MEDIA IMPROVEMENT FOR *IN VITRO* CULTURING AND HARDENING OF TC BANANA CV. NENDRAN.

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Media improvement for *in vitro* culturing and hardening of TC banana cv. Nendran"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Padannakkad

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Vinayak Lamani

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

%	-	per cent
^0C	-	Degree Celsius
@	-	at the rate of
AMF	-	Arbuscular Mycorrhizal Fungi
B	-	Boron
Ca	-	Calcium
CD	-	Critical difference
cm	-	Centimeter
Cu	-	Copper
cv.	-	Cultivar
DAT	-	Days after transplanting
et al	-	And others
Fe	-	Iron
Fig.	-	Figure
FYM	-	Farm yard manure
g	-	Gram
ha^{-1}	-	Per hectare
i.e.	-	That is

K	-	Potassium
KAU	-	Kerala Agricultural University
Kg	-	Kilogram
kg ha^{-1}	-	Kilogram per hectare
KNO_3	-	Potassium nitrate
L	-	Litre
m	-	Meter
Mg	-	Magnesium
mg g^{-1}	-	Milli gram per gram
mg kg^{-1}	-	milligram per kilogram
ml	-	Milli litre
m^2	-	Squre meter
mm	-	Milli meter
mmol g^{-1}	-	Milli moles per gram
Mn	-	Manganese
MT/ha	-	Million tonnes per hectare
N	-	Nitrogen
$(NH_4)_2H_2PO_4$	-	Di-ammonium hydrogen Orthophosphate

NH_4NO_3	-	Ammonium nitrate
NS	-	Not significant
pH	-	Soil reaction
ppm	-	parts per million
PSB	-	Phosphate solubilizing bacteria
SE	-	Standard error
TC	-	Tissue culture
t ha^{-1}	-	Tonnes per hectare
TSS	-	Total soluble solids
viz.	-	namely
Zn	-	Zinc

Introduction

1. INTRODUCTION

Edible bananas (*Musa sp*) are the major staple food for rural and urban consumers in the tropical and subtropical countries of the world. Banana occupies prominent position among tropical fruits as that of apples among temperate fruits. It is a rich source of carbohydrates, vitamins and mineral nutrients and by virtue it is an important component of human diet. This is the only tropical fruit which is exported in large quantities and is leading fruit in the international trade for its fresh and processed form (Thomas *et al.*, 1968). With the increasing demand and vast export potential coupled with the farmers desire to grow banana on a large area, *in vitro* propagated plants are becoming increasingly important as planting material for rapid multiplication of economically important commercial varieties.

In terms of gross value production it ranks fourth position after rice, wheat and maize globally. In India, among the fruit crops it is the second most important fruit crop after mango. India is the highest producer of banana in the world with a share 39.04% of production compared with other countries, with cultivated area is around 858.1million hectares, production of 29162.6 million tonnes and productivity of 34.0 MT/Ha (NHB, 2017- 2018).

Among several varieties of banana cultivated in Kerala, cultivar Nendran occupies the first choice among Keralites. Vitamins B_1, B_2 and B_3, vitamin C, amino acids and mineral nutrients like iron, calcium and phosphorous and proteins are rich in fruit pulp which are needed daily in human diet (Das, 2010).

Conventional propagation generally leads to production of 5-10 suckers from single plant and is also affected by some diseases (Rahman *et al.*, 2004). But through *in vitro* propagation, a large amount of true to type and disease free planting materials can be obtained. Micropropagation allows rapid production of high quality, disease free and uniform planting material irrespective of the season and weather. From single explant large number of uniform and disease free plants could be produced (Martin *et al.*, 2006). Micropropagated plants are vigorous and of uniform growth and early emergence of bunch and through this technique 39 % higher yield was obtained in comparison to conventional sword suckers (Frahani

et al., 2008). *In vitro* culturing of banana through shoot tip culture and meristem culture are reported by many authors (Wong, 1986; Arinaitwe *et al.*, 2000).

Most commonly used basal medium for the micropropagation of banana is MS (Murashige and Skoog, 1962), in which inorganic nitrogen sources consist of KNO_3 and NH_4NO_3. Nitrate and ammonium ions are the most common inorganic nitrogen sources used as mineral salts in tissue culture media for *in vitro* propagation. Ammonium ions are very essential for the utilization of nitrate by plant tissue. If the media contain nitrate as single nitrogen source, it is not very successful *in vitro* culture (Halperin and Wetherell, 1965; Selby and Harvey, 1990).

Tissue culture plantlets have very divergent leaf anatomy and physiology (Pierik, 1987) and therefore require an acclimatization process before transition of culture vessel to greenhouse or field conditions. *In vitro* grown plants had shown high mortality when it is transferred to *ex vitro* condition due to its have non functional stomata, weak cuticle development and poor root system (Mathur *et al.*, 2008). Before transfer to field condition gradual acclimatization is very essential (Hazarika, 2003). Primary and secondary hardening is an important process in tissue culture. The acclimatization is the last and most critical step of *in vitro* culture. For that particular practice is required for maximization of survival and good growth of plantlets during acclimatization (particular climatic condition). Nowdays "Biotization" (plants inoculated with beneficial microorganisms) is an advanced technique to maximize survival and growth of tissue cultured plants during hardening (Gosal *et al.*, 2010).

Objective of the research programme entitled 'Media improvement for i*n vitro* culturing and hardening of tissue culture banana cv. Nendran' is a) to study the effect of nitrogen sources on shoot and root regeneration in tissue cultured Nendran banana, and b) to enhance the growth and survival of plantlets during primary and secondary hardening.

Review of literature

2. REVIEW OF LITERATURE

MS media is the most commercially used basal medium for the micropropagation of banana, in which inorganic nitrogen sources KNO_3 and NH_4NO_3 are major macro nutrients in MS media. Because of the non availability of NH_4NO_3 due to security reasons, an alternate source needs to be found out. Hardening is the final stage in micropropagation and its very important stage for success of plantlets in field establishment and for that increase the survival and growth of plantlets with the application of different potting media and bio-fertilizers during hardening process is necessary.

The present study on "Media improvement for *in vitro* culturing and hardening of tissue culture banana cv. Nendran" was undertaken during the period from 2017 to 2019 at Regional Agricultural Research Station, Pilicode, Kasargod (Dt.) for standardization of MS media in *in vitro* culturing and different potting media and various bio-fertilizers for primary and secondary hardening respectively.

2.1. Banana and plantain

Banana is the second most important fruit crop in India after mango and it is widely cultivated in tropical and sub tropical regions in world. Hot tropical region of South East Asia is regarded as origin of banana (Spiden, 1926; Suar, 1952). It belongs to Musaceae family. Banana is the staple food of millions of people globally because of its delicious taste, high energy and pleasant aroma (Anonymous, 1969). It is an important source of carbohydrates, vitamins, proteins, potassium, iron, calcium and ascorbic acid and also contains moderate amount of thiamine, riboflavin, nicotinic acid and folic acid (Rasheed, 2003).

2.2. Nendran

The cultivar Nendran ranks first in commercial value. The cultivators of Agasthiamalai ranges call this variety as "King of banana". The shelf life of Nendran is more than that of other cutivars. So, the fruit of Nendran have been exported to Arabian and European countries (Das, 2010). This belongs to plantain

group. It is a dual purpose cultivar of Kerala. The fruit is relatively longer and thicker than other bananas with very good keeping quality. Average bunch weight is about 10-15 kg. This is the best variety suitable for chips making. Nendran is susceptible to bunchy top disease and banana bract mosaic virus (Radha and Mathew 2007).

Venugopal (2008) described that Nendran banana have excellent fruit quality, multiple use and sustainable income, due to which Nendran is most popular commercial cultivar which is most loved by small and marginal farmers of Kerala. Nendran banana is mostly cultivated in homesteads and in well-drained rice fields.

2.3. Tissue culture banana

In banana, the difficulty to obtain large number of uniform disease free plants with high yield potential by the conventional propagation using suckers, this technique is a one of important limiting factor for increasing the productivity of banana. Staggered flowering (variability in time of flowering) is another problem with conventional propagation technique. Tissue culture technology allows rapid production of disease free, uniform and high quality planting material from single plant showing good genetic potential with respect to season and weather (Sheela and Nair, 2006).

Success in micro propagation of banana through shoot tip culture or meristem culture was standardized (Bower and Fraser, 1982; Swamy *et al.*, 1983). Major problem of micro propagated plantlets are high mortality initially during transfer from lab to direct field condition due to altered environmental factors (temperature, light intensity and humidity) and biotic stress like soil micro flora. Therefore, to reduce high mortality and to increase the survival and growth of plantlets and successful establishment in field condition, hardening process is essential (Deb and Imchen, 2010).

2.4. Tissue culture in Nendran banana

Though Nendran is the leading variety of Kerala, not many studies have been conducted for development of protocol for *in vitro* culture. Study conducted by Jayasree *et al.* (2018) for optimization of media for the meristem culture of five Nendran ecotypes, revealed that mixture of BAP (0.5 mgL^{-1}) and NAA (0.1 mgL^{-1}) in MS media is the best media for micropropagation of Nendran banana.

2.5 Role of nitrogen sources in tissue culture

Nitrate and ammonium ions are the most common inorganic nitrogen sources used as mineral salts in tissue culture media for *in vitro* propagation (Murashige and Skoog, 1962). Formation of adventitious bud is affected by ratio of KNO_3: NH_4NO_3 in micro propagation of some fruit trees (Li and Han, 2003). Different forms and their proportion of nitrogen in the culture media alter the endogenous levels of cell metabolites as well as of proteins, organic acids, and plant hormones (Preece, 1995).

Formation of adventitious bud is affected by the ratio of NH_4^+: NO_3. When supplied with proper concentration of sucrose and growth regulators (NAA, IBA), modified basal medium with KNO_3 as sole nitrogen source at the concentration of 17.80 to 19.78 mmol/l could significantly improve the quality of rooted plantlets (Yuanli *et al.*, 2005). The effects of different N-sources (KNO_3 + NH_4NO_3 = control, KNO_3, NH_4NO_3 and $NH_4H_2PO_4$) on growth and nutritional status of apple rootstock MM 106 shoots cultured *in vitro* was studied. In comparison with all the other treatments, control explants grown on MS medium containing KNO_3 + NH_4NO_3 had the highest fresh mass, chlorophyll content, net photosynthetic rate, transpiration rate, and stomatal conductance (Sotiropoulos *et al.*, 2005).

Explants of the apple rootstock MM106 *in vitro* treated with KNO_3 + NH_4NO_3 (control) produced the highest fresh mass (0.72g/plant) while those treated with $NH_4H_2PO_4$ the lowest fresh mass (0.58g/plant) (Sotiropoulos *et al.*, 2005). The most favourable ammonium to nitrate ratio for adventitious bud

regeneration was between 1:2 and 1:4 for nitrogen level balancing that of ½ MS nutrients. The maximum weight was recorded in the medium with a 1:4 ammonium to nitrate ratio, a result at least partly related to the number of buds differentiated on this medium, which was highest (Nowak *et al.*, 2007).

The better uptake of NH_4^+ at high pH causes medium acidification, which in turn results in the more uptake of NO_3^- and become greater in medium pH (Martin and Rose, 1975). Reinbothe *et al.* (1990) studied that the optimum ratio of NO_3^- to NH_4^+ was 11:1 in the production of somatic embryos from suspension cultures of *Digitalis lanata*. Mixture of nitrate and ammonium is considered to be a complete source of nitrogen for tissue culture (Kirby *et al.*, 1987).

Sathyanarayana and Blake (1994) observed that nitrogen in the mixed form of ammonium and nitrate had shown a higher percentage of rooting than either ammonium or nitrate supplied as a single source of nitrogen in MS media. Compared with different nitrogen sources, ammonium nitrate was the best nitrogen source for bud differentiation in callus tissue of *Dioscorea deltoidea.* Ammonium source of nitrogen is essential for pro embryo initiation in callus tissue after that it needs nitrate source of nitrogen for the formation of embryoids and shoot bud (Singh, 1978).

2.6 Role of acclimatization media

Acclimatization is the last step in *in vitro* culture. The plantlets of tissue culture are very delicate; hence, they are not planted in the field without hardening in poly house. For that, particular practice is required for maximization of survival and growth of plantlets during acclimatization, tissue culture plantlets have very divergent leaf anatomy and physiology and therefore, require hardening process in order to transfer from lab to field conditions (Pierik, 1987). A large number of factors directly or indirectly affect the survival and acclimatization of plantlets under *ex vitro* condition (Pospíšilová *et al.*, 1999). Initial root establishment in the potting mixture is a very essential factor of *in vitro* grown plantlets during hardening stage (Vasane and Kothari, 2006).

Study conducted on primary hardening of red banana, with the application of different potting media like cocopeat, sand and vermicompost showed that cocopeat was superior with high survival per cent (95), plantlet height (5.58 cm), plantlets diameter (4.59 mm), number of leaves (3.20), primary roots (5.20), secondary roots (25.50) and length primary of roots (5.18 cm) than sand and vermicompost (Uzaribara *et al.*, 2015). Primary hardening of banana *cv* Malbhog plantlets grown under different potting media cocopeat, soil and sterile soilrite and their proportion showed that cocopeat was the best with respect to survival per cent (89), plantlet height (7.98 cm), girth (2.20 cm), number of leaves (4.20), leaf length (6.10 cm) and leaf width (4.12 cm) compared with soil and soilrite and their proportions (Bharati *et al.*, 2018).

In nursery stage, potting media play important role in growth and development of tissue cultured plantlets. Primary hardening of different banana cultivars like Grand Naine, Mahalaxmi, Shrimanti and Basrai, with different potting media like coco peat, vermiculite and mixture of clay sand and organic manure showed that coco peat was the best medium with respect to survival per cent and plantlet height in all cultivars compared with other potting media (Patel *et al.*, 2015).

Saraswathi *et al.* (2014) at NRC Banana Tiruchirapalli conducted trial on primary hardening of tissue cultured banana *cv* Udhayam (ABB), observed that combination of cocopeat and vermiculite recorded maximum number of roots (4.25) and root length (8.70 cm) compared with other treatments. Effect of different media like red soil, sand, FYM (Farm yard manure), vermicompost and cocopeat and different proportion of these media were invesigated on secondary hardening of red banana. Survival per cent was 100 in all media but mixture of red soil : sand : cocopeat (1:1:1) shown best result in terms of plantlet height (20.50 cm), plantlet diameter(11.60 cm), number of leaves (5.80), length of leaves (15.43), width of leaves (6.47 cm), number of primary roots (12.30) and secondary roots (331.0) compared with other media mixtures. But length of primary root was highest in media containing red soil: sand: FYM (1:1:1) (Uzaribara *et al.*, 2015).

