IN VITRO PLANTLET REGENERATION OF RUCOLA (*Eruca sativa*)

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CERTIFICATE

*This is to certify that the thesis entitled "**IN VITRO** **PLANTLET REGENERATION OF RUCOLA** **(Eruca sativa)**" submitted to the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biotechnology, embodies the result of a piece of bona fide research work carried out by **SADIA AFRIN Reg. No.:11-04563** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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IN VITRO PLANTLET REGENERATION OF RUCOLA (*Eruca sativa*)

ABSTRACT

The present experiment was carried out in the Laboratory of Biotechnology, Department of Biotechnology, Sher-e-Bangla Agricultural University,Dhaka-1207 during the period of February, 2016 to June, 2017 to study the effect of BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l),IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and NAA (0.5, 1.0, 1.5, 2.0 mg/l) either alone or in combination for direct and indirect *in vitro* regeneration of Rucola. The cotyledonary nodes of Rucola were used as explants which were sterilized with 70% ethanol and 0.2% $HgCl_2$.The highest frequency of shoot (80.00%) was observed in MS medium augmented with BA 3.0 mg/l in minimum (6.40 days). Maximum number of shoots(2.60, 3.60 and 4.60)were obtainedat 14, 21 and 28 days after shoot induction (DAI), respectively in BA 4.0 mg/L where the control treatment gave the lowest result.In combined effect, BA 4.0 mg/l+ IBA 3.0 mg/lshowed the highest percentage of shoot (76.00%) in 6 days and the maximum number of shoot (2.40, 3.0 and 3.40) at 14, 21 and 28 DAI, respectively. Besides, the highest number of leaves (3.20, 7.20 and 11.00) andlength of leaves (2.60, 4.42 and 5.00 cm) were observed in BA 4.0 mg/l+ IBA 4.0 mg/l and BA 4.0 mg/l+ IBA 2.0 mg/l at 14, 21 and 28 DAI, respectively. For root regeneration, the best result was recorded with BA 4.0 mg/l +IBA 2.0 mg/l in case of percentage of root(68.00%) and maximum number of roots(3.60) in BA 4.0 mg/l+ IBA 4.0 mg/lwhere the lowest result observed in control. On the other hand, BA 4.0 mg/l+ NAA 1.0 mg/l gave the optimum results in case of frequency of callus (68.00%) and weight of callus (1.71 g, 2.15 g and 2.80 g) at 20 , 30 and 40 DAI, respectively. Further, minimum days for shoot induction (10.80 days) and the highest number of shoots (3.00) were obtained from callus with BA4.0 mg/l+ NAA 1.0 mg/l. Then, IBA 3.0 mg/l produced the highest percentage of root(72.00%) in 14.40 days.The maximum number of root(2.60, 3.60 and 4.60) and the highest length of root (1.26 cm, 2.32 cm and 3.16 cm) at 14, 21 and 28 DAI, respectively was recorded with IBA 4.0 mg/l.Finally, the survival rate of *in vitro* regenerated plantlets was recorded 75 % innatural condition. Overall, feasible protocol has been developed for *in vitro* rapid regeneration of Rucola having potential application in breeding programfor qualities improvement in commercial use.

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ABBREVIATIONS ANDACRONYMS

Agril.	:	Agriculture
Biol.	:	Biological
Cm	:	Centimeter
CRD	:	CompletelyRandomizedDesign
DMRT	:	Duncan's Multiple Range Test
Conc.	:	Concentration
DAI	:	Days After Induction
et al.	:	And others (at elli)
FAO	:	Food and Agricultural Organization
g/L	:	Gramperlitre
BAP	:	6-BenzylAminoPurine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indoleaceticacid
IBA	:	Indolebutyricacid
NAA	:	*a*-Napthaleneaceticacid
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
MS	:	MurashigeandSkoog
Int.	:	International
2-ip	:	2-isopentenyladenine
J.	:	Journal
Mol.	:	Molecular
mg/2	:	Milligramperlitre
µM	:	Micromole
MS	:	MurashigeandSkoog
PGRs	:	PlantGrowthRegulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera

CHAPTER I
INTRODUCTION

Eruca sativa commonly known as rocket, rucola, arugula, rucoli, etc. is an edible annual plant under the family Brassicaceae is used as a leafy vegetables for its fresh peppery flavor and is geographically confined to the Mediterranean region (Chopra *et al.*, 1996 and Yaniv *et al.*, 1998). Rocket was used medicinally at once, although it is now used commonly as a salad herb (Lamy *et al.*, 2008). Rucola is a cool loving crop and grows best in winter and can reach about 20–100 cm in height. The leaves of this plant are formed in lobe like structure in both lateral sides. All parts of the plant except root are edible (leaves, flowers, siliquas, young and mature seeds). High temperature during early seed formation affects the seed oil content and quality adversely. The plant is able to withstand temperature up to 40ºC. It has photoperiod ranges of 10h. Maturity of the plant takes place from 60 to 340 days. Brassicaceae family contributes fifth major portion amongst the oil seed crops (Yaniv, Z. *et al.*, 1998).

The plant is reported to have antiscorbutic, diuretic, stimulant, stomachich, and ribefacient activities (Songsak and Lockwood, 2002). Alqasoumi *et al.* (2009) recently found anti-ulcer activity and anti-inflammatory effects of *Eruca* extracts (Yehuda *et al.*, 2009). Oil extracted from its seed is reported to have aphrodisiac properties. An economically important compound, 4-methylthiobutyl glucosinolates have been extracted from the seeds of *E. sativa* (Iori *et al.*, 1999 and Zhang *et al.*, 2005). A major compound extracted from *Eruca* is erucic acid that is commonly used as lubricant (Kumar and Tsunoda, 1980). It contains health promoting natural phytochemicals and possesses a great medicinal value. Rucola controls human diseases and disorders like diabetes, cancer, cardiovascular diseases have already been proven from scientific research. According to National Nutrient Database (Anon., 2016) of the United States, 100 g of fresh leaves contain only 25 Kcal energy while rich in folic acid (97 µg or 24%), vitamin A (2373 IU), vitamin C (15 mg), vitamin K (108.6 µg) and vitamin B-complexs. Like other Brassicas, it contains glucosides such as allylsulphonocyanate while the seed oil contains erucic acid (Nuez and Hernandez, 1994).

Rucola is traditionally grown in home gardens. It is now grown commercially in many places and is available for purchase in supermarkets and farmers' markets throughout the world. Although it has so many medicinal values, its supply from conventional method is not up to the satisfactory level. The conventional techniques used the seed as planting material which shows the low viability and less regeneration capacity. The traditional method is also time consuming and slow growing process. Therefore, Rucola is also susceptible to many diseases and insect attack. For solving this problem and large scale production, tissue culture technology can be the best way. It will give disease free healthy seedling for higher yield. Therefore, inspite of the recalcitrant nature of certain oil seed crops like Brassica and Sesamum, reports are available on their *in vitro* regeneration (Batra and Dhingra, 1991; Khan *et al.*, 2009). However, clonal propagation through *in vitro* culture can enhance the multiplication rates (Sauvaire and Galgy, 1978). The producer can provide this Rucola plant to the farmers quickly.

Plant biotechnology, a modern technique is mainly rooted on plant cell culture. This technique has been used in the multiplication of "good clones" of agricultural crops and medicinal plants. Plant cell culture is considered as an effective system for the study of the biological importance of bioactive metabolites in *in vitro* (Yanpaisan *et al.,* 1999). Rucola is amenable to tissue regeneration and both shoot organogenesis and somatic embryogenesis from a variety of explants, including cotyledons, hypocotyls, zygotic embryos, and mesophyll protoplast, have been achieved on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with either a-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) or indole-3-acetic acid (IAA) and BA (Ahloowalia, 1987; Sikdar *et al.,* 1987; Parkash *et al.,* 1989; Batra and Dhingra, 1991; Zhang *et al.,* 2005; Chen *et al.,* 2011; Slater *et al.,* 2011). *Eruca* undergoes androgenesis by either anther culture (Branca and Cacopardo, 1998) or microspore culture (Leskovsek *et al.,* 2008).

A balance between auxin and cytokinin determines the *in vitro* regeneration of plants grown in artificial medium. Generally, cytokinin helps in shoot proliferation and auxin helps in callus formation and rooting of proliferated shoots. The presence of auxin in defined combinations with cytokinins in the culture medium is also necessary to obtain adventitious shoot formation (Caboni and Tonelli, 1999). However, the requirement of

cytokinin and auxin depends on the plant species, genotype, explant type and culture conditions. Requirement of growth regulators depends on type of explants and its physiological condition also. The best media with optimum growth regulator, growth condition and suitable explants are needed to be standardized for large scale.

As Rucola has so many uses but in Bangladesh no commercial production is reported yet. So, for starting large scale commercial production, tissue culture method (micro propagation) should be used. It is possible now to obtain a large number of plants from one explant *in vitro*. In Bangladesh till now no evidence has been reported on the *in vitro* rapid multiplication capacity of Rucola which is very much essential to be known for its commercial production. So, considering the above problem and prospects, the experiment was designed to fulfill the following objectives.

Objectives:

i. Establishment of *in vitro* regeneration protocol of Rucola.
ii. Assessment of hormonal effect for *in vitro* response of Rucola.
iii. Identification of best hormonal concentration for *in vitro* regeneration of Rucola.
iv. To study the rapid regeneration of Rucola within a short period of time.

CHAPTER II
REVIEW OF LITERATURE

The present investigation involved *in vitro* micropropagation of *Eruca sativa* plant regeneration from callus culture of leaf explants followed by genetic stability of plantlets seems to be meager. However, information available in this aspects have been reviewed and presented in this section.

In vitro Regeneration of Rucola

Abbasi *et al.* (2016) were established feasible regeneration protocol for economically important plant *Eruca sativa* and 1, 1-diphenyl- 2-picrylhydrazyl scavenging activity of regenerated tissues was evaluated and compared with plant material collected from the wild leaf portions inoculated onto Murashige and Skoog (MS) medium responded to all plant growth regulators exploited. Optimum callus production was achieved on a combination of 2.0 mg/l 6- benzyladenine (BA) and 1.0 mg/l a-naphthalene acetic acid (NAA) and the lowest response was recorded for 0.5 mg/l gibberellic acid (GA_3) and 1.0 mg/l NAA. The callus was sub-cultured on similar composition/concentrations of plant growth regulators after 4 weeks of culture time. A 5.0 mg/l BA and 1.0 mg/l NAA produced optimum percentage shoot organogenesis after 4 weeks of sub-culturing. However, optimum number of shoots per explant was recorded for moderate concentrations (1.0 and 2.0 mg/l) of kinetin. Incorporation of NAA into MS medium-containing GA_3 also produced a feasible number of shoots/explant. Similar mean shoot length was recorded for 2.0 mg/l kinetin and 1.0 mg/l NAA and optimum concentrations (2.0, 5.0, and 10.0 mg/l) of GA_3 and 1.0 mg mg/l NAA. *In vitro* generated shoots were shifted to MS medium augmented with indole acetic acid (IAA) for rooting after 4 weeks of sub-culturing. Moderate concentrations (5.0 mg/l) of IAA produced feasible rooting.

Cucel *et al.* (2017) were determined micropropagation protocol of *Eruca sativa* Mill. In order to specify the most effective basal medium where two basal media, Murashige and Skoog (MS) and Gamborg B5 medium (B5) were used each supplemented with 1.0 mg/l BAP) and without plant growth regulators (PGRs) for seed germination. MS, supplemented with BAP, was found to be the most favored basal medium tested with

90% seed germination and 29.80 ± 2.30 shoot length success. Since shoot proliferation ability highly depends on the medium and cytokinin concentration, lateral buds were selected as explants, which were then individually cultured in MS, each supplemented with 1.0 mg/l kinetin, thidiazuron (TDZ), BA, and 6-(y,y-dimethylallylamino)-purine (2iP) in combination with 0.1 mg/l (IBA). MS containing 1.0/0.1 mg/l BA/IBA was chosen to be the most effective basal media among tested. Therefore, MS medium each individually supplemented with 0, 0.25, 0.5, 1.0 and 2.0 mg/l BA in combination with 0.1 mg/l IBA was again tested for most suitable 6-BA concentration. The highest shoot multiplication successes were obtained in the lowest BA treatments with 37.88 mm shoot length.

Explants such as apical buds, axillary buds, cotyledons, cotyledonary nodes, leaves, hypocotyls and immature embryonal axes from *in vitro*-grown plantlets were inoculated on the Murashige and Skoog (MS) medium supplemented with 4.44 μM BAP in combination with 2.85 μM indole-3-acetic acid (IAA) for *Eruca sativa*. Best multiple shoots formation was obtained with cotyledonary nodes. Each inoculated explant produced 18.10 ± 0.66 shoots within 2 to 3 weeks. These shoots were separated carefully and were transferred to the fresh half strength MS solid medium with indole-3-butyric acid (4.90 μM) for the development of the roots. These *in vitro*-developed plantlets produced flowers on the same medium with supplementation of 6-furfuril kinetin (0.23 μM). These plantlets were successfully transferred to the soil where they grew well for 8 to 10 weeks with 80% survivability (Sharma *et al.*, 2012).

