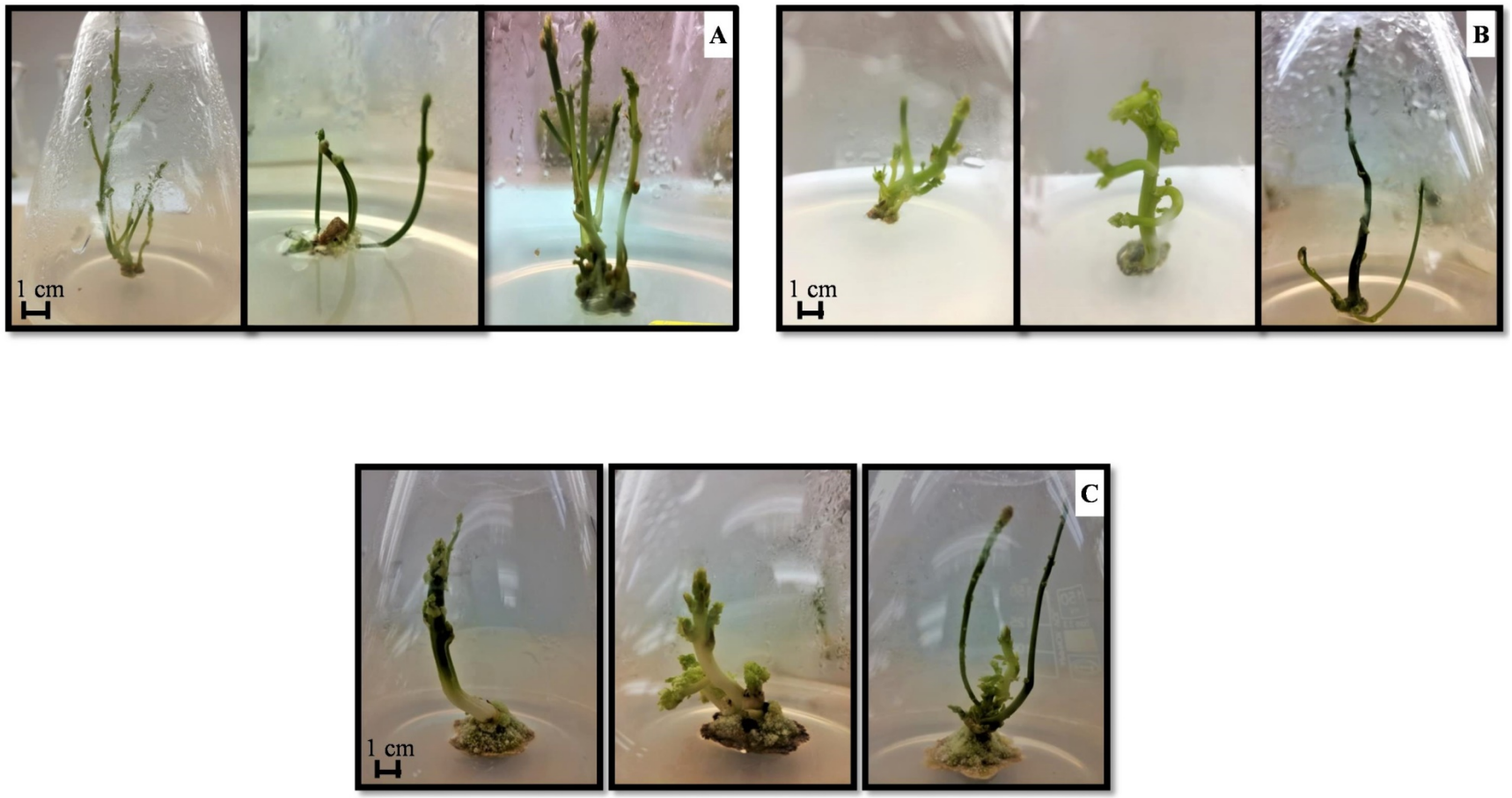


Plate 4.1 Effects of different explant types on number of shoots per explant after six weeks in shoot induction of asparagus; (A) apical buds, (B) lateral buds, and (C) spear segments

