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IN VITRO PROPAGATION OF *PTEROLOBIUM STELLATUM* FROM SHOOT TIP EXPLANTS

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By

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LIST OF ABBREVIATION

BAP	6-Benzyl amino purine
IBA	Indol-3- Butryric acid
ILDIS	International Legume Data Base and Information Service
FTEA	Flora of Tropical East Africa
NAA	α- Naphthalene Acetic acid
MS	Murashige and Skoog
LSD-	Least Significance Difference
SPSS	Statistical Package for Social Science
ANOVA	Analysis of variance

ABSTRACT

Pterolobium stellatum i*s ever green perennial dicotyledonous woody shrubs commonly known as redwing. It belongs to Fabaceae family and distributed in tropical and subtropical climates of South Africa to Eastern Africa. It is used as traditional medicine against various diseases in some Africa countries and as source of tannins for leather industries to produce ink. This plant is highly destructed or action of destroying the plants locally and has prolonged seed dormancy and low seed germination rate. Therefore, the objective of this study is to develop micro propagation protocol for* Pterolobium stellatum *from shoot tip explants. The seeds of* P. stellatum *were de-coated and surface sterilized with 70% ethanol for 5 minutes followed by 30% ,15% and 5% Clorox for 25, 15 and 5 minutes respectively. The de-coated and sterilized seeds were cultured on Murashige and Skoog medium containing different concentrations of 6-Benzylaminopurine (0.0, 0.5, 1.0.1.5, 2.0, 2.5, 3.0 and 3.5 mg/l), Kinetin (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) alone and combination of 6-Benzyl aminopurine (0.5- 3.0 mg/l) with α- Naphthalene Acetic acid (0.1, 0.25, 0.5 mg/l) on MS medium for shoot tip initiation and multiplication. Rooting was achieved by using half strength MS medium containing different concentrations of Indol-3-Butryric acid (0.0, 0.5, 1.5, 1.0, 2.0 mg/l) and α- Naphthalene Acetic acid (0.5, 1.0, 1.5, 2.0 mg/l). The highest percentage of shoot initiation (86.7%) and maximum numbers of shoots per explants (3.76±0.32) were obtained on MS medium fortified with 2.0 mg/l BAP alone. The highest shoot length was obtained on MS medium containing 1.5 mg/l KN combined with 0.1 mg/l NAA (4.45±.20 cm). The highest mean numbers of roots per explants was (1.93±0.21) and mean root length (1.73±0.17 cm) was obtained on MS medium containing 1.0 mg/l NAA alone. Among acclimatized plantlets, 43% survived in greenhouse. This protocol can be used as a starting protocol for further optimization for production of large number of planting materials.*

Key words: Micro propagation, shoot multiplication, rooting, shoots tips, *Pterolobium stellatum.*

1. INTRODUCTION

Pterolobium stellatum (Forssk) Brennan is a perennial tall scan dent woody medicinal shrubs belonging to Fabaceae family**.** It is semi erect dicotyledonous flowering plants with long size shrub reaching 2-15 m long, sometimes 20 m high. It is multipurpose plant commonly known as red wing (Mutshinyalo, 2009; Fern, 2014) and in Ethiopia called Kenitaffa (Amharic). The shrubs have very sharp, curved spines and large plants develop long coiled stems bearing thorny, woody swelling armed with recurved prickles plants. The stem of *P. stellatum* are rope like bearing prominent thorny projections, densely covered with hairs or puberulous when young on their leaves and stems with down wardly 6 mm long thorns or conical knobs paired at node and also scattered along internodes or surface (Schmidt *et al.,* 2002).

The genus *Pterolobium* comprises 11 species with distinctive geographical ranges. Among species of the genus ten of them naturally occur in Asia like Philippines, Indonesia, Malaysia and peninsular Arabia. *P. stellatum* is the only native species and distributed throughout tropical and sub-tropical region of Africa, where it has extensive but easterly range from South to Eastern Africa like Sudan, Ethiopia, Kenya, Eritrea, Zambia, Tanzania and Yemen (Jonson and Cardon 2005; Thulin ,1989). The shrub found along forest margins and dry forest, bush land riverine; deciduous wood land sometimes they grow on termite mounds (Demel Teketay, 1997; Edeline *et al.,* 2016). *P. stellatum* is multipurpose plant that usually grows best on acidic red loam soil areas at altitude between 750 to 2500 m above sea level (Mutshinyalo, 2009).

Pterolobium stellatum contains different chemicals that are used to treat against different diseases in Ethiopia and other Eastern and South Eastern Africa traditionally using fresh leaves, fruits and roots. It is used to treat tuberculosis, pneumonia and other related respiratory diseases, to treat diarrhea (Amare Getahun, 1979), epilepsy and neuroglia. In Malawi, the plant is used to treat infertility, persistent cough (asthma), and spleenomegally (Kigen *et al.,* 2016), to avoid the vomit by eating the fruits (Abiyu Enyew *et al*., 2014). In Kenya, it is used to treat sexually transmitted disease (Njoroge and Bussmann, 2007).

The root of *P. stellatum* is used to remove retained placenta during deliver, to avoid tumor, treat snake bite and used to treat stomach-ache, headache, and rheumatic pain locally called kurtumati (Amharic).

Generally the plant produces different secondary metabolites such as tannins, terpenoids, saponins, flavonoids and phenolics. These compounds have been shown to possess cytotoxic and anti-diarrhea properties of the plants (Gizachew Alemu *et al.*, 2014).

In Ethiopia, the leaves and bark of red wing is a good renewable natural source for ink production as it contains 20% tannins and yields a dark red and black dye when crushed, which has been used in textiles, basketry and as ingredients used for leather ground (Jonson and Cardon, 2005). The red wing plant is also used for animal fodder, live fence, fire wood, charcoal, ornamental value, shade and shelter for wild animals and soil conservation for healthy environment (Getu Alemayehu *et al*., 2015; Haileab Zegeye *et al.*, 2005).

P. stellatum sets flower in autumn that produce fruit. The plants flower sweetly scents that the honey bees and butterflies collect pollen and nectar from flowers very frequently. It is an important for bees that produce a surplus of honey. Ecologically shrubs are highly favored by wild life for refuge because of the canopy (Mutshinyalo, 2009). The young fruits are brick red and attractive when observed from distance, contains 6-8 seeds. The plants can be propagated by seed. The seeds are dispersed from mother plant and display prolonged dormancy due to hard seed coat (Demel Teketay, 1998).

Despite its great importance, red wing has attracted low research attention like other indigenous leguminous woody species in Ethiopia. Farmers cut down the plants to create open fields to reduce wild animals from the canopy like pig, monkey ape and hedgehogs before extracting the medicine from their roots, leaves, and barks and of the plants organ (Tola Gemechu *et al.*, 2014). The seeds covered by hard seed coat that require to break dormancy in order to enhance the rate of germination by using hot water, concentrated sulfuric acids, and mechanically remove the coats or scarification (Demel Teketay, 1998). Propagation through seeds is time consuming due to nature of hard seed coat that is dormant to accomplish large scale production for conservation and cultivation of the species. Therefore, it is important to develop effective propagation techniques for rapid in vitro multiplication using the shoot tip explants of *P. stellatum.*

This promotes scientific activities including pharmaceutical studies, extraction of important medicinal compounds and industrial renewable natural raw material of tannin using different organ of the plants.

In vitro propagation method gives option for initiation, multiplication and conservation of the plant. No reports of in vitro propagation of *P. stellatum.* Therefore, this study was designed to develop *in vitro* propagation protocol for *P. stellatum* from shoot tip explants.

2. LITERATURE REVIEW

2.1. Taxonomy

Pterolobium stellatum (Forssk) Brenan belongs to Fabaceae family that forms canopy evergreen perennial dicotyledonous and semi erect multi stemmed climbing woody shrubs. It has high branches armed with prickles paired at node and often also scattered on internodes (Janson and Cardon, 2005).The chromosome number of *P. stellatum* is *(*2n=24) (Spellenberg and Ward, 1988).

The young stem of *Pterolobium stellatum* is covered with hairs and scrambling or climbing shrubs armed branches with recurved prickles about 6 mm long, pairs near nodes otherwise scattered along surface. Leaves of *P. stellatum* are alternate and bipinnately compound consists of 5-13 paired primary leaflets (pinnae) and 7-16 paired leaflets per pinna. The leaflets in large (6-10x2-4mm), the leaflets tip rounded, the lower surface hairy or smooth, the latter armed with mostly paired reflexes prickles straight ascending one also being often present on the upper side of rachis at the insertion of pinnae (Janson and Cardon, 2005 ; Thulin , 1989).

