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Effects of Genotype and Growth Regulators for the Callus Initiation and Regeneration of Mustard (*Brassica napus)*

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ABSTRACT

The present study is conducted to develop an efficient protocol for shoot and plant regeneration using four cultivars grown under the agricultural conditions of Bangladesh. Seed explants from four varieties were cultured in vitro and callus formation and swelling as well as the mode of shoot and root regeneration was evaluated. A highly positive response to in vitro culture, i.e., callus formation or swelling, was observed in all the varieties tested. Effect of two different callusing media (2 mg/L and 3 mg/L 2, 4-D with 2 mg/L BA) and shoot regeneration media (2 mg/L and 3 mg/L NAA with 2 mg/L BA) and for root (1 mg/L IBA and 1 mg/L NAA) were used for callus induction and regeneration of four cultivars of Brassica napus, viz., Hoiro, Rye, Shewto Sarisa and Tori-7. Parameters like callus formation and number of shoots and roots per explants were highly variable among genotypes. A high frequency of callus was obtained in the variety Tori-7 (83.33%) seed followed by Shewto Sarisa. Between the two concentrations, 2 mg/L of 2, 4-D showed the highest number of callus. 100% shoot regeneration was seen in Rye in the concentration of 2 mg/L BA with 3 mg/L of NAA. The shoots were then rooted and best results were obtained on media having 1 mg/L of IBA in MS medium. Rooted plantlets were successfully established into the soil. The developed system relies on somaclonal variation for further research and the high regenerative capacity of seed explants of B. napus cutivar.

Keywords: *Mustard, callus, MS-medium, 2, 4-D, NAA, IBA*

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1. INTRODUCTION

The term *Brassica* genus covers a large group of plants that include radish, turnip, rutabagas, cabbage, cauliflower, canola, rape and kale. As a vegetable and oilseed crop rapeseed is attracting great attention not only of breeders using conventional methods but also of those concerned with biotechnological methods; tissue culture is the initial process for advanced biotechnology. Non-conventional genetic improvement programs based on tissue culture are essential as a complement to standard breeding. It is well known that improvement of plant through conventional breeding methods is slow, time-consuming and labor-intensive [1]. Plant tissue culture is a practice used to propagate plants under sterile conditions and success in plant tissue culture can pivot on two critical factors, i.e., choice of explants and culture medium used. The tissue obtained from the plant to culture is called an explant. The explants may be as large as a seedling or an organ such as ovule, embryo, etc., and as small as a single cell and protoplasts [2]. Plant tissue culture cannot work without plant growth regulators in the growth medium. Among plant growth

regulators, auxins and cytokinins (CKs) are widely used for callus induction. Auxins promote both cell division and cell growth. Auxin (2, 4-D) when combined with cytokinin (BA), callogenesis, and cell division stimulated faster and better in rapeseed cultivars whereas NAA (with BA or without it) stimulated root formation and rhizogenesis. Higher concentration of 2, 4-D (2mg/L), resulted in low rhizogenesis and root formation but induced callus formation [3].

The effect of culture media, explants and genotypes on adventitious shoot regeneration in spring rapeseed (*Brassica napus* L.) was examined in a study using hypocotyls and cotyledons as explants. Different concentrations of 6-benzylaminopurine (BA) and α-naphtylacetic acid (NAA) or zeatin and 2, 4-dichlorphenoxyacetic acid (2, 4-D) were supplemented with MS medium. 2.0 mg/L zeatin + 0.15 mg/L 2, 4-D promoted a high callus induction frequency. On a medium supplemented with 4.0 mg/l BA and 0.05 mg/L NAA showed the bud regeneration frequency 21.2%, 25.6% and 13.1% respectively in different varieties. The results indicate that shoot regeneration ability is strongly influenced by the genotype [4]. For regeneration *B. napus L*., cultivars, viz., Star, Cyclon and Westar shoots were then rooted and best results were obtained on media having 0.3 mg/L IBA in half-strength MS medium [5]. In Bangladesh, six genotypes of oilseed *Brassica*, viz., Tori-7, Sampad, Kallyania, BARI Sarisha-7, BARI Sarisha-8, and MM 20-3 using petioles as explants for callus induction and regeneration with the media supplemented with 2 mg/L BAP, 0.1 mg/L NAA and 2.0 mg/L AgNO3 were observed; the highest percentage of callus induction (91.43%) was observed in Tori-7. Root formation from the regenerates was found best in Tori-7 with half MS medium supplemented with 0.5 mg/L NAA in genotype [6].