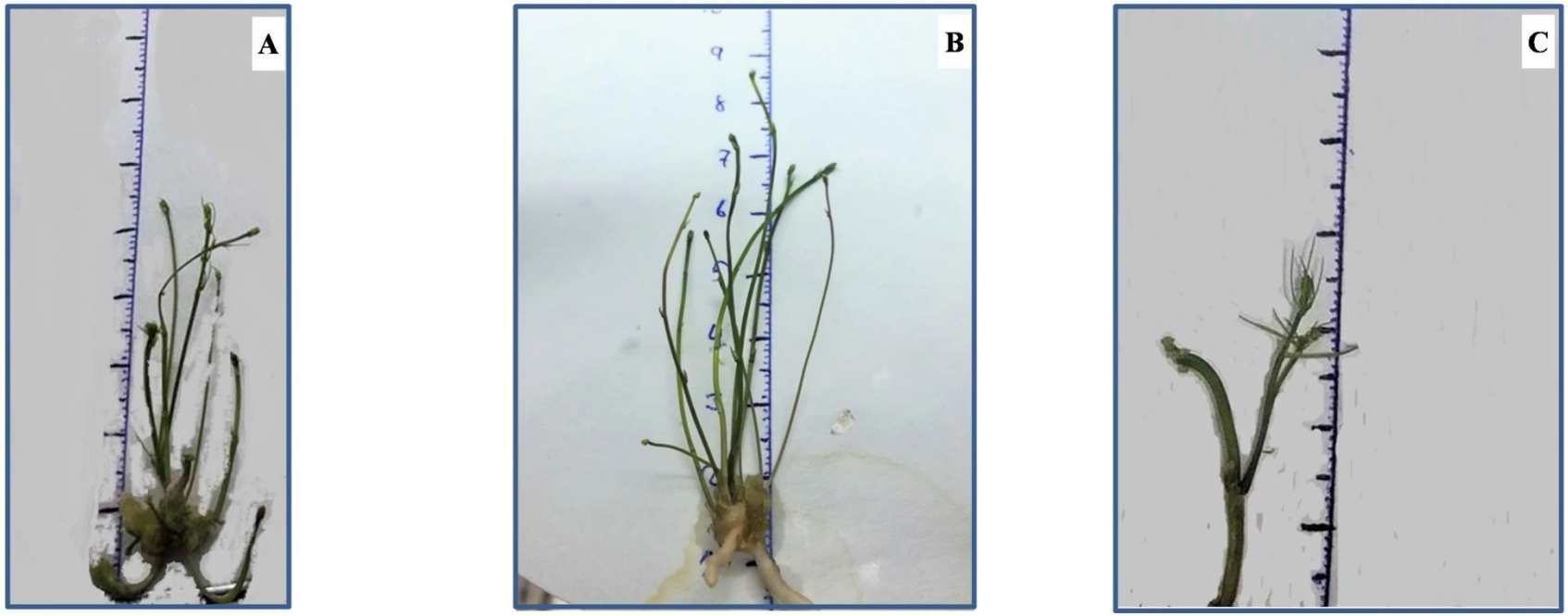


Plate 4.2 Shoot length for (A) apical buds, (B) lateral buds, and (C) spear segments in shoot induction of asparagus after six weeks culture

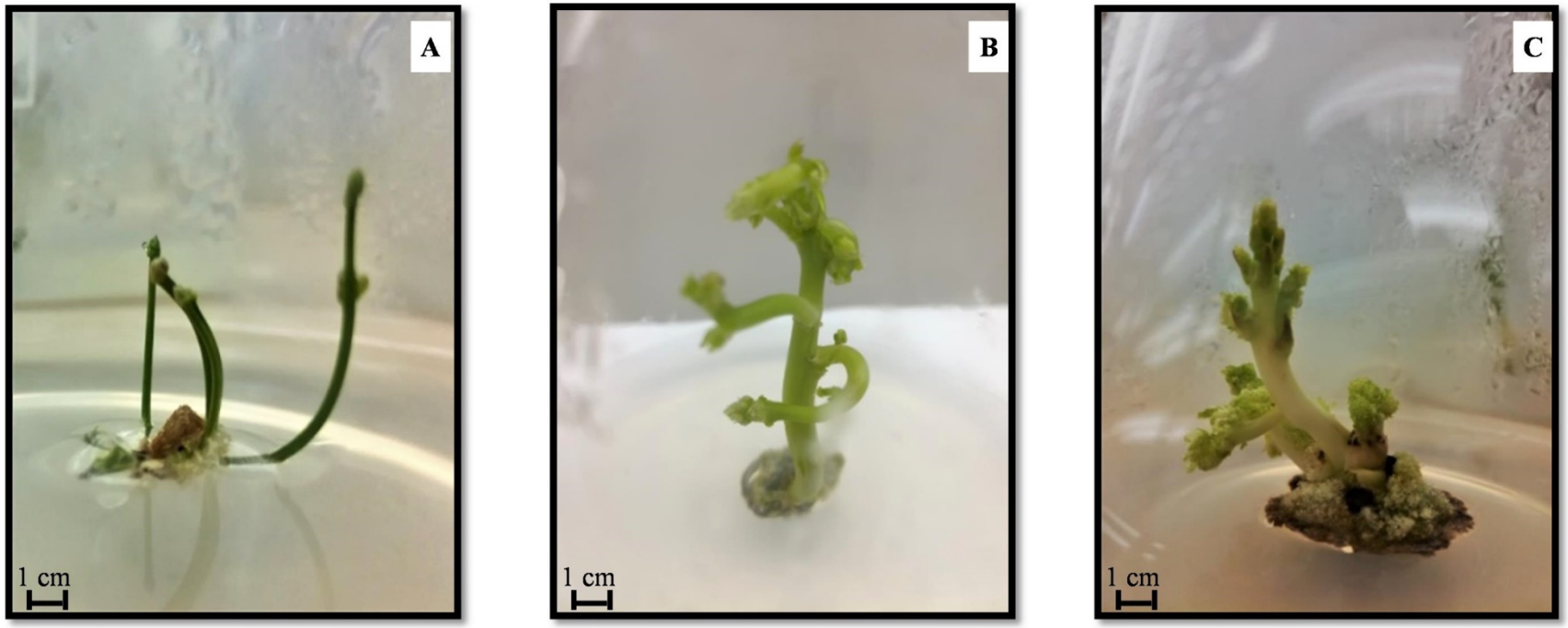


Plate 4.3 Effects of different explant types on number of nodes per shoot after six weeks in shoot induction of asparagus; (A) apical buds, (B) lateral buds, and (C) spear segments

4.1.4 Number of shoots per explant

The effects of different explants on number of shoots per explant were described (Table 4.2). There was highly significant effect of explant types on number of shoots per explant. The maximum number (2.71 per explant) was obtained from apical bud explants. The minimum number of shoots (1.34 per explant) was obtained from spear segment explants.