Flowers are small and sweetly scented with a pale yellowish white color except for the pale green calyx .The flower is bisexual slightly zygomorphic with five small petals are creamy white and are borne on several terminal spikes 5-13 cm long that are seen in clusters (FTEA, 1971). The creamy colored inflorescence composed of dense compounds racemes (panicle) that is 50-80 mm long. The petals have almost equal oblanceolate oblong 3mm long and 1.5mm wide, pubescent towards base. It contains irregular calyx with shallow basin shape tube, slightly sometimes unequal. Stamen-filament 4-5mm long, it contains 10 stamens sometimes unequal. Petal longer than sepal usually villous at base anther ± quadrates concave terminal stigma. Ovary is free shortly stipulate and pubescent-1-ovuled, style short or elongated and clavate with truncate (Gillett *et al.,* 1971). The fruits of this plant are bright red winged pods, ultimately brown 30-60mm long with tiny pubescent and pod winged up to 1.7mm.

The pods are flat and papery with bean located at the attachments end and result is a winged samara that has a pair of thorns below the rachis of their bipinnate leaves. The pod with stalked -1-seeded basal portion and much prolonged upper surface. Its bright pink spray resembles flower when seen from distance (Bate-smith, 1973).

The seeds are covered with hard coats and with very small helium that hampers inhibition of water prevents maximum uniform and rapid germination. Ellipsoid seed shape and brown smooth seed surface is an ovoid–ellipsoid 11x6.5mm, height of the seed is 8.10-9.55 mm. The seeds are containing basal part up to 2x1cm, pubescent at maturity. Due to hard seed coat, it requires severe treatments to enhance the germination of seed by breaking dormancy (Demel Teketay, 1997; Demel Teketay, 1998). Mechanically removing the coat or scarification is used for rapid and higher percentage of germination than other treatments. The seeds are sensitive to high temperature so hot water treatments cause death of the embryo of the seeds then not recommended for seed germination of *P. stellatum in vitro*. The seed is viable for more than a year if stored properly and placed in air tight containers, cool, dry, areas and insect free place without loss of viability (Demel Teketay, 1997; Bein *et al.,* 1996).

2.2. Growth and Development of *Pterolobium stellatum*

Pterolobium stellatum (Forssk), Brennan is perennial plant native to Africa and Yemen. It is grown throughout all season at altitude between 750-2500 m a.s.l. The plants well grow in acidic red loam soil, in high light intensity and germinate naturally at average temperature between 23-25° C in 3-6 weeks .The growth type of roots is tap root system. In Ethiopia, the flowering time is May to October (Hyde *et al*., 2008; Demel Teketay, 1998).

Red wing disperses fruits from parent plants to other place by animals and water. The transfer of pollen grain from anther to stigma is through biotic and abiotic factor. The plant develops fruits from April to November based on period of flowering time and seeds of red wing are covered by very hard seed coat; smooth seed surface and brown seed color. The plant has a hard and thick seed coat that hampers imbibitions of water and prevents maximum uniform, rapid germination and difficulty of raising seedling from seeds (Demel Teketay, 1997; Thulin, 1989; Jonson and Cardon, 2005; Gillett *et al.,* 1971).

2.3. Geographical distribution of *P. stellatum*

Pterolobium stellatum (Forssk) Brennan is a perennial shrub that grows in different African regions. The habitat is predominantly terrestrial at lower elevation of tropical and subtropical rain forest near to the equator, where warmth of sun and length of day light are constant (Janson and Cardon, 2005).

The genus *Pterolobium* belongs to the tribe Caesalpinieae and comprises about 11 species. The only single member of the genus, *P. stellatum* is naturally native to Africa where it has an extensive range from South to Eastern Africa including Sudan, Ethiopia, Eritrea and Yemen (Janson and Cardon, 2005; Demel Teketay, 1998). It is also widespread in Zimbabwe, Zambia, Tanzania, Mozambique, Malawi, Kenya, Rwanda, and South Africa (ILDIS, 2007; FTEA, 1971). The other species of the genus are occurring in Asia including Indonesia, Philippines and Malaysia Philippines (Bate-Smith, 1973). It is also characterized by gentle slope closely associated with stream, forest margin riparian vegetation, re growth near to waterfall or riparian flood plains (Edeline *et al*., 2016). In Ethiopia, it grows in all regions at boundaries of field of farmer and sometimes grows on termite mound (Roux, 2003; Hebson *et al.*, 2017).

2.4. Importance of *Pterolobium stellatum*

2.4.1. Medicinal value

P. stellatum is very important medicinal plant for different infections in some places in Africa. Some purposes of the plants are**:**

The fresh leaves and roots are chewed for medicinal purpose to treat tuberculosis, pneumonia, common cold and other related respiratory diseases (Amare Getahun, 1976; Hebson *et al.*; 2017).

According to Ethno botanical surveys of Endale Amenu (2007) reported the leaves of plant are crushed and mixed with butter and applied to the paste around swollen neck to cover it and used to treat goiter, powdered root cream applied and used to treat external haemoriod. The whole plant juice is given orally for one month to treat epilepsy and neuralgia (Ragunathan and Solomon Mequenente, 2009).

Root is dried and the powder is mixed with water, three spoons of the powdered mixture is given for three days to treat intestinal parasite like tape worm. Plant organ contains terpenoids, saponins, and flavonoids. Then the presence of these compounds have been shown to possess cytotoxic and anti-diarrheal properties and used to treat amoebic dysentery and vomiting (Abiyu Enyew *et al.,* 2014; Gizachew Andualem *et al.,* 2014).

In northern region of Ethiopia around Tigray(Kunema ethnic group), the pounding root mix with water wait for two days and drink a cup of concoction that used to remove retained placenta during delivery (Meaza Gidey *et al*., 2015). In Kenya, the roots are boiled with water and used to treat sexually transmitted diseases (Njoroge and Bushmann, 2007). The roots are used to treat rheumatic pain of legs and fruits are used to treat sore and mixed with butter repairing the damaged parts and dislocated bone of livestock (Abraha Tekelay, 2015).

Wubayehu Kahaliw *et al*., (2017) reported genotoxicity assay of *P. stellatum* caused damage of DNA at low concentration. Necessary precautions should be taken during utilizations of the plants. Therefore, the chloroform and methanol extracts from the roots of *P.stellatum* was the most effective plant that is used as adjuvant therapy and high activity against anti-mycobacterium activities like *Sallemonella typhi, S. paratyphi, S. aureus and E. coli* that inhibit their growth due to the presence of terpenoids, saponins and flavonoids (Mulegeta Kuma, 2014; Endale Balcha *et al.,* 2014; Hesbon *et al.,* 2017).

2.4.2. Industrial application of *P. stellatum*

In Ethiopia, infusion of the pounded bark of *P. stellatum* has been major ingredient of tanning Morocco leather at the same time provides a bright red color. The leaves contain 20 % tannins used for producing a dark red dye for leather ground and boiled in water with little oil or butter to help fix the color. It is used to dye clothes for mourning and does not need mordanting process due to having tannins which has been used in textiles, basketry and as ingredients in black ink production. The main gradients for making black ink based on a chemical reaction of tannins with iron slag or iron oxide in water. This leather dyed with mixture of dried leaves and iron a filling is water resistant (Janson, 2018; Demel Teketay; 1997; Janson and Cardon, 2005).

2.4.3 Other uses

Pterolobium stellatum is very important for bees and butter flies due to its attractive flowers. The shrubs are covered with butterflies and bees during the flowering season mostly in autumn. In Ethiopia, the honey bees collect pollen and nectar from flowers very frequently and produce high yields of honey (Tedrowose Alemu *et al.,* 2013). It is also used as animal fodder (Amare Getahun, 1976). Ecologically shrubs are used to keep birds in a region for roosting due to ever green leaves (Hailemariam Araya *et al.,* 2013). People grow it densely at boundary of fields interconnected with wood for live fences due to impenetrable and erecting thorn nature that is used for shielding the crops or young seedlings from domestic and wild animals like cattle, pigs' hedgehogs (Tola Gemechu *et al.,* 2014; Demel Teketay, 1997). Red wing grows fast after germination and provides environmental protection against soil erosion and contributes to soil conservation. In low land part of Ethiopia, it is used as building material (Haileab Zegeye *et al*., 2005).