In this study, seeds of *B. napus* genotypes like local variety, Hoiro, Rye, Shewto Sarisa and Hybrid variety Tori 7 were used as explants for efficient callus induction and regeneration. This study throws some light on the behavior of cultured *B. napus* varieties that grow with satisfactory regenerative capacity and attempts to develop an efficient and reliable protocol for future experiments.

2. MATERIALS AND METHODS

This research work was conducted at the Plant Genetic Engineering Laboratory of Department of Genetic Engineering and Biotechnology, Shahjalal University of Science & Technology (SUST), Sylhet, during the period of March–June, 2011. The detail of materials used and analytical methods employed during this study is given below.

2.1. Preparation of Seed Materials

Four mustard varieties (*B. napus*) Hoiro, Rye, Shweto Sarisa and (Hybrid) Tori-7 were used for callus formation and regeneration

experiments. Mustard was used as explant for callus induction. Seeds were placed in an autoclaved beaker and washed with sterile distilled water, surface sterilized in 70% ethanol for 1 min, followed by immersion in 30% commercial sodium hypochlorite (Clotech) for 20 min and finally by washing with sterile distilled water three times. The seeds were then placed on a sterilized petriplate having sterile filter papers with the help of forceps to remove excess water [7].

2.2. Media Preparation

The cultures were incubated in culture room at 25 ± 3 ºC to white florescent light under 16 h photoperiod. MS media with different concentrations of 2, 4- D (2, 3 mg/L) and 3% sucrose were used to select the efficient medium for embryogenic callus induction. The plant shoot regeneration ability of four varieties was assessed using MS [8] medium with 1 mg/L of BA and different concentrations of NAA, 3% sucrose. The root regeneration ability of four varieties was assessed using MS [8] medium with only 1 mg/L of IBA and only 1 mg/L of NAA and 3% sucrose.

2.2.1. Sterilization of Media

Media was autoclaved at 15 psi for 20 min at 121 ºC. Data was recorded for the following parameters: The basal medium MS was used for callus induction. The proposed medium was supplemented with concentration of growth hormone 2, 4-D (2, 3 mg/L). The pH of the media was adjusted to 5.8 ± 2 before autoclaving. After inoculation, the surface sterilized seeds of four mustard varieties were transferred and maintained in an environmentally controlled growth room for 2 weeks for callus induction and growth. The cultures were positioned away from continuous light provided by general electric white florescent tubes. Temperature was maintained at 25 ± 3 °C throughout the growth period. Callus induction frequency for four varieties was recorded 7–9 days after inoculation. Experiments were replicated three times and eight conical flasks were inoculated with six seeds used per replication for each genotype. All the calli originated from a single seed were considered as one.