In this experiment, the apical bud explants produced more number of shoots per explants than lateral bud and spear segment explants (Plate 4.1). It might be due to the cell division of asparagus spear. In this experiment, apical buds used as explant were located in the portion of spear tip (0-15 mm), which is the apical meristem and sub apical meristem region. In asparagus, cell division occurs in the apical meristem of spear tip according to Culpupper and Moon (1939). And then, the apical meristem is responsible for most of the organogenesis phenomena normally associated with shoot morphogenesis (Sachs 1965, Brown and Sommer 1992).

4.1.5 Shoot length (cm)

The effects of different explants on shoot length were also described in table 4.2. There was highly significant in the effect of different explants on shoot length. The longest shoot length (2.7 cm) was recorded from lateral bud explants which was significantly higher than apical bud (1.8 cm) and spear segment (1.3 cm) explants.

Among three different explants, the shoot length from lateral bud explants was observed to be longer than that from apical bud and spear segment explants in shoot induction culture (Plate 4.2). It may be due to the cell elongation zone in the asparagus spear. In this experiment, lateral buds used as explant were located in the lower portion of apical and sub apical meristem region, which is belong to the elongation zone. In asparagus spear, the cell elongation zone appears to be immediately below the spear tip and is about 20 mm long (Kojima et al. 1993).

4.1.6 Number of nodes per shoot

Effect of different explants on number of nodes per shoot was also shown in table 4.2. The results showed that the effects of different explants on number of nodes per shoot were highly significant.

In this experiment, the maximum number of nodes (2.4 per shoot) was obtained from lateral bud explants followed by number of nodes (2.1 per shoot) from apical bud explants while the minimum number of nodes (1 per shoot) was obtained from spear segment explants.

In this study, it was found that apical bud and lateral bud explants produced more number of nodes per shoot than spear segment explants (Plate 4.3). It may be linked to the cell division and elongation zone of different explants located from spear. In this experiment, spear segment used as explant was older part of spear with separated spear leaf scales, which has little elongation growth. Lill et al. (1990) described that the older cells are gradually swept into the elongation zone below the tip after cell division and, finally, into a zone below the elongation zone comprised of non-elongating, lignified, and vascular tissues. Therefore, spear segment explants were reduced number of nodes per shoot when compared apical bud and lateral bud explants.

4.2 Effects of Different Explant Types and Different Concentrations of 6-benzylaminopurine (BAP) on *In Vitro* Shoot Multiplication of Asparagus

4.2.1 Multiple shoots formation percent

Effects of different explant sources and different concentrations of BAP on shoot multiplication were shown in figure 4.2. Both explant types responded to the different BAP concentrations compared with lack of BAP treatment. At two weeks after culture, many of the explants occurred extending and swelling from the cultured shoot tips (0.5 mm). George (1993) reported that the cytokinins are used to stimulate cell division in the culture medium and induce shoot formation or axillary shoot proliferation.

The maximum shoot formation percent was 92.3 % from shoot tips derived from apical bud culture on BAP free medium. For shoot tips derived from lateral bud culture, maximum shoot formation (82.1 %) was recorded from BAP free medium. Minimum shoot formation percent was recorded on the medium containing 3 mg. L^{-1} BAP in both explant sources. Hussey (1982) also found that some plant species have enough levels of endogenous hormones and do not require a high level of exogenous growth regulators for plant regeneration.

It was observed that shoot tips derived from apical bud cultures have higher shoot formation frequency than shoot tips derived from lateral bud cultures. It may be to the nature of the explant source and types of tissue. Brown and Sommer (1992) also reported that apical meristem is normally associated with shoot morphogenesis.

4.2.2 Number of shoots per explant

The effect of different explant types and concentrations of BAP on number of shoots per explant were described in table 4.3. The number of shoots (6.3 per explant)

was more produced by shoot tips derived from lateral bud cultures than those derived from apical bud cultures (5.5 per explant). Among two different explant sources, shoot tips derived from lateral bud cultures were produced number of shoots per explant than shoot tips derived from apical bud cultures (Plate 4.4). However, there was no significantly difference on number of shoots per explant.

There was significantly difference effect of different concentrations of BAP on the number of shoots per explant (Table 4.3). The maximum number of shoots (8.92 per explant) was obtained from 2 mg. L^{-1} BAP containing media. The minimum number of shoots per explant was 4.18 from 4 mg. L^{-1} BAP containing media, and followed by number of shoots (4.4 per explant) from 3 mg. L^{-1} BAP containing media.