2.5. Vernacular Names of *Pterolobium stellatum*

Pterolobium stellatum species has become infamous for the vicious character of its thorns. It was first described from Africa by Bruce Travels (1790) under Abyssinian vernacular name kantuffa. The thorns in society has got itself in degree of reputation and respect from noxious qualities and power of doing ill which passes constant exertion of the power and soldiers tested by plant thorns (Janson, 2018).

Pterolobium is derived from two Greek words "*Pteros"* and "*lobous*."*Ptero means* wing, *lobous* means pod or capsule then collectively '*Pterolobium'* alluding to winged fruit whereas '*stellatum'* it is Latin word meaning starry or star like possibly alluding to flower, the radial arrangements of inflorescence (Bate- Smith, 1973; Schmidt *et al*., 2002).

In different countries *Pterolobium stellatum* was named by different language. In Ethiopia Kenitaffa or kontir(Amharic), Kentetefe or kwentefetefe (Tigregna) , Harengema ,cheka kamale or Gora (Afaan Oromo), Qajimaa (Borena- Afaan Oromo), Kuku (Kunema), Qudu(Berta-Gumuz), Gomoryya (Welayita),Libah-halalalis(Somaligna), Snduko(Gamo).

and other African countries Shona (Batu-Zimbabwe),Tshivenda (Gwambazi);Chishona(Gado) ,Mutanda (Kiswahili) (Janson, 2018; Amare Getahun,1997).

2.6. Plant Tissue culture and its applications

Tissue culture is the *in vitro* aseptic cultures of cells, tissue organs or whole plant under controlled nutritional and environmental conditions often produce the clones of plants (Thorpe, 2007).It is a method based on vegetative propagation of plant material on defined media (Oggema *et al.,* 2007). The plant cells or tissue growth and multiplication may be in liquid or solid medium under controlled environment. Tissue culture is protocols used to preservation of plants tissue when the targets conservation are clones instead of seeds to keep the genetic background of a plant to avoid the loss of conserved patrimony due to natural disasters whether biotic or abiotic stress (Filho *et al.,* 2005; Tyagi *et al.,* 2007).

Plant tissue culture is a science of growing plant cells, tissue or organ by selecting from stock plants and cultured on artificially prepared medium that states a cell is capable of autonomy and potentially totipotent (George, 1993**)**.This concept is enhanced plant growth by presence of hormone in culture media that ability to plant cells, tissue and organ to develop into fertile whole new plants (Fowler *et al.,* 1993).

Plant tissue culture is also used to produce secondary metabolites which are used as pharmaceuticals, bio pesticides, agrochemicals, colors, flavors and fragrance (Al Snafi, 2016). The technique consists of removing tissue under sterile condition from living organism and incubating them in an environment under favorable culture conditions.

It involves the establishment of different cells, in vitro cell proliferation and subsequent regeneration of plants (Vasil *et al.,* 1982). Therefore, plant tissue culture is carried out through aseptic technique in the absence of invading microorganisms by using sterile instruments and culture media in order to avoid the contamination.

2.6.1. Micro propagation

Micro propagation is the process of vegetative growth and multiplication from pieces of plant tissue or organs through aseptic conditions on growth media, using various plant tissue culture techniques by means of asexual propagation (Zhou and Wu, 2006; Hartman *et al*., 2004, Conger BV,1980 ; George EF, 1996; Herman EB,1995).

Micro propagation protocols are aimed to the rapid multiplication of plantlets of true type to the original material. It is one form of tissue culture which allows the production of large number of plants from small pieces of the mother plant in relatively limited space and short period of time without seasonal interruption. It is carried out in aseptic process which requires sophisticated laboratory procedures and special skills (Hartman *et al.,* 2004).

Micro propagation is means of vegetative reproduction by avoiding whole life cycle of seed development in very limited space areas with very few resources aseptic and sterile technique (Debnth *et al.,* 2006 ; Hussain *et al*., 2001).

The method is mainly used for multiplication, ornamental plants (Conger, 1980 ; Harman, 1991), endangered threatened or highly destructed plants, difficult to propagate plants, for producing chemicals for pharmaceutical and industrial interests without requirement of seed germination (West and Mika, 1957). It has high commercial potential due to the spread of propagation, clonal propagation, germplasm conservation, genetic transformation and its high quality and ability to produce disease free plants (Tileye Feyissa *et al*., 2005; Hartman *et al.,* 2004).The technique is used for rapid production of high quality, disease free and uniform planting material. The high quality of plants can be produced under controlled environment to produce healthy plants. Micro propagation process that produces copy of plants in large numbers through developmental stages usually classified into five stages.

Stage 0: Mother Plant selection-To enhance the probability of success, the mother plant should be *ex vitro* cultivated under optimal conditions to minimize contamination in the *in vitro* culture.
Stage I: Establishment of aseptic culture. In this stage an explants are surface sterilized and transferred into nutrient medium (Husain MK, and Anis M, 2009).
Stage II: Production of suitable propagules. The number of propagules is multiplied by repeated subcultures until the desired

Stage III: Preparation for growth in natural environment. The rooting stage necessary to change media, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth

Stage IV: Transfer to the natural environment. At this stage, the *in vitro* plants are weaned and hardened. Hardening is done gradually from high to low humidity and from low light intensity to high light intensity.

In vitro propagation of plants are advantage over the conventional method because it is possible to produce a millions of clones from single explants in short period, produce high quality and disease free plants, cultures plantlets without seasonal interruptions, multiply sexually derived sterile hybrid plants organs and long term storage of valuable germplasm (Khanna, 2003; Tileye Feyissa *et al.,* 2005; Hartman *et al.,* 2004).

2.7 Media composition and Culture conditions

Plants may require different composition of nutrients for rapid growth of new shoots and roots by adding plant growth hormones. Plant growth nutrients are composed of macronutrients, micronutrients, vitamins, plant growth regulators, carbon source and other organic substance (Bhojwani and Razadan, 1996). The growth supplements used to optimize for its specific type, when the growth supplements are added to the basal medium that forms a growth culture (Pierik, 1997).

Plant growth regulator contains carbon, gelling agent agar and each growth supplements are added to be basal medium, it forms the growth culture (Murashige and Skoog, 1962). Agar is not essential media components but used for gelling agent that prevents cultured cells from death by submerged and lack of oxygen in liquid medium (Rayaneet *et al*.,1993).

Carbohydrates play important role in vitro culture of plants and other organisms as source of carbon, used as energy as well as osmotic agent and to modulate gene expression in plants (Koch,1976). The sugar mostly used in tissue culture is sucrose as source of carbon at concentration of 30 mg/l. The concentration of sugars depends on the type and age of growth material. Juvenile embryo requires relatively high concentration of sugars for growth and development until reached optimum level. In addition to sucrose, glucose and fructose are used for tissue culture of plants (Bhojwani and Razdon, 2004). Vitamins including myoinositol,

thiamine, nicotinic acid, and pyridoxine are ingredients of (Murashige and Skoog, 1962) medium and have been used in varying proportions for the culture of tissues of many plant species.

2.8 Plant growth regulator

Plant growth regulator is defined as organic compound other than nutrients used in small concentration that affects the physiological and morphological process of plants. These numerous chemicals profoundly influence the growth and differentiation of plant cells, tissue, organs (Gasper *et al.,* 1996). The presence of plant growth hormone in media is necessary for shoot and root initiation (Aggarwal and Barna, 2004). Therefore, without adequate concentration of exogenous hormone, shoot and root induction in vitro is not achieved (Sasikumar *et al.,* 2009). Based on their chemical structures and effects, plant hormones are divided in to five main groups of compounds. The group includes cytokinin, auxin, gibberellins, abscisic acid and ethylene.

The hormone growth regulators belonging to cytokinin is immediate effects on undifferentiated cells that stimulation of DNA synthesis, induce adventitious bud formation, increasing and stimulate cell division of plants. It also used in leaf expansion, retard leaf senescence promote chlorophyll synthesis and enhance chloroplast development (Ting, 1982, kuhnle *et al.,* 1977).
The most common cytokinin used *in vitro* growth of cells, tissue and organs includes 6-Benzyl amino purine (BAP), kinetin (KN), zeatin , Gibberellic acid(GA) and Thidiazuron(Pierik., 1994).

BAP-is most active cytokinin than others that used to promote axilliary bud growth, for callus, shoot induction and multiplication of shoots (Wareing and Phillips, 1981).
KN is a plant growth regulator that used to increases the rate of cell division, differentiation and growth. It delays senescence in plant tissues, increases flower set, fruit formation and side branching.
Gibberellic acid(GA)-is often referred to as inhibitory rather than stimulatory hormone. It involved in closure of stomata, bud and seed dormancy and it known to inhibit other hormonal actions. It used to enhance growth of callus and help to elongation of dwarf plantlets (Torres KC.1989).

