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A quick method for micro-propagation of *Aloe vera* L. from leaf explants via callus induction

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Abstract
Micropropagation or regeneration of plants depends on an efficient protocol. An efficient protocol for plant regeneration was developed in present experiment using leaves and lateral shoots explants of *Aloe vera*. Investigation was conducted to assess the suitability of leaf explants for callusing using different concentration of auxin (2, 4-D and NAA) cytokinin (BAP and Kinetin) in semi-solid Murashige and Skoog (MS) media. Both shoot induction and elongation were better on MS medium supplemented with 4.0 mg/L BAP+ 1.0 mg Kin highest percentage (93.33%). All cultures showed shoot regeneration in this medium with multiple shoots/callus. Root regeneration was found maximum 66.67% in half basal MS medium with combination of 3.0 mg/L 2, 4 D+ 2.0 mg NAA in all combinations of both PGRs. On the other hand, the lowest number of root per inoculated shoot was zero without PGRs. Further the regenerated plants with well-developed roots were transferred to sterilized cockpit in poly house. By this procedure in future the cost of *Aloe vera* production will be decreased which will lead the decrease of aloe product prices in market and the standardization of the media and PGRs combinations are very useful for future studies and micropropagation of *Aloe vera* L.

Keywords: *Aloe vera*, tissue culture, explants, PGRs and callus induction

Introduction
Nature provides us free of cost many sources to fulfill the requirement for the life on earth in which one of the beautiful as well as special sources is green plants they provide different components for the growth and development of animals as well as human being. The requirement of various human and animal's nutrition are as old as mankind itself therefore trees are playing very important role from ancient time to fulfill these requirements. However, trees are commonly used for pharmaceutical properties, in modern era synthetic chemistry has tremendous advancement but has several human health problems are dominantly also, which can solved by herbal drugs and having no or rare side effects. The need of trees increases with increasing world population for nutritional and anthapogenic activities. *Aloe vera* it is a plant that have different medicinal importance for human being.

Aloe vera is a monocotyledonous plant and it is belongs to the family liliaceae. It is a xerophytic medicinal plant and grows even in rainfall condition. It is also used in decoration purposes in houses and gardens as well as medicinal. Although *Aloe vera* originated in the warm, dry climates of Africa, the plant is readily adaptable and grows worldwide [13]. The genus *Aloe* has more than 500 species but only a few are having medicinally important reported in 1999 [5]. Among all these species, *Aloe vera* is the plant of greatest interest and uses. Its leaves have been found to contain over 200 bioactive constituents [18]. *Aloe vera* having or contain different bioactive materials such as saponins, anthraquinones, mucopolysaccharides, steroids, vitamins and glucomannans observed in 2006 and 2007 [10 & 11]. Plant tissue culture technique play more important role in production of new plants where seed formation is not necessary. In *Aloe vera* micropropagation technique is more useful for its cultivation of seed production for cultivation. In present study a method has developed for micropropagation of *Aloe vera* plant. By the protocol in future it may be helpful for plant regeneration of *Aloe vera* L.

2. Methods and Materials
Considering the medical importance and slower propagation rate of *Aloe vera* L., this micro propagation research work was conducted during 2016-2017 academic session in the Plant Tissue Culture Laboratory, RGSC, BHU, Barkachha, Mirzapur, Uttar Pradesh, India.

The detail of materials used and analytical methods employed during this study is given below:

2.1 Plant materials

Lateral shoots (suckers) and leaf parts of *A. vera* (more than one month old) collected from experimental *Aloe vera plants from* medicinal park(garden) of RGSC, BHU, Barkachha, Mirzapur, Uttar Pradesh, leaves used as the explants for experiment. In Plant Biotechnology Laboratory, Explants were prepared by removing roots, soil particles and brown colored tissues and extending leaf portions to give an average size of 2-5cm. They were washed thoroughly with running tap water for about 7-10 minutes till all soil and other foreign materials washed off. Sets of sixteen explants were then washed with tap water containing a few drops of Tween 20 and rinsed in 70% ethanol for 25-30 seconds followed by initial soaking in sodium hypochlorite containing approximately 4% available chlorine for 10 minutes and then in freshly prepared mercuric chloride solution (0.2%) for 10 minutes. Finally they were washed 3-5 times with sterile double distilled water before culturing.