Chen *et al.* (2011) were established a high-efficiency plant regeneration protocol based on somatic embryo formation for Huining Roquette, an interesting ecotype of *Eruca sativa* Mill, for future transgenic applications. On Murashige and Skoog (MS) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), alone or in combination with 6-benzylaminopurine (BA) or kinetin (KT), the cotyledon explants, cotyledon petioles, and hypocotyls all produced embryogenic callus (ECs) or somatic embryos (SEs) to different extents. After transferring onto hormone-free MS medium, the ECs or SEs from the different explants and media, all of them developed shoots with a frequency of 6–48%, and then produced roots with a frequency of 2–29%. As regards the probability of shoot differentiation, cotyledon explants appeared similar to hypocotyls, but superior to

cotyledon petioles; 2,4-D + KT worked more effectively than 2,4-D alone and 2,4-D + BA for callus induction and shoot differentiation. The optimal hormone combinations for plant regeneration of cotyledon, cotyledon petiole, and hypocotyl explants were 1.0 mg/l 2,4-D + 0.1 mg/l KT, 0.8 mg/l 2,4-D + 0.3 mg/l BA, and 1.0 mg/l 2,4-D + 0.3 mg/l KT, respectively. MS medium with 60–80 g/l sucrose was the most effective for improving SE maturation and germination.

Liqaa *et al.* (2013) were studied a plant regeneration protocol of *Eruca sativa* found the green callus was sub-cultured on the same media to regenerate shoots and roots. The callus continued growing without any response for shooting even at different concentrations of the extract. The white callus was also sub-cultured with different concentrations of the extract. The results indicate the regeneration of roots with different number and length. The best response was on 4mg/l extract and hormone. These results will give us a clear response for male tendency callus (white callus) to the extract treatment when we compare the results.

El-Nagar and Mekawi (2014) were conducted a laboratory study to evaluate the oil contents and its fatty acids, total phenolic compounds and total flavonoid compounds in seed and callus induced from tissue culture of two rocket genotypes. The influence of genotype, explants, and growth regulators on callus induction was investigated. The highest values of all parameters were obtained from Coltivata genotype. Callus was induced from two explants type on MS medium containing different combinations of 2,4-D and Kinetin as well as NAA and BA. Growth of the callus of cotyledonary explants was more vigorous than that formed from explants of stem segments. Media containing NAA in combination with BA stimulated the formation of green compact calli while soft, friable, yellow calli were formed on media containing 2,4-D and kinetin. Coltivata genotype had the highest amount of total phenolic and flavonoid content in green and yellow callus induced from tissue culture as well as in seed with exception of seed of Balady genotype had relatively phenolic compounds higher than seed of Coltivata genotype.

Zhang *et al.* (2005) were developed a protocol for high frequency somatic embryogenesis and plant regeneration from cotyledon and hypocotyl explants of *Eruca*

sativa. Explants grown on Murashige and Skoog (MS) medium supplemented with 4.52 pM 2,4-D formed embryogenic callus after 4 wk of culture. Secondary somatic embryos were also produced from primary somatic embryos on MS medium containing 0.56 pM 2,4-D. Somatic embryos developed into mature embryos on MS medium in the presence of 45 g 1- polyethylene glycol. After desiccation, somatic embryos developed into plantlets by culturing the mature somatic embryos on 1/2 x MS medium containing 0.24 pM indole-3-butyric acid (IBA).

Daud *et al*. (2015) were carried out a experiments to establish optimum culture condition and to identify the most responsive explants to regenerate *Brassica oleraceae* L. Var *Capitata* through plant tissue culture system. The explants were induced with manipulation of growth regulators during organogenesis. Plant growth regulators that were used are NAA and BAP. Different combinations and concentrations of plant growth regulators were added into the MS medium. The formations of callus were initiated after two weeks of subculture. The new plantlets were raised in a short period of time when stem and root explants were cultured on MS medium containing 1.0mg/1 BAP and 0.5 mg/1 NAA. The explants of petiole and leaves showed that there were slower growths of callus formation for most of the concentration. The well rooted in vitro raised plantlets were successfully transferred to soil and their survival rate under natural environment was 90%.

Javeed *et al.* (2016) were carried a study entitled "*In vitro* Regeneration Studies in *Brassica napus*, variety GS/L and DG/S out in the Biotechnology Laboratory, Division of Genetics and Plant Breeding SKUAST. *Brassica napus*, variety GS/L and DG/S were cultured on MS Media supplemented with different concentrations of growth regulators. Highest callus induction was observed in GS/L where MS medium augmented with 2,4-D at 2-2.5 mg/l. Different concentrations of growth regulators in media showed a significant difference in the callus induction frequency (CIF) and regeneration frequency (RF) for both the genotypes tested. MS medium supplemented with BAP (5.0 mg/l) and 2, 4-D (0.5 mg/l) showed highest shoot regeneration frequency in GS/L.

Ali *et al.* (2007) were established a regeneration protocols for *Brassica napus* L., using star, cyclon and westar cultivar. Callus induction ability was evaluated by using different

concentration of 2, 4-D in combination with 0.5mg/l BAP and 0.5mg/l Silver nitrate, which was used for the first time in MS medium and efficient callus was produced using 0.5mg/l 2, 4-D. Silver nitrate in callus induction media resulted in green callus. Shoots were regenerated on callus using different concentrations of NAA with 2mg/l BAP and 5mg/l Silver nitrate in MS medium. 67-82% shoots were regenerated on media having 0.1mg/l NAA.

CHAPTER III

MATERIALS AND METHODS

3.1 Time and Location of the experiment

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher-e-Bangla Nagar, Dhaka-1207 from the period of February 2016 to June 2017.

3.2 Experimental materials

3.2.1 Source of materials

The source materials of *Eruca sativa* were collected from Department of Genetics and Plant Breeding and Department of Horticulture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207.

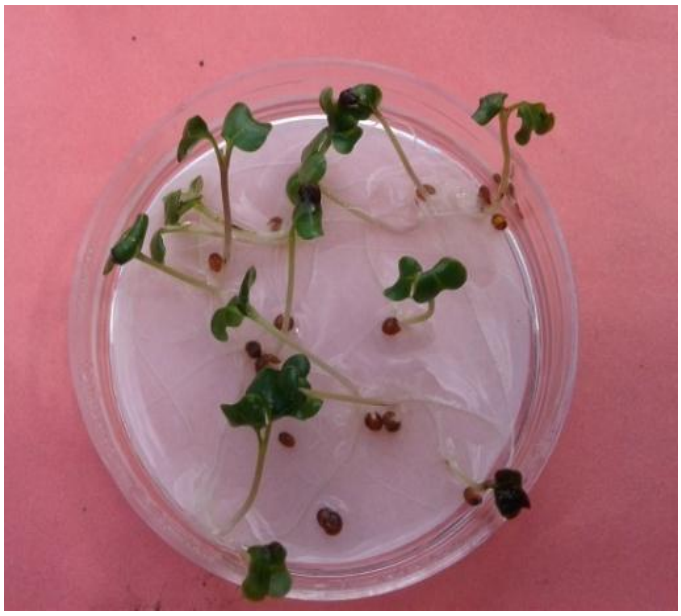

Plate 1. Source materials of Rucola

3.2.2 Plant materials

The cotyledonary nodes of *Eruca sativa* were used as plant materials in the present investigation.

3.2.3 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121ºC for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glass ware

The Borosil glasswares were used for all the experiments. Oven dried (250ºC) Erlesnmeyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Finally they were rinsed with distilled water and sterilized in oven at 160-180ºC for 3- 4 hours.

3.2.5 Culture media

The degree of success in any technology employing cell, tissue and organ culture is related to few major factors. A significant factor is the choice of nutritional components and growth regulators. MS (Murashige and Skoog, 1962) medium supplemented with different phytohormones as per treatments were used as culture medium for callus and shoot induction, shoot multiplication and maintenance and regeneration of roots from multiplied shoots. Hormones were added separately to different media according to the requirements. And for that stock solutions of hormones were prepared ahead of media preparation and stored at 4ºC temperature.

3.2.6 Plant growth regulators (PGRs)

Following stock solutions of hormones were prepared ahead of media preparation and stored at 4ºC temperature.

1. BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) for shoot induction
2. BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) combination with IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) respectively for shoot and root formation.
3. BA 4.0 mg/l with NAA (0.5, 1.0, 1.5, 2.0 mg/l) respectively for callus and shoot induction
4. IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) for root formation

3.3 Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions. As different ingredients were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins, growth hormones, etc, were used.

3.3.1 Stock solution "A" (Macronutrients)

Stock solution of macronutrients was prepared up to 10 times the concentration of the final medium in 1000 ml of distilled water (dw). Ten times the weight of the salts required per litre of the medium were weighed properly and dissolved by using a magnetic stirrer in about 750 ml of distilled water and then made up to 1000 ml by further addition of distilled water (dw). To make the solution free from all sorts of solid contaminants, it was filtered through Whatman no. I filter paper. Then it was poured into a plastic container, labeled with marker and stored in a refrigerator at 4ºC for later use.

3.3.2 Stock solution "B" (Micronutrients)

The stock solution of micronutrients was made up to 100 times the final strength of necessary constituents of the medium in 1000 ml of distilled water (dw) as described for the stock solution of macronutrients. The stock solution was filtered, labeled and stored in a refrigerator at 4ºC.

3.3.3 Stock solution "C" (Iron sources)

This was prepared at 100 times the final strength of Fe_2SO_4 and Na_2-EDTA in 100 ml of distilled water and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4ºC.

3.3.4 Stock solution "D" (Vitamins)

Each of the desired ingredients except myo-inositol were taken at 10 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots and stored at –20ºC.

3.4 The preparation of the stock solution of hormones

In order to prepare hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Cytokinins were dissolved in few drops of acidic solutions (1N HCl) and auxins were dissolved in few drops of basic solutions (1N NaOH).

Hormones (Solute)	Solvents used
BA	1 N NaOH
IBA	1 N NaOH
NAA	1 N NaOH

The stock solution of hormones was prepared by following a general procedure.10 ml of 70% ethyl alcohol or 1 (N) NaOH solvent and 100 mg of solid hormone was placed in a small beaker and then dissolved with the addition of sterile distilled water using a measuring cylinder and the volume was made up to 100 ml. The prepared hormone solution was then labeled and stored at 4±1°C for use up to two month. Growth regulators were purchased from Sigma, USA.

3.4.1 Preparation of culture media from stock solutions

To prepare 1 litre of MS (Murashige and Skoog, 1962) medium the following steps were followed:

1. About 400 ml distilled water was taken in a flask.
2. 100 ml of macronutrients, 10 ml of micronutrients, 100 ml of irons and 10 ml of vitamins were taken from each of these stock solutions into a 2 litre beaker on a heater cum magnetic stirrer.
3. 30 gm of sucrose was added to this solution and gently agitated to dissolve completely.
4. Required volume of hormone solutions was directly added to the solutions in the beakers.
5. The solution was poured into a 1000 ml measuring cylinder and the volume was made up to 1000 ml with addition of distilled water.
6. pH of the medium was adjusted to 5.8 with a digital pH meter with the help of adding 0. 1 N NaOH or 0.1 N HCl as necessary.

7. To solidify the medium, 8 gm (0.8%) of Difco-brand Bacto-Agar was added to the solution and the whole mixture was heated (avoiding boiling) by micro oven for 5 to 10 minutes till complete dissolution of the agar.

8. Required volume of hot medium was dispensed into culture vessels. After dispensing and proper cooling of the medium, the culture vessels were plugged with cork and marked with different codes with the help of a glass marker to indicate specific hormonal combinations.

3.5 Sterilization

Fixed volume of medium was dispensed into 100 ml conical flask or bottle. The flask or bottle was plugged with aluminium foil and marked with different codes with the help of a glass marker to indicate specific hormonal supplement. The conical flasks were then autoclaved at 15 psi of pressure at 121°C for 20 minutes.

3.5.1 Sterilization of culture medium

The media contained in glass vials were autoclaved at 15 psi and 121°C for 20 minutes. After autoclaving, the culture media were allowed to cool under normal condition.

3.5.2 Sterilization of glasswares and instruments

Beakers, test-tubes, conical flasks, pipettes, metallic instruments, like forceps, scalpels, needles and spatula were sterilized in an autoclave at a temperature of 121°C for 40 minutes at 15 psi of pressure.

3.5.3 Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all over the floors and walls with detergent or lysol (germicide) followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals. Generally, switching on the laminar airflow cabinet (LAC) and sterilized the cabinet by wiping the working surface with 70% ethyl alcohol and then UV light was on for 30 minutes so that the working area of the (LAC) is sterilized. After switch off UV light at least 5 minutes was waited to ensure safe environment.

3.6 Precaution to ensure aseptic condition

The instruments like forceps, scalpels, needles etc. were pre-sterilized by autoclaving and subsequent sterilization was done by dipping in 70% ethylalcohol followed by flaming and cooling. Hands were also sterilized by wiping with 70% ethylalcohol. Aseptic conditions were followed during each and every operation to avoid the contamination of culture.

3.7 Explants preparation and culture

3.7.1 Preparation of explants

The cotyledonary node was used as explants. It was obtained from developing shoots of rucola grown under field conditions and was brought to the preparation room .The cotyledonary nodes were washed with tap water in a beaker. The nodal segment then cut in 0.5-1.0 cm size required for inoculation in culture vial.

3.7.2 Surface sterilization of explants

The cotyledonary nodes of 2 to 3 cm size were taken in a beaker. Surface sterilization of explants was done as follows:

i. The cotyledonary nodes were cut as small size (2 to 3 cm) and rinsed with water.
ii. The nodes were soaked with Tween-20 solution having 10% concentration for 5 min.
iii. Washing with distilled water was done for several times.
iv. The explants were sterilized with 70% ethanol for 1 min.
v. Then the explants were sterilized with 0.2% $HgCl_2$ for 2 min.
vi. The explants were rinsed with sterilized distilled water for at least 4 times.
vii. The final size of explants were made 0.5-1.0 cm.
viii. Finally the explants were transferred to the MS media carefully.

3.8 Culture of explants

3.8.1 Inoculation of explants in culture vial

The isolated and surface sterilized nodal segment was collected carefully through maintaining aseptic condition inside the laminar air flow cabinet. The individual nodal segments were directly inoculated to each of the culture vial containing 25 mL of MS medium supplemented with different concentrations of hormones as per treatment.