Frequency of callus induction was calculated according to the following formula:

Callus induction frequency (%)

$$= \frac{\text{No.of seeds produced calli}}{\text{No.of seeds cultured}} \times 100$$

For plant shoot regeneration, the embryogenic part of calli was cut into small pieces by removing non-embryogenic part. Calli were then inoculated on regeneration media, i.e., MS salts and vitamins [8] with different combination and concentrations of hormone. The MS basal medium was supplemented with 3% sucrose and NAA (2, 3 mg/L) while maintaining 2 mg/L of BA as constant. The pH of media was adjusted to 5.8 ± 2 before autoclaving. The culture was performed at 25 ± 3 ºC under a cycle of 16 h light/8 h dark for 4 weeks, after which the frequencies of

plant regeneration were calculated based on the appearance of shoot. For plant root regeneration media, the calli were inoculated on the regeneration media, i.e., MS salts and vitamins [8] with different combinations and concentrations of hormones. The MS basal medium was supplemented with 3% sucrose containing 1 mg/L of NAA or 1 mg/L of IBA. The pH of media was adjusted to 5.8 ± 2 before autoclaving. The culture was performed at 25 ± 3 ºC under a cycle of 16 h light/8 h dark for 4 weeks, after which the frequencies of plant root regeneration were calculated based on the appearance of hairy roots. The callus with shoots and the callus without shoots both are inoculated on the root regeneration medium.

3. RESULTS

3.1. Callus Induction

Mature dehusked mustard was used for callus initiation and after 3 days, callus began to form at the scutellum of the cultured seed in all the four media. Both embryogenic and non-embryogenic calli were initiated. Embryogenic calli were found to be dark to light greenish, dry, compact, hard and nodular. On the other hand, non-embryogenic calli appeared to be watery, light yellow and soft. After 2 weeks, the first subculture was carried out and calli were removed from the seed and shoots were transferred onto fresh media.

3.1.1. Effect of Variety

The explants exhibited an initial swelling followed by callus formation within 2 weeks of inoculation. In the present study, media with different combinations of auxin (2, 4-D) to cytokinin (BA) were used to induce callus in the seed explants of the *B. napus*: Hoiro, Rye, Shewto Sarisa and Tori-7. The effects of varieties on callus induction from rape seeds are shown in Figure 1. From the second week, it became more round and formed hard, light greenish callus. From the above seeds, Tori-7 showed the best performance on callus induction (83.33%) followed by Shweto Sarisa (66.50%), Rye (49.9%) and Hoiro (41.66%)

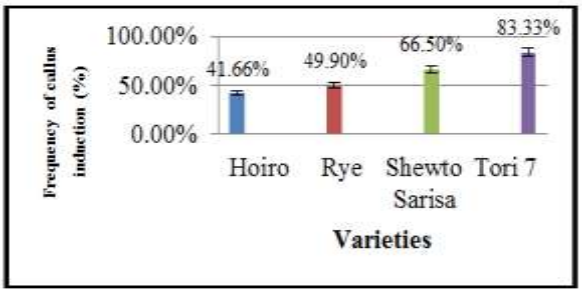

Fig. 1: *Effect of Variety on Callus Induction.*

3.1.2. Effect of Growth Regulators

External hormones act as triggers in regulating explant differentiation, which in turn results in the production of extensive and friable callus. In cultures, the ratio of external to internal auxin concentrations is essential for regulation of the phases of the standard growth cycle. Combination of auxin and cytokinin is important for callus induction [9]. Application of 2, 4-D was very effective on callus formation, especially when combined with cytokinin (BA) [3]. In this experiment, callus induction frequency of four varieties like

Hoiro, Rye, Shewto Sarisa and Tori-7 varied with the variation of the concentration of 2, 4-D. At the lowest concentration of 2, 4-D (2 mg/L) shows the highest frequency of callus induction (62.50%). When the concentration of 2, 4-D is increased (3 mg/L), callus induction frequency goes downwards (58.33%) (Figure 2).