Two types of media, i.e., light textured media like vermicompost and coir pith and heavy textured media like red earth + sand and red earth + perlite were assessed during primary hardening of silk banana 'Nanjanagud Rasabale'. Survival per cent was 100 in both light and heavy textured media. Light textured media coir pith shown highest shoot length, shoot girth, number of leaves, width of leaf and root length followed by vermicompost (Waman *et al.*, 2015).

Desai *et al.* (2018) studied the effect of various hardening mixtures on primary hardening of pomegranate cultivar Bhagwa plantlets. Among the various potting mixture coco peat was the best media, which resulted in highest number of shoots (4.80), length of shoot (12.70 cm) number of leaves (32.00) number of roots (10.40) length of roots (6.10 cm) than other hardening mixtures like soil, organic manure, perlite and vermiculite.

Effect of different potting media on the survival and average increase in height of banana cv. Meitei Hei was analysed by Larlisangha *et al.* (2013). Among different potting media like river sand, FYM and vermicompost, they found that river sand gave 100% survival and increased the height in plantlets grown under combination of soil and vermicompost (4.11 cm).

2.7Role of biotization in secondary hardening

Biotization (application of beneficial micro-organisms to plantlets) is an emerging trend in tissue culture technology to increase survival, growth and development and to enhance resistants to biotic and abiotic stress of micropropagated plantlets during hardening (Nowak, 1998). Various plant growth promoting microorganisms like Arbuscular Mycorrhizal Fungi (AMF), nitrate and phosphate solubilizers etc. have been reported by various scientists in hardening phase on different crops. Treatment of *in vitro* grown plantlets with AMF induced tolerance to transplant stress and increased growth and mineral nutrient status of plantlets (Gianinazzi *et al.*, 1989).

Micropropagated avocado *(Persea americana* Mill.) plantlets were treated with Arbuscular mycorrhizal fungus *Glomus fasciculatum* with substrate media as

soil and sand (1:1). Significant improvement was noticed in survival (72.5), shoot weight (9.3 g), number of leaves (16.1), dry weight of shoot (5.2 g) and root (3.0g) and increase in nutrient status in plantlets particularly N (86.1mg/plant), P (4.8 mg/plant) K (86.1 mg/plant) compared to non treated plantlets (Vidal *et al.*, 1992).

Vasane and Kothari (2006) conducted a study on optimization of secondary hardening process of banana plantlets variety Grand Naine with three different biofertilizers (Arbuscular mycorrhizal fungus, *Azotobacter* and *Aspergillus*). Plantlets treated with AMF, *azotobacter* and *Aspergillus* have showed best results in terms of plantlet height (18.44 cm), plantlet diameter (6.45 cm), number of leaves (7.84), length of leaves (22.00), width of leaves (9.32 cm), number of primary roots (18.00), maximum root length (38.60 cm) and weight of roots (34.20 g).

Inoculation of bacterial culture supernatant (*Pseudomonas flourescens* R68) during hardening of micropropagated banana cultivar Grand Naine in weekly intervals for five weeks. Plantlets inoculated with *Pseudomonas flourescens* R68 bacterial culture showed significant increase in leaf number (7.10), leaf length (14.76 cm), leaf width (6.34 cm), plant height (23.77 cm), number of roots (13.20), maximum root length (32.30cm), fresh weight of plant (17.44 g) and fresh root weight (12.39 g) compared with plantlets not treated with any beneficial microorganisms (Suada *et al.*, 2015).

Effects of Arbuscular mycorrhizal fungi on plant, leaf and root characteristics of acclimatized micropropagated banana plantlets cultivar Pacovan, were increased plant height, shoot fresh weight and dry weight, leaf area and root fresh weight and dry weight over non inoculated plantlets (Yano-Melo *et al.*, 1999).

Plantlets treated with AMF showed enhanced nutrient uptake in shoots, there was increase the concentration of nutrients like P (0.0439 g), K (3.75 g), Mn (82.4 mg), Cu (21.4 mg) and Zn (32.2 mg) than non inoculated plantlets (Yano-Melo *et al.*, 1999).

Krishna *et al.* (2005) at IARI, New Delhi conducted trial on treatment of seven different Arbuscular mycorrhizal fungi species with*in vitro* grown grape vine plantlets during hardening stage. Among the seven different treatments, *Acaulospora scrobiculata* was shown best result in terms of increased chlorophyll content than control.

Biotization with *Pseudomonas fluorescens* to improve survival rate, shoot system, root system, chlorophyll content and micro nutrient uptake in micropropagated *Chlorophytum sp.* was reported by Gosal *et al.*, (2010). Biotized plantlets provided best result than non biotized plantlets in terms of shoot length, shoot dry weight, root length, root dry weight and there was increased chlorophyll and micro nutrient contents (Mn, Zn, Cu and Fe). Thaker and Jasrai (2002) reported in micropropagated banana plantlets inoculated with AMF during hardening period, exhibited the progressive increase in chlorophyll content, leaf length, leaf width and leaf area over control.

Micropropagated plantlets of banana cultivars Dwarf Cavendish and Robusta were inoculated during the secondary hardening stage with Arbuscular Mycorrhizal Fungi (AMF). All the AM fungi increased the growth of both the cultivars, but the positive influence of the fungi was more evident for Dwarf Cavendish plantlets inoculated with *Glomus fasciculatum* which resulted in higher plantlet height (60.7%) and leaf area (2.2 times) besides increasing the pseudo stem girth (39.6%) and shoot biomass production and this also increased the shoot P concentration than control (Mathews *et al.*, 2002).

Influence of beneficial microorganisms (*Azospirillum brasilense*) on *in vitro* grown tea (*Camellia sinensis*) plants during hardening stage, plantlets treated with *Azospirillum brasilens*e exhibited enhanced survival rate and progressive growth in terms of shoot length, number of leaves, number of roots and fresh weight of plants over control and there was improvement in nutrient concentration particularly macro nutrients 2.41% N, 0.15% P and 1.19% K than non inoculated plants (control) (Thomas *et al.*, 2010).

Azotobacter chroococcum is a beneficial micro organism for nitrogen fixation in plants and widely used in acclimatizing micropropagated plantlets. There was trial on *in vitro* raised pineapple plantlets which were treated with *Azotobacter chroococcum* during acclimatization for four month. Treated plantlets exhibited highest plant height, plantlet fresh and dry weight and root length and more total chlorophyll content over control and increased mineral composition particularly macro nutrients (N, P and K) and micro nutrients (Mn, Zn, Cu and Fe) compared with control (Rodrı´guez *et al.*, 2013). Madaan *et al.* (2013) reported beneficial effect of *Azotobacter chroococcum* on micropropagated banana cultivar Grand Naine during hardening stage under glass house for two months, observed that maximum survival rate (84.6%), root length (15.5 cm), shoot length (33.3 cm), root dry weight (2.0 g), and shoot dry weight (3.6 g).

A study was conducted on effect of *Azotobacter chroococcum* on *in vitro* grown pineapple plantlets during acclimatization, the bacterial treated plantlets exhibited best result in terms of plantlet height, root length and plant fresh and dry weight besides control (González *et al.*, 2011).

A study on secondary hardening of micropropagated banana cultivar Grand Naine plantlets with different bio-fertilizers like nitrogen fixer (*Azotobacter*) phosphate solubilizing microbes (*Aspergillus*) and AMF (*Glomus fasciculatum*) revealed that plantlets treated with bio-fertilizers resulted in progressive development of plantlets, maximum survival rate about 98.5% and increased plant height (23.6 cm), pseudostem girth (5.2 cm), improvement in leaf characters like number of leaves (8.4), leaf length (25.1 cm), leaf width (12.6 cm) and chlorophyll content (5.6 mg/g) and well developed root system in number of primary roots (11.9) and maximum root length (29.0 cm) compared with control (Vasane *et al.*, 2010).

Azospirillum significantly improved the performance of *in vitro* grown plantlets during acclimatization stage. There was study was on the effect of *Azospirillum brasilense* Sp245on the micropropagation of three fruit rootstocks: Mr.S 2/5plum (*Prunus cerasifera_P. spinosa*), GF 677 hybrid (*Prunus persica_P. amigdalus*), and MM 106 apple (Northern Spy_M1) was evaluated. Progressive

growth in bacterial treated plants was there with increased stem length and number of nodes 75% and 65% respectively than control (Vettori *et al.*, 2010).

Kaur *et al.* (2011) conducted a study on acclimatization of the *in vitro* raised plantlets of *Tylophora indica* with different potting media (soil and vermicompost) and biofertilizers (*Azotobacter* and *Pseudomonas*) in different mixture. Among different treatment the mixture of soil : vermicompost : *Azotobacter* (nitrogen fixer) : *Pseudomonas* (phosphate solubilizer) (1:1:1:1) showed highest survival rate about 92%, shoot length (19 cm) and number of leaves (31).

Arbuscular mycorrhizal fungi are known to form symbiosis with many fruit crops. Microbial associations with plant roots are beneficial and positively stimulate rhizosphere expansion which results into increased absorption of water and immobile nutrients, drought tolerance and reduced disease incidence. Plantlets inoculated with the AMF strain *Glomus mosseae* showed the maximum per cent survival (90.40), plant height (24.96 cm), root length (23.42 cm) and fresh weight (7.86 g) compared with control (Singh *et al.*, 2012).

Vasane and Kothari (2008) conducted study on integrated approach on secondary hardening of banana cv. Grand Naine. With an objective of enhancing survival and growth of the plantlets, different bio-fertilizers like AMF with different concentration (V_1 = 5 g, V_2= 10 g and V_3 = 15 g/ plant), phosphate solubilizers (*Asperigillus*) (P1=0.5 g, P2 = 1 g, P3 = 1.5 g/ plant) and nitrogen fixer (*Azotobacter*) (N) were applied. After secondary hardening, macro and micro elements were analyzed. Analysis has shown that there was no significant effect in N uptake by plants but there was significant effect for the uptake of P, K and micronutrients like Mn, Cu, Zn and Fe.

Materials and methods

3. MATERIALS AND METHODS

The present study on "Media improvement for *in vitro* culturing and hardening of tissue culture banana cv. Nendran" was conducted at the Department of Pomology, College of Agriculture, Padannakkad and Regional Agricultural Research Station, Pilicode, Kasargod (Dt.) during the period of 2017-2019.

3.1. EXPERIMENTAL SITE:

Experiment 1 was conducted at tissue culture laboratory, RARS, Pilicode. Experiment 2 and 3 were conducted at Horticulture Nursery, primary hardening was done in mist chamber and secondary hardening was done in shade house at RARS, Pilicode, located at $12^{0}12'$N latitude and $75^{0}10'$E longitude and at an altitude of 15m above mean sea level.

3.2. AGROCLIMATIC CONDITION:

Experiments were conducted at Regional Agricultural Research Station, Pilicode, Kasargod, which comes under tropical humid region. It comes under NARP Northern zone of the state of Kerala and AZ 109th climatic zone of the country.

3.3. EXPERIMENTAL MATERIAL:

For *in vitro* culture experiment of banana cultivar Nendran, sword suckers (1.50 to 2.0 kg) were collected from banana orchard, RARS, Pilicode. For conducting primary hardening, various potting mixtures cocopeat, vermicompost and sand were collected and sterilized using autoclave and used. Different bio-fertilizers like Arbuscular Mycorrhizal Fungi (AMF), *Pseudomonas fluorescens* and *Azospirillum* were collected from Instructional farm, College of Agriculture, Padannakkad and *Azotobacter* was collected from Department of Agricultural Microbiology, College of Horticulture, Vellanikara, Thrissur and treatments were imposed during secondary hardening.

3.4. EXPERIMENT: 1

Effect of nitrogen sources on shoot and root regeneration of tissue cultured Nendran banana.

Design of experiment: CRD

Replications: 4

Treatments: 5 (Six subculture per treatment)

T_1 - MS media (control) (NH_4NO_3 1650mg/l and $KNO_3$1900mg/l)

T_2 - Modified MS media (2850mg/l KNO_3 + 825mg/l NH_4NO_3)

T_3 - Modified MS media (1900mg/l KNO_3)

T_4 - Modified MS media (3800mg/l KNO_3)

T_5 - Modified MS media (1900mg/l KNO_3 +1361mg/l $(NH_4)_2H_2PO_4$)

Required growth regulators 2ppm BAP for initiation, 4ppm BAP for sub culturing, 0.5 IBA along with half the strength of respective media for rooting was used along with the treatments.

3.4.1. Media preparation

The four different treatments based on the ionic composition of MS media, where in the changes were in terms of KNO_3, NH_4NO_3 sources and their content. One treatment NH_4NO_3 was excluded and $(NH_4)2H_2PO_4$ was included as the ammonium source. For all treatments other macronutrients, micronutrients, iron stock, vitamins, myoinositol, sucrose were same as normal MS media(Chemical Composition of MS medium and stock given in Annexure 1) and agar was added at the rate of 6.5g/ L, pH was adjusted to 5.7. The medium was sterilized by autoclaving at 121 ^{0}C for 15 minutes.

3.4.2. *In vitro* culturing

For this experiment, shoot tips excavated from sword suckers (1-2 kg) were used as explant. For each treatment fifty explants were inoculated, totally 250 sword suckers were used for removal of explants. Shoot tips were cleaned with tap water to remove soil particles. Primary cutting of suckers about 8 - 10 cm was done then they were dipped in flask containing soap water. Secondary

cutting of explants was done into 4 – 5 cm length. They were then dipped in flask containing sterilized water. After that, explants were washed with sterilized water for three times and treatment of explants with 5% SAAF (5g in 1000ml of distilled water) for one hour time was done. After this treatment washed with ultra-filtered water four times, then clean explants were taken to laminar air flow chamber. In laminar air flow chamber explants were treated with 0.8% mercuric chloride for 10 minutes for complete surface sterilization of explants. Sterilized explants were again cut into 1 – 1.5 cm length for final use for inoculation in to bottle. Initiation of culture took 30 days then subsequent subculture was done for every 21 days interval for total six subculture. After that plants were put for rooting which lasted for about 45 days for complete rooting.

3.4.3. Observations

The important biometric observations were made after completion of rooting stage.

3.4.3.1. Number of multiple shoots

Initial two sub cultures did not result in any multiple shoot. After third subculture, multiple shoots are formed, every 4^{th}, 5^{th} and 6^{th} subculture number of multiple shoots are recorded and expressed as number of multiple shoots per explant.

3.4.3.2. Shoot length (cm)

After rooting stage bottles containing multiple shoots with roots, plantlets were taken out from the bottle and washed with tap water, cleaned it well. The shoot length was measured with the help of scale, individual plantlet was measured from base of plantlet to growing tip of the plantlet, for every replication four plantlets were measured, average was computed and expressed in centimeter.

3.4.3.3. Number of leaves

The number of leaves from four selected plantlets from each replication was recorded.

3.4.3.4. Fresh weight of shoot (g)

The fresh weight of shoot from selected plantlets separated from roots and individual shoot was weighed with electronic balance and expressed in gram per plant.