Explants were transferred to culture vials with the help of sterile forceps under strict aseptic conditions. The surface sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. The mouth of culture vial was flamed before and after positioning of the explant on the medium.

Plate 2. Inoculation of cotyledonary node as explant in MS medium

3.8.2 Incubation

The culture vials transferred to culture racks and allowed to grow in controlled environment. The temperature of the growth room was maintained within 25±1ºC by an air conditioner and a 16 hour photo period was maintained along with light intensity of 3000 lux for proper growth and development of culture.

3.8.3 Maintenance of proliferating shoots

Initial sub-culturing was done after 15-25 days when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or brownish basal tissues were removed. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of 10-20 days.

3.8.4 Root induction of regenerated shoots

When the shoots grew about 3-4 cm in length with 4-5 well developed leaves, they were removed aseptically from the culture vials and were separated from each other and again cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

3.9 Treatments

Five sub experiments were conducted to assess the effect of different concentrations of BA and IBA on shoot proliferation and subsequent rooting of the multiplied shoot and BA with NAA for callus induction.

Sub-experiment 1.

Effect of BA on *in vitro* shoot induction potentiality in Rucola

Five levels of BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and control (0.0 mg/l) were used. The experiments were arranged in Completely Randomized Design (CRD) with five replications.

Sub-experiment 2.

Combined effect of BA and IBA on *in vitro* shoot and root induction potentiality in Rucola

Treatments:

In this sub-experiment, Five levels of IBA were practiced with each level of BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l). Total 25 combinations of BA and IBA were examined in this experiment and control treatment also practiced. The combinations were as follows:

T_1 = BA 1.0 mg/l + 1.0 mg/l IBA

T_2 = BA 1.0 mg/l + 2.0 mg/l IBA

T_3 = BA 1.0 mg/l + 3.0 mg/l IBA

T_4 = BA 1.0 mg/l + 4.0 mg/l IBA

T_5 = BA 1.0 mg/l + 5.0 mg/l IBA

T_6 = BA 2.0 mg/l + 1.0 mg/l IBA

T_7 = BA 2.0 mg/l + 2.0 mg/l IBA

T_8 = BA 2.0 mg/l + 3.0 mg/l IBA

T_9 = BA 2.0 mg/l + 4.0 mg/l IBA

T_{10} = BA 2.0 mg/l + 5.0 mg/l IBA

T_{11} = BA 3.0 mg/l + 1.0 mg/l IBA

T_{12} = BA 3.0 mg/l + 2.0 mg/l IBA

T_{13} = BA 3.0 mg/l + 3.0 mg/l IBA

T_{14} = BA 3.0 mg/l + 4.0 mg/l IBA

T_{15} = BA 3.0 mg/l + 5.0 mg/l IBA

T_{16} = BA 4.0 mg/l + 1.0 mg/l IBA

T_{17} = BA 4.0 mg/l + 2.0 mg/l IBA

T_{18} = BA 4.0 mg/l + 3.0 mg/l IBA

T_{19} = BA 4.0 mg/l + 4.0 mg/l IBA

T_{20} = BA 4.0 mg/l + 5.0 mg/l IBA

T_{21} = BA 5.0 mg/l + 1.0 mg/l IBA

T_{22} = BA 5.0 mg/l + 2.0 mg/l IBA

T_{23} = BA 5.0 mg/l + 3.0 mg/l IBA

T_{24} = BA 5.0 mg/l + 4.0 mg/l IBA

T_{25} = BA 5.0 mg/l + 5.0 mg/l IBA

The experiments were arranged in Completely Randomized Design (CRD) with five replications. Each of replications consisted of five culture vials.

Sub-experiment 3.

Effect of BA with NAA on *in vitro* callus induction potentiality in Rucola

In this sub-experiment, cotyledonary nodes of rucola were used as source materials to investigate the effect of BA and NAA.

Treatments: BA 4.0 mg/l with NAA (0.5, 1.0, 1.5 and 2.0 mg/l) and control (0.0 mg/l) respectively were used.

Sub-experiment 4.

Effect of IBA on root induction potentiality of *in vitro* regenerated Rucola

Five levels of IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and control (0.0 mg/l) were used. The experiments also practiced as sub-experiment 1.

Sub-experiment 5.

***Ex vitro* acclimatization and establishment of plantlets on soil**

Tissue culture derived plantlets were acclimatized in shade house and natural condition to find out the survival percentage.

3.10 Data collection

The observations on development pattern of callus, shoots and roots were made throughout the entire culture period. Five replicates (single shoot per culture bottle) were used per treatment. Data were recorded after 14, 21 and 28 days of induction on culture media in case of shoot and root proliferation. In case of callus formation, data was recorded at 20, 30 and 40 days after callus initiation (DAI). The following observations were recorded in cases of callus, shoot and root formation under *in vitro* condition.

1. Days to callus induction
2. Percentage of callus induction
3. Weight of callus (g)
4. Days to shoot induction from callus
5. Number of shoots from callus
6. Days to shoot induction
7. Percentage of shoot induction
8. Number of shoot
9. Number of leaves per shoot
10. Length of leaves per shoot (cm)
11. Days to root induction
12. Percentage of root induction
13. Number of roots per shoot
14. Length of roots per shoot (cm)

3.10.1 Percentage of callus induction

Number of explants formed callus were recorded and the percentage of callus induction was calculated as

$$\text{Percentage of callus induction} = \frac{\text{Number of explants induced callus}}{\text{Number of explants inoculated}} \times 100$$

3.10.2 Weight of callus

The fresh weight of callus was measured in (g) using analytical balance.

3.10.3 Percentage of shoot induction

The number of shoots were produced per explant were recorded and the percentage of shoot regeneration was calculated as

$$\text{Percentage of shoot induction} = \frac{\text{Number of explants induced shoots}}{\text{Number of explants inoculated}} \times 100$$

3.10.4 Days to shoot induction

Days to shoot induction were calculated by counting the days from explants inoculation to the first induction of shoots.

3.10.5 Number of shoot per explant

Number of shoot per explant was calculated by using the following formula,

$$\text{Number of shoot per explant} = \frac{\text{Number of shoots per explant}}{\text{Number of observation}}$$

3.10.6 Number of leaf

Numbers of leaves produced on the plantlet were counted and the mean was calculated.

3.10.7 Percent of explants showing root induction

The number of roots were produced per explant were recorded and the percentage of root regeneration was calculated as

$$\text{Percentage of root induction} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot incubated}} \text{ X } 100$$

3.10.8 Days required for root initiation

Number of days required for initiation of root from the day of inoculation was recorded.

3.10.9 Number of roots/plantlet

Average number of roots/plantlet was calculated as the following formula -

$$\bar{x} = \frac{\sum xi}{n}$$

Where,

$\overline{X}$ = Mean no. of roots/plantlet

∑=Summation

xi=No. of roots in i th observation

n = No. of observation

3.10.10 Length of roots

Root length was determined in centimeter (cm) from the base to tip of the roots. Average length of the root was calculated by the following formula-

$$\bar{x} = \frac{\sum xi}{n}$$

Where,

$\overline{X}$ = Mean no. of roots/plantlet

∑=Summation

xi = length of roots in i th observation

n = No. of observation

3.10.11 Percentage of established plantlets

The percentages of established plantlets were calculated based on the number of plantlets placed in the plastic pots and the number of plants finally survived. The percentages of established plantlet were calculated by using the following formula:

$$\text{Percentage of established plantlets} = \frac{\text{Number of established plantlets}}{\text{Total number of plantlets}} \text{ X } 100$$

3.11 Transfer of plantlets from culture vials to soil

After two and half months, the culture vials with well-developed plantlets were transferred to normal room temperature. After 2-3 days, the rooted plantlets were removed from culture vials and the medium attached to root was gently washed out with tap water.

Plantlets were individually transplanted in Plastic pot containing the mixture of soil, sand and cow dung (1:1:1). Immediately after transplantation, the plants along with pot were covered with moist and transparent poly bag for 7 days to prevent desiccation. To reduce sudden shock, the plantlets were kept in shade house for 12 days. Then after 12 days plantlets were transferred to the field.

3.12 Statistical analysis of data

Data recorded for different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The means for all the treatments were calculated and analyses of variance of all the characters were performed. Experiment was conducted in laboratory and arranged in Completely Randomized Design (CRD) with five replications. The significant difference between the pair of means was evaluated at 1% level of significance by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

CHAPTER IV

RESULTS AND DISCUSSION

Different investigations were made on callus induction, shoot regeneration, root formation and establishment of plantlets in the field condition. Major findings were presented in different sub headings.

4.1 Sub-experiment 1. Effect of BA on *in vitro* shoot induction potentiality in Rucola

The result of different concentrations of BA has been presented under following headings with Figure (1.1-1.2) and Table (1-3).

4.1.1 Days to shoot induction

Significant variations were observed among different concentrations of BA on days to shoot induction. The maximum (14.00 days) to shoot induction were recorded in control followed by 1.0 mg/l (11.60 days) and 2.0 mg/l (10.80 days). On the other hand, minimum (6.40 days) was required in 3.0 mg/l BA followed by 5.0 mg/l (7.20 days) and 4.0 mg/l (9.00) days (Figure 1.1).

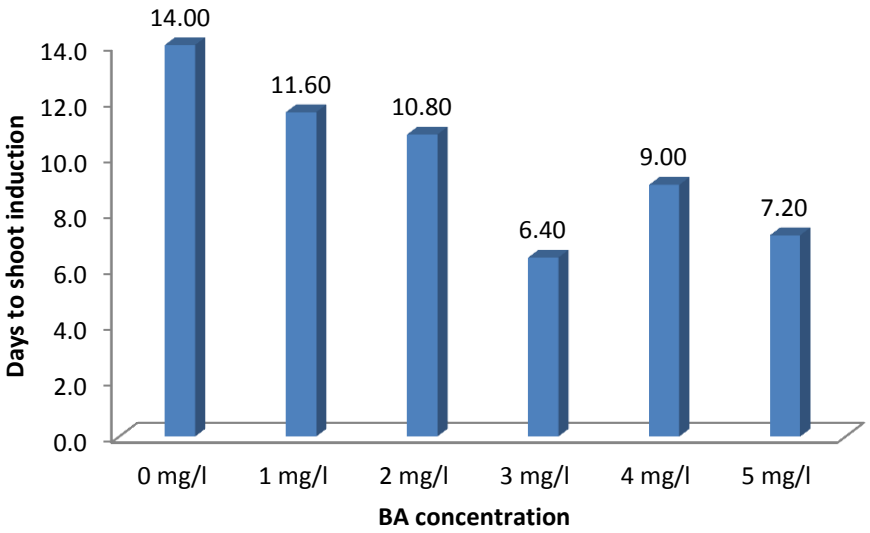

Figure 1.1 Effect of BA on days to shoot induction in Rucola

4.1.2 Percentage of shoot induction

The different concentrations of BA showed the significant variations on percentage of shoot induction. BA 3.0 mg/l had produced the highest frequency of shoot (80.00%), while the lowest percentage (36.00%) in control treatment (Figure 1.2).

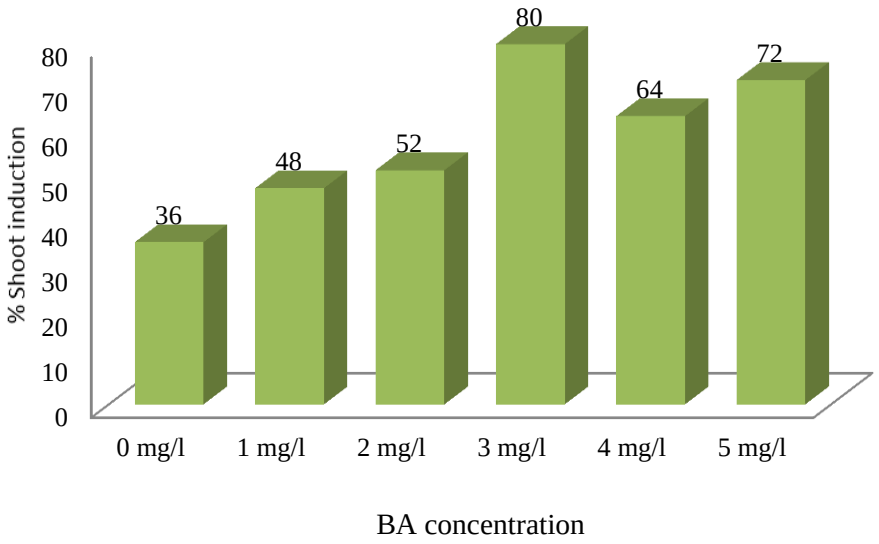

Figure 1.2 Effect of BA on percentage of shoot induction in Rucola

4.1.3 Number of shoot

There was a significant influence of different concentrations of BA on the number of shoot at 5% level of significance in laboratory condition. The highest number of shoot (2.60, 3.60 and 4.60) at 14 , 21 and 28 DAI respectively (Plate 3) was noticed from the 4.0 mg/l BA which was statistically similar with 3 mg/l (2.40, 3.00 and 4.00) and 5.0 mg/l (2.20, 3.20 and 4.00) at 14, 21 and 28 DAI (Table 1). Whereas the lowest number of shoot (1.00, 1.60 and 1.60) at 14, 21 and 28 DAI, respectively were noticed in control without hormone (Table 1). Cucel *et al.* (2017) found that the highest shoot multiplication successes were obtained in the lowest BA treatments with 37.88 mm shoot length. The findings of this study are partially similar with their results.