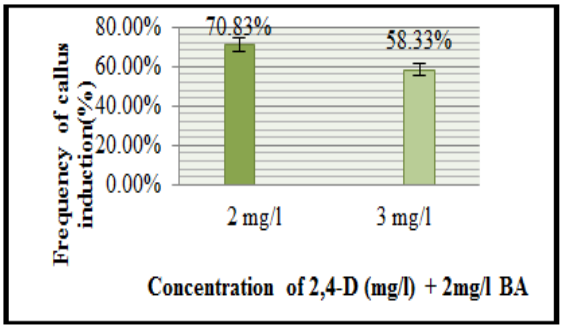

Fig. 2: *Effect of Growth Regulators.*

3.1.3. Effect of Variety and Plant Growth Regulators Interaction

The result obtained from Table I is the callus induction frequency of Rye (K_1R – 66.66%), Shewto Sarisa (K_2Sh – 100%), and Tori-7(K_1T – 83.33%) but the frequency of Hoiro (K_1H – 33.33%) only. But when the concentration of 2, 4-D is (3 mg/L) with the same basal medium and constant concentration of BA (2 mg/L), the callus induction frequency gets suppressed. In Tori-7 (K_2T – 83.33%) variety, callus induction frequency remains unchanged with the different concentration of 2, 4-D. In Hoiro (K_2H – 83.33%) variety, callus induction frequency gets stimulated through increasing concentration of 2, 4-D beyond 2 mg/L. In the present study, it may be concluded that 2, 4-D concentration (2 mg/L) was chosen as the optimum level for callus induction and growth for the variety of Rye and Shewto Sarisa. Hoiro shows a minimum result and Tori-7 shows an average result.

Table I: *Effect of Variety and Plant Growth Regulators Interaction.*

Calli derived from the Combination	**Frequency of Callus Induction (%)**
K_1R	66.66
K_2R	33.33
K_1H	33.33
K_2H	83.33
K_1Sh	100
K_2Sh	66.66
K_1T	83.33
K_2T	83.33

Here, K_1 = 2 mg/L 2, 4-D, K_2 = 3 mg/L 2, 4-D, H = Hoiro, R = Rye, Sh = Sweto Sarisha, T = Tori-7.

3.2. Shoot Initiation

3.2.1. Effect of Variety

The percentage of explants that formed shoots in shoot regeneration medium differed with the cultivars (Figure 3). During this study, local variety Hoiro showed the highest frequency of shoot regeneration (75%) followed by hybrid (Tori-7) variety (50%), Rye (45%). Among the four varieties, the lowest shoot regeneration frequency was

Shewto Sarisa (25%) (Figure 3). For the regeneration of viable shoots from cultured tissues, genetic engineering of crop plants relies on the development of efficient methods [10]. In shoot regeneration, the most important factors were explants type and genotype and the influence of the hormone regime was negligible [11].

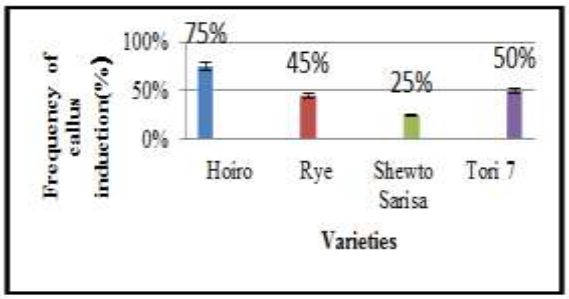

Fig. 3: Effect of Variety.

3.2.2. Effect of Plant Growth Regulators

In this experiment as the auxin concentration increased, the number of shoots from the explant increased with the constant concentration of cytokinin BA (2 mg/L). Shoot regeneration frequency became 57.5% at the concentration of NAA (3 mg/L), but when NAA concentration was lowered (2 mg/L),shoot regeneration showed a minimal frequency (40%) (Figure 4).

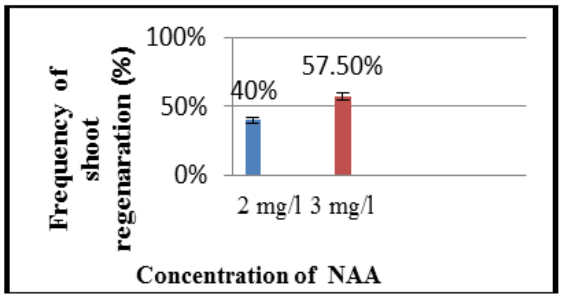

Fig. 4: Effect of Plant Growth Regulators.