Thidiazuron (TDZ)-exhibits strong cytokinin like activity promotes the proliferation of axillary shoots, stimulates adventitious organ regeneration and induce somatic embryogenesis (Hutteman C.A & Preece J.E., 1993).

Auxin is a phytohormone that influence on physiological process cause of cell division, root initiation, adventitious bud formation, the inhibition of axilliary shoot formation, and cell enlargements of rooting depend on either endogenous or exogenous hormone (Hartman *et. al.*, 1997). It includes indole -3- acetic acid (IAA), Indole-3-butyric acid (IBA), α-Naphalene acetic acid (NAA) and 2-4-dichlorophenoxy acetic acid (2-4 D) (Pieick, 1997).

IBA is the most commonly plant growth hormone used in tissue culture that tend to denatured in media and rapidly metabolized with in plant tissues contributes formation of rooting in very manure concentration(Gaspar *et al.*, 1994).

IAA is a root hormones that promoter rooting for higher plants and plays formation of adventitious roots that most naturally detected and involved in many of physiological process of plants and NAA is effective growth regulator for rooting(Stefancic *et al.,* 2005).

2-4 dichlorophenoxy acetic acid (2, 4 D) is synthetic auxin commonly used plant tissue culture for callus induction and suspension cultured. It is also an important factor for the induction of somatic embryogenesis (Bhojwani & Razdan, 1996).

Abscisic acid (ABA)-a plant hormone that important roles in seed development and maturations, in the synthesis of protein and compatible osmolytes, which enable plants to tolerate stresses due to environmental or biotic factors and as a general inhibitor. It enhances shoot proliferations and inhibits later stages of embryo development (Anagnostakis SL., 1974).

All kinds of plant tissue cultures produce ethylene, and the rate of production increases under stress conditions. In cultures, ethylene is also produced when the organic constituents of the medium are subjected to heat, oxidation, sunlight or ionizing radiation.

3. Objective of the study

3.1 General objective

- To develop *in vitro* propagation protocol for *Pterolobium stellatum* from shoo tip explants

3.2. Specific objectives

- To initiate shoot culture of *P. stellatum*
- To evaluate the combined effects of different cytokinins and auxins on shoot multiplication
- To identify optimum growth regulators concentration for *in vitro* rooting
- To acclimatize *in vitro* developed plantlets.

4. Materials and methods

4.1. MS stock solution preparation

Murashige and Skoog (1962) MS medium was used throughout the experiments. The stock solution of macronutrient, micronutrient, vitamins, Fe-Na-EDTA and iron mixture were prepared separately. Each nutrient was weighed and dissolved with double distilled water. After all components were completely dissolved using magnetic stirrer, the solution was poured in to plastic bottles and stored in refrigerator at temperature of 4°C until used. The prepared stock solutions were used within a maximum of one month.

4.2. Preparation of growth regulators stock solution

In this study different plant growth regulator namely 6-benzyl aminopurine (BAP), kinetin (KN) α-naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) were used. Each of the growth regulator was prepared by weighing powder to prepare concentration of 1mg/ml by dissolving in few drops (2-3) 1M NAOH or 1M HCl based on the requirements of the plant growth regulator followed by dissolving in double distilled water. 1M of HCl was used for dissolving BAP and KN and 2-3 drops of NaOH was used to dissolve IBA and NAA. The prepared growth regulators were stored in refrigerator at temperature of 4°C until used.

4.3. Culture media preparation

Culture media were prepared by taking the appropriate volume of stock solutions; 50 ml/l of macronutrient, 5ml/l micronutrient, 5ml/l vitamins and 5 ml/l Fe Na-EDTA with double distilled water, 30g/l sucrose, and different growth regulators were added according to the need for shoot initiation, shoot multiplication and rooting. The pH of the medium was adjusted to 5.8 with 1M HCl or 1N NaOH and 8g/l agar. The medium was heated with microwave oven until the agar was melted and completely dissolved, then 50 ml was dispensed into each culture vessel. The medium was autoclaved at 121°C for 15 minutes.

4.4. Plant material and surface sterilization of seeds

The mature fresh seeds of *Pterolobium stellatum* were collected from western Ethiopia Oromia regional state, Horo Guduru Wollega zone, Jima Genet woreda, Ballibala Sorgo kebele about 320 km West of Addis Ababa.

The collected seeds were washed thoroughly under running tap water and detergent (Omo) followed by double distilled water for ten minutes. Then the seeds were categorized in to two conditions coated and de coated. Coated seeds were immersed in 70% ethanol for 5 minutes and washed five times with autoclaved double distilled water. The coated seeds were treated with sodium hypochlorite (30%, 15 % and 5%) for 25, 15 and 5 minutes respectively, followed, by washing with autoclaved double distilled water**.**

In the other experiment, the de-coated seeds were soaked in tap water for three-seven days. Three to four days soaked and swollen seeds were washed thoroughly with tap water and detergent (Omo) for 15 minutes followed washing ten times by autoclaved double distilled water. The seed coat was removed mechanically from cotyledons using scalpels and forceps and washed ten times thoroughly with double distilled water.

Both treated coated and de-coated seeds were cultured on growth regulators free MS medium containing 30g/l sucrose and 8g/l agar with macro nutrients, micronutrients and vitamins. The medium was autoclaved at 121°C at 105 KPa pressure for 15 minutes. Then seeds were cultured in to 50 ml medium in culture vessels with 5 replications and 6 seeds per culture vessel. The vessels containing cultured seeds were properly sealed with parafilm and maintained in culture room.

4.5. Shoot initiation

The shoot tips that were excised from in vitro seedling were used for culture initiation. The shoot tips were cultured in 50 ml of MS medium containing different concentrations of BAP (0.0, 0.5 1.0, 1.5, 2.0 2.5, 3.0, 3.5 mg/l) alone and KN (0.5, 1.0, 1.5, 2.0 2.5 mg/l) alone. The medium was supplemented with 30g/l sucrose and the pH was adjusted to 5.8 before addition of 8g/l agar. Thirty explants were used per treatments, six explants per vessel in five replications. The number of initiated shoots and dead shoots were recorded after four weeks.

The cultured shoot tips were transferred to the same fresh medium after four weeks. The cultures were maintained in culture room at temperature of 25±2°C under light intensity of 20 µ mol $m^{-2}s^{-1}$ and 16 hours photoperiod provided by white fluorescent lamp.

4.6. Shoot multiplication

The in vitro initiated shoots were cultured in full strength MS medium containing different concentrations of BAP (0.0, 0.5,1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/l) alone , KN (0.5 ,1.0, 1.5, 2.0, 2.5 mg/l) alone, combinations of BAP (0.5,1.0, 2.0 mg/l) with NAA (0.1, 0.25 0.5 mg/l) and KN (0.5, 1,0,1.5 2.0 mg/l) combined with NAA (0.1 and 0.25mg/l). Shoots from initiation medium were cultured in culture vessel containing 50 ml medium. For each treatment, a total of 30 explants (six explants per culture vessel) a five replication for each treatments. Number of shoots per explants, shoot length and number of leaves per shoot were recorded. The culture was maintained in the culture room at the same condition as for shoot initiation.

4.7. Rooting

In vitro rooting was done in half strength MS medium containing different concentrations of IBA (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) and NAA (0.5, 1.0, 1.5 and 2.0 mg/l). Growth regulator free MS medium was used as control. Six explants per culture vessel in baby food jars in five replications were used for each treatment. Number of roots and root length per shoot was recorded after six weeks. The cultures were maintained in growth room under the same conditions as of shoot initiation.

4.8. Acclimatization

Shoots with well-developed roots were removed gently from culture vessels and washed under autoclaved doubled distilled water and transferred in to plastic pots containing sterilized garden soil composition of compost and sand in ratio of 3:1, respectively. The planted plantlets were covered with polyethylene bags and watered as necessary for two weeks then the polyethylene bag was removed. The survived plants were recorded after 4 weeks of acclimatization.

4.9. Data Analysis

Data were subjected to Analysis of Variance (ANOVA). The mean values of the parameters were subjected to analysis of variance using the SPSS Software Packages Version 20 and Tukey's multiple range tests were used. Significant differences among mean values were compared using least significant difference (LSD) test using statistical data analysis software SPSS version 20 at 0.05 probability level.