Table 1. Effect of different concentration of BA on number of shoot at different days after shoot induction

BA (mg/l)	Number of shoot		
	14 DAI	21 DAI	28 DAI
0	1.00f	1.60e	1.60d
1.0	1.60e	2.00d	2.60c
2.0	2.00d	2.60c	3.20bc
3.0	2.40b	3.00b	4.00ab
4.0	2.60a	3.60a	4.60a
5.0	2.20c	3.20b	4.00ab
CV (%)	30.60	24.54	17.75
LSD (0.05)	0.134	0.357	0.772

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

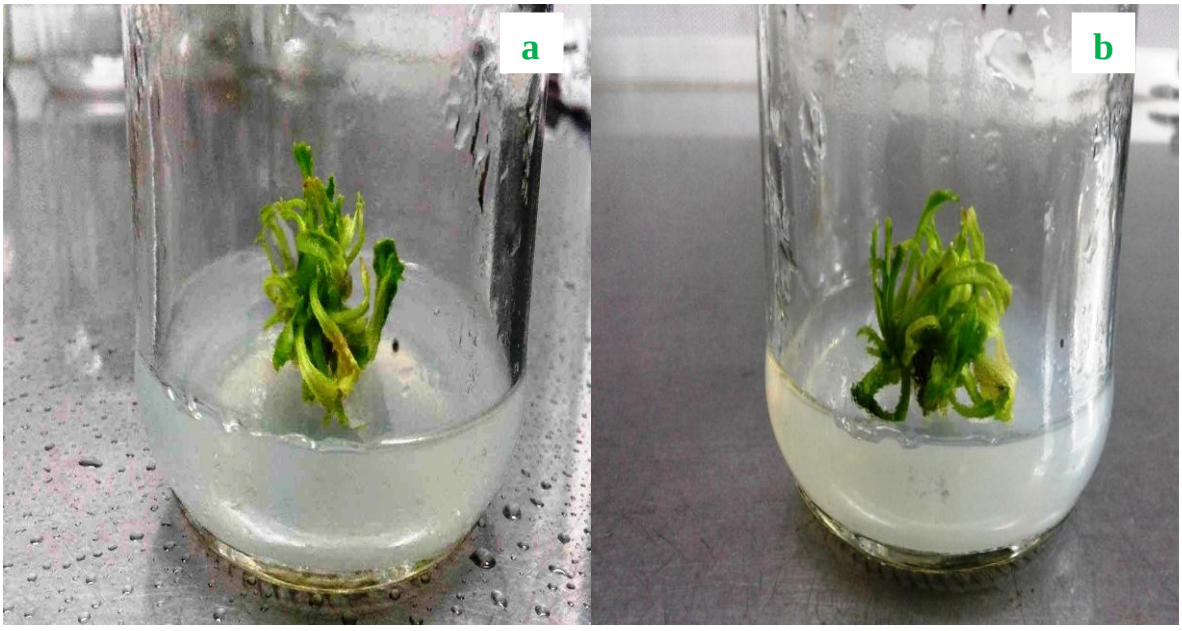

Plate 3. Highest number of shoot induction in MS medium supplemented with BA 4.0 mg/l at a) 21 DAI and b) 28 DAI

4.1.4 Number of leaves per shoot

There was a significant influence of different concentrations of BA on the number of leaves per shoot. BA 4.0 mg/l gave the maximum number of leaves (2.60, 4.20 and 6.40) (Plate 4) and the second highest leaves number (1.80, 3.20 and 5.20) was found in BA 5.0 mg/l at 14, 21 and 28 DAI, respectively (Table 2) whereas, the control treatment showed the lowest number of leaves (1.00, 1.20 and 1.60) at 14, 21 and 28 DAI (Table 2) respectively.

Table 2. Effect of different concentration of BA on number of leaves at different days after induction (DAI)

BA (mg/l)	Number of leaves/shoot		
	14 DAI	21 DAI	28 DAI
0	1.00c	1.20c	1.60e
1.0	1.20c	1.80c	2.20de
2.0	1.20c	1.80c	2.80d
3.0	2.20ab	3.40ab	4.20c
4.0	2.60a	4.20a	6.40a
5.0	1.80b	3.20b	5.20b
CV (%)	25.69	26.27	20.16
$LSD_{(0.05)}$	0.558	0.892	0.982

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

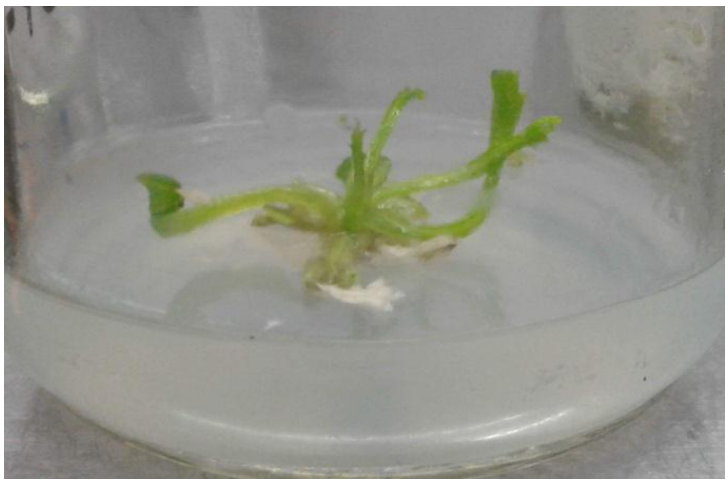

Plate 4. BA 4.0 mg/l showing highest number of leaves at 28 DAI

4.1.5 Length of leaves (cm)

Significant variation of different concentrations of BA on average length of leaves was found. The highest length of leaves (2.56 cm, 4.12 cm and 5.24 cm) at 14, 21 and 28 DAI, respectively was noticed from the 4.0 mg/l BA (Plate 5) which was statistically different from rest of treatments. Whereas, the minimum length (1.40 cm, 2.16 cm and 2.34 cm) at 14, 21 and 28 DAI, respectively were noticed in control treatment (Table 3).

Table 3. Effect of different concentration of BA on length of leaves at different DAI

BA (mg/l)	Length of leaves (cm)		
	14 DAI	21 DAI	28 DAI
0	1.40e	2.16f	2.34f
1.0	1.62d	2.60e	3.20e
2.0	1.92c	3.38d	4.08d
3.0	2.18b	3.62c	4.60c
4.0	2.56a	4.12a	5.24a
5.0	2.26b	4.00b	5.08b
CV (%)	9.76	4.96	4.67
LSD (0.05)	0.189	0.108	0.139

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

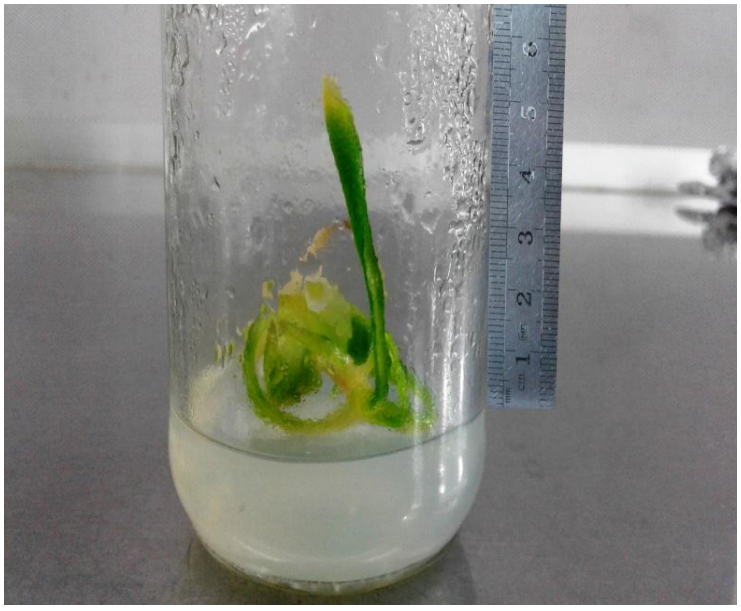

Plate 5. Effect of BA 4.0 mg/l showing highest length of leaves at 28 DAI

4.2 Sub-experiment 2. Combined effect of BA and IBA on shoot and root induction potentiality in Rucola

The results of the combined effect of different concentrations of BA + IBA have been presented under following headings with Table (4-10) and Plate (6-10).

4.2.1 Days to shoot induction

Significant variations were observed among different concentrations of BA and IBA on days to shoot induction. The minimum duration (6.00 days) was obtained in BA 4.0 mg/l+ IBA 3.0 mg/l than rest of the treatments. On the other hand, the maximum days (18.80 days) to shoot induction was recorded in control (Table 4). Daud *et al*. (2015) showed that the new plantlets were raised in a short period of time when explants were cultured on MS medium containing 1.0 mg/1 BAP and 0.5 mg/1 NAA. The variation may be due to influence of genetic and environmental factors (Sen, J. *et al*., 2002).

4.2.2. Percentage of shoot induction

There was a significant influence of different concentrations of BA and IBA on the percentage of shoot induction per explant. The optimum percentage (76.00%) of shoot induction was noticed in treatment BA 4.0 mg/l+ IBA 2.0 mg/l which was best than others and minimum percentage (36.00%) was noticed in control hormone free media (Table 4). Abbasi *et al.* (2016) observed that the treatment 5.0 mg/l BA and 1.0 mg/l NAA produced optimum percentage of shoot organogenesis after 4 weeks of sub-culturing. This variation may be due to growth regulators in the culture media, genetic, physiological and morphological change *in vitro* (Narayanswamy, S. *et al*., 1977).

Table 4. Combined effect of different concentration of BA and IBA on days to shoot induction and percent of shoot induction

Treatment	Days to shoot induction	Percent of (%) Shoot Induction
Control	18.80a	36.00g
BA 1.0 mg/l + IBA 1.0 mg/l	13.80de	52.00ef
BA 1.0 mg/l + IBA 2.0 mg/l	14.20d	52.00ef
BA 1.0 mg/l + IBA 3.0 mg/l	15.40c	52.00ef
BA 1.0 mg/l + IBA 4.0 mg/l	15.40c	48.00f
BA 1.0 mg/l + IBA 5.0 mg/l	16.20b	48.00f
BA 2.0 mg/l + IBA 1.0 mg/l	13.00f	52.00ef
BA 2.0 mg/l + IBA 2.0 mg/l	12.00g	64.00b-d
BA 2.0 mg/l + IBA 3.0 mg/l	12.40fg	52.00ef
BA 2.0 mg/l + IBA 4.0 mg/l	13.20ef	52.00ef
BA 2.0 mg/l + IBA 5.0 mg/l	12.80f	52.00ef
BA 3.0 mg/l + IBA 1.0 mg/l	10.20jk	60.00c-e
BA 3.0 mg/l + IBA 2.0 mg/l	10.60ij	60.00c-e
BA 3.0 mg/l + IBA 3.0 mg/l	10.00jk	68.00bc
BA 3.0 mg/l + IBA 4.0 mg/l	11.80gh	60.00c-e
BA 3.0 mg/l + IBA 5.0 mg/l	11.20hi	60.00c-e
BA 4.0 mg/l + IBA 1.0 mg/l	7.80no	68.00a-c
BA 4.0 mg/l + IBA 2.0 mg/l	6.60pq	76.00a
BA 4.0 mg/l + IBA 3.0 mg/l	6.00q	68.00a-c
BA 4.0 mg/l + IBA 4.0 mg/l	8.60l-n	64.00b-d
BA 4.0 mg/l + IBA 5.0 mg/l	7.20op	72.00ab
BA 5.0 mg/l + IBA 1.0 mg/l	8.60l-n	60.00c-e
BA 5.0 mg/l + IBA 2.0 mg/l	8.60mn	68.00a-c
BA 5.0 mg/l + IBA 3.0 mg/l	8.80lm	56.00d-f
BA 5.0 mg/l + IBA 4.0 mg/l	9.60k	52.00ef
BA 5.0 mg/l + IBA 5.0 mg/l	9.40kl	56.00d-f
CV (%)	5.35	21.23
$LSD_{(0.05)}$	0.754	8.219

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

4.2.3 Number of shoot

Different concentrations of BA and IBA showed significant variations on the number of shoot. The highest number of shoot (2.40, 3.00 and 3.40) was noticed from the BA 4.0 mg/l + IBA 3.0 mg/l (Plate 6) and second highest number (1.80, 2.80 and 3.00) at 14, 21 and 28 DAI, respectively, were observed in 4.0 mg/l BA + 2.0 mg/l IBA. Whereas the lowest average number of shoot (1.00, 1.40 and 1.40) at 14 DAI, 21 DAI and 28 DAI, respectively were noticed in control treatment (Table 5). These findings are completely opposed to the results of Sharma *et al.* (2012). They found that each inoculated explant produced 18.10 ± 0.66 shoots within 2 to 3 weeks from *in vitro* grown plantlets inoculated on the Murashige and Skoog (MS) medium supplemented with 4.44 μM 6-benzylaminopurine (BAP) in combination with 2.85 μM indole-3-acetic acid (IAA). This variation is due to the age, nature, origin and the physiological state of the explant and environmental factors can play a crucial role in the establishment of cultures and subsequent plant regeneration (Bajaj, Y.P.S. *et al*., 1991).