In this experiment, the number of shoots per explant was found to increase when BAP concentration was increased to 2 mg. L^{-1} and the number of shoots per explant decreased when BAP concentrations became more than 2 mg. L^{-1} BAP (Table 4.3). Kamile et al. (2014) also found that MS medium with 2 mg. L^{-1} BAP and 0.5 mg. L^{-1} NAA was the best propagation medium for *in vitro* multiplication in *Asparagus stipularis* Forssk. The finding in this study agreed with the report of Strosse et al. (2008) that there was a decrease in survival percent with increasing plant growth regulators concentrations.

4.2.3 Shoot length (cm)

The effect of different explant sources and concentrations of BAP on shoot length was described (Table 4.3). Among two different explant sources, shoot tips derived from apical bud cultures were longer shoot length than shoot tips derived from lateral bud cultures. However, there was no significantly difference on shoot length.

There was significantly difference effect of different concentrations of BAP on shoot length (Table 4.3). The longest shoot length (2.1 cm) was obtained from 2 mg. L^{-1} BAP containing media. The shortest shoot length (1.24 cm) was recorded from 4 mg. L^{-1} BAP containing media.

In this experiment, shoot length was found to be longer when BAP concentration increased to 2 mg. L^{-1} and shoot length became shorter when BAP concentrations reached more than 2 mg. L^{-1} BAP. It might be due to the higher concentrations of BAP also resulted that the hyperhydricity was the adverse effects on shoot growth. These results confirmed that plant species have some endogenous hormones which generally do not require any exogenous growth regulators for regeneration (Paek and Hahn 2000 and Armstrong and Razdan 2001).

4.2.4 Number of nodes per shoot

Effect of different explant sources on number of nodes per shoot was also shown (Table 4.3). In this experiment, the maximum number of nodes (1.8 per shoot) was obtained from shoot tips derived from apical bud cultures while the minimum number of nodes (1.6 per shoot) was obtained from shoot tips derived from lateral bud cultures. However, they were not statistically significant each other.

There was highly significant effect of different concentrations of BAP on number of nodes per shoot (Table 4.3). The maximum number of nodes (2.2 per shoot) was obtained from 2 mg. L^{-1} BAP containing media. The minimum number of nodes per shoot was 1.47 from 4 mg. L^{-1} BAP containing media, and followed by number of nodes (1.49 per shoot) was obtained from 3 mg. L^{-1} BAP containing media.

In this study, it was found that 2 mg. L^{-1} containing media produced more number of nodes per shoot than other treatments. This finding agrees with that of Kamile et al. (2014). They reported that in *Asparagus stipularis* Forssk, MS medium with 2 mg. L^{-1} BAP and 0.5 mg. L^{-1} NAA was the best propagation medium for *in vitro* multiplication. This finding also confirmed to the report of Hussey (1982) that some plant species have enough levels of endogenous hormones and do not require a high level of exogenous regulators for plant regeneration.

Table 4.3 Effects of different explant types and BAP concentrations on number of shoots per explant, shoot length and number of nodes per shoot in shoot multiplication of asparagus

Treatments	Number of shoots per explant	Shoot length (cm)	Number of nodes per shoot
Explant types			
Shoot tips derived from apical bud culture	**5.52 a**	**1.69 a**	**1.79 a**
Shoot tips derived from lateral bud culture	**6.30 a**	**1.60 a**	**1.63 a**
LSD (0.05)	2.47	0.43	0.26
PGRs level			
0 mg.L^{-1} BAP	**6.13 ab**	**1.90 ab**	1.70 b
2 mg.L^{-1} BAP	**8.92 a**	**2.07 a**	**2.18 a**
3 mg.L^{-1} BAP	4.40 b	1.38 bc	1.49 b
4 mg.L^{-1} BAP	4.18 b	1.24 c	1.47 b
LSD (0.05)	3.49	0.61	0.37
Pr>F			
Explant type	ns	ns	ns
PGRs	*	*	**
Explant type × PGRs	ns	ns	ns
CV%	47.72	30.11	17.28

Means within a column followed by the same letter are not significantly difference at 5 % LSD.