5. RESULTS

5.1. In vitro seed germination

The coated seeds germinated from 22-45 days interval. Among tested concentrations of Clorox 5%, 15% and 30 % for 5, 15 and 25 minutes exposure times was used for *P. stellatum*. Maximum germinated clean coated seed per explants was (58.0%) recorded upon using 30% concentrations of commercial bleach (Clorox) for 25 minutes exposure time. About (35%) of coated seeds was contaminated from 30% concentration of commercial bleach for five minutes exposure time. The maximum percentage of contaminated coated seeds (100%) was obtained at 5% concentrations of commercial bleach (Clorox) for 5 minutes. Increasing the concentration of Clorox resulted in higher decontamination, but decreased the percentage of seed germination (Table 1).

This result showed that higher concentration sterilizing agent maximum effect against microbial contamination, but the survival percentage of seeds was low. When Clorox concentration decreases, the seeds became decayed and contaminated by microbial. No significance difference was observed among 5% and 15% Clorox concentration with respect to seed decontamination but the increasing concentration of Clorox towards 30% was affected seed germination.

The highest percentage cleaned seed germinated (91.4%) was obtained on de-coated seeds where washed the seeds with detergent (Omo) and removed mechanically seed coat from cotyledons after soaking seeds for three to four days. The de-coated seeds were germinated within four to seven days. The seeds developed seedlings after 4 weeks.

Table 1: Effects of different concentration of commercial bleach (Clorox) and exposure time *in vitro* germination of *Pterolobium stellatum.*

Seed Condition	Treatment	Clorox (%)	Exposure (min)	Clean explants (%)	Germination (%)	Contamination (%)
Coated	Alcohol - Clorox	5	5	0.0	0.0	100
		15	15	23.0	13	38
		30	5	54.3	49.9	35
		30	15	60.6	52	19
		30	25	93.2	58	5.0
	Hot water	Immersion	3 min	40.9	45.2	22.4
		Immersion	5min	24.2	14.0	28.8
De-coated	Discard	Soaked	3-4 days	98.7	91.4	5.8
		Soaked	5-7 days	72.0	56.6	12

N.B.In this study 820 seeds of *Pterolobium stellatum* were used for sterilizations and in vitro germination.

5.2. Shoot induction from shoot tip explants

The cultured shoots began responding from ten to fourteen days. Experiment of analysis of variance revealed that different level of BAP and KN had a highly significant difference effect on shoot initiation percentage, shoot number, shoot length and number of leaves per shoot on medium containing. No significance difference ($P \leq 0.05$) was observed in the number of shoots produced in the number of shoot produced per explants in medium containing 1.mg/l BAP and 2.0 mg/l BAP but highly significance difference between 2.0 mg/l BAP and 2.5mg/l BAP containing MS medium. The shoot induction efficiency of BAP was better than KN in this species.

During shoot induction cultured shoot tips of explants displayed physiological changes of plant that frequently released excessive phenolic compounds from explants was a problem for *in vitro* Propagation of *P. stellatum*. This compound converted the medium to brown and affected the metabolic system of plant and gradually caused death of plant *in vitro*. To avoid this problem, cultured shoots explants were transferred to new prepared medium within 14 days from starting from culture by washing thoroughly with autoclaved double distilled water which resulted in reducing the negative effect of compounds. It is a common problem on woody trees, transferred to new fresh medium is important to reduce the risk of death of explants.

As indicated in table 3, shoot tips cultured on MS medium supplemented with BAP and KN alone showed significance difference in terms of shoot number and length per explants **.**Among different concentrations of cytokinins, the maximum shoot induction per explants(2.76 ±0.31) with (86.7%) shoot induction was recorded on MS medium supplemented with 2.0 mg/l BAP alone. Lowest shoot mean number per explants (0.76 ± 0.47) was obtained on growth regulators free MS medium.

Highest mean shoot length was obtained on MS medium supplemented with 2.5 mg/l KN (3.68±0.41cm) followed by (3.33±0.29cm) on MS medium supplemented with 2.0 mg/l KN alone. The shortest mean shoot length (1.10 ± 0.23) was obtained on growth regulators free MS medium. Therefore the variance (ANOVA) analysis showed that no significance difference was observed in the shoot length produced per explants in medium containing 0.5mg/l and 1.0mg/l KN alone, but a significance difference among MS medium containing 1.0 mg/l KN and 2.0 mg/l KN. Shoots developed on MS medium containing various concentrations of KN were taller than

shoots obtained on medium containing BAP. This might be BAP reduced the apical dominance than KIN. Increasing the concentration of BAP resulted in the production of stunted shoots in *P, Stellatum.*

Table 2: Effect of different concentrations of BAP and KN on shoot initiation *P. stellatum* on MS medium after 4 week of culture.

BAP (mg/l)	KN (mg/l)	Shoot induction(%)	Number of shoots per explants	Shoot length per explants	Numbers of leaf per explants
0.0	0.0	24	0.76 ± 0.14^{c}	1.10 ± 0.23^{b}	3.90 ± 0.75^{b}
0.5	0.0	56.6	1.63 ± 0.29^{abc}	2.13 ± 0.33^{ab}	5.23 ± 0.98^{ab}
1.0	0.0	64.6	1.93 ± 0.29^{abc}	2.45 ± 0.35^{ab}	5.13 ± 0.85^{ab}
1.5	0.0	80	2.60 ± 0.31^{a}	3.00 ± 0.25^{a}	8.80 ± 1.06^{a}
2.0	0.0	86.6	2.76 ± 0.31^{a}	3.23 ± 0.30^{a}	8.46 ± 0.74^{a}
2.5	0.0	46.6	1.93 ± 0.20^{abc}	3.03 ± 0.25^{a}	7.20 ± 0.59^{ab}
3.0	0.0	36	1.66 ± 0.27^{abc}	3.33 ± 0.40^{a}	5.56 ± 0.84^{ab}
3.5	0.0	28	1.13 ± 0.21^{bc}	2.35 ± 0.33^{ab}	5.53 ± 0.86^{ab}
0.0	0.5	63.3	1.70 ± 0.28^{abc}	2.55 ± 0.35^{ab}	7.83 ± 1.02^{ab}
0.0	1.0	60	1.60 ± 0.24^{abc}	2.63 ± 0.36^{ab}	6.06 ± 0.98^{ab}
0.0	1.5	39	1.63 ± 0.22^{abc}	2.51 ± 0.35^{ab}	5.66 ± 0.79^{ab}
0.0	2.0	43	2.03 ± 0.33^{ab}	3.33 ± 0.47^{a}	8.13 ± 0.98^{a}
0.0	2.5	33	1.20 ± 0.19^{bc}	3.68 ± 0.41^{a}	9.20 ± 0.85^{a}

Mean shoot number followed by the same letter within a column were not significantly different at 5% probability ($p \leq 0.05$).Values are presented as mean ± standard deviation

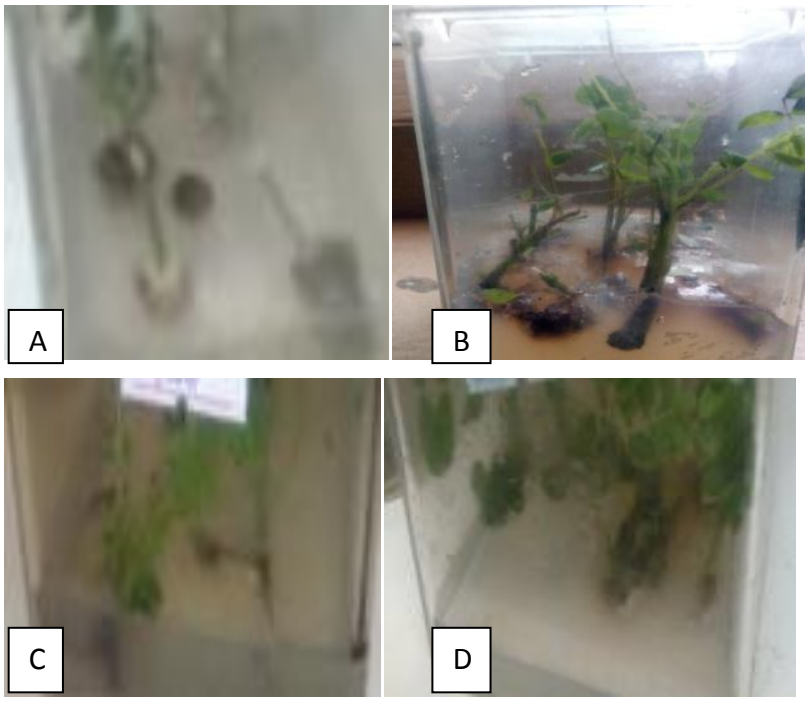

Figure 1: Effects of different concentrations of BAP and KN on shoot induction from shoot tips explants of *Pterolobium stellatum* A. control B. 3.5 mg/l BAP, C. 2.0 mg/l BAP & D.1.5 mg/l BAP

5.3. Shoot multiplication

5.3.1.Effect of BAP or KN and combined with NAA on shoot multiplication

A significant difference ($P < 0.05$) was observed among the different concentrations of BAP and KN in terms of shoot number per explants and shoot length after 4 weeks of culture. The result of the present study showed that using BAP alone for shoot multiplication was better than KN alone and their combinations.MS medium resulted varying degree of shoot proliferation based on different concentrations of growth regulators gave different responses based on the different hormonal composition presented (Tables 3 and 4)**.**

Results showed that medium supplemented with 2mg/l BAP in the absence of NAA produced the highest number of shoots (3.76±0.32) followed by (2.53±0.30) number of shoots per explants on MS medium supplemented with 1.5 mg/l BAP alone. Lowest number of shoots per explants (0.56±0.11) was recorded on growth regulators free MS medium.