Table 5. Combined effect of different concentration of BA and IBA on number of shoot at different DAI

Treatments	Number of shoot		
	14 DAI	21 DAI	28 DAI
Control	1.00c	1.40d	1.40d
BA 1.0 mg/l + IBA 1.0 mg/l	1.60bc	1.60cd	2.20b-d
BA 1.0 mg/l + IBA 2.0 mg/l	1.60bc	1.60cd	2.20b-d
BA 1.0 mg/l + IBA 3.0 mg/l	1.40bc	1.60cd	1.80c-e
BA 1.0 mg/l + IBA 4.0 mg/l	1.20bc	1.40d	1.60de
BA 1.0 mg/l + IBA 5.0 mg/l	1.20bc	1.40d	1.80c-e
BA 2.0 mg/l + IBA 1.0 mg/l	1.60bc	2.00b-d	2.20b-e
BA 2.0 mg/l + IBA 2.0 mg/l	1.40bc	1.60cd	2.00c-e
BA 2.0 mg/l + IBA 3.0 mg/l	1.60bc	2.00b-d	2.40b-d
BA 2.0 mg/l + IBA 4.0 mg/l	1.40bc	2.20a-d	2.60a-c
BA 2.0 mg/l + IBA 5.0 mg/l	1.40bc	1.60cd	2.40b-d
BA 3.0 mg/l + IBA 1.0 mg/l	1.40bc	1.60cd	1.80a-c
BA 3.0 mg/l + IBA 2.0 mg/l	1.60bc	2.00b-d	2.20b-e
BA 3.0 mg/l + IBA 3.0 mg/l	1.60bc	1.60cd	2.00c-e
BA 3.0 mg/l + IBA 4.0 mg/l	1.60bc	1.80cd	2.00c-e
BA 3.0 mg/l + IBA 5.0 mg/l	1.60bc	2.20a-d	2.60a-c
BA 4.0 mg/l + IBA 1.0 mg/l	1.80ab	2.00b-d	2.60bc
BA 4.0 mg/l + IBA 2.0 mg/l	1.80ab	2.80ab	3.00ac
BA 4.0 mg/l + IBA 3.0 mg/l	2.40a	3.00a	3.40a
BA 4.0 mg/l + IBA 4.0 mg/l	1.60bc	2.40a-c	2.60bc
BA 4.0 mg/l + IBA 5.0 mg/l	1.60bc	2.20a-d	2.60bc
BA 5.0 mg/l + IBA 1.0 mg/l	1.40bc	2.20a-d	2.40b-d
BA 5.0 mg/l + IBA 2.0 mg/l	1.40bc	1.60cd	2.00c-e
BA 5.0 mg/l + IBA 3.0 mg/l	1.40bc	2.00b-d	2.00c-e
BA 5.0 mg/l + IBA 4.0 mg/l	1.80ab	2.40a-c	2.60a-c
BA 5.0 mg/l + IBA 5.0 mg/l	1.60bc	1.80cd	2.40b-d
CV (%)	16.06	9.61	15.72
LSD (0.05)	0.523	0.716	0.729

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 6. Effect of BA 4.0 mg/l + IBA 3.0 mg/l on maximum number of shoot observed at 28 DAI

4.2.4 Number of leaves per shoot

The number of leaves per shoot showed significant difference with combined concentrations of BA and IBA. The treatment BA 4.0 mg/l+ IBA 4.0 mg/l gave the highest number of leaves (3.20, 7.20 and 11.00) at 14, 21 and 28 DAI, respectively (Plate 7) whereas the lowest number of leaves (1.20, 1.20 and 1.40) at 14, 21 and 28 DAI respectively was found with hormone free media (Table 6).

Table 6. Combined effect of different concentration of BA and IBA on number of leaves at different DAI

Treatment	Number of leaves/shoot		
	14 DAI	21 DAI	28 DAI
Control	1.20e	1.20o	1.40l
BA 1.0 mg/l + IBA 1.0 mg/l	1.60de	2.00kl	2.80jk
BA 1.0 mg/l + IBA 2.0 mg/l	1.40e	1.60mn	2.40kl
BA 1.0 mg/l + IBA 3.0 mg/l	1.60de	1.80lm	2.40kl
BA 1.0 mg/l + IBA 4.0 mg/l	1.20e	1.60mn	2.20kl
BA 1.0 mg/l + IBA 5.0 mg/l	1.20e	1.40no	2.20kl
BA 2.0 mg/l + IBA 1.0 mg/l	1.40e	2.40ij	3.80h-j
BA 2.0 mg/l + IBA 2.0 mg/l	1.40e	1.80lm	3.80h-j
BA 2.0 mg/l + IBA 3.0 mg/l	1.60de	2.60hi	3.80h-j
BA 2.0 mg/l + IBA 4.0 mg/l	1.40e	2.00kl	3.00jk
BA 2.0 mg/l + IBA 5.0 mg/l	1.60de	2.20jk	3.40i-k
BA 3.0 mg/l + IBA 1.0 mg/l	1.60de	3.20g	4.40g-i
BA 3.0 mg/l + IBA 2.0 mg/l	1.40e	3.20g	5.00f-h
BA 3.0 mg/l + IBA 3.0 mg/l	1.80c-e	3.40g	5.00f-h
BA 3.0 mg/l + IBA 4.0 mg/l	1.80c-e	3.40g	5.20fg
BA 3.0 mg/l + IBA 5.0 mg/l	1.40e	2.80h	3.80h-j
BA 4.0 mg/l + IBA 1.0 mg/l	2.40bc	4.40de	8.00c
BA 4.0 mg/l + IBA 2.0 mg/l	1.80c-e	4.40de	7.20cd
BA 4.0 mg/l + IBA 3.0 mg/l	2.80b	6.80b	9.40b
BA 4.0 mg/l + IBA 4.0 mg/l	3.20a	7.20a	11.00a
BA 4.0 mg/l + IBA 5.0 mg/l	2.40bc	4.80c	8.20c
BA 5.0 mg/l + IBA 1.0 mg/l	1.80c-e	4.60cd	7.60cd
BA 5.0 mg/l + IBA 2.0 mg/l	2.60ab	4.60cd	5.80ef
BA 5.0 mg/l + IBA 3.0 mg/l	2.20b-d	4.00f	5.80ef
BA 5.0 mg/l + IBA 4.0 mg/l	1.80c-e	4.20ef	6.60dg
BA 5.0 mg/l + IBA 5.0 mg/l	1.80c-e	4.00f	5.20fg
CV (%)	31.95	22.38	18.27
$LSD_{(0.05)}$	0.583	0.302	1.141

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 7. Effect of BA 4.0 mg/l + IBA 4.0 mg/l on maximum number of leaves observed at 28 DAI

4.2.5 Length of leaves (cm)

There was a significant influence of different concentrations of BA with IBA on the length of leaves at 5% level of significance in laboratory condition. The highest length of leaves (2.60 cm, 4.42 cm and 5.00 cm) at 14, 21 and 28 DAI, respectively was noticed from the BA 4.0 mg/l + IBA 2.0 mg/l (Plate 8) followed by BA 4.0 mg/l + IBA 3.0 mg/l (2.50 cm, 4.12 cm and 4.72 cm) and BA 4.0 mg/l+ IBA 1.0 mg/l (2.20 cm, 4.14 cm and 4.64 cm) at 14, 21 and 28 DAI, respectively. Whereas the minimum length of leaves (0.94 cm, 1.50 cm and 1.62 cm) at 14, 21 and 28 DAI, respectively were noticed in control treatment (Table 7).

Table 7. Combined effect of different concentration of BA and IBA on length of leaves at different DAI

Treatments	Length of leaves (cm)		
	14 DAI	21 DAI	28 DAI
Control	0.94l	1.50o	1.62p
BA 1.0 mg/l + IBA 1.0 mg/l	1.06jk	1.86kl	2.06lm
BA 1.0 mg/l + IBA 2.0 mg/l	1.00kl	1.66mn	2.00l-n
BA 1.0 mg/l + IBA 3.0 mg/l	1.00kl	1.74lm	1.88m-n
BA 1.0 mg/l + IBA 4.0 mg/l	1.04kl	1.68mn	1.80n-p
BA 1.0 mg/l + IBA 5.0 mg/l	0.94l	1.58no	1.70op
BA 2.0 mg/l + IBA 1.0 mg/l	1.08jk	2.22j	2.44ij
BA 2.0 mg/l + IBA 2.0 mg/l	1.10j	2.28ij	2.48i
BA 2.0 mg/l + IBA 3.0 mg/l	1.00kl	2.28ij	2.40i
BA 2.0 mg/l + IBA 4.0 mg/l	1.08jk	1.98k	2.22i-k
BA 2.0 mg/l + IBA 5.0 mg/l	1.12j	1.96k	2.22kl
BA 3.0 mg/l + IBA 1.0 mg/l	1.10j	2.40hi	2.76h
BA 3.0 mg/l + IBA 2.0 mg/l	1.24i	2.44h	2.90gh
BA 3.0 mg/l + IBA 3.0 mg/l	1.46h	2.72g	3.06fg
BA 3.0 mg/l + IBA 4.0 mg/l	1.26i	2.60g	2.80h
BA 3.0 mg/l + IBA 5.0 mg/l	1.10j	2.20j	2.52i
BA 4.0 mg/l + IBA 1.0 mg/l	2.20c	4.14b	4.64b
BA 4.0 mg/l + IBA 2.0 mg/l	2.60a	4.42a	5.00a
BA 4.0 mg/l + IBA 3.0 mg/l	2.50b	4.12bc	4.72b
BA 4.0 mg/l + IBA 4.0 mg/l	2.10d	3.98c	4.36c
BA 4.0 mg/l + IBA 5.0 mg/l	2.00	3.58d	4.12d
BA 5.0 mg/l + IBA 1.0 mg/l	1.90f	3.46d	4.04d
BA 5.0 mg/l + IBA 2.0 mg/l	1.84f	3.30e	3.72e
BA 5.0 mg/l + IBA 3.0 mg/l	1.62g	3.04f	3.22f
BA 5.0 mg/l + IBA 4.0 mg/l	1.62g	3.00f	3.20f
BA 5.0 mg/l + IBA 5.0 mg/l	1.54g	3.04f	3.14f
CV (%)	6.98	6.82	6.62
LSD (0.05)	0.079	0.143	0.206

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

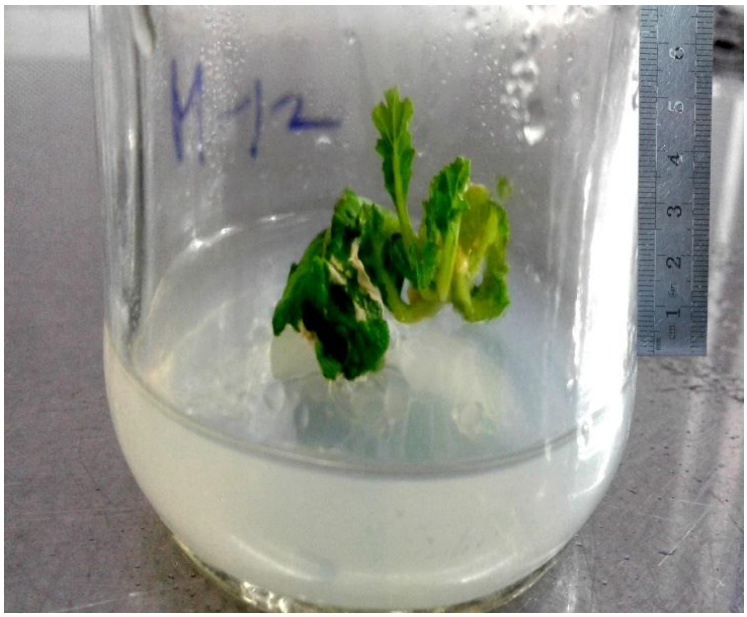

Plate 8. Effect of BA 4.0 mg/l + IBA 2.0 mg/l on length of leaves observed at 28 DAI

4.2.6 Days to root induction

Significant variation was observed among different concentrations of BA and IBA on days to root induction. The maximum (26.60 days) to root induction was recorded in control treatment and minimum (16.20 days) was required in BA 4.0 mg/l + IBA 2.0 mg/l concentration (Table 8).

4.2.7 Percentage of root induction

Different concentrations of BA and IBA showed the significant variations on percent of explants showing root induction. The highest percentage (68.00%) of root induction was recorded with BA 4.0 mg/l + IBA 2.0 mg/l, whereas the lowest percentage (36.00%) of root induction was recorded in control condition (Table 8).

Table 8. Combined effect of different concentration of BA and IBA on days to root induction and percent of root induction

Treatments	Days to root induction	(%) Root induction
Control	26.60a	36.00f
BA 1.0 mg/l + IBA 1.0 mg/l	22.00cd	44.00de
BA 1.0 mg/l + IBA 2.0 mg/l	21.80cd	44.00de
BA 1.0 mg/l + IBA 3.0 mg/l	22.20cd	44.00de
BA 1.0 mg/l + IBA 4.0 mg/l	22.60bc	44.00de
BA 1.0 mg/l + IBA 5.0 mg/l	23.80b	40.00ef
BA 2.0 mg/l + IBA 1.0 mg/l	19.60f-i	44.00de
BA 2.0 mg/l + IBA 2.0 mg/l	20.80d-f	48.00cd
BA 2.0 mg/l + IBA 3.0 mg/l	21.20c-e	48.00cd
BA 2.0 mg/l + IBA 4.0 mg/l	21.20de	52.00c
BA 2.0 mg/l + IBA 5.0 mg/l	20.80d-f	52.00c
BA 3.0 mg/l + IBA 1.0 mg/l	20.80d-f	52.00c
BA 3.0 mg/l + IBA 2.0 mg/l	18.60h-j	52.00c
BA 3.0 mg/l + IBA 3.0 mg/l	18.00jk	52.00c
BA 3.0 mg/l + IBA 4.0 mg/l	19.60f-i	52.00c
BA 3.0 mg/l + IBA 5.0 mg/l	19.40f-j	48.00cd
BA 4.0 mg/l + IBA 1.0 mg/l	17.00kl	60.00b
BA 4.0 mg/l + IBA 2.0 mg/l	16.20l	68.00a
BA 4.0 mg/l + IBA 3.0 mg/l	16.60l	60.00b
BA 4.0 mg/l + IBA 4.0 mg/l	17.20kl	52.00c
BA 4.0 mg/l + IBA 5.0 mg/l	18.00jk	48.00cd
BA 5.0 mg/l + IBA 1.0 mg/l	18.80g-j	48.00cd
BA 5.0 mg/l + IBA 2.0 mg/l	18.20i-k	52.00c
BA 5.0 mg/l + IBA 3.0 mg/l	19.80e-h	52.00c
BA 5.0 mg/l + IBA 4.0 mg/l	19.20g-j	52.00c
BA 5.0 mg/l + IBA 5.0 mg/l	20.20e-g	44.00de
CV (%)	5.01	21.25
LSD (0.05)	1.257	6.433

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

4.2.8 Number of root per shoot

There was a significant influence of different concentrations of BA and IBA on the number of root per shoot. The treatment BA 4.0 mg/l+ IBA 4.0 mg/l gave the highest number of root (1.80, 2.60 and 3.60) (Plate 9) and second best result (1.60, 2.40 and 3.40) was noticed from BA 4.0 mg/l + IBA 3.0 mg/l at 14, 21 and 28 DAI whereas the lowest number of root (1.00, 1.00 and 1.40) at 14, 21 and 28 DAI was found with hormone free media (Table 9).