3.4.3.5. Dry weight of shoot (g)

The shoots after fresh weight were kept in hot air oven at 60 ^{0}c for drying until constant weight was obtained. The dry weight of each shoot was weighed after drying and stated in gram per plantlet.

3.4.3.6. Number of roots

From four selected plantlets per replication, the numbers of roots per plantlet were counted and mean was calculated and expressed in number per plantlet.

3.4.3.7. Root length (cm)

After counting number of roots and selected maximum length root and it was measured with help of scale and expressed in centimeter.

3.5. EXPERIMENT: 2

Effect of different media on primary hardening of tissue cultured Nendran banana.

Primary hardening studies were conducted in mist chamber of Horticulture nursery, RARS, Pilicode. For primary hardening, different media, sand, cocopeat and vermicompost were sterilized by autoclaving at 121^0C for15 minutes. During primary hardening, culture vessels or bottles were taken out from culture room, and the plantlets were washed with tap water for removing the adhering agar medium. After cleaning plantlets were subjected to fungicidal treatment with 20% SAAF for period of one hour. After fungicidal treatment plantlets are transferred to pro trays. Pro trays of 98 cells were used for filling different media and their combinations. Pro trays were kept in mist chamber and water supplied through misting daily three times for 15 days. Relative humidity about more than 90% was maintained. During primary hardening each replication 26 plantlets, 80 plantlets per treatment and in total 720 plantlets for nine treatments were used for primary hardening.

Design of experiment: CRD

Replications: 3

Treatments: 9

T_1: Sand (control)

T_2 - Cocopeat

T_3 - Vermicompost

T_4 - Sand + Cocopeat (1:1)

T_5 - Sand +Vermicompost (1:1)

T_6 - Cocopeat +Vermicompost (1:1)

T_7 - Sand + Cocopeat + Vermicompost (1:1:1)

T_8 - Sand + Cocopeat + Vermicompost (1:2:1)

T_9 - Sand + Cocopeat + Vermicompost (1:1:2)

3.6. EXPERIMENT 3:

Effect of bio- fertilizers for secondary hardening of tissue cultured Nendran banana.

After 15 days of primary hardening, plantlets were transferred to secondary hardening process. During secondary hardening, different bio-fertilizers (AMF, (PSB) *Pseudomonas fluorescens*, *Azospirillum* and *Azotobacter*) were used with basic potting mixture sand, soil, FYM, cocopeat and vermicompost. The container used was 8 × 6 inch size of polybags. During transplanting 10 g of powdered form of bio-fertilizers were applied to root zone of per plantlet. Secondary hardening process was conducted under shade net house and 60 – 70% relative humidity was maintained.60 plantlets per treatment, for each replication 20 plantlets, totally 540 plantlets were used for this experiment.

Design of experiment: CRD

Replications: 3

Treatments: 9

T_1 - AMF (Arbuscular mycorrhizal fungi)

T_2 - *Azotobacter*

T_3 - *Azospirillum*

T_4 - PSB (Phosphate solubilizing bacteria)

T_5 - AMF + *Azotobacter*

T_6 - AMF + *Azospirillum*

T_7 - PSB + *Azotobacter*

T_8 - PSB + *Azospirillum*

T_9 - Potting mixture only (Control)

3.6.1. OBSERVATIONS

Duration of primary hardening was 15 days and that of secondary hardening was 60 days. Observations were at 15 days for primary hardening and 30 and 60 days for secondary hardening. Similar observations were made except NPK and micro nutrients content at 60 days after transplanting.

3.6.1.1. Percentage of survival

Survival percentage was calculated by following formula

Survival % of plantlets = $T - M_P / T \times 100$

Where,

T = Total number of rooted plants

Mp = Mortal plants

3.6.1.2. Plant height (cm)

The plant height was measured from base of the pseudostem to growing tip of the plant.

3.6.1.3. Girth of pseudostem (cm)

Collar region of shoot was used for measurement pseudostem girth and it was expressed in centimeter.

3.6.1.4. Leaf length (cm)

Leaf length was measured from petiole to tip of the leaf.

3.6.1.5. Leaf width (cm)

Leaf width was measured from widest portion of the leaf

3.6.1.6. Leaf area (cm^2)

Leaf area of all leaves of selected plantlets was calculated using portable leaf area meter, LI-COR Model LI-3000A model and expressed in square centimeter per plantlet.

3.6.1.7. Number of leaves

The photo synthetically active leaves were counted and expressed as number of leaves per plantlet.

3.6.1.8. Number of primary roots

Plants were taken out from polybags and cleaned the root portion with tap water then number of primary roots were counted and expressed as number of primary roots per plantlet.

3.6.1.9 Length of primary roots (cm)

After counting primary roots, root with maximum root length was selected and measured with scale and expressed in centimeter.

3.6.1.10 Number of secondary roots

Secondary roots in individual primary root were counted and expressed in number of secondary roots per plant.

3.6.1.11 Weight of roots (g)

Fresh roots were weighed with help of electrical balance and expressed as gram per plant.

3.6.1.12. Chlorophyll content (mg g^{-1})

Third leaf from the top of of the plant was collected and estimation of total chlorophyll content in leaf by DMSO method and readings were taken from spectrophotometer at 663, 652 and 645 nm (Hiscox and Israel. 1979) and it expressed as milligram per gram of leaf sample.

3.6.1.13. Shoot fresh weight (g)

Fresh weight of shoot was weighed through electrical balance and it was indicated in gram per plant.

3.6.1.14. Shoot dry weight (g)

After taking fresh weight, shoots were kept in hot air oven at 60 ^{0}c temperature until constant weight is obtained. After drying shoots are weighed and it was indicated in gram per plant.

3.6.1.15. NPK and micro nutrient content in plants (60 DAP)

After secondary hardening (60 days after transplanting), plant samples are collected and dried in hot air oven, after drying plant samples are used for estimation of nutrients N, P,K, Mn, Zn, Cu and B as per the standard procedures as given in the table 1.

3.8 STATISTICAL ANALYSIS

The data collected from lab experiment and green house experiments were statistically analyzed and tasted significance level by using WASP 2.0 software prepared by ICAR Research Complex for GOA.

Table 1: Analytical methods followed for plant analysis

S. No	Parameters	Method	Reference
1.	Total N	Modified Kjeldhal digestion method	Jackson (1958)
2.	Total P	Vanadomolybdate yellow colour method	Piper (1966)
3.	Total K	Flame photometry	Jackson (1958)
4.	Total Zn	Atomic absorption spectroscopy	Emmel *et al.*, (1977)
5.	Total B	Azomethine- H colorimetric method	Bingham (1982)
6.	Total Fe	Atomic absorption spectroscopy	Piper (1966)
7.	Total Cu	Atomic absorption spectroscopy	Emmel *et al.* (1977)
8.	Total Mn	Atomic absorption spectroscopy	Piper (1966)

Plate 1: General view of Thalassery Nendran banana plant

Plate 2: Primary hardened tissue cultured Nendran banana plants

T_1: Sand (control), T_2 – Cocopeat, T_3 – Vermicompost, T_4 - Sand + Coco peat (1:1), T_5 - Sand +Vermicompost (1:1), T_6 - Cocopeat +Vermicompost (1:1), T_7 - Sand + Cocopeat + Vermicompost (1:1:1), T_8 - Sand + Cocopeat + Vermicompost (1:2:1) and T_9 - Sand + Coco peat + Vermicompost (1:1:2)

Plate 3a: Secondary hardened tissue cultured Nendran banana plants at 30 DAT

T_1 - AMF (Arbuscular rmycorrhizal fungi) T_2 – *Azotobacter* T_3 – *Azospirillum*

T_4 - PSB (Phosphate solubilizing bacteria) T_5 - AMF + *Azotobacter* T_6 - AMF + *Azospirillum* T_7 - PSB + *Azotobacter* T_8 - PSB + *Azospirillum*

T_9 - Potting mixture only (Control)

Plate 3b: Secondary hardened tissue cultured Nendran banana plants at 60 DAT

T_1 - AMF (Arbuscular rmycorrhizal fungi) T_2 – *Azotobacter* T_3 – *Azospirillum*

T_4 - PSB (Phosphate solubilizing bacteria) T_5 - AMF + *Azotobacter* T_6 - AMF + *Azospirillum* T_7 - PSB + *Azotobacter* T_8 - PSB + *Azospirillum*

T_9 - Potting mixture only (Control)

Results

4. RESULTS

4.1. EXPERIMENT: 1

Effect of nitrogen sources on shoot and root regeneration of tissue cultured Nendran banana

In this experiment the basal MS medium was modified with altering KNO_3 and NH_4NO_3 concentration. $(NH_4)_2H_2PO_4$ is another nitrogen source for ammoniacal nitrogen for replacing NH_4NO_3. Totally there were four different treatments including modified MS media wherein the macro nutrients were halved and normal MS medium as control. The experiment was carried out with an objective of finding the best media for *in vitro* culture of banana cultivar Nendran (ABB) which could give results on par with control *ie*; Normal MS Medium. The observed results are furnished hereunder.

4.1.1. Number of multiple shoots

The results of effect of nitrogen sources on number of multiple shoots per explants presented in Table 2. After 4^{th}, 5^{th} and 6^{th} subculture, number of multiple shoots per explant were counted as multiple shoot production did not occur in first three sub cultures. Maximum number of multiple shoots per explant was recorded in T_1 (control) followed by T_2 and T_5. Multiple shoots were not observed in T_3 and T_4 which showed negative results with regard to number of multiple shoots per explants. Plate 4a shows number of multiple shoots per explant of treatments and control. Plate 4b shows number of multiple shoots after rooting stage.

4.1.2. Number of leaves

Number of leaves was counted after rooting stage. Highest number of leaves was observed in T_2 which was on par with T_1 (control) followed by T_5. T_5 has shown lowest number of leaves. There was no result in T_3 and T_4. Data on number of leaves is presented in Table 3.

4.1.3. Shoot length

Shoot length was maximum in T_1 (control) followed by T_2 and T_5. T_3 and T_4 recorded negative results regarding shoot length. Data regard to shoot length given in Table 3.

4.1.4. Shoot fresh and dry weight

Results of Shoots fresh and dry weight are given in Table 3. T_1 (control) has recorded increased shoot fresh and dry weight followed by T_2 and T_5. T_3 and T_4 have no data.

4.1.5. Number of roots and root length

Number of roots counted and maximum root length was measured. Maximum number of roots and root length is found in T_1 (control) followed by T_2 and T_5. T_3 and T_4 showed no results. Number of roots and root length are furnished in Table 3.

Table 2: Effects of treatments on number of multiple shoots of *in vitro* cultured banana cv. Nendran

Treatments	Number of multiple shoots / explant		
	4th subculture	5th subculture	6th subculture
T_1	2.83	2.60	2.40
T_2	1.20	1.53	1.13
T_3	0.00	0.00	0.00
T_4	0.00	0.00	0.00
T_5	0.62	0.63	0.35
SE (m)	**0.02**	**0.02**	**0.01**
CD (0.05)	**0.32**	**0.32**	**0.25**

Table 3: Effects of treatments on shoot, leaf and root characters of *in vitro* grown banana cv. Nendran

Treatments	Shoot length (cm)	No of leaves	No of roots	Root length (cm)	Fresh weight of shoot (g)	Dry weight of shoot (g)
T_1	7.18	2.28	6.96	17.56	2.48	0.16
T_2	6.10	2.40	4.23	21.68	1.50	0.08
T_3	0.00	0.00	0.00	0.00	0.00	0.00
T_4	0.00	0.00	0.00	0.00	0.00	0.00
T_5	3.38	1.70	2.82	17.08	0.68	0.04
SE (m)	**0.03**	**0.03**	**0.02**	**0.35**	**0.01**	-
CD (0.05)	**0.46**	**0.42**	**0.40**	**1.49**	**0.19**	**0.02**

Note:

T_1: MS media (control) (NH_4NO_3 1650mg/l and $KNO_3$1900mg/l)

T_2: Modified MS media (2850mg/l KNO_3 + 825mg/l NH_4NO_3)

T_3: Modified MS media (1900mg/l KNO_3)

T_4: Modified MS media (3800mg/l KNO_3)

T_5: Modified MS media (1900mg/l KNO_3 +1361mg/l $(NH_4)_2H_2PO_4$)

Plate 4a: Multiple shoots of *in vitro* cultured Nendran banana

T_1

T_2

T_5

Plate 4b: Rooting stage of *in vitro* cultured Nendran banana

4.2. EXPERIMENT: 2

Effect of different media on primary hardening of tissue cultured Nendran banana

This experiment involved imposing treatments with three different potting media in three different proportions resulting in nine treatments. Trial was conducted to find out which is best treatment (potting media) for primary hardening of tissue cultured Nendran banana. The results observed on various characteristics are presented below.

4.2.1. Survival percentage

Survival of plantlets during primary hardening was significantly influenced by the potting media (Table 4). Treatments T_1 (control), T_2, T_6, T_7 and T_9 recorded 100 % survival. Treatments T_4 and T_8 showed more than 95 % survival but T_3 and T_5 recorded minimum survival. Among the treatments T_5 has shown lowest survival (18%).

4.2.2. Plant height

Plant height was recorded for every treatments and control (Table 4). Among the treatments, T_6 recorded highest plant height (8.53 cm) which was on par with T_1 (control) (8.27 cm) and T_2 (8.17 cm). Treatment T_3 (5.30 cm) and T_5 (5.33 cm) have shown lowest plant height compared with other treatments.

4.2.3. Pseudostem girth

Pseudostem girth of plantlets has shown significant differences among different treatments (Table 4). Girth of pseudostem was highest in treatment T_6 (1.73 cm) which was on par with T_9 (1.57 cm) and T_1 (1.53 cm) (control). Treatment T_3 recorded minimum girth 1.13 cm.

Table 4: Effects of acclimatization media on survival percentage, plant height and pseudostem girth of primary hardened banana cv. Nendran plants

Treatments	Survival percentage (%)	Plant height (cm)	Pseudostem girth (cm)
T_1 (Sand) (control)	100.00 (89.45)	8.27^{ab}	1.53^{ab}
T_2 (Cocopeat)	100.00 (89.45)	8.17^{abc}	1.50^{b}
T_3 (Vermicompost)	47.33 (43.47)	5.30^{f}	1.13^{d}
T_4 (S + C 1:1)	95.00 (77.12)	7.53^{d}	1.43^{b}
T_5 (S + V 1:1)	18.00 (25.09)	5.33^{f}	1.17^{cd}
T_6 (C + V 1:1)	100.00 (89.45)	8.53^{a}	1.73^{a}
T_7 (S + C + V 1:1:1)	100.00 (89.45)	6.83^{e}	1.47^{b}
T_8 (S + C + V 1:2:1)	95.67 (78.10)	7.83^{cd}	1.37^{bc}
T_9 (S + C + V 1:1:2)	100.00 (89.45)	8.10^{bc}	1.57^{ab}
SE (m)	0.46	0.02	0.01
CD (0.05)	1.70	0.42	0.21

4.2.4. Number of leaves

Numbers of leaves were significant differences among the treatments (Table 5). Treatment T_6 recorded maximum number of leaves (4.00) which was on par with T_1 (3.67) (control), T_9 (3.67) and T_4 (3.33). Minimum numbers of leaves were observed in T_5 (2.00).