3.2.3. Effect of Variety and Plant Growth Regulators Interaction

Shoot regeneration is highly affected by the genotype of explants. At the concentration NAA 2 mg/L, shoot regeneration frequency became 50% but it became 100% when the concentration 3 mg/L only for the Rye variety (Table II). It is the highest frequency for shoot regeneration in 2 mg/L NAA concentration. On the other hand, in the variety of Hoiro (A_1H and A_2H) 50% and 40% shoot induction frequency was found respectively at the concentration of MS + 2mg /L BA + 2 mg/L NAA and MS + 2 mg/L BA + 3 mg/L NAA. At the lower concentration of NAA (2 mg/L) + 2 mg/L BA higher shoot induction frequency was shown by the variety of Tori-7 60% (A_1T), in correspondence with minimum 40% (A_2T) that resulted from the concentration of 3 mg/L NAA + 2 mg/L BA. At the concentration of MS + 3 mg/L NAA + 2 mg/L BA for the variety of Sweto Sarisha (A_2Sh) for the shoot was 50% but at the lower concentration MS + 2 mg/L NAA + 2 mg/L BA (A_2Sh) no shoot was observed.

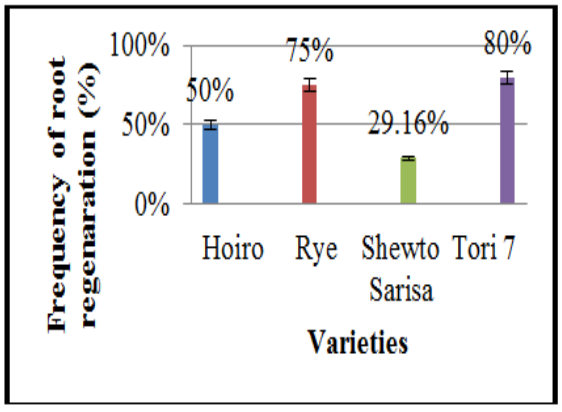

Fig. 5: Effect of Variety on Root Regeneration.

Table II: *Effect of Variety and NAA Interaction on Shoot Regeneration.*

Shoot Derived from the Combination	Frequency of Shoot Regeneration (%)
A_1R	50
A_2R	100
A_1H	50
A_2H	40
A_1Sh	00
A_2Sh	50
A_1T	60
A_2T	40

Here, A1 = 2 mg/L 2, 4-D + NAA, A_2 = 3 mg/L 2, 4-D + NAA.
H = Hoiro, R = ye, Sh = Shewto Sarisha, T = Tori-7.

3.3. Root Regeneration

3.3.1. Effect of Variety

The combination of growth regulators for root induction was genotype dependent. Lower concentration of NAA induces rhizogenesis or root formation [3]. In the present study, genotype Tori-7 (hybrid variety) performed high value on responding in root induction medium 80%. With the following Rye showed 75%. Average result was found from the local genotype Hoiro with 50%. A minimum response was got from the Shewto Sarisa 29.16% (Figure 5).

3.3.2. Effect of Different Plant Growth Regulators

The increase in length of root may be due to enhanced hydrolysis of carbohydrates, accumulation of metabolites at the site of application of auxins, cell enlargement and cell division. This was due to synergistic effect of IBA and NAA, which induce faster root extension in the medium. In the present study, 1 mg/L IBA showed the best result with the frequency of 58.33% and 1 mg/L NAA showed 47.91% for root regeneration frequency (Figure 6).