2.2 Sterilization

Among the treatment given good response for surface sterilization of explants was seen in explants when dipped in 1.0% bavistin for 30 minutes followed by 0.1% HgCl2 for 45 minutes followed by washing with double sterilized water followed by dipping in 2% NaOCl for 20 min. then dipping in 70% ethanol for 25-30 second. This sterilization procedure gave contamination free explants. The explants leaf tips of 2-5 cm were subjected to the MS medium supplemented with different concentration of PGRs for their response.

2.3 Culture media

The basal medium used for the culture is MS medium [16] with 3% sucrose and 0.8% agar with growth hormones.

2.4 Shooting and culture conditions

The pH of the medium was adjusted to 5.7 and then cultures were incubated at 25± 00C, under cool white fluorescent tubes with 16 h photoperiod. Fifteen calli were cultured on MS nutrient medium supplemented with eleven different concentrations of BAP and Kinetin (Table 3). The callus showing shoots proliferation on the basis of number of total shoots per callus. The data was recorded for shoot proliferation in suitable medium and further sub culturing of proliferated shoots for rooting. Explants devoid of contaminations were then inoculated on the MS basal medium supplemented with different concentrations of BAP (2.0 mg/L), Kn (1.0mg/L) and BAP (1.5 mg/L) along in combination with Kn (2.0 mg/L). Shoots amplified from callus of leaf explants in shoot induction media were detached from callus and transferred on to a shoot elongation medium containing 1.0 gm/L activated charcoal.

2.5 Rooting of Micro shoots

After growing of shoot from callus (8 weeks old), 4-7 cm tall shoots was inoculated into MS medium containing different concentrations of 2, 4-D, NAA and in combinations of both. For 2,4-D, NAA and in combinations the concentration were 2.0 mg, 4.0 mg and 6 mg/L, while in combinations 3mg/L 2,4-D constant and 1.0 mg, 2.0 mg, 3.0 mg and 4.0 mg/L NAA was add in half basal MS medium.

2.6 Hardening of plantlets

About 4-5cm height of shoots and well rooted plantlets were taken out from the culture vessels about one month after from root induction media into small pots with sterile soil were used for acclimatization of new regenerated plants. The roots were treated with 0.2% bavistin for 30 to 45 seconds and then transferred to sterilized cockpit in green house with automatically controlled temperature, relative humidity, proper misting, irrigation and air exhaustion. Further ten days, plants were transferred to sterilized earthen pots containing a mixture of sand, FYM, soil at a ratio of 1:2:1. The potted plants were kept in the conventional net house for acclimatization before transfer to the open field. Plants were watered at two days interval.

2.7 Calculation of percent of shoots and roots induction from culture

Number of shoots was recorded and the percentage of shoot regeneration was calculated as:

$$\textbf{(i)}\ \text{Per cent (\%) of Callus Induction} = \frac{\text{Number of callus induction in explants}}{\text{Total number of explants innoculated}} \times 100$$

Number of shoots was recorded and the percentage of shoot regeneration was calculated as:

$$\textbf{(ii)}\ \text{Per cent (\%) of Shoot Induction} = \frac{\text{shoot induced explants of Number}}{\text{Number of explants incubated}} \times 100$$

The percentage of root formation was calculated as:

$$\textbf{(iii)}\ \text{Per cent (\%) of Root formation} = \frac{\text{Root induced shoots of Number}}{\text{Incubated shoots of Number}} \times 100$$