4.2.5. Leaf length and leaf width

The observations on leaf length and leaf width revealed that treatments are significantly different from each other. Potting media positively influenced the leaf length and leaf width. Highest leaf length was recorded in T_6 (9.83 cm) which was significantly different from other treatments followed by T_7 (8.67 cm), T_9 (8.67 cm), T_4 (8.23 cm) and T_8 (8.00 cm). Lowest leaf length was observed in T_5 (6.50 cm). Maximum leaf width was recorded in treatments T_6 (2.70 cm) and T_9 (2.60 cm) followed by T_8, T_7, T_4, T_1 (control) and T_2. But T_5 recorded

minimum leaf width 1.43 cm followed by T_3. Results of leaf length and leaf width are presented in Table 5.

4.2.6. Leaf area

Leaf area was significantly affected by different hardening media (Table 5). Treatment T_6 was the best which recorded maximum leaf area of 49.37 cm^2 followed by T_9, T_2, T_1 (control) and T_4 whereas T_5 shown lowest leaf area (30.80 cm^2) followed by T_3.

Table 5: Effects of acclimatization media on number of leaves, leaf length, leaf width and leaf area of primary hardened banana cv. Nendran plants

Treatments	Number of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
T_1 (Sand) (control)	3.67^{ab}	8.00^{cd}	2.27^{b}	42.83^{c}
T_2 (Cocopeat)	3.00^{bc}	7.70^{cd}	2.27^{b}	44.76^{bc}
T_3 (Vermicompost)	2.67^{cd}	7.50^{d}	1.67^{c}	36.90^{d}
T_4 (S + C 1:1)	3.33^{abc}	8.23^{bc}	2.40^{b}	42.80^{c}
T_5 (S + V 1:1)	2.00^{d}	6.50^{e}	1.43^{d}	30.80^{e}
T_6 (C + V 1:1)	4.00^{a}	9.83^{a}	2.70^{a}	49.37^{a}
T_7 (S + C + V 1:1:1)	3.00^{bc}	8.67^{b}	2.37^{b}	37.67^{d}
T_8 (S + C + V 1:2:1)	2.67^{cd}	8.00^{cd}	2.37^{b}	38.53^{d}
T_9 (S + C + V 1:1:2)	3.67^{ab}	8.67^{b}	2.60^{a}	46.60^{b}
SE (m)	**0.09**	**0.06**	**0.01**	**0.98**
CD (0.05)	**0.73**	**0.61**	**0.19**	**2.49**

4.2.7. Number of primary roots and root length

The data regarding number of primary roots and root length are given in Table 6. Potting media significantly influenced the number of primary roots and root length. More number of primary roots were observed in treatment T_2 (3.67) which was on par with T_6, T_9, T_1 and T_4 while minimum number of primary roots was recorded in T_5 (1.33). With regard to root length, maximum root length was shown in T_6 (13.20 cm) which was significantly on par with T_9 (12.17 cm). These two treatments were significantly higher compared with other treatments whereas lowest root length observed in T_5 (3.00 cm).

4.2.8. Number of secondary roots

The observations on number of secondary roots revealed that hardening media could exert on significant influence, which is presented in Table 6. Treatment T_2, which recorded highest number of secondary roots of 26.00, significantly different than other treatments and followed by T_6 (18.33) which was on par with T_1, T_7, T_9 and T_4. Treatment T_5 showed poor result with lowest number of 1.33 secondary roots.

4.2.9. Weight of roots

The fresh weight recorded is furnished in table 6. Treatment T_2 which recorded highest root weight of 0.38g was significantly the best one. This was on par with T_4 whereas treatments T_5 (0.10 g) and T_3 (0.13 g) have recorded minimum weight.

Table 6: Effects of acclimatization media on number of primary and secondary roots, primary root length and root weight of primary hardened banana cv. Nendran plants

Treatments	Number of primary roots	Length of primary roots (cm)	Number of secondary roots	Weight of roots (g)
T_1 (Sand) (control)	3.00^{ab}	8.50^{e}	17.67^{b}	0.29^{bc}
T_2 (Cocopeat)	3.67^{a}	9.30^{de}	26.00^{a}	0.38^{a}
T_3 (Vermicompost)	1.66^{cd}	5.10^{f}	9.33^{c}	0.13^{e}
T_4 (S + C 1:1)	2.67^{abc}	7.83^{e}	15.33^{b}	0.31^{ab}
T_5 (S + V 1:1)	1.33^{d}	3.00^{g}	1.33^{d}	0.10^{e}
T_6 (C + V 1:1)	3.33^{ab}	13.20^{a}	18.33^{b}	0.29^{bc}
T_7 (S + C + V 1:1:1)	2.33^{bcd}	11.10^{bc}	16.33^{b}	0.29^{bc}
T_8 (S + C + V 1:2:1)	2.33^{bcd}	10.60^{cd}	14.67^{b}	0.17^{de}
T_9 (S + C + V 1:1:2)	3.33^{ab}	12.17^{ab}	15.67^{b}	0.23^{cd}
SE (m)	**0.19**	**0.38**	**2.34**	**0.01**
CD (0.05)	**1.10**	**1.55**	**3.82**	**0.07**

4.2.10. Shoot fresh and dry weight

The effects of treatments on shoot fresh and dry weight are presented in Table 7. Treatment T_6 was the significantly superior from other treatments which recorded highest shoot fresh weight of 3.33 g. T_6 was followed by T_9, T_7, T_2, T_4 and T_1. T_3 has resulted in lowest weight 1.34 g followed by T_5. With respect to shoot dry weight, T_6 recorded highest weight about 0.21 g which was on par with T_2, T_9 and T_7. T_3 had lowest dry weight 0.07 g.

4.2.11. Chlorophyll content

Chlorophyll content was affected by treatments resulting in significant differences among them. The figures regarding total chlorophyll content were given in Table 7. Treatment T_7 was superior one which recorded highest total chlorophyll content of 2.07 mg/g. This was on par with T_4 (1.93 mg/ g). T_1

(control) recorded minimum total chlorophyll content compared with other treatments.

Table 7: Effects of acclimatization media on shoot fresh and dry weight and chlorophyll content in primary hardened banana cv. Nendran plants

Treatments	Shoot fresh weight (g)	Shoot dry weight (g)	Chlorophyll content (mg g^{-1})
T_1 (Sand) (control)	2.46^{c}	0.11^{cd}	1.40^{d}
T_2 (Cocopeat)	2.78^{bc}	0.19^{ab}	1.70^{bc}
T_3 (Vermicompost)	1.34^{e}	0.07^{d}	1.67^{c}
T_4 (S + C 1:1)	2.56^{bc}	0.15^{bc}	1.93^{ab}
T_5 (S + V 1:1)	1.36^{e}	0.08^{d}	1.77^{bc}
T_6 (C + V 1:1)	3.33^{a}	0.21^{a}	1.80^{bc}
T_7 (S + C + V 1:1:1)	2.62^{bc}	0.16^{ab}	2.07^{a}
T_8 (S + C + V 1:2:1)	1.99^{d}	0.10^{cd}	1.63^{cd}
T_9 (S + C + V 1:1:2)	2.91^{b}	0.18^{ab}	1.80^{bc}
SE (m)	0.03	0.01	0.01
CD (0.05)	0.40	0.05	0.23

4.3. EXPERIMENT: 3

Effect of bio-fertilizers during secondary hardening of tissue cultured Nendran banana

The treatments comprised of nine different media enriched with four different bio-fertilizers (AMF, *Pseudomonas fluorescens*, *Azospirillum* and *Azotobacter*) and their combination and control without any bio-fertilizer. Experiment was conducted to find out the best treatment for enhancing survival and growth of plantlets during secondary hardening. Results observed on various growth parameters are presented below.

4.3.1. Survival percentage

With respect to survival percentage, the treatment differences were statistically insignificant. The data are presented in Table 8. After 30 and 60 days of transplanting, survival percentage of 100 % were recorded in all treatments. This indicates that there is no effect of bio-fertilizers on survival percentage during secondary hardening.

4.3.2. Plant height

The data regarding plant height is presented in Table 8. There were significant differences among treatments and control. Treatment T_8 recorded highest plant height of 16.33 cm and 31.63 cm after 30 and 60 days of transplanting respectively. This was on par with T_5, T_7, T_1, T_4 and T_6 during 30 days after transplanting and T_8 only on par with T_5, T_7, T_1, T_4, T_6 and T_3 during 60 days transplanting. Control had shown lowest height in both condition (14.00 cm and 27.30 cm). All treatments were superior over control.

4.3.3. Pseudostem girth

There were significant differences among the treatments and treatments were better than control. After 30 days of transplanting among treatments T_1 has recorded maximum pseudostem girth (4.23 cm) which is followed by T_4, T_7, T_8,

T_5, T_2 and T_2 whereas T_9 (control) shown minimum girth 2.96 cm. The data are presented in table 8.

At 60 days after transplanting, maximum pseudostem girth was recorded in T_6 (4.83 cm) which was on par with T_5, T_1, T_7 and T_4 whereas minimum girth found in T_9 (4.27 cm). Data are shown in table 8.

Table 8: Effect of bio-fertilizers on survival percentage, plant height and pseudostem girth of secondary hardened banana cv. Nendran plants

Treatments	30 days after transplanting			60 days after transplanting		
	Survival percentage (%)	Plant height (cm)	Pseudo stem girth (cm)	Survival percentage (%)	Plant height (cm)	Pseudo stem girth (cm)
T_1 (AMF)	100.00	15.67^{ab}	4.23^{a}	100.00	30.43^{ab}	4.70^{ab}
T_2 (*Azotobacter*)	100.00	14.33^{cd}	3.63^{b}	100.00	29.40^{b}	4.40^{cd}
T_3 (*Azospirillum*)	100.00	14.67^{bcd}	3.47^{b}	100.00	30.33^{ab}	4.60^{bc}
T_4 (PSB)	100.00	15.67^{ab}	3.80^{b}	100.00	30.83^{ab}	4.63^{ab}
T_5 (AMF + *Azotobacter*)	100.00	16.00^{a}	3.63^{b}	100.00	30.67^{ab}	4.73^{ab}
T_6 (AMF + *Azospirillum*)	100.00	15.33^{abc}	3.57^{b}	100.00	30.63^{ab}	4.83^{a}
T_7 (PSB + *Azotobacter*)	100.00	15.66^{ab}	3.70^{b}	100.00	30.70^{ab}	4.63^{ab}
T_8 (PSB + *Azospirillum*)	100.00	16.33^{a}	3.67^{b}	100.00	31.63^{a}	4.53^{bc}
T_9 (Control)	100.00	14.00^{d}	2.96^{c}	100.00	27.30^{c}	4.27^{d}
SE (m)	-	0. 18	0.02	-	0.38	0.01
CD (0.05)	NS	1.05	0.34	NS	1.55	0.20

Note:

- PSB- Phosphate solubilizing bacteria (*Pseudomonas fluorescens*)
- AMF - arbuscular mycorrhizal fungi
- Every treatment 10 g/plant bio-fertilizers

4.3.4. Number of leaves

Numbers of leaves per plant are presented in Table 9. Results revalued that there was no significance different among treatments in both condition. It

indicates that there is no impact of treatments on number of leaves per plant during secondary hardening.

4.3.5. Leaf length and leaf width

There was positive impact on leaf length and width. Results revealed that significant differences were there among the treatments (Table 9). All treatments were superior to control. Among the treatments T_1 recorded maximum leaf length (25.03 cm) which was on par with T_8 and T_5 whereas T_9 (control) recorded minimum leaf length (20.60 cm) during 30 days after transplanting. At 60 days after transplanting treatment T_7 (33.20 cm) showed maximum leaf length which was on par with T_4 and T_9 (control) recorded minimum length of 28.33 cm.

With respect to leaf width, there were significant differences between the treatments and control (Table 9). At 30 days after transplanting among the treatments, T_7 showed maximum width (10.23 cm) which was on par with T_8 and T_1 while T_9 (control) recorded lowest width (8.30 cm). During 60 days after transplanting, treatment T_3 given highest leaf width (14.13 cm) which is statistically on par with T_7 (14.07 cm) whereas T_9 (control) (12.10 cm) had the lowest width compared with other treatments.

4.3.6. Leaf area

Impacts of treatments on leaf area are given in Table 9. Among the treatments, T_7 (662.90 cm^2) had shown maximum leaf area which was on par with T_5, T_8, T_1 and T_2, while T_9 (control) had recorded the minimum leaf area (487.20 cm^2) during 30 days after transplanting.

At 60 days after transplanting, T_7 (891.80 cm^2) recorded maximum leaf area. T_7 was on par with T_6, T_8, T_1, T_5 and T_4. There were significant differences between the treatments and control, T_9 (control) recorded lowest leaf area (833.40 cm^2). Data are given in table 9.

Table 9: Effect of bio-fertilizers on number of leaves, leaf length, leaf width and leaf area of secondary hardened banana cv. Nendran plants

Treatments	30 days after transplanting				60 days after transplanting			
	No of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	No of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
T_1 (AMF)	4.33	25.03^{a}	9.50^{ab}	621.20^{abc}	5.00	30.57^{bc}	12.93^{b}	877.367^{abc}
T_2 (*Azotobacter*)	4.33	22.70^{cd}	8.63^{cd}	600.50^{abc}	5.00	31.13^{b}	12.50^{c}	865.83^{c}
T_3 (*Azospirillum*)	4.33	22.13^{d}	8.50^{cd}	565.40^{c}	5.00	30.57^{bc}	14.13^{a}	870.27^{bc}
T_4 (PSB)	5.00	22.53^{cd}	9.43^{b}	574.13^{bc}	5.00	32.23^{ab}	13.20^{b}	877.17^{abc}
T_5 (AMF + *Azotobacter*)	4.66	23.77^{abc}	9.17^{bc}	641.93^{ab}	5.00	31.27^{b}	12.90^{b}	877.50^{abc}
T_6 (AMF + *Azospirillum*)	4.00	22.73^{cd}	9.43^{b}	574.93^{bc}	5.00	29.36^{cd}	12.50^{c}	884.13^{ab}
T_7 (PSB + *Azotobacter*)	4.66	23.10^{bcd}	10.23^{a}	662.90^{a}	5.00	33.20^{a}	14.07^{a}	891.80^{a}
T_8 (PSB + *Azospirillum*)	4.33	24.10^{ab}	9.83^{ab}	626.00^{abc}	5.00	31.23^{b}	12.97^{b}	882.13^{abc}
T_9 (Control)	4.33	20.60^{e}	8.30^{d}	487.20^{d}	5.00	28.33^{d}	12.10^{d}	833.40^{d}
SE (m)	**0.12**	**0.27**	**0.10**	**873.70**	**-**	**0.49**	**0.02**	**45.99**
CD (0.05)	**NS**	**1.30**	**0.78**	**73.85**	**NS**	**1.74**	**0.38**	**16.95**

Note:

- PSB- Phosphate solubilizing bacteria (*Pseudomonas fluorescens*)
- AMF - arbuscular mycorrhizal fungi
- Every treatment 10 g/plant bio-fertilizers

4.3.7. Number of primary roots

Data regarding number of primary roots were displayed in Table 10. There was a positive impact of treatments on primary roots production. T_1 (10.20) had more number of roots compared with control while T_9 (control) recorded minimum number of roots of 4.73. T_1 was on par with T_6 and T_2 followed by T_7, T_3, T_4 and T_8 at 30 days after transplanting.