In terms of shoot length, the highest mean shoot length (4.45±0.20cm) and number of leaves per explants (10.6±0.62) were obtained on MS medium supplemented with 1.5 mg/l KN + 0.1 mg/l NAA followed by (3.91±0.47cm) on medium containing 1.5 mg/l BAP in combination with 0.25 mg/l NAA.

The lowest mean shoot length (1.30±0.33 cm) was recorded on growth regulators free MS medium. The study showed KN in combination with lower concentrations of NAA was resulted better shoot height and number of leaves than MS medium containing BAP in combination with NAA**.** The variance (ANOVA) analysis showed that no statistically difference ($P \leq 0.05$) was observed in the shoot number produced per explants in MS medium supplemented 2.5mg/l and 3.0 mg/l BAP alone, but it is high significance difference between MS medium containing KN and BAP on formation of shoot length per explants.

Table 3: Effects of different concentrations of BAP and IN shoot multiplication of *P.stellatum*.

BAP (mg/l	KN (mg/l)	Shoot number per explants	Shoot length per explants	Leaf number of per explants
0.0	0.0	0.56 ±0.11^{c}	1.30±0.33^{c}	4.34±0.82^{b}
0.5	0.0	1.43±0.22^{bc}	2.13±0.29^{bc}	5.43±0.74^{ab}
1.0	0.0	1.93±0.25^{b}	2.10±0.26^{bc}	5.93±0.70^{ab}
1.5	0.0	2.53 ±0.30^{ab}	2.40±0.28^{abc}	6.50±0.92^{ab}
2.0	0.0	3.76±0.32^{a}	**2.03± 0.29^{bc}**	5.96 ±0.76^{ab}
2.5	0.0	1.96±0.26^{b}	2.86±0.49^{ab}	6.80±0.87^{ab}
3.0	0.0	1.83±0 29^{b}	2.84±0.37^{ab}	6.56.01^{ab}
3.5	0.0	1.83±0.28^{b}	2.15±0.28^{bc}	5.36±0.75^{ab}
0.0	0.5	2.25±0.29^{bc}	2.25±0.27^{abc}	5.56±0.71^{ab}
0.0	1.0	1.63 ±0.19^{bc}	2.40±0.27^{abc}	5.66±0.72^{ab}
0.0	1.5	2.33±0.25^{ab}	2.66±0.41^{ab}	6.68±0.50^{ab}
0.0	2.0	2.00±0.27^{b}	2.66±0.32^{abc}	5.00±0.75^{ab}
0.0	2.5	1.50±021^{bc}	3.65±0.32^{a}	8.66 ±1.06^{a}

Mean values with the same letter in columns are not significantly different at ($P \leq 0.05$). The values represent mean ± standard deviation.

Table 4:Effectof different concentrations of BAP and KN and combinations of BAP or KN with different concentrations of NAA on shoot multiplication.

BAP (mg/l)	KN (mg/l)	NAA (mg/l)	Shoot numbers per explants	Shoot length per explants(cm)	Leaf number per explants
0.1	0.0	0.5	0.66 0.13^{c}	1.60±0.31^{d}	4.03±0.82^{c}
0.5	0.0	0.25	1.46±0.20^{ab}	2.65 ±0.35^{bcd}	7.56±1.02^{abc}
1.0	0.0	0.1	1.26±0.21^{ab}	2.85±0.46^{abcd}	6.16±0.95^{bc}
1.5	0.0	0.25	1.62 ± 0.25^{ab}	3.91± 0.47^{ab}	9.24 ±1.07^{ab}
2.0	0.0	0.1	2.00 ± 0.31^{a}	2.56±0.36^{bcd}	7.16±1.18^{abc}
2.0	0.0	0.25	1.90 ± 0.25^{a}	2.78 ±0.30^{abcd}	7.76 ±1.02^{abc}
0.0	0.5	0.25	1.56±0.17^{ab}	3.25 ± 0.33^{abcd}	8.03±0.77^{abc}
0.0	1.0	0.25	1.30±0 .24^{ab}	3.41±0.48^{abc}	7.73±1.18^{abc}
0.0	1.5	0.1	1.33±0.08^{ab}	4.45 ±0 .20^{a}	10.6±0 .62^{a}
00	2.0	0.25	1.54±0.24^{abc}	3.77±0.44^{abc}	7.38±0.99^{abc}

Mean values with the same letter in columns are not significantly different at P≤0.05. The values represent mean ± standard deviation.

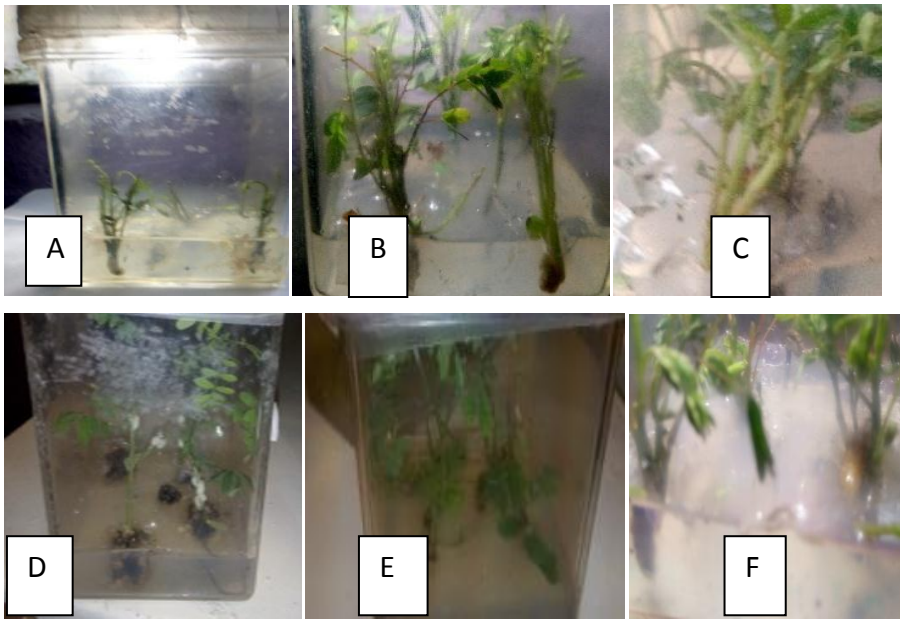

Figure 2. Multiple shoots of *Pterolobium stellatum* on MS medium supplemented with different concntrations of growth regulators A. Control B.2.0 mg/l BAP, C.0.5 mg/l KN D.1.5 mg/l BAP E. 1.5 mg/L KN. F.2.0 mg/l + 0.10 mg/l NAA

5.4. Rooting and acclimatization

The cultured shoots produced roots on medium containing IBA and NAA (Table 6). Root was developed after six weeks of culturing in root induction medium. The cultured medium containing NAA showed highest root formation in half strength MS medium. Analysis of variance (ANOVA) showed that the effect of NAA & IBA on, root number& root length were significant ($p \leq 0.05$). No the effect of IBA on the root number was significant except 0.5mg/l IBA. NAA had significant effect ($p \leq 0.05$) on all measured treatment (Table 5).