Table 9. Combined effect of different concentration of BA and IBA on number of roots at different DAI

Treatments	Number of root/shoot		
	14 DAI	21 DAI	28 DAI
Control	1.00b	1.00e	1.40g
BA 1.0 mg/l + IBA 1.0 mg/l	1.20b	1.40c-e	1.60fg
BA 1.0 mg/l + IBA 2.0 mg/l	1.40ab	1.60c-e	2.20d-g
BA 1.0 mg/l + IBA 3.0 mg/l	1.20ab	1.40c-e	1.80e-g
BA 1.0 mg/l + IBA 4.0 mg/l	1.20ab	1.20c-e	1.60fg
BA 1.0 mg/l + IBA 5.0 mg/l	1.00b	1.40de	1.60fg
BA 2.0 mg/l + IBA 1.0 mg/l	1.20ab	1.40c-e	2.00d-g
BA 2.0 mg/l + IBA 2.0 mg/l	1.20ab	1.40c-e	2.00d-g
BA 2.0 mg/l + IBA 3.0 mg/l	1.40ab	1.60c-e	2.40c-f
BA 2.0 mg/l + IBA 4.0 mg/l	1.20ab	1.60c-e	2.20d-g
BA 2.0 mg/l + IBA 5.0 mg/l	1.20ab	1.60c-e	2.00d-g
BA 3.0 mg/l + IBA 1.0 mg/l	1.20ab	1.40c-e	2.00d-g
BA 3.0 mg/l + IBA 2.0 mg/l	1.20ab	1.40c-e	2.00d-g
BA 3.0 mg/l + IBA 3.0 mg/l	1.20ab	1.60c-e	2.00d-g
BA 3.0 mg/l + IBA 4.0 mg/l	1.20ab	1.40c-e	1.60fg
BA 3.0 mg/l + IBA 5.0 mg/l	1.20ab	1.40c-e	2.20d-g
BA 4.0 mg/l + IBA 1.0 mg/l	1.40ab	2.40ab	3.20a-c
BA 4.0 mg/l + IBA 2.0 mg/l	1.40ab	2.00a-c	2.80a-d
BA 4.0 mg/l + IBA 3.0 mg/l	1.60ab	2.40ab	3.40ab
BA 4.0 mg/l + IBA 4.0 mg/l	1.80a	2.60a	3.60a
BA 4.0 mg/l + IBA 5.0 mg/l	1.20ab	1.60c-e	2.60b-e
BA 5.0 mg/l + IBA 1.0 mg/l	1.20ab	1.60c-e	2.60b-e
BA 5.0 mg/l + IBA 2.0 mg/l	1.20ab	1.80b-d	2.00d-g
BA 5.0 mg/l + IBA 3.0 mg/l	1.20ab	1.80b-d	2.00d-g
BA 5.0 mg/l + IBA 4.0 mg/l	1.20ab	1.60c-e	2.20d-g
BA 5.0 mg/l + IBA 5.0 mg/l	1.20ab	1.60c-e	2.00d-g
CV (%)	16.01	32.87	30.20
LSD (0.05)	0.519	0.613	0.783

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 9. Effect of BA 4.0 mg/l + IBA 4.0 mg/l on number of root observed at 28 DAI

4.2.9 Length of root (cm)

There was significant influence of different combined concentrations of BA and IBA on the length of root. There was a significant variation at 14, 21 and 28 DAI among different concentration of BA and IBA. The highest length of root (1.12 cm, 2.00 cm and 2.68 cm) at 14, 21 and 28 DAI, respectively was found in BA 4.0 mg/l+ IBA 3.0 mg/l (Plate 10) which is statistically similar with BA 4.0 mg/l+ IBA 2.0 mg/l (0.98 cm, 1.62 cm and 2.58 cm) at 14, 21 and 28 DAI, respectively. (Table 10). The control treatment found the lowest number of root (0.36 cm, 0.48 cm and 0.56 cm) at 14, 21 and 28 DAI (Table 10).

Table 10. Combined effect of different concentration of BA and IBA on length of root at different DAI

Treatments	Length of root		
	14 DAI	21 DAI	28 DAI
Control	0.36k	0.48l	0.56k
BA 1.0 mg/l + IBA 1.0 mg/l	0.52fg	0.62jk	0.72j
BA 1.0 mg/l + IBA 2.0 mg/l	0.50f-h	0.58k	0.68j
BA 1.0 mg/l + IBA 3.0 mg/l	0.50f-h	0.58k	0.68j
BA 1.0 mg/l + IBA 4.0 mg/l	0.44h-j	0.56kl	0.66jk
BA 1.0 mg/l + IBA 5.0 mg/l	0.42i-k	0.56kl	0.66jk
BA 2.0 mg/l + IBA 1.0 mg/l	0.64d	0.78i	0.94hi
BA 2.0 mg/l + IBA 2.0 mg/l	0.62de	0.76i	0.92hi
BA 2.0 mg/l + IBA 3.0 mg/l	0.62de	0.76i	0.94hi
BA 2.0 mg/l + IBA 4.0 mg/l	0.54fg	0.76i	0.90hi
BA 2.0 mg/l + IBA 5.0 mg/l	0.56ef	0.72i	0.84i
BA 3.0 mg/l + IBA 1.0 mg/l	0.54fg	0.74i	0.92hi
BA 3.0 mg/l + IBA 2.0 mg/l	0.56ef	0.92h	1.02h
BA 3.0 mg/l + IBA 3.0 mg/l	0.48f-i	0.96gh	1.16g
BA 3.0 mg/l + IBA 4.0 mg/l	0.48f-i	0.70ij	0.96hi
BA 3.0 mg/l + IBA 5.0 mg/l	0.64d	0.78i	0.94hi
BA 4.0 mg/l + IBA 1.0 mg/l	0.94b	1.68b	2.30b
BA 4.0 mg/l + IBA 2.0 mg/l	0.98b	1.62b	2.58a
BA 4.0 mg/l + IBA 3.0 mg/l	1.12a	2.00a	2.68a
BA 4.0 mg/l + IBA 4.0 mg/l	0.80c	1.60bc	2.20b
BA 4.0 mg/l + IBA 5.0 mg/l	0.66d	1.52cd	2.08c
BA 5.0 mg/l + IBA 1.0 mg/l	0.54fg	1.44d	1.98cd
BA 5.0 mg/l + IBA 2.0 mg/l	0.56ef	1.24e	1.90d
BA 5.0 mg/l + IBA 3.0 mg/l	0.46g-j	1.10f	1.66e
BA 5.0 mg/l + IBA 4.0 mg/l	0.44h-j	1.04fg	1.48f
BA 5.0 mg/l + IBA 5.0 mg/l	0.40jk	0.96gh	1.40f
CV (%)	9.59	7.22	6.44
LSD (0.05)	0.065	0.088	0.104

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

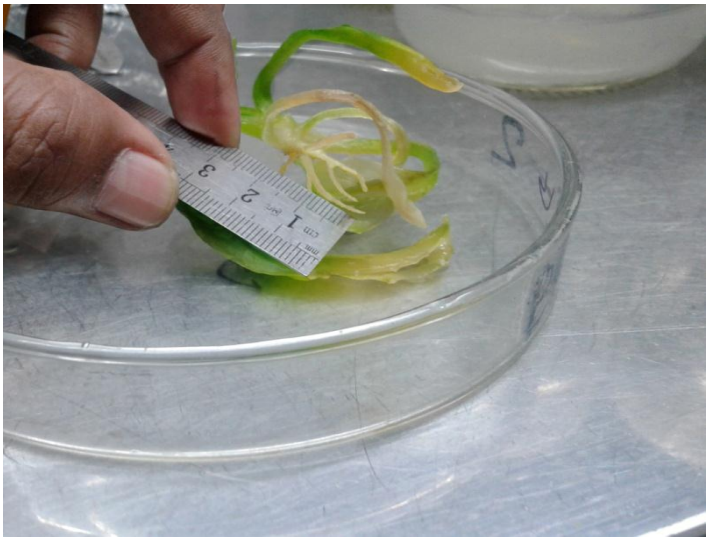

Plate 10. Effect of BA 4.0 mg/l + IBA 3.0 mg/l on length of root observed at 28 DAI

4.3 Sub-experiment 3. Effect of BA and NAA on callus and shoot induction potentiality in Rucola

From previous experiment, BA 4.0 mg/l alone or combined with IBA 3.0 mg/l gave the highest response in most of the parameters of shoot. So, in this sub-experiment, BA 4.0 mg/l was used with NAA (0.5, 1.0, 1.5 and 2.0 mg/l), respectively to know the callus and shoot induction performance. The result of the effect of different concentrations of BA with NAA has been presented under following headings with Figure (2.1-2.3) and Table (11-12).

4.3.1 Days to callus induction

Significant variations were observed among different concentrations of BA and NAA on days to callus induction. The maximum (23.40 days) to callus induction were recorded in BA 4.0 mg/l + NAA 2.0 mg/l treatment where minimum (15.60 days) was needed treatment BA 4.0 mg/l + NAA 1.0 mg/l (Figure 2.1). Zhang *et a1*., 2005, Chen *et al.*, 2011 and Slater *et al.*, 2011 used cotyledon as explants for callus induction in *Eruca sativa*. Our results confirm that rocket has a high a capacity to produce callus. Besides, *in vitro* growth and regeneration is a complex phenomenon and is influenced by a number of genetic and environmental factors (Sen, J. *et al*., 2002).

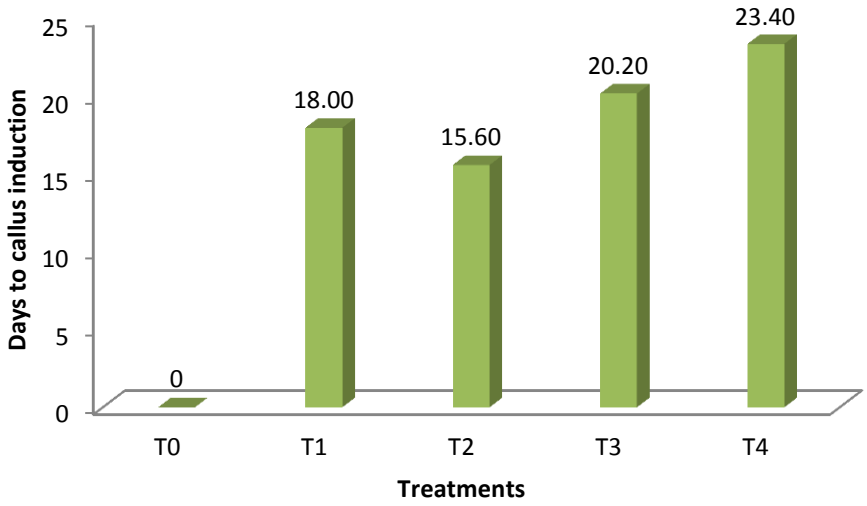

Figure 2.1 Effect of different treatments on days to callus induction in Rucola

T_0 (control)
T_1 (BA 4.0 mg/l + NAA 0.5 mg/l)
T_2 (BA 4.0 mg/l + NAA 1.0 mg/l)
T_3 (BA 4.0 mg/l + NAA 1.5 mg/l)
T_4 (BA 4.0 mg/l + NAA 2.0 mg/l)

4.3.2 Percentage of callus induction

The effective variation was noticed with combine dose of BA and NAA at 5% level of significance on percentage of callus. Treatment BA 4.0 mg/l + NAA 1.0 mg/l had produced the highest frequency of callus (68.00%) (Plate 11), while the lowest percentage (52.00%) of callus was produced in BA 4.0 mg/l + NAA 2.0 mg/l (Figure 2.2). Parkash *et al.* (1989) reported that 2.0 mg/1 BA + 0.5 mg/1 NAA and 2.0 mg/1 Kinetin + 0.2 mg/1 NAA produced 93.3% callus from cotyledonary explants of *E. sativa.* Similar percentage callus induction was recorded for 5.0 mg/1BA and 10.0 mg/1 Kinetin and combination of 10.0 mg/1 Kinetin with 1.0 mg/l NAA by Abbasi *et al.* (2013). Sikdar *et al.* (1987) found that 5.0 mg/1 of BA with 0.75 mg/1 NAA produced optimum morphogenic response in mesophyll protoplasts of *E. sativa*. There was no callus in control treatment. These above findings partially supported our experiment results in case of callus induction. Callus induction and morphogenic response of different genotype tested were controlled by interaction between genotype, explants type and growth regulators (El-Nagar *et al*. 2014).