At 60 days after transplanting treatments had showed significant difference. T_5 (14.27) recorded maximum number of roots compared to T_9 (control) which recorded lowest number of primary roots. T_5 was on par with T_6. It was followed by T_1, T_7, T_8, T_3 and T_2.

4.3.8. Primary root length

With respect to primary root length data is presented in Table 10. All treatments were superior to control, significant differences between treatments and control are seen. At 30 days after transplanting treatment T_5 recorded maximum root length (31.7 cm) which was on par with T_6. T_1 was on par with T_7 and T_8. T_9 (control) showed minimum root length (18.47 cm).

At 60 days after transplanting treatments were found significantly different (Table 10). Treatment T_5 was superior one compared to other treatments which recorded maximum root length (51.17 cm) followed by T_6, T_1, T_3 and T_7. T_9 (control) showed minimum root length 32.27 cm.

4.3.8. Number of secondary roots

Treatments were positively influenced on number of secondary roots in plantlets. Results have shown that there were significant differences between treatments (Table 10). Treatment T_5 had shown maximum number of secondary roots at 30 and 60 days after transplanting (171.33 and 326.90) respectively. T_5 was on par with T_6 and T_1 was on par with T_7 and T_8 in both conditions. T_9 (control) recorded minimum number of secondary roots at 30 and 60 days after transplanting 103.67 and 153.77 respectively.

4.3.9. Weight of roots

With regarding to root weight results revealed that there were significant differences between treatments and control (Table 10). At 30 days after transplanting all treatments were superior to control. Among the treatments T_5 (4.17 g) showed highest root weight compared with control. T_9 (control) recorded lowest root weight of 1.43 g. T_5 was on par with T_6 and T_3 and T_8 was on par with T_7, T_1 and T_2.

During 60 days after transplanting, T_5 recorded the maximum weight about 12.97g which was followed by T_6. T_6 was on par with T_7, T_3, T_1 and T_8 whereas T_9 (control) recorded lowest root weight about 5.23 g.

Table 10: Effect of bio-fertilizers on number of primary and secondary roots, primary root length and root weight of secondary hardened banana cv. Nendran plants

Treatments	30 days after transplanting				60 days after transplanting			
	No of primary roots	Length of primary roots (cm)	No of secondary roots	Weight of roots (g)	No of primary roots	Length of primary roots (cm)	No of secondary roots	Weight of roots (g)
T_1 (AMF)	9.20^{ab}	27.87^{bc}	149.67^{bc}	2.77^{cd}	13.10^{bc}	42.33^{bc}	298.27^{abc}	9.23^{bc}
T_2 (*Azotobacter*)	7.83^{abc}	21.97^{e}	131.33^{d}	2.80^{cd}	12.03^{d}	35.53^{e}	195.90^{d}	7.37^{d}
T_3 (*Azospirillum*)	7.63^{bc}	24.23^{de}	136.67^{cd}	3.83^{ab}	12.10^{cd}	42.00^{bc}	289.37^{c}	10.27^{bc}
T_4 (PSB)	6.57^{cd}	23.83^{de}	135.33^{cd}	2.67^{d}	12.23^{cd}	35.60^{e}	212.07^{d}	7.53^{d}
T_5 (AMF + *Azotobacter*)	10.20^{a}	31.17^{a}	171.33^{a}	4.17^{a}	14.27^{a}	51.17^{a}	326.90^{a}	12.97^{a}
T_6 (AMF + *Azospirillum*)	8.37^{abc}	29.70^{ab}	156.67^{ab}	3.63^{ab}	13.40^{ab}	44.60^{b}	320.56^{ab}	10.66^{b}
T_7 (PSB + *Azotobacter*)	6.70^{cd}	26.20^{cd}	145.67^{bcd}	3.30^{bc}	12.73^{bcd}	39.47^{cd}	292.57^{bc}	10.13^{bc}
T_8 (PSB + *Azospirillum*)	7.37^{bc}	25.40^{cd}	142.00^{bcd}	3.43^{b}	12.17^{cd}	38.37^{de}	289.13^{c}	8.97^{cd}
T_9 (Control)	4.73^{d}	18.47^{f}	103.67^{e}	1.43^{e}	10.93^{e}	32.27^{f}	153.77^{e}	5.23^{e}
SE (m)	**0.98**	**1.71**	**44.19**	**0.06**	**0.16**	**1.50**	**140.89**	**0.43**
CD (0.05)	**2.48**	**3.27**	**16.60**	**0.62**	**1.02**	**3.06**	**29.66**	**1.64**

Note:

- PSB- Phosphate solubilizing bacteria (*Pseudomonas fluorescens*)
- AMF - arbuscular mycorrhizal fungi
- Every treatment 10 g/plant bio-fertilizers

4.3.10. Shoot fresh and dry weight

The treatment effects on shoot fresh and dry weight were statistically significant (Table 11). At 30 days after transplanting treatments found to be better than control, T_5 recorded highest weight of 33.23 g and T_7 were on par with T_1 and T_8 whereas T_9 (control) had shown lowest weight of 22.60 g. After 60 days of transplanting, T_5 recorded maximum weight about 71.40 g which is on par with T_6, T_7 and T_8 while T_9 (control) recorded minimum weight 50.70 g.

With respect to shoot dry weight there were significant differences among treatments (Table 11). Results of 30 days after transplanting have shown that T_5 (2.20 g) recorded highest dry weight compared with other treatments and it is statistically similar as T_6 and T_1 which in turn was on par with T_7, T_8, T_2 and T_3 whereas T_9 (control) shown the lowest weight 1.00 g. At 60 days after transplanting T_5 (5.37 g) had given highest dry weight which is on par with T_6 and T_1 which was on par with T_7 and T_8 while T_9 (control) showed the lowest weight 3.30 g.

4.3.11. Chlorophyll content

The data related to total chlorophyll content were presented in Table 11. There were significant differencesamong the treatments. At 30 days after transplanting results had shown that T_7 recorded higher total chlorophyll content about 1.37 mg g^{-1} which is statistically on par with T_8, except these two treatments other treatments did not show any significant different compared with control. T_2 and T_9 (control) recorded minimum chlorophyll content 1.00 mg g^{-1}.

At 60 days after transplanting small increase in total chlorophyll content was recorded (Table 11). Treatment T_4 shown highest chlorophyll content 1.60 mg g^{-1}which is followed by T_8, T_7, T_2, T_1, T_3, T_9 and T_5. T_5 had showed lowest chlorophyll content (1.00 mg g^{-1}).

Table 11: Effect of bio-fertilizers on shoot fresh and dry weight and chlorophyll content in secondary hardened banana cv. Nendran plants

Treatments	30 days after transplanting			60 days after transplanting		
	Shoot fresh weight (g)	Shoot dry weight (g)	Chlorophyll content (mg g^{-1})	Shoot fresh weight (g)	Shoot dry weight (g)	Chlorophyll content (mg g^{-1})
T_1 (AMF)	28.63^{bc}	1.83^{bc}	1.03^{c}	64.13^{bc}	4.96^{bc}	1.23^{cd}
T_2 (*Azotobacter*)	27.30^{c}	1.60^{cd}	1.00^{c}	60.43^{cd}	4.10^{ef}	1.33^{bc}
T_3 (*Azospirillum*)	27.30^{c}	1.60^{cd}	1.16^{b}	58.33^{d}	4.43^{de}	1.13^{de}
T_4 (PSB)	27.03^{c}	1.53^{d}	1.03^{c}	56.50^{d}	3.90^{f}	1.60^{a}
T_5 (AMF + *Azotobacter*)	33.23^{a}	2.20^{a}	1.10^{bc}	71.40^{a}	5.37^{a}	1.00^{e}
T_6 (AMF + *Azospirillum*)	30.63^{ab}	2.00^{ab}	1.07^{bc}	69.40^{ab}	5.13^{ab}	1.07^{e}
T_7 (PSB + *Azotobacter*)	29.93^{bc}	1.70^{cd}	1.37^{a}	68.57^{ab}	4.83^{bc}	1.37^{bc}
T_8 (PSB + *Azospirillum*)	27.97^{bc}	1.63^{cd}	1.30^{a}	67.30^{ab}	4.73^{cd}	1.40^{b}
T_9 (Control)	22.60^{d}	1.00^{e}	1.00^{c}	50.70^{e}	3.30^{g}	1.13^{de}
SE (m)	**1.41**	**0.02**	**0.01**	**4.54**	**0.03**	**0.01**
CD (0.05)	**2.96**	**0.29**	**0.13**	**5.33**	**0.39**	**0.15**

Note:

- PSB- Phosphate solubilizing bacteria (*Pseudomonas fluorescens*)
- AMF - arbuscular mycorrhizal fungi
- Every treatment 10 g/plant bio-fertilizers

4.3.12. Nitrogen

After completion of secondary hardening (60 days after transplanting) plant samples are used for analyzing nutrient content. With respect to nitrogen content plant samples showed significant differences among treatments. The data related to nitrogen in plant sample presented in Table 12. Treatment T_3 (2.30%) recorded highest nitrogen content in plant which is significantly superior than other treatments. T_5 was on par with T_2, T_6, T_7 and T_8 whereas T_1 (1.27 %) recorded lowest nitrogen content

4.3.13. Phosphorus

Phosphorus content in plant was significantly influenced by treatments. Results of phosphorus content in plant is depicted in Table 12. Treatment $T_1$was significantly superior one with respect to phosphorus uptake in which recorded maximum phosphorus content of 0.45 % followed by T_5 and T_6 whereas T_9 (control) recorded minimum phosphorus content of 0.21 %.

4.3.14. Potassium

With respect to potassium content in plant data is presented in Table 12. Significant differences among treatments were noticed. Treatment T_1 (8.70%) only recorded increased the potassium content in plant which is significantly superior and is followed by T_6 and T_4 whereas T_3 (4.50%) had showed reduced content of potassium in plant.

Table 12: Effect of bio-fertilizers on N, P and K content in secondary hardened banana cv. Nendran plants

Treatments	Nitrogen (%)	Phosphorous (%)	Potassium (%)
T1 (AMF)	1.27^{e}	0.45^{a}	8.70^{a}
T_2 (*Azotobacter*)	1.97^{bc}	0.24^{cd}	7.20^{b}
T_3 (*Azospirillum*)	2.30^{a}	0.23^{cd}	4.50^{e}
T_4 (PSB)	1.83^{bcd}	0.27^{bc}	7.03^{b}
T_5 (AMF + *Azotobacter*)	2.03^{b}	0.30^{b}	6.57^{c}
T_6 (AMF + *Azospirillum*)	1.83^{bcd}	0.30^{b}	7.30^{b}
T_7 (PSB + *Azotobacter*)	1.80^{bcd}	0.26^{bcd}	5.20^{d}
T_8 (PSB + *Azospirillum*)	1.73^{cd}	0.29^{b}	4.80^{de}
T_9 (Control)	1.70^{d}	0.21^{d}	6.43^{c}
SE (m)	**0.01**	**0.01**	**0.04**
CD (0.05)	**0.24**	**0.05**	**0.46**

Note:

- PSB- Phosphate solubilizing bacteria (*Pseudomonas fluorescens*)
- AMF - arbuscular mycorrhizal fungi
- Every treatment 10 g/plant bio-fertilizers

4.3.15. Zinc

With respect to zinc content in plant results are presented in Table 13. Significant differences were reported among the treatments. The treatment T_5 had increased content of zinc (33.27 ppm) which is on par with T_6 while T_9 (control) (18.97 ppm) recorded lowest zinc content in plant.

4.3.16. Iron

Information related to iron content in plant is presented in Table 13. Highest iron content was reported in T_5 (215.27 ppm) which is on par with T_6 and T_7 and decreased content of iron in T_9 (control) (109.63 ppm).

4.3.17. Manganese

Results revealed that there were significant differences among the treatments. In treatment T_5, maximum content of manganese (115.47ppm) was seen which is significantly superior and is followed by T_6, T_7, T_8, T_1 and T_4 whereas T_9 (control) recorded minimum uptake of manganese in plant (55.97 ppm) (Table 13).

4.3.18. Copper

Copper content in plant was influenced by treatments. Data were presented in Table 13. Copper content was enhanced by treatments. T_7 recorded maximum content of copper (25.46ppm) which is on par with T_8. Copper content was less in T_9 (control) (12.63 ppm) which recorded minimum content.

4.3.19. Boron

Boron content was enhanced by treatment T_1 (16.43 ppm) which recorded highest content of boron in plant, this is significantly superior compared with other treatments and control. T_2 was on par with T_7, T_8, T_3, T_4 and T_5 whereas T_9 (control) recorded lowest uptake of boron 12.50 ppm (Table 13).

Table 13: Effect of bio-fertilizers on micro nutrients content in secondary hardened banana cv. Nendran plants

Treatments	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	B (ppm)
T_1 (AMF)	26.83^{d}	161.97^{c}	93.93^{de}	18.97^{de}	16.43^{a}
T_2 (*Azotobacter*)	21.83^{f}	138.27^{cd}	72.33^{g}	16.47^{f}	14.97^{b}
T_3 (*Azospirillum*)	24.20^{ef}	133.00^{de}	80.53^{f}	17.73^{ef}	14.60^{bc}
T_4 (PSB)	26.30^{de}	139.23^{cd}	93.27^{e}	20.70^{cd}	14.57^{bc}
T_5 (AMF + *Azotobacter*)	33.27^{a}	215.27^{a}	115.47^{a}	21.70^{c}	14.50^{bc}
T_6 (AMF + *Azospirillum*)	31.10^{ab}	200.33^{ab}	108.40^{b}	22.20^{bc}	14.00^{c}
T_7 (PSB + *Azotobacter*)	30.37^{bc}	192.63^{ab}	101.67^{bc}	25.46^{a}	14.80^{bc}
T_8 (PSB + *Azospirillum*)	28.17^{cd}	189.17^{b}	100.23^{cd}	24.03^{ab}	14.77^{bc}
T_9 (Control)	18.97^{g}	109.63^{e}	55.97^{h}	12.63^{g}	12.50^{d}
SE (m)	**1.03**	**105.50**	**7.30**	**0.85**	**0.13**
CD (0.05)	**2.53**	**25.67**	**6.76**	**2.30**	**0.89**

Note:

- PSB- Phosphate solubilizing bacteria (*Pseudomonas fluorescens*)
- AMF - arbuscular mycorrhizal fungi
- Every treatment 10 g/plant bio-fertilizers

Discussion

5. DISCUSSION

Investigation was carried out on "Media improvement for *in vitro* culturing and hardening of TC banana cv. Nendran", with three separate experiments. The results obtained during course of experimentation are discussed hereunder.