The maximum mean number of roots per shoot was obtained in medium containing 1.0 mg/l NAA alone (1.93±0.21).The lowest mean root per shoot (0.46±0.14) was obtained on MS medium supplemented with 2.0 mg/l NAA. No root induction was observed on growth regulators free and full strength MS medium. The highest mean root length (2.18±0.23 cm) was observed on half strength MS medium containing 1.0 mg/l NAA followed by (1.10±0.29 cm) on MS medium fortified with 0.5 mg/l NAA.

The maximum shoot induction (43%) was obtained at MS medium supplemented 1.0mg/l of NAA. After acclimatization,(36.62 %) of explants was survived. This low percentage of explants survival during acclimatization was caused due to highly contaminated by fungal, viral infection.

Table 5: Effects of different concentrations of IBA and NAA

IBA (mg/l)	NAA (mg/l)	Percentage of root induction	Number of root per explants	Mean length of roots (cm)
0.0	0.0	0.0	0.00 ± 0.00^{d}	0.00 ± 0.0^{d}
0.50	0.0	34	1.13 ± 0.23^{b}	1.06 ± 0.18^{b}
1.0	0.0	0	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
2.0	0.0	0	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
0.0	0.5	28	1.10 ± 0.29^{ab}	1.10 ± 0.29^{b}
0.0	1.0	43	1.93 ± 0.21^{a}	2.18 ± 0.23^{a}
0.0	1.5	28	0.70 ± 0.14^{ab}	1.26 ± 0.24^{b}
0.0	2.0	23	0.46 ± 0.14^{cd}	0.83 ± 0.23^{b}

Mean values with the same letters in columns are not significantly different at $P \leq 0.05$. The values represent mean ± standard deviation

Figure.3: Effects of NAA and IBA on rooting and acclimatization A and B half strength MS medium containing 1.0 mg/l NAA C.0.5mg/l IBA, D. During transplanting E. After two weeks F. After four weeks.

6. Discussion

6.1. Seed sterilization

This study used various sterilization agents to avoid the contamination of microorganisms from culture of *P. stellatum*. Among seeds categories, de-coated seeds washed with detergent (Omo) thoroughly followed double distilled water and removed the coat mechanically was the most effectively reduced percentage of contamination.

The coated category seeds were surface sterilized with 70% ethanol for five minutes and 30% Clorox for 25 minutes showed highest percentage of clean seeds. The present study showed that the highest mean percentage of clean survived explants (58.0%) was obtained from 30% Clorox concentrations for 25 minutes exposure time resulted but reduced the seed viability. All seeds surface sterilized with 15% and 5% Clorox for 15 and 5 minute exposure time, respectively showed highest microbial contamination.

Analysis of variance (ANOVA) revealed that concentration of Clorox, exposure time, and their interaction result in significant difference overcoming contamination of growth media and improving survival and germination level of *P. stellatum in vitro* seed culture.

The prolonged exposure time and increasing concentrations to Clorox (NaOCl) decreased the percentage of microbial contamination and rate of germination on *P. stellatum*. However, less exposure time of Clorox showed increased rate of germination and death of shoots of explants by microbial contaminants. In the present study commercial bleach is effective to remove surface contaminants from *P. stellatum* seed explants. This is in agreement with work of (Chanie Dereso and Tileye Feyissa (2015) on *Caurdexia edulis* and Keredin Mohamed (2017) on *Ximenia americana.*

6.2. Germination rate of seeds

In present study De-coated seeds showed better germination than coated seeds. The removing the hard seed coat mechanically was found to be more effective treatment than culturing coated seeds on *P. stellatum.* Therefore, tap water immersion for 3-4 days facilitated removal of the seed coat from cotyledon. This reduced contamination and increased cleaned percentage of seeds germination on *P. stellatum*. De-coated seeds begun to germinate within 4-7 days on growth regulators free MS medium about (91.4%) cleaned germinated seed per explants was recorded.

This finding was similar to the work of Ganaie *et al.*,(2011) who reported (96 %) of germination by removing the seed coat with in 4-7 days on critically endangered medicinal plant, *Arnebia benthamii.*

In addition Legesse Negash (2004) who obtained best percentage of germination (91±2.7%) on *Prunus africana* full strength of MS medium**.** This mechanical removed seed coat from cotyledon is used to remove pathogenic infections from surface of seeds (Desai, B.B.*et .al*., 1997b).

Coated seeds germination in all media was less effective than de coated seeds. This may be caused by exogenous factor of seed coat impermeability water and oxygen to embryo. Hot water immersion treatment was the least effective application of germination of *P. stellatum in vitro* caused decayed and grows of moulds on seeds. The coated seeds germinated from 22 to 45 days and had deep green leaves than de-coated seeds. Maximum cleaned seed germination per explants (58.0%) was observed in full strength MS medium of coated seeds. This finding agrees with the report of (Demel Teketay, 1998) on similar species.

In general mechanical removed seed coat treatments from cotyledons resulted higher percentage of germination than coated seeds (hot water).This is due to avoid seed coat impermeability as a mechanism which delays germination.

6.3 Shoot induction

In vitro growth and organogenesis are regulated by interaction and balance between the growth regulators supplied in medium and growth substance produced endogenously in plants (George, 1993).In this study, the effect of BAP and KN was compared. Application of BAP alone induced maximum shoot number than KN alone and best shoot induction of *P. stellatum* from shoot tips culture was obtained.

The percentage shoot induction, shoot numbers, shoots length and leaves varied according to concentrations of growth regulators and type of treatments used. In the present study, optimum percentage of shoot induction (86.6%) was recorded on MS medium supplemented with 2.0 mg/l BAP alone. This finding was similar with work of (Mengistu Fentahun *et al.,* 2017) who got (86.7%) on *Alloysia tripylla.* The lowest percentage (24%) of shoot induction was observed in growth regulators free MS medium that showed short shoots with low number of leaves.

From this study, maximum mean shoot number (2.76±0.31) with (3.08±0.29 cm) mean shoot length and (8.46±0.74) mean number of leaves per explants were recorded from shoot tips of *Pterolobium stellatum* on MS medium fortified with 2.0 mg/l BAP alone. These findings were in agreement with the work of Indravathi and Pulluiah (2013) on *Albizia amara (*Fabaceae*)* (2.80±0.45) shoot numbers and (2.10±0.25cm) shoot length were obtained using shoot tip explants.

Birhanu Kassaye (2017) reported (3.00± 0.58) mean shoot number with (3.33±0.29cm) shoot length was obtained using axilliary bud of *Prunus domesticum.* Jemal Ali *et, al.*,(2017) also in agreement with the result of our present study using 2 mg/l of BAP alone.

The present study didn't agree with the work of Banchiayehu Gelan (2015) who obtained (5.47±3.29) maximum mean shoot number with (0.65±0.64 cm) shoot length on *Taverniera abyssinca* on MS medium supplemented with 0.4 mg/l BAP. In addition, (Park *et.al*.,2012) obtained (3.60±0.10) numbers of shoots on MS medium containing 0.1 mg/l BAP alone on *Liriope platyphylla* from meristem shoot culture. Both author obtained maximum shoot numbers relatively at lower concentrations of BAP. Generally, MS medium containing KN resulted in higher mean shoot length than MS medium containing BAP.

In the present study, the highest shoot length (3.68±0.41cm) was obtained on MS medium supplemented with 2.5mg/l KN. This result is similar with work of Indravathi and Pullaiah (2013) who reported (3.20±0.54 cm) shoot length of *Albizia amara* (Fabaceae) on MS medium containing 2.0 mg/l BAP from cotyledonary node. In this study highest number of leaves(9.20±0.85) per explants was obtained on MS medium supplemented with 2.5mg/l KN. As shoot length increased number leaves per explants was also increased.

In general, mean shoot number increased towards 2.0 mg/l BAP alone then decreased beyond optimum as concentration of BAP increased. This increasing concentration of BAP caused a reducing length of shoots due to reduced apical dominance and inhibiting shoot proliferations (Rahimeal *et al.,* 2013).Therefore BAP is the most widely used and effective cytokinin in legumes species **(**Gulati and Jaiwal .1994**).**

6.4 Shoot multiplication

In shoot multiplication, there were twenty four different concentrations of BAP or KN alone and combinations of BAP or KN with NAA were compared. Among all different concentration treatments, application of BAP alone induced more shoot number than KN. In present study, the highest mean number of shoots per explants was (3.76±0.32) with (3.65±0.32 cm) mean shoot length and (7.96±0.76) numbers of leaves per explants were recorded on MS medium supplemented with 2.0 mg/l BAP alone.