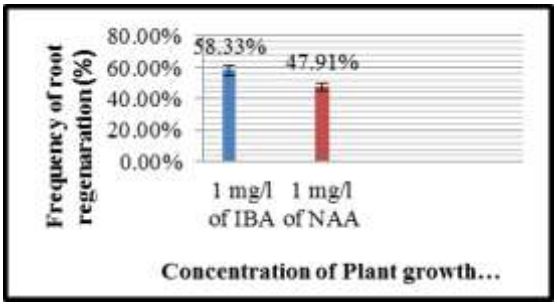

Fig. 6: *Effect of Different Plant Growth Regulators.*

3.3.3. Effect of Variety and IBA Interaction on Root Regeneration

In the present study, the different experimented varieties like Rye, Hoiro, Shewto Sarisa and Tori-7 were transferred into root induction medium after initiation of shoot. Callus contained in MS + 3 mg/L 2, 4-D showed the best response in MS + 1 mg/L IBA. 100% root induction was obtained from both Rye (K_2R) and Tori-7 (K_2T) in the medium MS + 1 mg/L IBA with the following 66.66% root induction obtained from Hoiro and there was no result from Shewto Sarisa. On the other hand, callus formed in 2 mg/L 2, 4-D showed a minimum response on average. 33.33% result was obtained from Shewto Sarisa (K_1Sh) and there was no result obtained from Hoiro (K_1H) genotype (Table III).

Table III: *Effect of Variety and IBA Interaction on Root Regeneration.*

Root Derived from the Combination	Frequency of Root Regeneration (%)
K_1H	00
K_2H	66.66
K_1R	100
K_2R	100
K_1Sh	33.33
K_2Sh	00
K_1T	66.66
K_2T	100

Here, K_1 = 2 mg/L 2,4-D, K_2 = 3 mg/L 2,4-D, H = Hoiro, R = Rye, Sh = Shewto Sarisa, T = Tori-7.

3.3.4. Effect of Variety and NAA Interaction on Root Regeneration

The response from NAA in different varieties was also studied in the present work. Callus formed in MS + 2 mg/L 2,4-D + 2 mg/L BA and MS + 3 mg/L 2,4-D + 2 mg/L BA of different varieties like Rye, Hoiro, Shewto Sarisa and Tori-7 were successfully transferred in root formation medium MS + 1 mg/L NAA. In 3 mg/L 2, 4-D + 2 mg/L BA callus showed the best result (100%) that was obtained from Rye (K_{2R}) following 50% root formed from the variety Rye (K2R), Sweto Sarisha (K2Sh) and Tori-7 (K_2T). The minimum result obtained was from (2 mg/L 2,4-D) with the variety like 33.33% from Shewto Sarisa (K_1sh) and no result was found from Rye (K1R) and Hoiro (K1H) (Table IV).

Table IV: *Effect of Variety and Plant Growth Regulators (NAA) Interaction.*

Root Derived from the Combination	Frequency of Root Regeneration (%)
K_1R	00
K_2R	100
K_1H	00
K_2H	50
K_1Sh	33.33
K_2Sh	50
K_1T	100
K_2T	50

Here, K_1 = 2 mg/L 2, 4-D, K_2 = 3 mg/L 2,4-D, H = Hoiro, R = Rye, Sh = Shewto Sarisa, T = Tori-7.

4. DISCUSSION

The plant growth regulators intravarietal variability of the organogenic and callogenic response may be as great as the variability between *Brassica* species [13]. The cultivars of this study were local variety Rye, Hoiro and Sweto Sarisha with the high responsive hybrid variety (Tori-7) of the *B. napus* species. Assessment on callus induction was studied through three quantitative traits depending on explant, the average callus induction frequency of different genotypes, comparative study with the best highly responsive cultivar, and auxin concentration variation with the genotype response.