***** Significant at 5 % level, ****** Significant at 1 % level

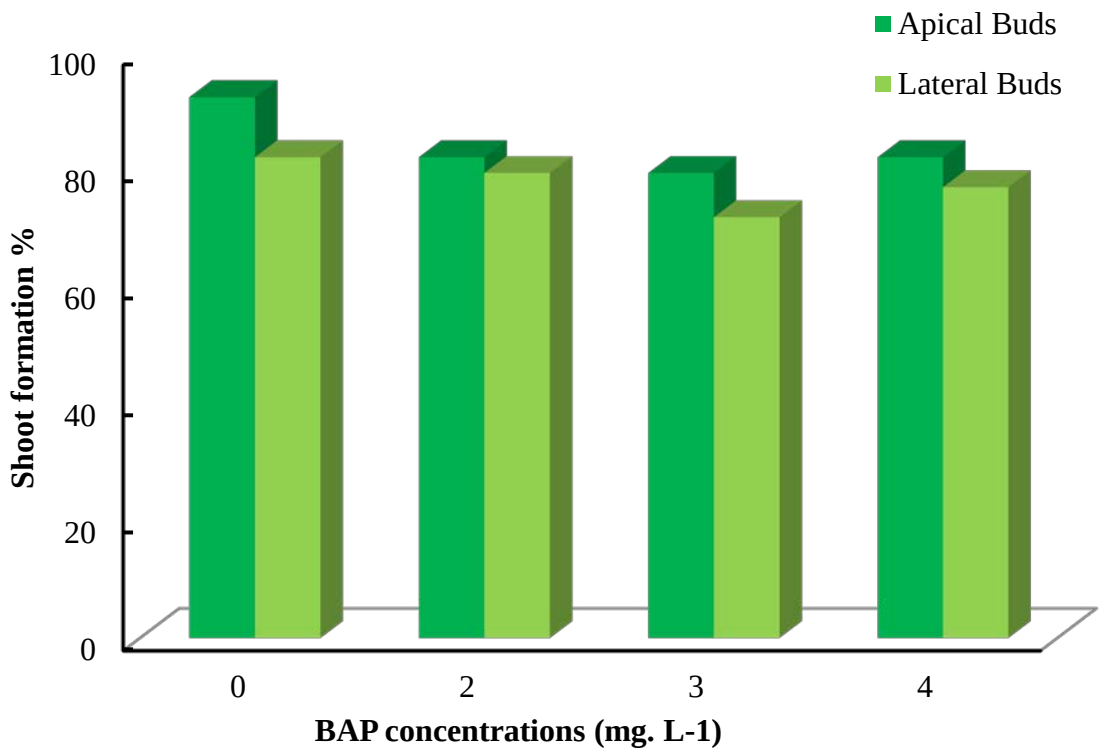


Figure 4.2 Effects of different explant types and plant growth regulators on shoot formation % in shoot multiplication of asparagus