5.1. Role of nitrogen sources on *in vitro* culture

Nitrogen is one of the most important macro nutrient content in any tissue culture media. Nitrogen can be added to the medium either as NO_3^- ion or as NH_4^+ ion or a combination of both. The quantity of both ions and their ratios determine shoot induction, growth and differentiation (Grimes and Hodges 1990; Leblay *et al.*, 1991; Ramage and Williams 2002; Niedz and Evens 2008; Wada *et al.*, 2015).

In this experiment, *in vitro* culture of banana *cv* Nendran with four media with basic MS media composition, different with respect to inorganic nitrogen sources like nitrate and ammonia and control (normal MS media) have been tried. Compared to all treatments, control (normal MS media) observed as significantly best one with regard to number of multiple shoots per explants, shoot length, number of leaves per plant, number of roots, root length and shoot fresh and dry weight.

The treatments T_3 and T_4 media containing nitrogen source as nitrate only (1900mg/l KNO_3 and 3800mg/l KNO_3 respectively), in these two conditions no response was observed with respect to multiple buds and shoots. The explants become blackened after sub culturing, this might be due to toxicity of nitrates and it might be due to variation of pH after sterilization of media and one observation was that in these two condition media became solid after sterilization. Also these observations reiterate necessity of NH_4^+ for maintaining the ionic balance and ensuring proper nitrogen availability.

Response only obtained from those media containing NH_4^+ ion nitrogen source. From these results we can conclude that presence of NH_4^+as nitrate

source is essential for successful *in vitro* culture of banana cv. Nendran. Nitrate has been in general considered as the most crucial form of nitrogen for culture of plant tissues but contrary cases in which nitrate alone as a source of nitrogen in plant cell culture has resulted in unsuccessful plant cell cultures are there as well (Halperin and Wetherell, 1965; Selby and Harvey 1990 and Smith and Krikorian, 1990). Sathyanarayana and Blake (1994) have stated that a reduced nitrogen source such as ammonium is essential for optimal use of nitrate ion by tissue cultures.

The ratio of KNO_3 and NH_4NO_3 mainly influence on the formation of adventitious buds in micropropagated fruit trees (Li and Han, 2003). In i*n vitro* culture, production of endogenous cell metabolites, proteins, organic acid and plant hormones from tissue might be depending on forms of nitrogen source and their proportion in culturing media (Preece 1995). It also affects on chlorophyll content, electron transport rate, photosynthetic rate, anthocyanin production, fresh mass, soluble protein concentration, and osmotic pressure of the cell sap of various cultures *in vitro* (Guidi *et al.,* 1998). NH_4^+ to NO_3^- ratio in the medium affects not only the growth of plant cell cultures (Velky and Rose, 1973) but also the production of secondary compounds (Smetanska, 2008). The ammonium/nitrate ratio controls the pH of growth media, stimulates morphogenesis and embryogenesis, and thus it is important in inducing callus formation in many woody plant cultures (Velky and Rose, 1973).

The biomass of plant which is higher in MS media supplemented with balanced KNO_3 and NH_4NO_3 other than those supplied with sole nitrogen source this is the combination effect of NH_4^+ - N and NO_3^- -N (Abbes *et al.*, 1995). Inorganic nitrogen source and their proportion might have an impact on the cell division, differentiation, growth and development of somatic embryos in *in vitro* culture (Mordhorst and Lorz, 1993). Mengel (1991) explained that the nitrogen uptake was improved by plant *in vitro* culture when the KNO_3 and NH_4NO_3 were balanced in culturing media.

Nitrate has been regarded as the basic form of nitrogen for culture of plant tissue and in several studies it was the only source of nitrogen for morphogenesis

(Bayley *et al.*, 1972). It was observed that as ammonium could not be completely excluded from the medium for culture (Abu-Qaoud *et al.*, 1991), in most cases a balanced mixture of ammonium and nitrate should have resulted a good growth and nitrate should predominate as suggested by Abu-Qaoud *et al.* (1991).

MS media prepared with modified nitrogen sources such as 1900mg/l KNO_3 + 1361mg/l $(NH_4)_2H_2PO_4$ after preparing media it goes for sterilization by autoclaving, after sterilization media becomes full liquid which is not suitable for proper establishment of culture in bottle. A very important character of the N-forms used is the effect on the pH of the culture medium. Due to the release of H^+ in the $(NH_4)_2H_2PO_4$ treatment, the pH of the culture medium was reduced, while in the KNO_3 treatment, the pH of the medium was increased, due to OH- release (Mashayekhi-Nezamabadi, 2000).

Similar results were found by Sotiropoulos *et al.* (2005) in apple rootstock MM 106 grown in *in vitro* with modification of nitrogen source like KNO_3, NH_4NO_3 and $NH_4H_2PO_4$ in MS media. Sathyanarayana and Blake (1994) reported that *in vitro* culture of jack fruit showed higher rooting percentage in mixture of ammonium and nitrates and ammonium citrate as nitrogen source in half strength MS media.

There was no such previous report for the modified inorganic nitrogen source in MS media for micropropagation of banana. This study could conclude that both ionic forms of nitrogen are required for successful i*n vitro* culture of banana cv. Nendran and optimization of media requires further studies in detail regarding the ratio of both ions, pH changes and varietal responses as differential response could be obtained from different varieties as observed in pear (Vojnich *et al.*, 2017).

5.2. Primary hardening studies

5.2.1. Influence of hardening media on survival percentage

With respect to survival percentage, treatments T_1, T_2, T_6, T_7 and T_9 recorded 100 % but T_3 (vermicompost) and T_5 (vermicompost + sand) showed

poor survival. Cocopeat gives better aeration, it has good water holding capacity and provide micronutrients though in smaller quantity and significant level of phosphorous and potassium to plant growth and development. Plants grown only in vermicompost performed poor in growth and had low survival rate of *in vitro* grown plantlets during hardening stage because heavier and compact structure of vermicompost explained by Dewir *et al.* (2005).

Also this might be the presence of some component in vermicompost which could hamper the growth of banana plantlets. Heavier media like vermicompost and combination of vermicompost and sand had heavy and compact structure. Plantlets had shown wilting symptoms and root die back occurred due to water logging condition in this media which had led to very low survival percentage of plantlets during primary hardening stage. In this stage, maintaining high relative humidity more than 95% by misting or fogging had been done in hardening chamber. Sharma *et al.* (1997) found that 97% survival observed in Dwarf Cavendish banana plants which were grown in sand as hardening media. Figure 1 shows effect of treatments on survival percentage.

5.2.2. Effect of treatments on plant growth parameters

Various growth parameters were recorded during growth of plantlets, with respect to which, treatment T_6 (cocopeat + vermicompost) (1:1) was significantly superior treatment compared with other treatments. This treatment had highest plant height, girth of pseudostem, number of leaves, leaf length, leaf width, leaf area, maximum primary root length and shoot fresh and dry weight. T_2 (cocopeat) recorded maximum number of primary and secondary roots whereas T_3 (vermicompost) and T_5 (vermicompost + sand) had shown poor performance with respect all growth parameters. Hence it could be concluded from this experiment that rather than using a single media, the mixture of cocopeat + vermicompost in 1:1 ratio is the best primary hardening media ensuring maximum survival along with better growth parameters which could bring down the period of acclimatization. These plantlets might survive well in the main field as well. Figures 2 and 3 shows effect of treatments on leaf area and root weight respectively.

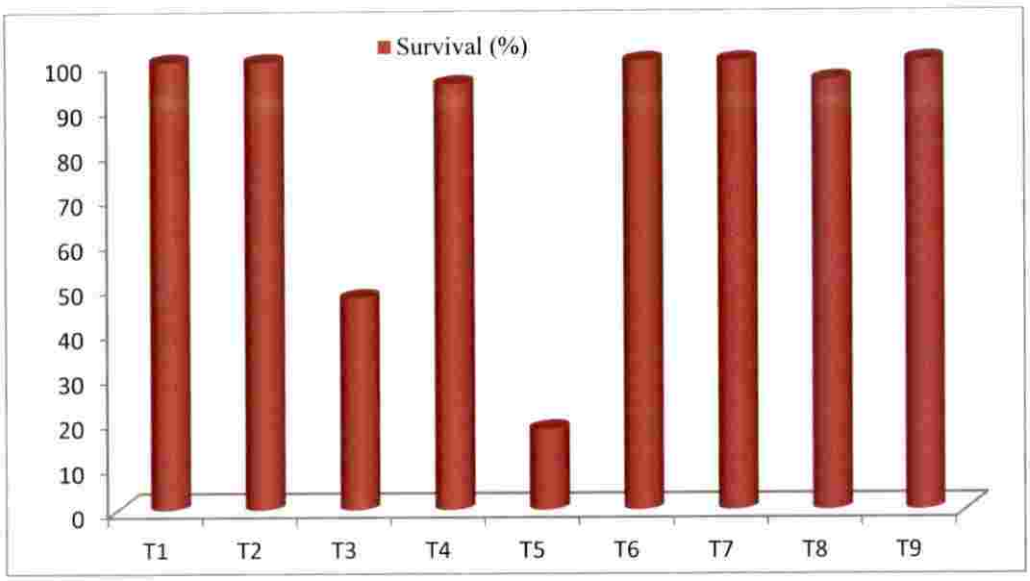

Figure 1: Effect of treatments on survival percentage of primary hardened plantlets

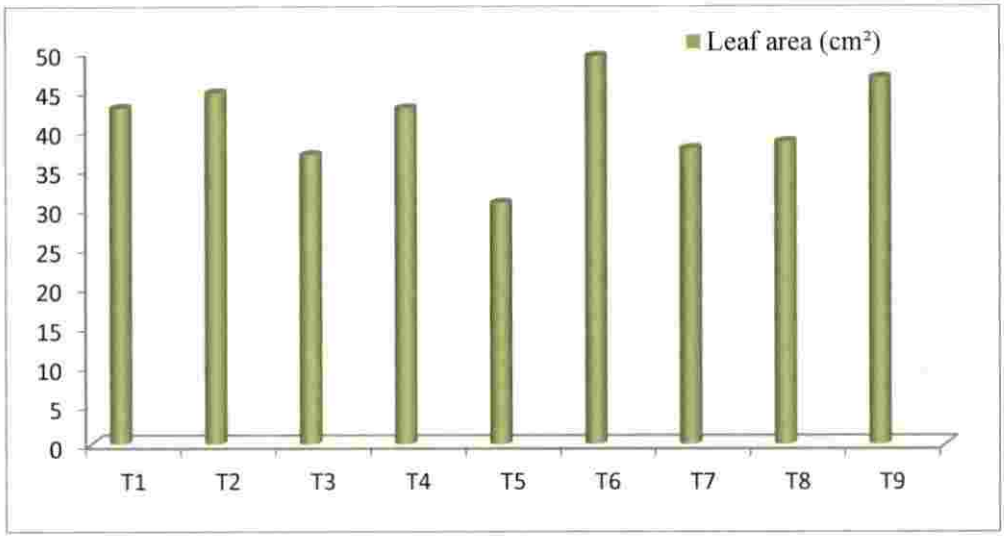

Figure 2: Effect of treatments on leaf area of primary hardened plantlets

With regard to chlorophyll content, maximum amount of total chlorophyll observed in treatment T_7 (cocopeat + vermicompost + sand) (1:1:1) compared with other treatments. Figure 4 shows effect of treatment on chlorophyll content.

Most of the nurserymen are using coirpith as basic media because of its better texture, physical and chemical properties (Savathri and Khan, 1993). Earlier Abad *et al.* (1992) explained that particle size of coir pith was mostly in between 0.25 to 2.5 mm because of which coir pith was favorite for nursery grown plants. Vermicompost contains micro and macro nutrients which is helpful for better uptake of nutrients for better root development in plants (Sharma *et al.*, 1997). There was study conducted by Robinson and Sauco (2009) which stated that the plantlets of banana cultivars like Williams and Grand Naine were grown in media containing higher proportion of heavy textured organic materials which resulted in poor growth of plants because condensed media and water logging occurred which leads to the root die back and wilting symptoms observed in plants. This might be one of the reasons for poor performance of vermicompost.

Savathri and Khan (1994) reported that whenever the coir pith was incorporated with heavy clay soil, sand and vermicompost which improves the drainage, water holding capacity and proper aeration near root zone of plants due to its spongy structure and physical properties like bulk density, pore space, infiltration rate and hydraulic conductivity were improved by adding coir pith to heavy soil or media for proper growth and development of plants. Coir pith has such ability to provide oxygen provide to the root zone of plants for better growth and development (Nagarajan *et al.*, 1985). Savathri and Khan (1994) and Abad (2002) observed that the cocopeat has higher amount of phosphorous and potassium compared with other organic media.

Edward and Burrows (1988) described that vermicompost prepared from especially animal waste which has higher amount mineral nutrients like nitrates, exchangeable phosphorous, soluble potassium, calcium and magnesium which were easily available to plants for better growth and development. Vermicompost has very important biological property that was it has higher population of

beneficial micro organisms like bacteria, actinomycetes and fungi (Werner and Cuevas, 1996). During vermicomposting it increases microbial population and their activities produce some growth regulators by interaction of micro organisms and earthworms, so for that reason vermicompost is a source of growth regulators. Garvilov (1963) first reported that earthworms also produce growth regulators during composting.

Whenever humic materials used as the basic media or their application to soil increased the dry matter production in corn seedlings (Lee and Bartlett, 1976) and increased root length of tobacco (Mylonas and Mccants, 1980), resulted in more shoot dry weight of soybean (Tan and Tantiwiramanod, 1983) and enhanced the growth of shoot and root of tissue cultured plantlets (Goenadi and Sudharama, 1995).