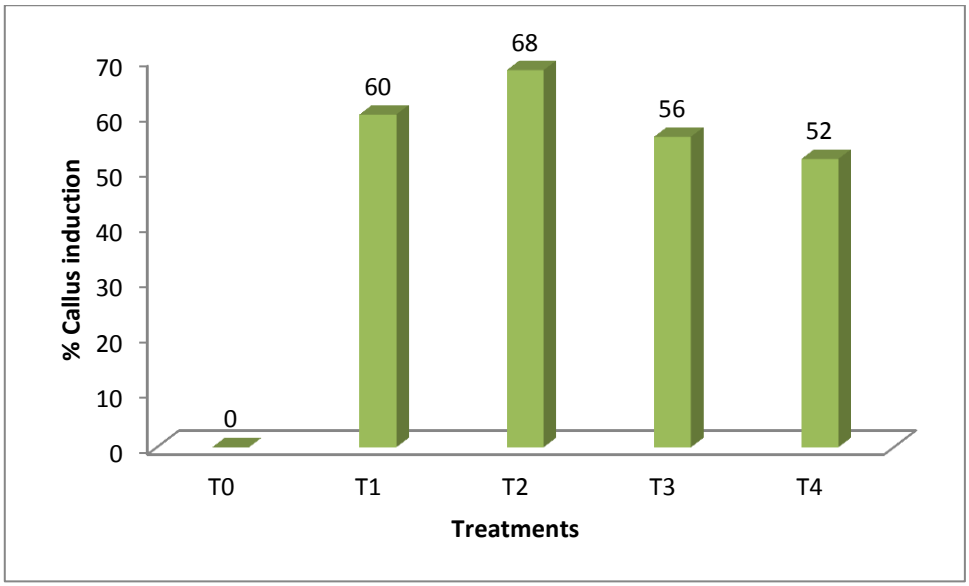

Figure 2.2 Effect of different treatments on percentage of callus induction in Rucola

T_0 (control)
T_1 (BA 4.0 mg/l + NAA 0.5 mg/l)
T_2 (BA 4.0 mg/l + NAA 1.0 mg/l)
T_3 (BA 4.0 mg/l + NAA 1.5 mg/l)
T_4 (BA 4.0 mg/l + NAA 2.0 mg/l)

Plate 11. Effect of BA 4.0 mg/l + NAA 1.0 mg/l on maximum percentage of callus induction from cotyledonary node

4.3.3 Weight of callus

There was a significant influence of different concentrations of BA and NAA on the weight of callus. The maximum weight of callus (1.71 g, 2.15 g and 2.80 g) at 20, 30 and 40 DAI, respectively was noticed from BA 4.0 mg/l+ NAA 1.0 mg/l which was statistically different from others treatments. Whereas the minimum weight of callus (0.94 g, 1.24 g and 1.49 g) at 20, 30 and 40 DAI found from BA 4.0 mg/l+ NAA 2.0 mg/l (Table 11).

Table 11. Effect of BA and NAA on weight of callus at different DAI

Treatment	Weight of callus (g)		
	20 DAI	30 DAI	40 DAI
Control	-	-	-
BA 4.0 mg/l + NAA 0.5 mg/l	1.21b	1.81b	2.14b
BA 4.0 mg/l + NAA 1.0 mg/l	1.71a	2.15a	2.80a
BA 4.0 mg/l + NAA 1.5 mg/l	1.09bc	1.28c	1.79c
BA 4.0 mg/l + NAA 2.0 mg/l	0.94c	1.24c	1.49d
CV (%)	7.47	4.71	3.97
LSD (0.05)	0.15	0.083	0.084

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

4.3.4 Days to shoot induction

Significant variations were observed among different concentrations of BA and NAA on days to shoot induction. The maximum (15.60 days) were recorded for shoot induction in BA 4.0 mg/l + NAA 2.0 mg/l treatment where minimum (10.80 days) was required in MS medium supplemented with BA 4.0 mg/l + NAA 1.0 mg/l (Figure 2.3).

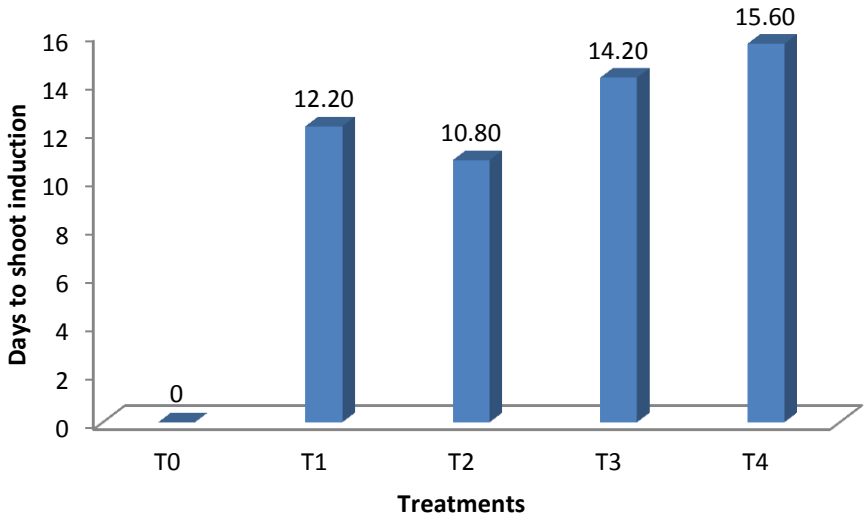

Figure 2.3 Effect of different treatments on days to shoot induction in Rucola

T_0 (control)
T_1 (BA 4.0 mg/l + NAA 0.5 mg/l)
T_2 (BA 4.0 mg/l + NAA 1.0 mg/l)
T_3 (BA 4.0 mg/l + NAA 1.5 mg/l)
T_4 (BA 4.0 mg/l + NAA 2.0 mg/l)

4.3.5 Number of shoot

The number of shoot at different concentrations of BA and NAA showed meaningful variation. The highest number of shoot (1.60, 2.20 and 3.00) at 20 DAI, 30 DAI and 40 DAI (Plate 12), respectively was obtained from the BA 4.0 mg/l + NAA 1.0 mg/l which was statistically different from rest of others. Whereas, the lowest number of shoot (1.20, 1.60 and 2.00) at 20, 30 and 40 DAI was found from BA 4.0 mg/l + NAA 2.0 mg/l. No shoot was produced from control treatment (Table 12). Abbasi *et al.* (2016) reported that the callus was sub-cultured on 5.0 mg/l BA and 1.0 mg/l NAA concentrations of plant growth regulators and produced optimum percentage shoot organogenesis after 4 weeks of sub-culturing. However, optimum number of shoots per explant was recorded for moderate concentrations (1.0 and 2.0 mg/l) of kinetin.

Table 12. Effect of BA and NAA on number of shoot induction at different DAI

Treatment	Number of shoot		
	20 DAI	**30 DAI**	**40 DAI**
control	-	-	-
BA 4.0 mg/l + NAA 0.5 mg/l	1.60a	1.80b	2.60b
BA 4.0 mg/l + NAA 1.0 mg/l	1.60a	2.20a	3.00a
BA 4.0 mg/l + NAA 1.5 mg/l	1.40b	1.80b	2.40c
BA 4.0 mg/l + NAA 2.0 mg/l	1.20b	1.60c	2.00d
CV (%)	40.43	41.65	28.28
LSD (0.05)	0.15	0.181	0.174

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

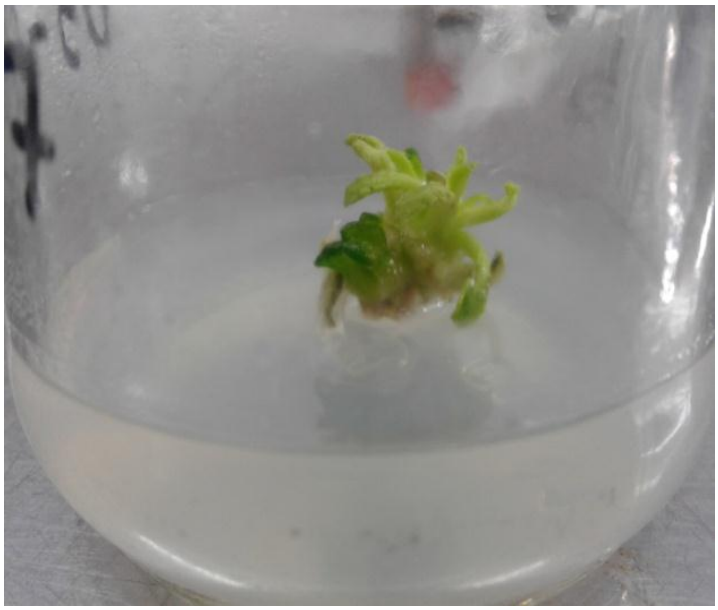

Plate 12. Effect of BA 4.0 mg/l and NAA 1.0 mg/l on number of shoot induction from callus of Rucola at 40 DAI

4.4 Sub-experiment 4. Effect of IBA on root induction potentiality in Rucola

The result of the effect of different concentrations of IBA has been presented under following headings with Figure (3.1-3.2) and Table (13-14).

4.4.1 Days to root induction

Significant variations were observed among different concentrations of IBA on days to root induction. The maximum days (21.20 days) to root induction were recorded in control followed by 1.0 mg/l (18.80 days) and 5 mg/l (18.00 days). On the other hand, minimum (14.40 days) was required in 3.0 mg/l IBA followed by 2.0 mg/l (15.80 days) and 4.0 mg/l (16.20 days) (Figure 3.1). Abbasi, *et al.* (2016) described that *in vitro* regenerated shoots were shifted to MS medium augmented with 5.0 mg/l indole acetic acid (IAA) for rooting after 4 weeks of sub-culturing. This result may be varied due to the differences of genotype and culture environments (Sen, J. *et al*., 2002).

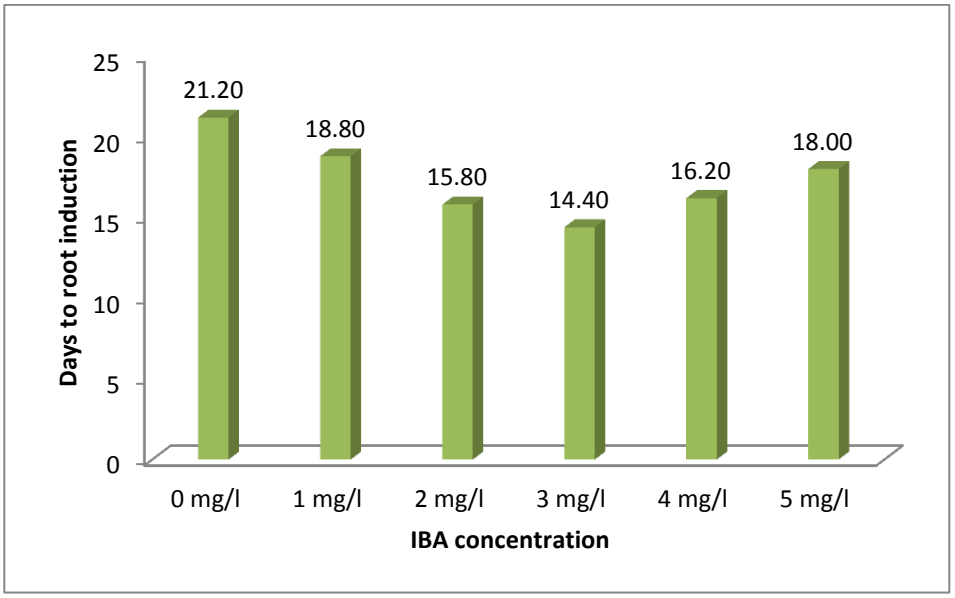

Figure 3.1 Effect of IBA on days to root induction in Rucola

4.4.2 Percentage of root induction

IBA 3.0 mg/l had produced the highest percentage of root induction (72.00%), while the lowest percentage (36.00%) of root induction was produced in control treatment (Figure 3.2). Sharma *et al.* (2012) observed rooting in IBA in Rucola and better development of the roots was in the fresh half strength MS solid medium with indole-3-butyric acid (4.90 µM). Best response at 4.90 µM of IBA and Kn (0.23µM) was 60 to 80% rooting. This

variation may be due to the age, nature, origin and the physiological state of the explant and seasonal variation play a crucial role in the establishment of cultures and subsequent plant regeneration (Bajaj, Y.P.S. *et al*., 1991).

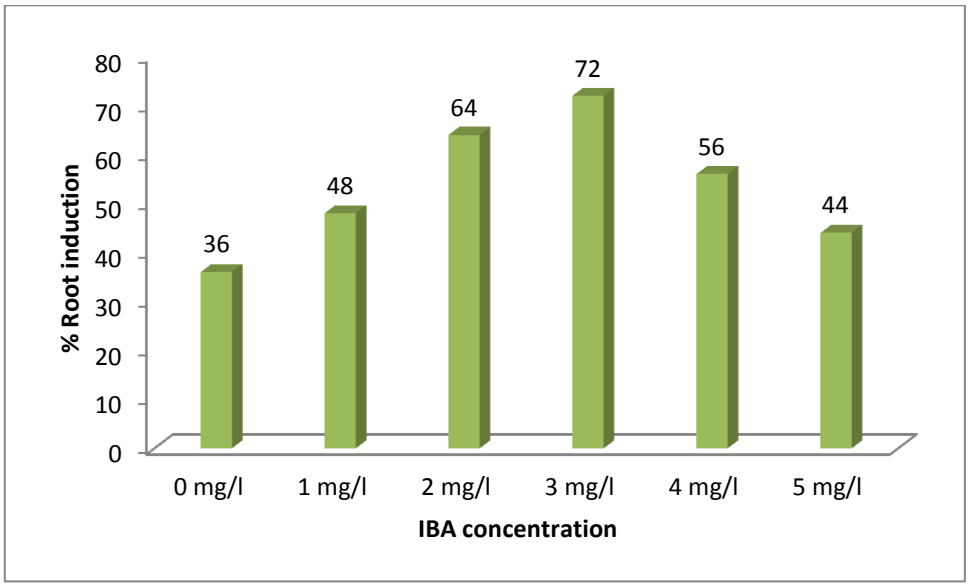

Figure 3.2 Effect of IBA on percentage of root induction in Rucola

4.4.3 Number of root per shoot

There was significant influence of different concentrations of IBA on the number of roots per shoot. IBA 4.0 mg/l gave the highest number of root (2.60, 3.60 and 4.60) at 14, 21 and 28 DAI (Plate 13) and the control treatment was found with the lowest number of root (1.00, 1.20 and 1.40) at 14, 21 and 28 DAI (Table 13). Sharma *et al.* (2012) stated that shoots were separated carefully and were transferred to the fresh half strength MS solid medium with indole-3-butyric acid (4.90 µM) for the development of the roots.