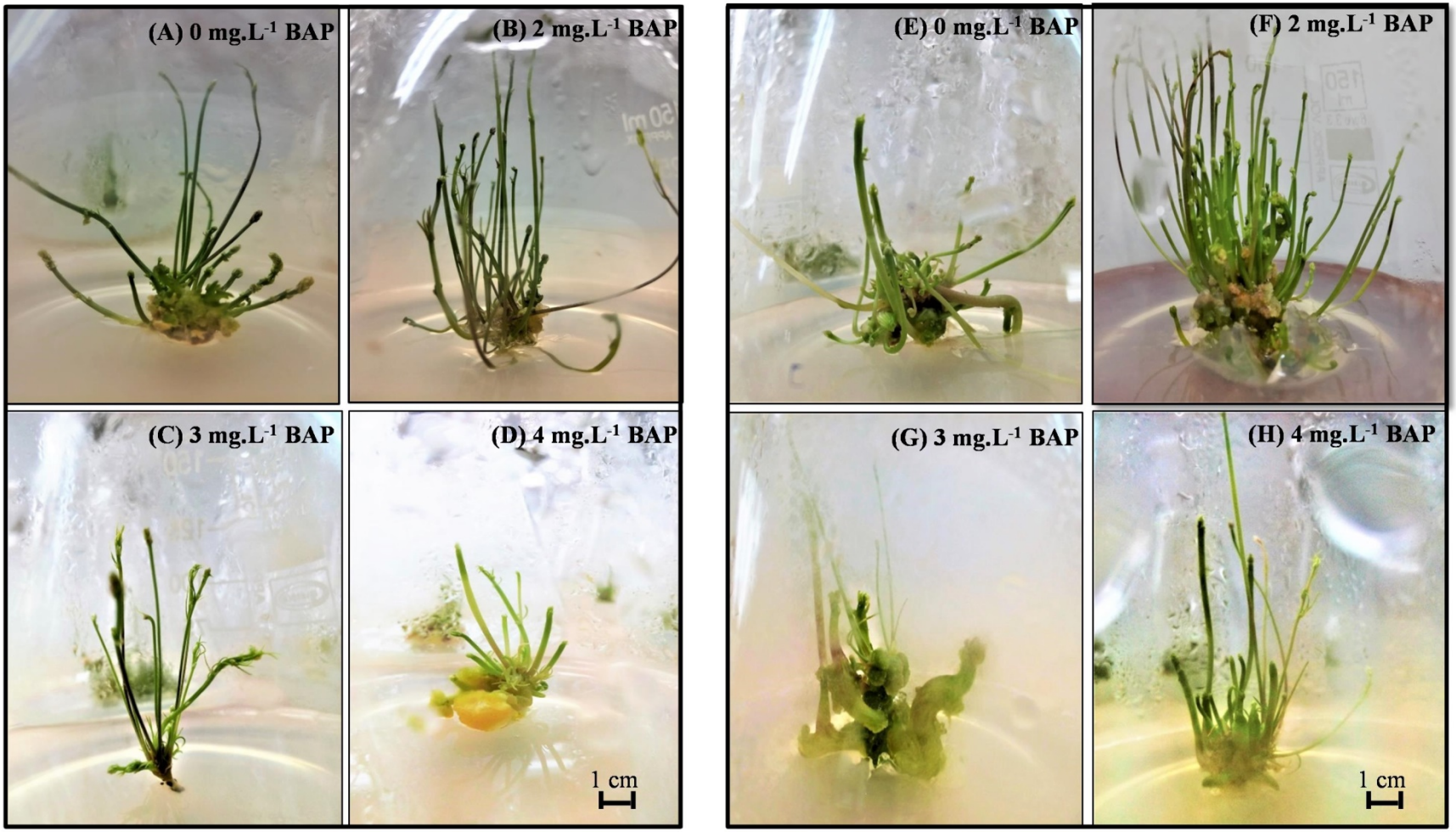


Plate 4.4 Shoot multiplication of shoot tips derived from apical buds (A, B, C and D) and lateral buds (E, F, G and H) on media containing 0.5 mg. L^{-1} NAA with 0, 2, 3 and 4 mg.L^{-1} BAP after six weeks culture

4.3 Effects of Shoot Density and Different Concentrations of indole-3-butyric acid (IBA) on *In Vitro* Root Induction of Asparagus

4.3.1 Root formation percent

Effects of shoot density and different concentrations of IBA were shown in figure 4.3. Single shoots and Cluster shoots were transferred onto MS basal medium supplemented with two different levels of IBA. George (1993) also stated that auxins are generally used to initiate shoots, particularly roots from shoot apices and shoot tip cultures.

The maximum root induction percent (40 %) was found in cluster shoots on the medium containing 2.5 mg. L^{-1} IBA. Minimum root induction (4.44 %) was recorded in single shoots on medium containing 1.5 mg. L^{-1} IBA. It was observed that single shoots have lower root induction frequency than the cluster shoots on two both media (Plate 4.5). It might be due to the nature of the explant type. Shen et al. (1995) found that when both cluster shoots (with 2–5 shoots) and a single shoot were cultured on 1/2 MS medium with 0.1 mg. L^{-1} NAA, a higher rooting percentage (78.9%) was observed from cluster shoots where 25.7 % was recorded in shoot culture. Benmoussa et al. (1997) and Mehta and Subramanian (2005) reported that roots were induced in *Asparagus* species by using media containing IBA. Negi et al. (2010) and Stajner (2013) reported that *in vitro* root induction of asparagus micro cuttings was difficult.

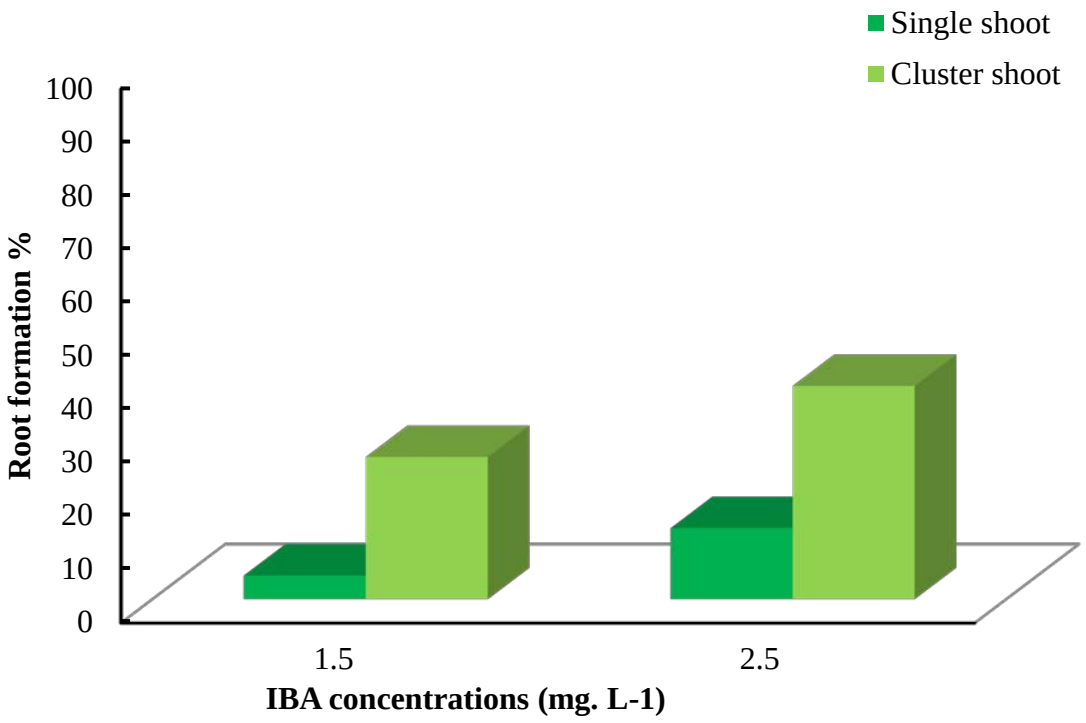


Figure 4.3 Effects of shoot density and IBA concentrations on root formation % in root induction of asparagus