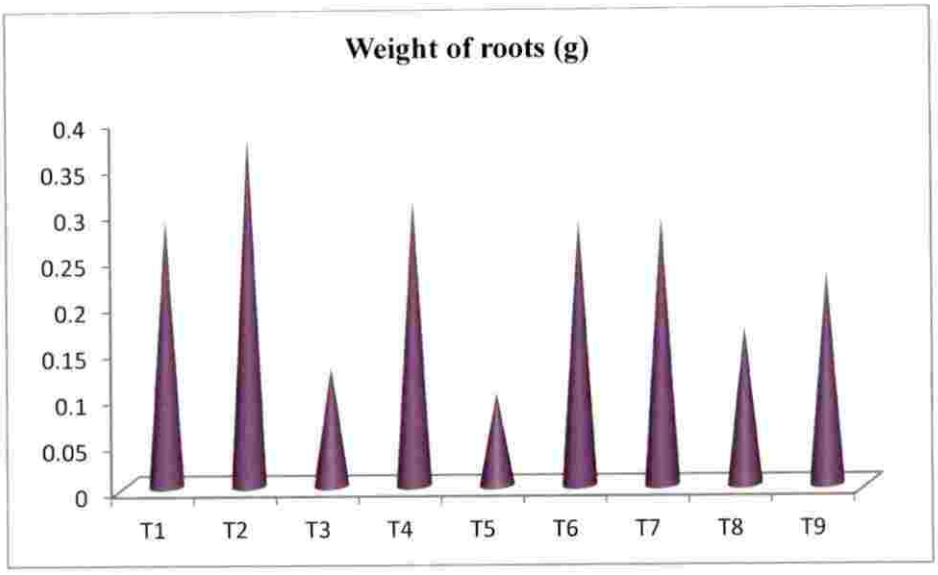

Figure 3: Effect of treatments on root weight of primary hardened plantlets

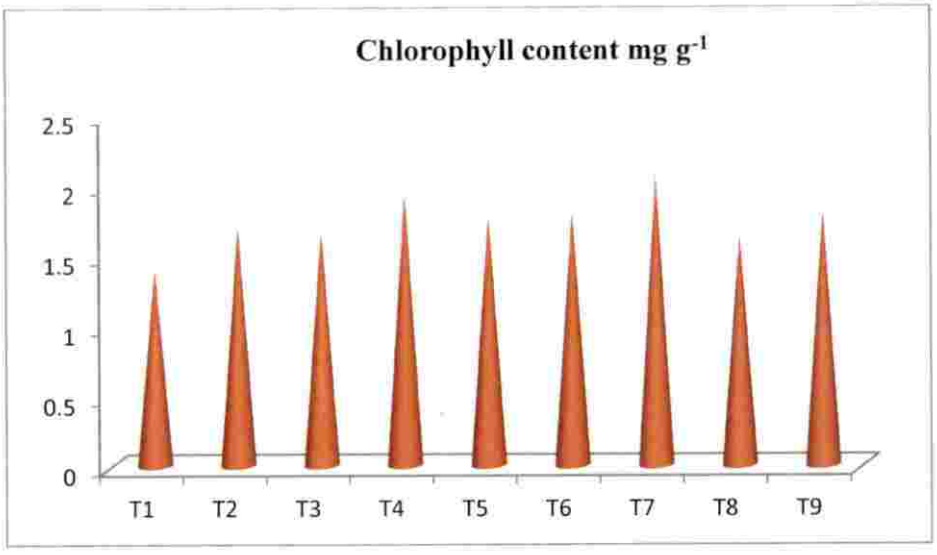

Figure 4: Effect of treatments on total chlorophyll content of primary hardened plantlets

5.3. Effect of biotization on secondary hardening studies

5.3.1. Survival percentage

With regard to survival percentage of the plants grown at 30 and 60 days after transplanting, there was no difference in treatments and control. Every treatments including control also recorded 100% survival in both stages. It clearly showed that there was no impact of treatments on plants with respect to survival percentage. This is because these plants have came the more critical primary hardening stage and the risk of losing plants during it less during the secondary hardening.

5.3.2. Plant growth parameters

With respect to plant height and pseudostem girth, treatment T_8 recorded the maximum plant height in both stages compared with other treatments. The maximum pseudostem girth was observed in T_1 and T_6 at 30 and 60 days after transplanting respectively.

Micropropagated plants treated with AMF during hardening stage, it was found to enhance growth and nutrients absorption and reduce transplant shock (Gianinazzi *et al.*, 1989). Similar findings were recorded by Vasane and Kothari 2006; 2008 and 2010. Vinutha (2005) reported *Ocimum sanctum* plants inoculated with *Azotobacter chroococcum* and *Glomus fasciculatum* either as single or in mixture had enhanced plant growth and biomass by increased absorption of nitrogen and phosphorus in plant.

5.3.3. Plant leaf characters

Regarding various leaf characters, the number of leaves per plant were observed to be unaffected by the treatments in both growth stages. The various microorganisms employed could not influence the rate of leaf production in the micropropagated plantlets. Leaf length was maximum in T_1 and T_7 at 30 and 60 days after transplanting respectively. Highest leaf width was observed with T_7 at both growth stages. T_7 recorded maximum leaf area. Regarding all leaf

characters of plant, treatment T_7 (PSB + *Azotobacter*) was the best one compared with others. This could be because the combination of both microbes with different mode of action has ensured better availability of nutrients and thereby enhancing growth. Figure 5 shows effect of treatments on leaf area at both conditions.

There was report from Panigrahi *et al.* (2013) which stated that enhanced growth of plant and increased primordial development in leaf the treatment of plant growth promoting rhizobacteria (PGPR) to *in vitro* plants during hardening stage. Gosal *et al.* (2010) recorded maximum leaf length of Chlorophytum plants inoculated with *Pseudomonas florescence* and *Pseudomonas indica*.

5.3.4. Plant root parameters

While assessing the impact of treatments on root system, it could be seen that the treatment T_5 was the best one with respect to more number of primary and secondary roots, maximum root length and root weight compared with others. Figure 6 shows effect of treatments on root weight at both stages.

AMF fungi produce some growth regulators, its help full for the well growth and development of roots in micropropagated avocado plantlets (Barea and Azon-anguilar, 1982). One of important effect of AMF application is to extension of roots to deeper layer for absorption of immobile phosphorous and other nutrients from soil and it transport to the host means plant (Gullemin *et al.*, 1992). Yano-melo *et al.* (1999) recorded highest fresh roots weight of micropropagated banana plants inoculated with Mycorrhizae. Mycorrized plants recorded highest root weight because the higher relative water content in root system (Hernandez-sebastia *et al.*, 1999). Richardson (2001) reported that phosphorous uptake improved the morphological and physiological characters of roots. *Azotobacter chroococcum* inoculation increased root volume (Ahmad *et al.*, 2008) and root length (Zayed, 2012). Plate 5a, 5b, 6a and 6b shows effect treatment T_5 (AMF + *Azotobacter*) and control at 30 and 60 days after transplanting.

Plate 5a: Plant root characters of T_5 (AMF + *Azotobacter*) at 30 DAT

Plate 5b: Plant root characters of T_9 (control) at 30 DAT

Plate 6a: Plant root characters of T_5 (AMF + *Azotobacter*) at 30 DAT

Plate 6b: Plant root characters of T_9 (control) at 30 DAT

5.3.5. Shoot fresh and dry weight

Treatment T_5 was observed as the superior one compared with other treatments and control as it recorded highest shoot fresh and dry weight. An increase of 47.03% was noted above control in case of shoot fresh weight in this treatment. This may because of the positive influence by the AM fungi on the growth rate.

AMF inoculated plants assimilates more CO_2 which leads to maximum biomass production and vigorous growth of plants. Earlier there was report from Mathur and Vyas (1995) that AMF inoculated plants had shown maximum photosynthetic rate. AMF treated plants are more vigorous and it leads higher biomass production (Luna and Davies, 2003). Application of AMF and *azotobacter* bio-fertilizers increases photosynthesis, root surface and better root development in plant which was helpfull for the more uptake of nutrients from soil it leads to increase biomass production of shoots and roots (Vasane and Kothari, 2010). Similar results were obtained by Vidal (1992) in micropropagated avocado plants at hardening.

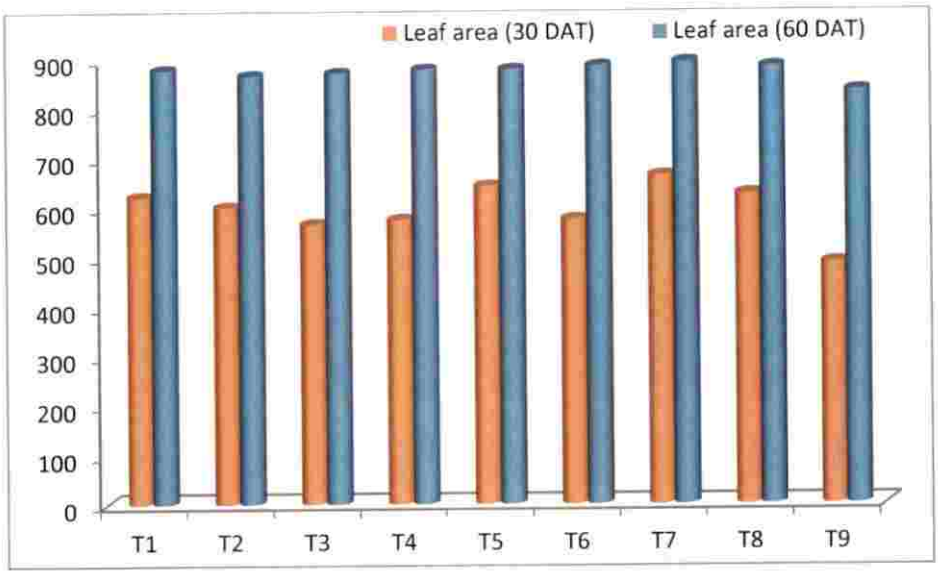

Figure 5: Effects of bio-fertilizers on leaf area of secondary hardened plants

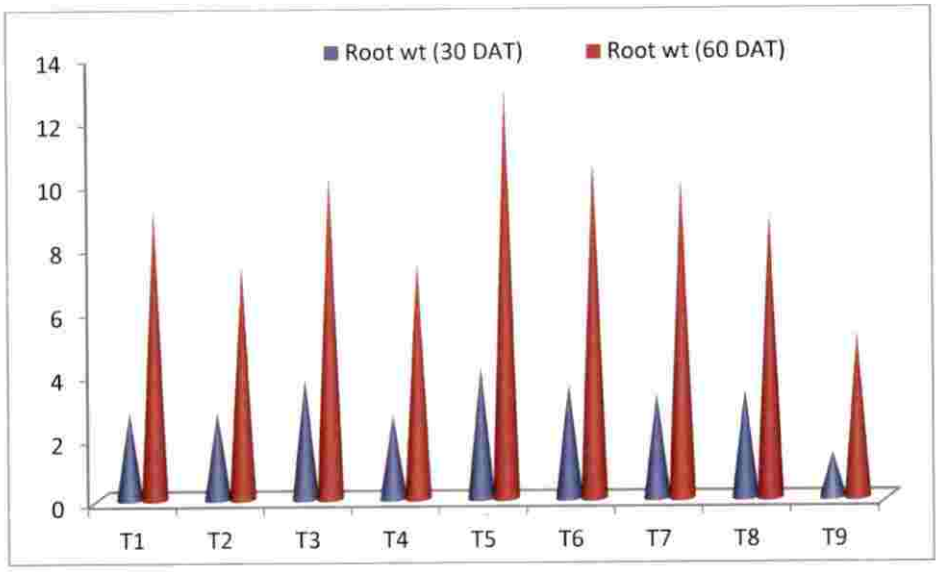

Figure 6: Effects of bio-fertilizers on root weight of secondary hardened plants

5.3.6. Chlorophyll content

With respect to total chlorophyll content, there was small increase from 30 to 60 days after transplanting. T_7 recorded maximum chlorophyll content at 30 days after transplanting and at 60 days after transplanting T_4 recorded highest content. Figure 7 shows effect of treatments on chlorophyll content at both conditions.

Gosal *et al.* (2010) reported that chlorophyll content was enhanced by application of *Pseudomonas florescence* and *Pseudomonas indica* to the *Chlorophytum sp* plants. Increased photosynthetic rate in plants which improved leaf area and chlorophyll content with application of bio-fertilizers has been earlier reported by Mathur and Vyas (1995).

5.3.7. Effect of treatments on nutrient status in plant

Regarding the nutrients status of plants after 60 days transplanting, it was observed that there was positive impacts of treatments on nutrients uptake by plants. With regard to available nitrogen T_3 recorded the maximum followed by T_5. Phosphorous content was maximum in T_1 followed by T_5. Potassium content was highest in T_1 followed by T_6. Figure 8 shows effect of treatments on NPK content in plant.

Estimation of micro nutrients status in plants, indicated that treatment T_5 was the best with respect to highest content of zinc, iron and manganese. Copper content was more in T_7 followed by T_8 and boron content maximum in T_1 followed by T_2. Superiority of AMF treatments for better hardened micro propagated plants have earlier reported in many other species as well.

Nutrients such as P, Zn, Fe and Cu are highly immobile in soil, plantlets inoculated with AMF absorbed more nutrients from soil to plant (Stribley 1987). Menge *et al.* (1978) explained micropropagated plantlets inoculated with AMF successfully established in field because of improvement of water and nutrients uptake of plant by Mycorrhizae. Yano-melo *et al.* (1999) found similar results that micro nutrients concentration significantly more in AMF inoculated banana

plants. Copper absorption higher in *chlorophytum sp* plants inoculated with *Pseudomonas florescence* and *Pseudomonas indica* (Gosal *et al.*, 2010). Srivastava *et al.* (2001) explained application of bio-fertilizers to micropropagated plants at hardening stage enhance biological nitrogen fixation (*Azotobacter* and *Azospirillum*), increased the absorption of water and mineral nutrients from soil to plant. Similar results were obtained by Krishna *et al.* (2006) in micropropagated grape vine where AMF inoculation increased the absorption of nutrients like P, Zn, Fe and Cu from soil.