The germinating seeds were used as an explant with the average callus induction frequency

60.35%. Six genotypes of oilseed *Brassica*, viz., Tori-7, Sampad, Kallyania, BARI Sarisha-7, BARI Sarisha-8, and MM 20-3 for *in vitro* regeneration with the petiole as an explant and the highest frequency obtained from the callus induction medium was 59.05% [2]. To establish a reproducible protocol or callus induction and regeneration of four rapeseed (*B. napus*) cultivars, viz., Dunkeld, Oscar, Rainbow and H-19 using Hypocotyls as explant source with the average callus induction 45.85% [2]. *In vitro* both Hypocotyls and Cotyledons were used as an explant and studied in *B. napus* L. cv. Kana with the average 88.7%–100% [13]. Hypocotyls and cotyledonary leaves of the cultivars Cyclone, Star and Wester (*B. napus* L) as explant for callus induction got an average callus induction frequency of 95%–96% [5]. The experimental cultivars were local variety Hoiro, Rye, Shewto Sarisa and Hybrid variety Tori-7 of *B. napus*. On this hybrid variety, Tori-7 showed the best performance in callus induction frequency of 83.33%. The type of auxin used in the medium influences culture morphology**,** for example, callus induction and maintenance. 2, 4-D is commonly used for callus induction. Application of 2, 4-D was very effective on callus formation, especially when combined with cytokinin [3]. In the present study, MS + 2 mg/L 2,4-D + 2 mg/L BA showed the best response with the callus induction frequency 70.83% with the following MS + 3 mg/L 2,4-D + 2mg/L BA, it showed an average response 58.33%. According to the review of Khan et al. [6], B5 + 2 mg/L 2,4-D showed the highest callus 96% in *B. napus* cv Oscar [12].

In this study, the percentage of explants forming shoots varied greatly between the *B. napus L.* cultivars Rye, Hoiro, Shewto Sarisa and Tori-7 where seeds were used as an explant on MS media with the auxin (NAA) to cytokinin (BA) ratio exhibiting a varied response (25%–75%) to shoot regeneration. The average shoot regeneration frequency 48.75% was inconsistence with average 38.17% shoot formed from the callus of the *B. napus* L cv Star, Cyclon and Wester [5]. It is clearly shown that shoot regeneration is highly dependent on explant type and genotype. On an average, the percentage of bud regeneration from hypocotyls derived callus of NL-611, NL-662 and NL-685 was 19.97% [4].

Shoot production efficiency was higher when the medium contained BA (2–4 mg/L). In the present study, combination of both auxin (NAA) and cytokinin (BA) growth regulators decreased shoot regeneration. The experimental cultivars like Rye, Hoiro, Shewto Sarisa and Tori-7 showed best response 57.5% at MS + 3 mg/L NAA + 2 mg/L BA with the correspondence 40% at MS + 2 mg/L NAA + 2 mg/L BA.

Root regeneration rate on average was 58.54% with the genotype Rye, Hoiro, Shewto Sarisa as well as Tori-7 with the root induction medium MS + 1 mg/L NAA or 1 mg/L IBA in

this experiment. Genotype is a limiting factor that severely limits the germplasm that can be manipulated or improved. Regeneration also depends on the age of the explants [14]. Average 44.44% root regeneration obtained in the medium contain ½ MS + 0.5 mg/L NAA [6]. In this experiment, Tori-7 showed 80% root regeneration frequency whereas 90% rooting was obtained in Wester from the medium MS + 0.3 mg/L IBA [5].

Fig. 7: *Callus Induction, Shoot and Root Initiation of Different Varieties of Brassica napus.*

5. CONCLUSIONS

The regenerated plantlets were washed off the media from their roots completely and transplanted into five-inch plastic pots using available potting mix like sterile soil sand and cowdung in a ratio 1:2:1 for acclimatization with moderate sunlight and humidity and after acclimatization the plantlets were successfully transferred into the soil. In conclusion, the present study describes an efficient and reproducible protocol for *in vitro* regeneration of plants. This report will help in mass propagation of the species with the maximum survival rate that can be used for commercial cultivation which is important for extraction of bioactive compounds.

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