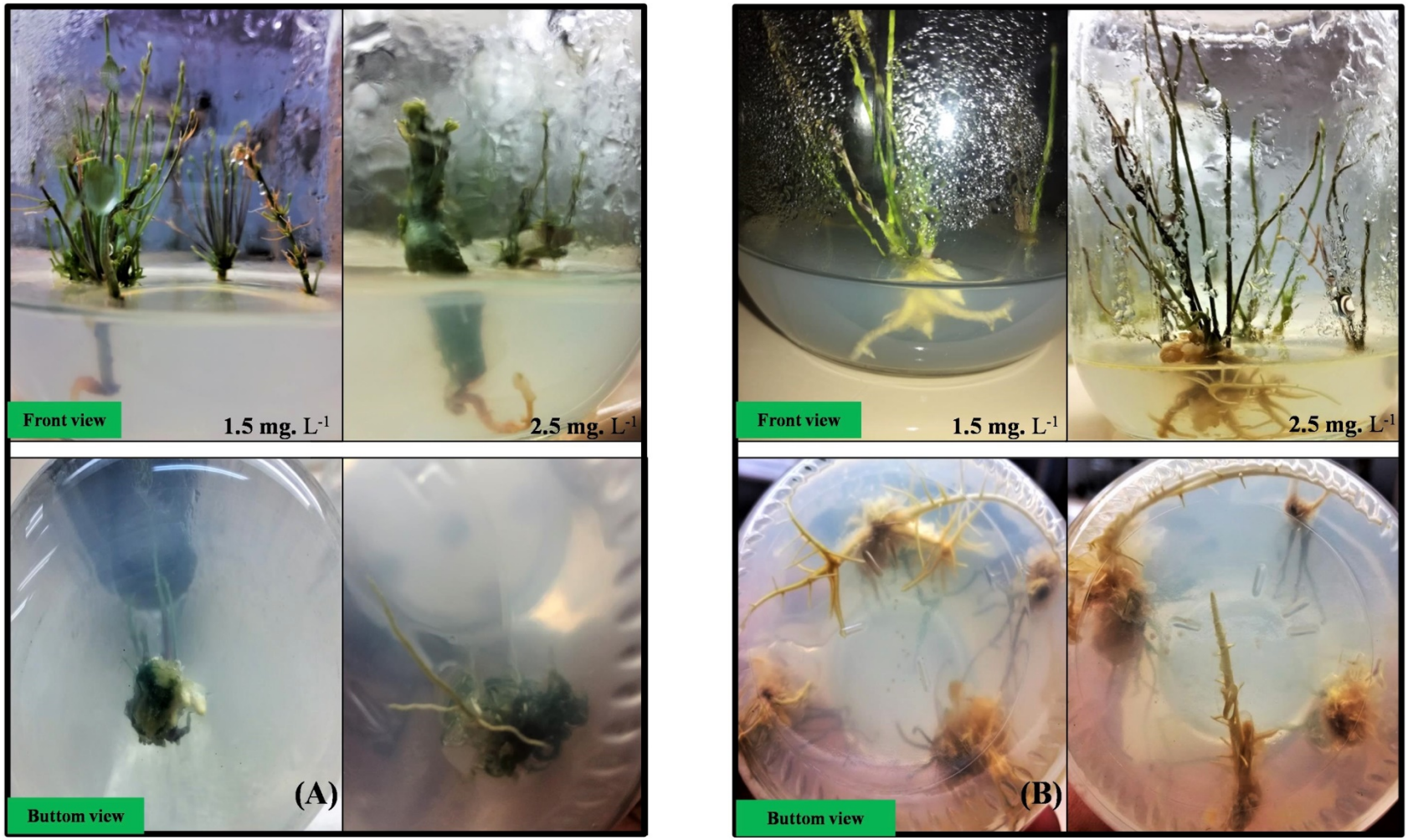


Plate 4.5 Root formation of (A) single shoot and (B) cluster shoot after eight weeks culture onto root induction media (1.5 mg. L^{-1} , 2.5 mg.L^{-1}) IBA

CHAPTER V
CONCLUSION

Based on the findings in this study, in shoot induction stage, results mentioned above revealed apical buds and lateral buds of asparagus spear have the potential to produce plantlets through *in vitro* cloning and explant type influenced on regeneration capability.

In shoot multiplication, BAP concentration plays a key role and it was noticeable that 2 mg. L^{-1} BAP was the best one. Since this medium give better results in number of shoots per explant, shoot length and number of nodes per shoot. Higher BAP concentrations tend to be abnormal shoots.

In root induction stage, not only IBA concentrations but also shoot density was pointed out as governing factors in this difficult to root species. Suitable concentration of IBA was needed to induce roots. Cluster shoots should be used for root induction culture.

To our knowledge, this was the first report for the protocol *in vitro* culture of edible green asparagus in Myanmar (Plate 4.6). Direct organogenesis was applied for the first time to obtain preliminary information on propagation of this plant. Further research was required to promote the efficiency of multiplication by using a wide range of concentrations and combinations in plant growth regulators, different genotypes of asparagus species, *in vitro* regeneration for male and female plants and the concentrations of media components.