The results of this study are similar with findings of Chanie Derso and Tileye Feyissa (2015) who obtained maximum mean shoot number per explants (4.20±0.85) of *Cordeauxia edulis.* Kumar and Sireelaja (2016) who obtained (4.62±1.60) shoot numbers and (5.81±1.10 cm) shoot length of *Adenthera pavonina* from shoot tips. This finding is similar with the present study but author obtained little more shoot number. This difference may be due to type and genotype difference of plants. Then mean numbers of shoots was increased towards 2.0 mg/l BAP alone and then decreased.

This increasing concentration of BAP caused a reducing length of shoots due to reduced apical dominance and inhibiting shoot proliferations (Rahimeal *et al.*,2013). MS medium supplemented with 2.0 mg/l BAP alone was optimum concentration that resulted in better shoot induction and multiplication than supra higher and lower concentrations of BAP.

In fact for *P. stellatum,* highest BAP concentration (3.5 mg/l) resulted in declined number of shoots. This finding is similar to the work of (Sudharson *et al*., 2014) on *Hybanthus enneasperms*. Therefore, BAP showed better results than KN on shoot multiplications because of BAP is not easily broken down in medium that stability and persists long time in medium due to conjugated in the medium in the form of smaller forms than KN ; existing in free or ionized forms where readily available for plant tissue from medium (Buah *et al.,* 2010). This agrees with the work of Kelem *et al.,* (2004) who reported BAP was the most stable cytokinin in plant tissue culture. MS medium containing KN showed dark green leaves and shoot elongations than BAP. This finding was similar with the work of Reddy *et al.,* (2012).

The finding of Kirika (2015) on *Erythrina abyssinica* showed that MS medium supplemented at supra-optimal concentration (8.6 mg/l) BAP resulted in maximum mean shoot number on nodal

explants and in addition Birhanu Kassay *et al.,* (2017) who reported (4.8±0.27) shoots number with (4.50±0.55cm) shoot length and (1.93+0.24) mean number of leaves was obtained on *Oxytenanthera abyssinica* (Poaceae) at 4.0 mg/l BAP alone from seed culture. The results of both authors are contrary with our present study who obtained maximum mean shoot number at supra optimum or at higher concentrations of BAP.

BAP combined with NAA was more effective than KN combined with NAA on shoot multiplication. The finding is similar with the work of Keredin Mohamed (2017) on *Ximenia americana* using shoot tips.

In terms of shoot length (4.45±0.20 cm) and (10.6±0.62) number of leaves was obtained on MS medium supplemented with 1.5 mg/l KN in combination with 0.1 mg/l NAA. The shortest shoot length was (1.30±0.33 cm) and (1.60±0.31 cm) obtained on growth regulators free MS medium and 0.1 mg/l BAP in combination with 0.5 mg/l NAA respectively.

The KN containing MS medium was better for shoot length than MS medium containing BAP and their combinations (Table 5). The study revealed that number of leaves per explants was increased as shoot length increased. The finding was in agreement with work Birhanu Kassay *et al.,* (2017) on micro propagation of *Oxytenathera abyssinca* from seed culture. As Indrias Teshome (2015) who obtained highest shoot length (2.62±0.17cm) on growth regulators free MS medium and lowest shoot length was obtained on MS medium containing BAP and KN on *Satureja punctata.* This is contrary with our present study that lowest shoot length was obtained on growth regulators free MS medium.

Generally, the present study showed that the explants of *Pterolobium stellatum* responded positively with multiple shoot numbers when BAP alone was used. It was comparatively more effective than KN and their combinations on shoot multiplication.

6.5 Rooting

There were ten different concentrations of treatments including the control group used for root induction. From the study, less percentage of root induction was observed *in vitro* propagation of *P. stellatum*. No roots induced in full strength and growth regulators free MS medium including

some IBA alone. This is may be due to unbalanced level effects of endogenous factors of cytokinin that probably cause inhibition of root formation.

Half strength MS medium containing NAA alone had better rooting response than MS medium containing IBA. From the present study, MS medium supplemented with 1.0 mg/l NAA resulted in maximum mean root number per explants (1.93±0.21) and (2.18±0.23cm) of root length was recorded on half strength MS medium. The maximum percentage of root induction (43%) was recorded. This finding is similar with the work of Biswas *et al.,* (2011) who reported (47%)root induction from nodal explants of *Stemon tuberose* were(1.23±0.06) mean number of roots and (0.77±0.05 cm) root length was obtained.

Alelegne Yeshamebel (2016) also reported (1.63±1.03) root number and (0.84±0.54 cm) root length from shoot explants of *Moringa stenopetala* on MS medium supplemented with 1.0 mg/l NAA.

Vinothkumar *et al.,* (2011) obtained (2.40±0.48) mean number of roots and (3.1± 0.13cm) root length from nodal culture of *Wattakaka volubilis* at lower concentrations (0.4 mg/l) of NAA. Rout (2004) also obtained (3.60±0.80) root numbers at lower concentrations of (0.25mg/l) NAA on *Clitoria ternatea* .L(Fabaceae).These findings contrast with our present study; both authors obtained maximum root number at lower concentrations of NAA. Unlike our present study Senthilkumar *et al.,* (2015) who obtained maximum root numbers at MS medium supplemented with 2.0 mg/l IBA on *Indigofera trita.*

In addition, Islam *et al*., (2005) contrary to our present study who obtained maximum mean root number on growth regulators free MS medium. Therefore, NAA containing MS medium was more effective growth regulator for rooting than MS medium fortified by IBA on *P. stellatum.* This finding was similar to that of Kristiansen *et al*., (1999).

In terms of length, the highest root length obtained (2.71±0.23cm) was recorded on MS medium supplemented with 1.0 mg/l NAA whereas the shortest root length (0.460±0.14 cm) that was fortified with 2.0 mg/l NAA (Table 5). In general, NAA alone exhibited more effects as compared to the effects IBA. From the study, there was no root induction on MS medium supplemented with IBA except 0.5 mg /l.

6.6 Acclimatization

Among 32 platelets acclimatized in the green house, 12 (36.62%) survived. The well grown roots *in vitro* plantlets were washed thoroughly by tap water successfully acclimatized. Most of plantlets didn't survive due to fungal and viral contamination.

7. CONCLUSION

Pterolobium stellatum is a multi-purpose perennial plant used as traditional medicine as Well as for ink production due to have tannins. The plant has low attention and highly destructs or action of destroying the plants by local people in Ethiopia. Results of the study indicate massive scale propagation *P. stellatum* from shoot tips explants. From the present study, it was concluded that a significance difference in mean number of shoots and length per explants using different concentrations revealed the effects of BAP, KN, in shoot induction and BAP, KN and BAP with NAA and KN with NAA for shoot multiplication. The application of BAP alone at 2.0 mg/l concentration was best and resulted in shoot induction and multiplication per explants than KN and their combinations (2.76±0.31) and (3.76±0.32), respectively. Most effective root formation was observed in shoots cultured on MS medium containing 1.0 mg/l NAA. This implies the development of protocol of mass propagation of *P. stellatum* from shoot tips explants. Generally results of this study indicate large scale propagation of *P. stellatum* through tissue culture technique with in short period. Therefore MS medium supplemented at 2.0 mg/l BAP alone was best for shoot induction and multiplication for *in vitro* propagation of *P. stellatum*.

8. RECOMMENDATIONS

Future perspectives, based on the present study:

- The protocol may require low cost mass propagation of plants by reducing the modern media components such as using table sugar or bulla instead of sucrose should be used for large scale micro propagation.
- It may require further optimization of this protocol.
- The effects of different concentrations of cytokinin on shoot induction and multiplications of shoots should be further studied.

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APPENDIX 1

Nutrient composition and Concentration of MS basal medium

Macronutrients g/l	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_20$	3.7
KH_2PO_4	1.7
Micronutrients	
$ZnSO_4.7H_2O$	0 .86
KI	0.083
$MnSO_4.4H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
H_3BO_3	0.62
Fe-Na-EDTA	4
$CoCl_2.6H_2O$	0.0025
$NaMoO_{4.}2H_2O$	0.025
Organic supplements (vitamins)	
Myoinositol	1.O
Nicotinic acidic	0.05
Thiamin (B1)	0.01
Glycine	0.2
Pyridoxin(B6)	0.05

DECLARATION

I the undersigned, declare that this thesis is my original work and has not been presented for any academic degree in any other university and all sources of materials used for the thesis has been correctly acknowledged.

Name :Kafane Jemaneh Signature ----------date----------

This thesis has been submitted for examination with my approval as an advisor:

Tileye Feyissa (PhD)

Signature----------

Date ----------