2.7(iv) Calculation of per cent (%) of plant establishment

The percentage of established plants was calculated based on the number of plantlets placed in the pot and the number of plants finally established or survived by the following equation:

$$\text{Per cent (\%) of plant establishment} = \frac{\text{Number of esteblished plantlets}}{\text{Total number of plantlets placed in plot}} \times 100$$

3. Results and Discussion

3.1 Callus induction Explants type and Different concentrations of PGRs in MS medium

After inoculation of leaf explants into MS Medium, it starts to show signs of callus initiation after four weeks of culturing. Explant starts to appear brownish from the green colour of natural leaves. Further callus sub-culturing into the same MS media with same combinations of the plant growth regulators for increasing size and amount of calls to the new experiment for shoots regeneration (Figure 1 a and b). Leaf explants were incubated on MS basal medium with different concentrations of 2,4-D, NAA, BAP and Kn and in combination with for callus initiation, shoot emergence and proliferation and root regeneration in Tables 2, 3 and 4 and Graph 2, 3 and 4. A number of factors such as genotype, culture medium (including growth regulators and their combinations), physical environment, explants develop-mental stage, etc affect adventitious shoot regeneration from tissue cultured explants [1]. Many factors such as genotype, culture medium (including growth regulators and their combinations), physical environment, explants develop-mental stage and callus etc affect adventitious shoot regeneration from tissue cultured explants [16].

3.2 Shoot induction, Explants type and Different Media concentrations of PGRs in MS medium

In the Table 3, showed callus used for shoot induction in half basal MS media with different plant hormones combinations. It was found that Kn gave better shoot Induction than BAP when both hormones were used separately in media. When callus were inoculated with only BAP, the best outcome of average number of shoots per explant was 4-15 in different hormone concentration. Half basal MS medium with 4.0 mg/L BAP+ 1.0 mg Kn was showed highest no of shoot regeneration in all inoculated culture bottles (93.33%) whereas half basal MS + 6.0 mg/L Kn hormone concentration exhibited 80% shoot induction. On the other hand, in case of Kn (6.0 mg/L) highest percentage of shoot induction were identify as 80%, BAP (4.0 mg/L) were 46% without combine to each other (Kn & BAP). Note that the least number of shoots per explant (nil) was shown in hormone-free medium.

Similar results with the present findings, [10] reported a better micropropagation rate with a combination of two cytokines (BAP with KN) in comparison to when BAP alone used. Both [4] for *Aloe barbadensis* and [9] for *Aloe vera* also indicated highest numbers of shoots (10 shoots per culture and 9.67) with 2 mg/L and 0.5 mg/L BAP along with 0.5 mg/L NAA respectively. Very lower numbers in comparison to the above-mentioned studies. In the study, shoot induction and proliferation occurred only in the presence of cytokinin BAP and KN. But the results of current research also showed the best ratio of Kn to BAP for shoot initiation, shoot induction was just double with Kn. Among the cytokinins tested, Kn proved to us more effective. These findings of the present research work are much closed to work off in [3, 7]. The current work is in contrast to earlier reports in *Aloe vera* by many other research workers in *Aloe vera*.

3.3 Rooting

In plant tissue culture programme rooting is the last step to complete the plant regeneration. Root induction mainly affected due to the presence or absence of auxin plant hormones, it was observed that basal MS medium devoid of phytohormones also induce rooting but percentage of rooting and number of roots per shoot increased by the presence of hormones. 2, 4-D was found best for induction of roots followed by NAA. 2, 4-D supplemented at 4.0 mg and 6.0 mg/l induced highest frequency of rooting 53.33% per cent also obtained in 2007 [3]. The best result was obtained by using basal MS medium with 3.0 mg/L 2, 4 D+ 4.0 mg NAA 100% per cent shoots showed root induction in high numbers then other hormones combinations and concentrations in experiments. Result of this found very coincides with the findings of Mehta [14] and among the all eleven combinations of hormones with same MS media both types of auxins 3.0 mg/L 2,4 D+ 4.0 mg NAA was found to be the best for root induction. In 2014 [7] also reported that the MS medium containing BA and NAA was found to be the best medium in Aloe micropropagation (Tables 4 and Graph 4). To the contrary, other studies indicated MS media with 0.5-1.0 mg/L NAA and IBA resulting in high rate of rooting and numbers of roots per shoots [4, 11, 16]. Rooting response of microshoots is reported [4], 95% rooting was obtained from plantlets cultured on half strength MS supplemented with 0.5 mg/L NAA.