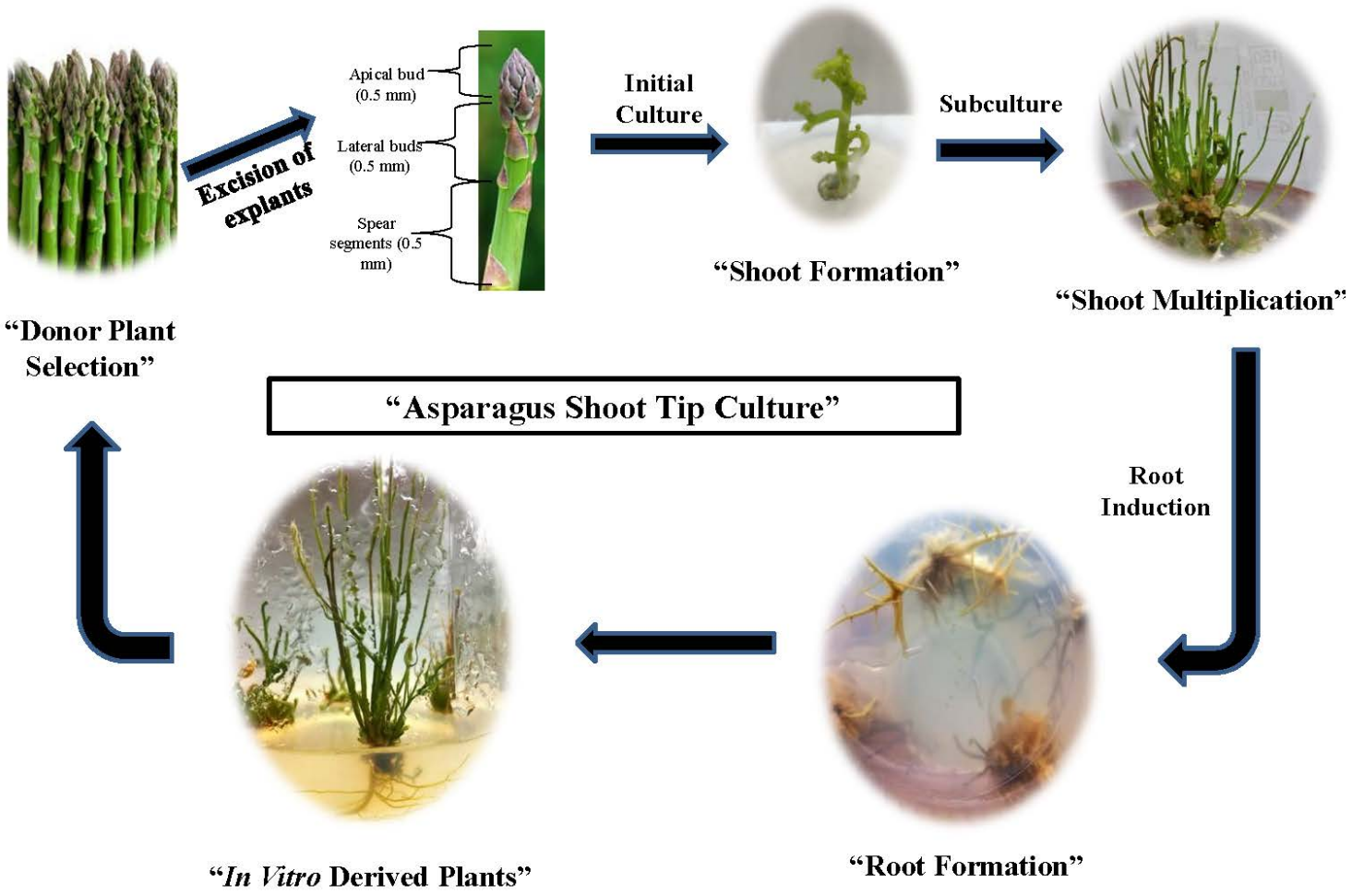


Plate 4.6 Stages of *In Vitro* Clonal Propagation established for Asparagus (*Asparagus officinallis* L.) by Shoot Culture

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APPENDIX

Appendix 1 Composition of Murashige and Skoog (1962) medium and preparation of its stock solution

Stock	Components	Amount ($mg.L^{-1}$)	Stock solution ($mg.L^{-1}$)	Stock solution concentration
Major	NH_4NO_3	1650	33000	20 X
	KNO_3	1900	38000	
	$CaCl_2.2H_2O$	440	8800	
	$MgSo_4.7H_2O$	370	7400	
	KH_2PO_4	170	3400	
Minor	KI	0.83	166	200 X
	H_3BO_3	6.2	1240	
	$MnSO_4.4H_2O$	22.3	4460	
	$ZnSO_4.7H_2O$	8.6	1720	
	$Na_2MoO_4.2H_2O$	0.25	50	
	$CuSO_4.5H_2O$	0.025	5	
	$CoCl_2.6H_2O$	0.025	5	
Iron	$FeSO_4.7H_2O$	27.8	5560	200 X
	$Na_2EDTA.2H_2O$	37.3	7460	
Organic	Myo-Inositol	100	20000	200 X
	Nicotinic Acid	0.5	100	
	Pyridoxine HCl	0.5	100	
	Thiamine HCl	0.1	20	
	Glycine	2	400	