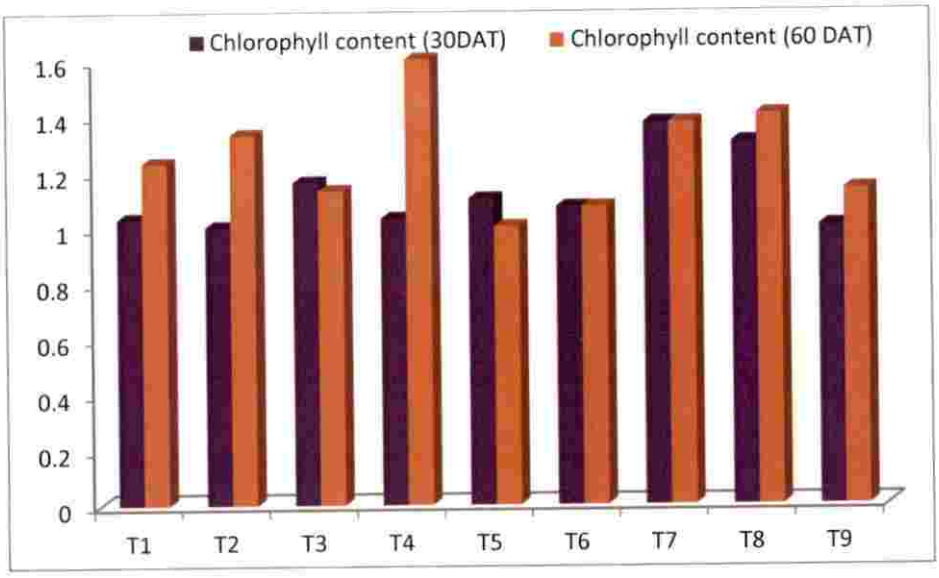

Figure 7: Effects of bio-fertilizers on chlorophyll content of secondary hardened plants

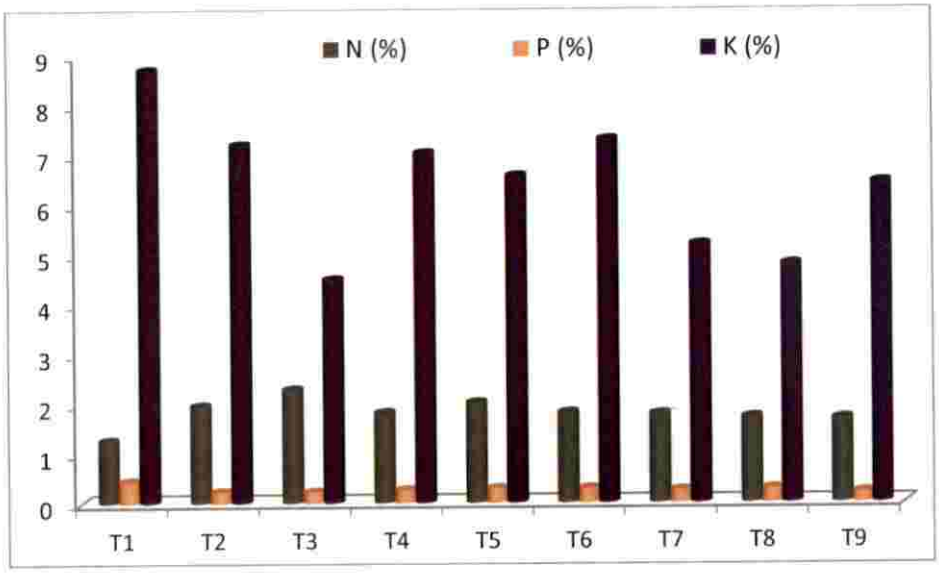

Figure 8: Effects of bio-fertilizers on NPK uptake in secondary hardened plants

Summary

6. SUMMARY

The salient observations of study conducted on "Media improvement for *in vitro* culturing and hardening of tissue culture banana cv. Nendran" are summarized in this chapter.

The objectives of this research work was to know the effect of nitrogen sources on shoot and root regeneration in tissue cultured Nendran banana, and to enhance the growth and survival of plantlets during primary and secondary hardening. The main aim was to find out best media for *in vitro* culture, primary and secondary hardening of tissue cultured Nendran banana. Trial was conducted at tissue culture laboratory, Regional Agricultural Research Station, Pilicode, Kasargod and Department of Pomology, College of Agriculture, Padannakkad during the period of 2017-2019. There were three experiments such as *in vitro* culture, primary hardening and secondary hardening. The *in vitro* culture experiment conducted at tissue culture laboratory, RARS, Pillicode, experiment was carried out with completely randomized design with five different treatments replicated with four times. Hardening studies were conducted at Horticultural nursery, RARS, Pillicode. Experiments were carried out in completely randomized design with nine treatments and three replications.

Experiment on *in vitro* culture consisted of five treatments likeT_1: MS media (control) (NH_4NO_3 1650mg/l and KNO31900mg/l), T_2: Modified MS media (2850mg/l KNO_3 + 825mg/l NH_4NO_3), T_3: Modified MS media (1900mg/l KNO_3), T_4: Modified MS media (3800mg/l KNO_3) and T_5: Modified MS media (1900mg/l KNO_3 +1361mg/l $(NH_4)_2H_2PO_4$). For each treatment six subculture cycles were done.

In the initial two sub cultures multiple shoots were not formed. After third subculture only multiple shoots were formed. Normal MS media, T_1 (control) only recorded maximum number of multiple shoots per explants compared with others because balanced ammonium and nitrate nitrogen sources essential for formation of multiple shoots and with respect to shoot length, number of leaves, number of roots, root length and shoot fresh and dry weight were significantly higher in normal MS

media (T_1) compared with modified MS media with respect to ammonium and nitrate nitrogen sources.

Primary hardening study was conducted with nine different treatments like T_1: sand (control), T_2: coco peat, T_3: vermicompost, T_4: sand + coco peat (1:1), T_5: sand +vermicompost (1:1), T_6: coco peat + vermicompost (1:1),T_7: sand + coco peat + vermicompost (1:1:1),T_8: sand + coco peat + vermicompost (1:2:1) andT_9: Sand + coco peat + vermicompost (1:1:2). Hardening media had significant effects on plant survival, growth and development of primary hardened plants.

Almost all treatments have shown similar results with respect to survival percentage. The treatments, T_1, T_2, T_6, T_7 and T_9 shown 100 percent survival but T_3 (47.33%) and T_5 (18.00%) recorded poorest survival. Acclimatization media significantly influenced plant growth parameters. All treatments showed significant results except T_3 and T_5. Among the treatments, combination of coco peat + vermicompost with proportion of 1:1 was superior which recorded maximum plant height, pseudostem girth, number of leaves, leaf length, leaf width, leaf area, root length and highest shoot fresh and dry weight and T_2 (cocopeat alone) was recorded maximum number primary and secondary roots and root weight which was on par with T_6.

The media combination of Sand + Coco peat + Vermicompost (1:1:1) recorded maximum chlorophyll content which was on par with media containing Sand + Coco peat (1:1) and this was significantly different from other treatments whereas sand alone as hardening media recorded minimum chlorophyll content.

The last experiment on secondary hardening with different bio-fertilizers application like T_1 (Arboscular mycorrhizal fungi), T_2 (*Azotobacter*), T_3 (*Azospirillum*) T_4 PSB (*Pseudomonas florescence*), T_5 (AMF + *Azotobacter*), T_6 (AMF + *Azospirillu*) T_7 (PSB + *Azotobacter*), T_8 (PSB + *Azospirillum*) and T_9 Potting mixture only (Control). All bio-fertilizers treatment enhanced nutrients uptake and growth of secondary hardened plants compared to control.

With respect to plant growth parameters, highest plant height was observed in T_8which was superior compared with other treatments at both 30 and 60 days after transplanting. Maximum pseudostem girth found in T_1 and T_6 at 30 and 60 days after transplanting respectively compared to other treatments.

Bio-fertilizers application had no influence on plants with respect to survival during secondary hardening stage. All treatment including control recorded 100 percent survival. Among the treatments T_7 (PSB + *Azotobacter*) was observed to be superior one which had recorded maximum leaf length, leaf width and leaf area whereas with regard to number of leaves treatment effects were insignificant. With respect to all leaf characters T_7 (PSB + *Azotobacter*) was superior compared to other treatments.

Bio-fertilizers application had significant effects on root system of plants. Among the treatments T_5 (AMF + *Azotobacter*) which was significantly different from other treatments and it recorded maximum number of primary and secondary roots, primary root length and root weight compared with other treatments. T_5 (AMF + *Azotobacter*) was superior one with respect root system at both stages.

Highest shoot fresh and dry weight observed in T_5 (AMF + *Azotobacter*) which was significantly different and bio-fertilizers application significantly influenced on chlorophyll content in plants, its maximum T_7 (PSB + *Azotobacter*) and T_4 PSB (*Pseudomonas florescence*) at 30 and 60 days after transplanting.

Nutrients status was analyzed at 60 days after transplanting. With respect to macro nutrients, phosphorous and potassium content was maximum in T_1 (Arbuscular mycorrhizal fungi) and nitrogen uptake was more in T_3 (*Azospirillum*) followed by T_5 (AMF + *Azotobacter*). Micro nutrients status in bio-fertilizers applied plants were significantly higher. T_5 (AMF + *Azotobacter*) had maximum content of Zn, Fe and Mn whereas Cu and B uptake was more in T_7 (PSB + *Azotobacter*) and T_1 (Arbuscular mycorrhizal fungi) respectively.

For successful *in vitro* culture of Nendran banana a balanced supply of nitrogen sources like ammonium and nitrates are required in MS media. To avoid transplant shock acclimatization is an essential process in tissue culture. Acclimatization consists two stages primary hardening and secondary hardening. T_6 - mixture of coco peat and vermicompost (1:1) was the best media for successful survival and growth of plants during primary hardening. Biotization was an important technique in secondary hardening stage, it enhance growth and development of plants through increased nutrients uptake from media. During secondary hardening T_5 - AMF and *Azotobacter* effectively enhanced plant growth and nutrients uptake from soil.

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MEDIA IMPROVEMENT FOR *IN VITRO* CULTURING AND HARDENING OF TC BANANA CV. NENDRAN.

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Abstract of the thesis

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Abstract

ABSTRACT

The research work "Media improvement for *in vitro* culturing and hardening of tissue culture banana cv. Nendran" was carried out 1) to study the effect of nitrogen sources (ammonium and nitrate) on shoot and root regeneration in tissue cultured Nendran banana and 2) to standardize methods to enhance the growth and survival of plantlets during primary and secondary hardening stage.

Experiments were conducted at the Regional Agricultural Research Station, Pilicode, Kasargod during 2017-2019. First experiment consisted of *in vitro* culture with five treatments- T_1 – MS media (control) (NH_4NO_3 1650mg/l and $KNO_3$1900mg/l), T_2 - Modified MS media (2850mg/l KNO_3 + 825mg/l NH_4NO_3), T_3 - Modified MS media (1900mg/l KNO_3), T_4 -Modified MS media (3800mg/l KNO_3) and T_5 - Modified MS media (1900mg/l KNO_3 + 1361mg/l $(NH_4)_2H_2PO_4$).

In the first experiment, normal MS media T_1 (control) was observed to be superior one compared with other treatments (modified MS media). It recorded maximum number of multiple shoots per explant after 4th, 5th and 6th subculture and highest shoot length, number of leaves, number of roots, root length and maximum fresh and dry weight of shoot after the rooting stage.

Second experiment on primary hardening was carried out with nine treatments such as T_1- sand (control), T_2 - coco peat, T_3 - vermicompost, T_4 - sand + coco peat (1:1), T_5 - sand + vermicompost (1:1), T_6 - coco peat + vermicompost (1:1), T_7 - sand + coco peat + vermicompost (1:1:1), T_8 - sand + coco peat + vermicompost (1:2:1) and T_9 - sand + coco peat + vermicompost (1:1:2) .

Primary hardening study was carried out with different hardening media. Plant growth and survival were effectively influenced by hardening media. T_1, T_2, T_6, T_7 and T_9 showed 100 percent survival but T_3 (47.33 %) and T_5 (18.00 %) had lower survival rates. With respect to various growth parameters T_6 recorded highest plant height, pseudostem girth, number of leaves, leaf length, leaf width, leaf area,

root length and highest shoot fresh and dry weight and T_2 had recorded maximum number primary and secondary roots and root weight which was on par with T_6.

Third experiment on biotization was done with nine treatments consisting of T_1 - Arbuscular mycorrhizal fungi, T_2 - *Azotobacter*, T_3 - *Azospirillum*, T_4 - PSB (*Pseudomonas fluorescens*), T_5 - AMF + *Azotobacter*, T_6 - AMF + *Azospirillum* T_7 - PSB + *Azotobacter*, T_8 - PSB + *Azospirillum* and T_9 - Control (Potting mixture only).

Investigation on biotization of plants, revealed significant effects on plant growth and development through enhanced nutrients uptake. All treatments showed 100 % survival. Among treatments, T_8 recorded highest plant height at 30 and 60 DAT, maximum pseudostem girth was found in T_1 and T_6 at 30 and 60 DAT. With respect all leaf characters T_7 was significantly superior which recorded maximum leaf length, leaf width and leaf area at both stages. T_5 recorded more number primary and secondary roots, primary root length and root weight and shoot fresh and dry weight compared to control. Chlorophyll content was maximum in T_7 and T_4 at 30 and 60 DAT respectively. In the nutrient status of plants, T_1 recorded maximum content of P and K and N content was more in T_3. Plants of T_5 recorded highest uptake of Zn, Fe and Mn. Cu and B content were more in T_7 and T_1 respectively.

For successful *in vitro* culture of banana cv. Nendran, both ammoniacal nitrogen and nitrate nitrogen are required as per the results obtained. For successful transplantation of tissue cultured plants, acclimatization is an integral process in tissue culture technique, which involves primary and secondary hardening. For primary hardening, T_6 - mixture of coco peat and vermicompost (1:1) was the best for successful survival and growth of plantlets. Biotization was found to be an important technique in secondary hardening stage, which enhanced the growth and development of plants through increased uptake of nutrients from media. Among the treatments, T_5 - AMF and *Azotobacter* combination was the best for secondary hardening.

Appendices

Annexure 1

Table 1: MS medium composition and stock preparation

	Ingredients	Quantity mg/ L	Concentration of stock	Quantity required	Volume of stock	Vol. req. for 1L media
Macro elements Stock A	NH_4NO_3	1650	40 X	33 g	500 ml	25 ml
	KNO_3	1900		38 g		
	$MgSO_4.7H_2O$	180.69		3.6 g		
	KH_2PO_4	170		3.4 g		
	CaCl2. 2H2O	440		8.8 g		
Micro elements Stock B	KI	0.83	200 X	83 mg	500 ml	5 ml
	H_3BO_3	6.20		620 mg		
	$MnSO_4.4H_2O$	16.90		2230 mg		
	$ZnSO_4.7H_2O$	8.60		860 mg		
	$Na_2MoO_4.2H_2O$	0.25		25 mg		
	$CuSO_4.5H_2O$	0.025		2.5 mg		
	CoCl2. 6H2O	0.025		2.5 mg		
Iron source	$FeSO_4.7H_20$	27.80	200 X	1.12 g	200 ml	5 ml
	Na_2 - EDTA	37.30		1.50 g		

Stock C						
Vitamins Stock D	Nicotinic acid	0.50	100 X	5 mg	100 ml	1 ml
	Pyridoxine HCl	0.50		5 mg		
	Thiamine HCl	0.10		1 mg		
	Glycine	2.00		20 mg		
	Myoinositol	100				
Carbon source	Sucrose	30g/ L