Table 13. Effect of different concentration of IBA on number of root at different DAI

IBA (mg/l)	Number of root/shoot		
	14 DAI	21 DAI	28 DAI
0	1.00d	1.20c	1.40e
1.0	1.40c	1.60bc	2.60cd
2.0	2.20b	3.00a	3.60b
3.0	1.40c	2.20b	2.80c
4.0	2.60a	3.60a	4.60a
5.0	1.00d	1.60bc	2.00de
CV (%)	26.76	24.90	19.86
LSD (0.05)	0.258	0.715	0.734

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 13. Effect of IBA 4.0 mg/l on maximum number of root induction at 28 DAI

4.4.4 Length of root

There was a significant influence of different concentrations of IBA on the length of root at 5% level of significance in laboratory condition. The highest length of root (3.16 cm) at 28 DAI (Plate 14) was noticed from the 4.0 mg/l IBA followed by 2.0 mg/l (2.70 cm) and 3.0 mg/l (2.50 cm). On the other hand, the lowest length of root (1.00 cm) at 28 DAI was noticed in control without hormone followed by 5.0 mg/l (1.64 cm) and 1.0 mg/l (1.98 cm). (Table 14)

Table 14. Effect of different concentration of IBA on length of root at different DAI

IBA (mg/l)	Length of root (cm)		
	14 DAI	21 DAI	28 DAI
0	0.38e	0.74e	1.00f
1.0	0.74c	1.20d	1.98d
2.0	1.14b	2.14b	2.70b
3.0	0.76c	1.72c	2.50c
4.0	1.26a	2.32a	3.16a
5.0	0.58d	1.10d	1.64e
CV (%)	6.38	5.31	3.16
LSD (0.05)	0.071	0.109	0.235

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$= Least significant difference.

Plate 14. Effect of IBA 4.0 mg/l on length of root at 28 DAI

Sub-experiment 5. *Ex vitro* acclimatization and establishment of plantlets on soil

After two and half months, a good number of root and shoot were found and plants were acclimatized under shade condition and in natural condition. 20 plants were transplanted in shade area after three weeks, 16 plantlets were survived and survival rate was 80% (Table 15). In natural condition, 16 plants were transplanted, 12 survived (Plate 15) and survival rate was 75%. Sharma *et al.* (2012) found that plantlets of Rucola were successfully transferred to the soil where they grew well for 8 to 10 weeks with 80% survivability. So considering the survival rate it can be said that acclimatization potentiality of Rucola is satisfactory.

Table 15. Survival rate of *in vitro* regenerated plantlets of Rucola

Acclimatization	No. of plants transplanted	No. of plants survived	Percentage of survival rate
In shade area with controlled atmosphere	20	16	80
In natural condition	16	12	75

Plate 15. Hardening of Rucola plantlet in shade condition (a) and in natural condition (b) & (c)

CHAPTER V

SUMMARY AND CONCLUSIONS

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of February 2016 to June 2017. The cotyledonary nodes of Rucola were used as experimental materials in the present investigation. BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) for shoot induction, BA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) with IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) combination doses, respectively for shoot and root regeneration, BA 4.0 mg/l with (0.5, 1.0, 1.5, 2.0 mg/l) of NAA, respectively for callus and shoot induction and IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) was applied for root formation. The experiments were arranged in Completely Randomized Design (CRD) with five replications. Each of replications consisted of five culture vials.

The minimum (6.40 days) was needed in 3.0 mg/l BA to the highest frequency (80.00%) of shoot. BA 4.0 mg/l gave the highest number of shoot (2.60, 3.60 and 4.60) at 14, 21 and 28 DAI and the control treatment found the lowest number of shoot (1.00, 1.60 and 1.60) at 14, 21 and 28 DAI. The highest number of leaves (2.60, 4.20 and 6.40) at 14, 21 and 28 DAI, respectively was noticed from the 4.0 mg/l BA. The length of leaves (2.56 cm, 4.12 cm and 5.24 cm) at 14, 21 and 28 DAI, respectively was noticed from the 4.0 mg/l BA and the minimum length was noticed in control treatment.

The maximum (18.80 days) to shoot induction was recorded in control and BA 4.0 mg/l + IBA 3.0 mg/l treatment required minimum (6.00 days). Maximum percentage (76.00%) of shoot induction was noticed in treatment BA 4.0 mg/l + IBA 2.0 mg/l and minimum percentage (36.00%) was in control hormone free media. The treatment BA 4.0 mg/l + IBA 3.0 mg/l gave the highest number of shoot (2.40, 3.00 and 3.40) at 14, 21 and 28 DAI, respectively. The highest number of leaves (3.20, 7.20 and 11.00) at 14, 21 and 28 DAI, respectively was noticed from the BA 4.0 mg/l + IBA 4.0 mg/l. The maximum length of leaves (2.60 cm, 4.42 cm and 5.00 cm) at 14, 21 and 28 DAI, respectively was noticed from the BA 4.0 mg/l + IBA 2.0 mg/l and the minimum length were noticed in control treatment.

The highest percentage (68.00%) of root induction was recorded with BA 4.0 mg/l + IBA 2.0 mg/l in minimum (16.20 days). The treatment BA 4.0 mg/l + IBA 4.0 mg/l gave the highest number of root (1.80, 2.60 and 3.60) at 14, 21 and 28 DAI, respectively and the highest length of root (1.12 cm, 2.00 cm and 2.68 cm) at 14, 21 and 28 DAI, respectively was found in BA 4.0 mg/l + IBA 3.0 mg/l and the control treatment found the lowest number of root (0.36 cm, 0.48 cm and 0.56 cm) at 14, 21 and 28 DAI.

Treatment BA 4.0 mg/l+ NAA 1.0 mg/l had produced the highest frequency of callus (68.00%), with minimum (15.60 days). The highest weight of callus (1.71 g, 2.15 g and 2.80 g) at 20, 30 and 40 DAI, respectively was noticed from the BA 4.0 mg/l + NAA 1.0 mg/l, while the lowest weight of callus (0.94 g, 1.24 g and 1.49 g) at 20, 30 and 40 DAI found from BA 4.0 mg/l + NAA 2.0 mg/l. No callus was produced in control treatment. The maximum (15.60 days) to shoot induction were recorded in BA 4.0 mg/l + NAA 2.0 mg/l treatment where minimum (10.80 days) was needed treatment BA 4.0 mg/l+ NAA 1.0 mg/l. The highest number of shoot (1.60, 2.20 and 3.00) at 20, 30 and 40 DAI, respectively was obtained from the BA 4.0 mg/l+ NAA 1.0 mg/l.

The maximum (21.20 days) to root induction were recorded in control treatment where minimum (14.40 days) was needed in 3.0 mg/l BA. IBA 3.0 mg/l had produced the highest percentage of root induction (72.00%) required minimum (14.40 days). IBA 4.0 mg/l gave the highest number of root (2.60, 3.60 and 4.60) at 14, 21 and 28 DAI and the control treatment found the lowest number of root at all DAI. The highest length of root (1.26 cm, 2.32 cm and 3.16 cm) at 14, 21 and 28 DAI, respectively was found in 4.0 mg/l IBA and the control treatment found the lowest length of root at all DAI.

From the above summary it is concluded that, the moderate dose of BA 4.0 mg/l gave the best results for shoot regeneration in comparison with combine dose of BA 4.0 mg/l with IBA 2.0 mg/l and 3.0 mg/l. Besides, BA 4.0 mg/l with NAA (0.5, 1.0, 1.5, 2.0 mg/l) showed the good performance for callus and shoot induction and IBA 4.0 mg/l gave the best performance in case of root. So, finally it can be concluded that, a convenient protocol of *in vitro* rapid regeneration of Rucola has been established which may contribute in breeding program and large scale virus free seedlings production throughout the year is possible.

RECOMMENDATIONS

Based on the summary and conclusion following recommendations can be made:

i. More explants such as shoot tip, meristem, nodal segment, leaf and root tip can be experimented other than cotyledonary nodes for *in vitro* regeneration of Rucola.
ii. Callus induction can be done with NAA or other callus induction hormone for large number of shoot induction.
iii. Accurate and details investigation on influence of other factors such as different elicitors, antioxidants should be considered.
iv. Further study can be done with different concentrations and combinations of auxins and cytokinins group of hormones for rapid proliferation of *Eruca sativa*.
v. As micropropagation techniques of Rucola is not so well established in Bangladesh. So, further research on this crop should be continued because of its commercial use.

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APPENDICES

Appendix I. Analysis of variance (ANOVA) of effect of different concentration of BA on days to shoot initiation, percent of shoot and number of shoot at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot Initiation	Number of Shoot		
				14 DAI	21 DAI	28 DAI
Treatment	4	40.833**	1333.333**	1.493**	3.413**	6.053**
Error	20	0.667	86.667	0.350	0.450	0.350
Total	24					

**= Significant at 1% level of Probability.

Appendix II. Analysis of variance (ANOVA) of effect of different concentration of BA on number of leaves at different DAI

Source of variance	d.f	Number of leaves		
		14 DAI	21 DAI	28 DAI
Treatment	5	2.053**	6.800**	17.253**
Error	24	0.183	0.467	0.567
Total	29			

**= Significant at 1% level of Probability.

Appendix III. Analysis of variance (ANOVA) of effect of different concentration of BA on length of leaves at different DAI

Source of variance	d.f	Length of leaves (cm)		
		14 DAI	21 DAI	28 DAI
Treatment	5	0.766**	2.075**	5.906**
Error	24	0.004	0.006	0.010
Total	29			

**= Significant at 1% level of Probability.

Appendix IV. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on days to shoot initiation, percent of shoot initiation and number of shoot at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot initiation	Number of Shoot		
				14 DAI	21 DAI	28 DAI
Treatment	25	51.280**	372.677**	1.536**	12.356**	29.957**
Error	104	0.362	150.769	0.331	0.558	0.827
Total	129					

**= Significant at 1% level of Probability.

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Appendix V. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on number of leaves at different DAI

Source of variance	d.f	Number of leaves		
		14 DAI	21 DAI	28 DAI
Treatment	25	0.332*	0.895**	0.956**
Error	104	0.308	0.327	0.338
Total	129			

**= Significant at 1% level of Probability.

Appendix VI. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on length of leaves at different DAI

Source of variance	d.f	Length of leaves		
		14 DAI	21 DAI	28 DAI
Treatment	25	1.282**	3.902**	5.020**
Error	104	0.004	0.505	0.017
Total	129			

**= Significant at 1% level of Probability.

Appendix VII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on days to root initiation, percent of root and number of roots at different DAI

Source of variance	d.f	Days to root initiation	Percent % Root initiation	Number of root		
				14 DAI	21 DAI	28 DAI
Treatment	25	28.104**	218.092**	0.137**	0.677**	1.544**
Error	104	1.004	110.769	0.204	0.285	0.438
Total	129					

**= Significant at 1% level of Probability.

Appendix VIII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on length of root at different DAI

Source of variance	d.f	Length of root		
		14 DAI	21 DAI	28 DAI
Treatment	25	0.169**	0.880**	2.138**
Error	104	0.003	0.005	0.007
Total	129			

**= Significant at 1% level of Probability.

Appendix IX. Analysis of variance (ANOVA) of effect of BA and NAA on days to callus initiation, percent of callus and weight of callus at different DAI

Source of variance	d.f	Days to callus	Percent of Callus	Weight of callus (g)		
				20 DAI	30 DAI	40 DAI
Treatment	4	413.7**	3656.00**	1.951**	3.359**	5.416**
Error	20	0.46	104.00	0.013	0.004	0.004
Total	24					

**= Significant at 1% level of Probability.

Appendix X. Analysis of variance (ANOVA) of effect of BA and NAA on Days to shoot and number of shoot at different DAI

Source of variance	d.f	Days to shoot initiation	Number of shoot		
			20 DAI	30 DAI	40 DAI
Treatment	4	191.140**	2.240**	3.660**	6.900**
Error	20	0.480	0.220	0.380	0.320
Total	24				

**= Significant at 1% level of Probability.

Appendix XI. Analysis of variance (ANOVA) of effect of different concentration of IBA on days to root initiation, percent of root and number of root at different DAI

Source of variance	d.f	Days to root initiation	Percent of (%) root initiation	Number of root		
				14 DAI	21 DAI	28 DAI
Treatment	5	29.760**	885.333**	2.160**	4.320**	6.513**
Error	24	0.767	93.333	0.183	0.300	0.317
Total	29					

**= Significant at 1% level of Probability.

Appendix XII. Analysis of variance (ANOVA) of effect of different concentration of IBA on length of root at different DAI

Source of variance	d.f	Length of root (cm)		
		14 DAI	21 DAI	28 DAI
Treatment	5	0.557**	1.950**	3.056**
Error	24	0.003	0.007	0.005
Total	29			

NS= Not Significant *= Significant at 5% level of Probability, **= Significant at 1% level of Probability.