After root regeneration next step is hardening and hardening was done when shoot length was about 4-5cm height. All shoots with well-developed roots were taken out from the culture vessels about one month old plantlets after root induction media into small pots with sterile soil were used for acclimatization of new regenerated plants for two weeks. Most of the new regenerated plants of *Aloe vera* were survive in the field without any problem. It's affected by hardening if hardening was proper and all plantlets were healthy there is no chance for die in the field.

Table 1: Contamination percentage in culture tubes of cultured explants (Leaf of *Aloe vera* L.) after 2-3 weeks of culture when MS medium containing different concentration of 2, 4-D, NAA and 2, 4-D with Kin.

Name of the hormone	Hormone concentration(mg/L)	No. of explants	Number of callus induction	Percentage of shoot induction
2,4-D	MS + 0.0mg/L 2,4 D	30	6.00	20.00
	MS + 2.0 mg/L 2,4 D	30	9.00	30.00
	MS + 4.0 mg/L 2,4 D	30	10.00	33.33
	MS + 6.0 mg/L 2,4 D	30	3.00	10.00
NAA	MS + 0.0 mg/L NAA	30	8.00	26.67
	MS + 2.0 mg/L NAA	30	2.00	6.67
	MS + 4.0 mg/L NAA	30	5.00	16.67
2,4-D & Kin	MS + 2.0 mg/L 2,4 D+ 2.0 mg Kin	30	2.00	6.67
	MS + 4.0 mg/L 2,4 D+ 2.0 mg Kin	30	4.00	13.33
	MS + 4.0 mg/L 2,4 D+ 4.0 mg Kin	30	3.00	10.00
	MS + 6.0 mg/L 2,4 D+ 2.0 mg Kin	30	5.00	16.67

Table 2: Effect of different concentration of 2,4-D, NAA and 2,4-D with Kn on callus induction (After 4 weeks of culture) from leaf explants of *Aloe vera* L.

Name of the hormone	Hormone concentration(mg/L)	No. of explants	Number of callus induction	Percentage of shoot induction
2,4-D	MS + 0.0 mg/L 2,4 D	15	0.00	0.00
	MS + 2.0 mg/L 2,4 D	15	3.00	20.00
	MS + 4.0 mg/L 2,4 D	15	8.00	53.33
	MS + 6.0 mg/L 2,4 D	15	8.00	53.33
NAA	MS + 0.0 mg/L NAA	15	0.00	0.00
	MS + 2.0 mg/L NAA	15	0.00	0.00
	MS + 4.0 mg/L NAA	15	1.00	6.67
2,4-D & Kin	MS + 2.0 mg/L 2,4 D+ 0.5 mg Kn	15	7.00	46.67
	MS + 2.0 mg/L 2,4 D+ 1.0 mg Kn	15	10.00	66.67
	MS + 2.0 mg/L 2,4 D+ 2.0 mg Kn	15	0.00	0.00
	MS + 2.0mg/L 2,4 D+ 2.5 mg Kn	15	15.00	100.00

*2, 4-D= 2, 4-dichlorophenoxyacetic acid, NAA= Naphthalene acetic acid, BAP=6-Benzylaminopurine and Kn= Kinetin

Table 3: Effect of different concentration of BAP, Kn and BAP with Kin on shoot induction and proliferation (After 6 weeks of culture) from callus inoculation**.**

Name of the hormone	Hormone concentration(mg/L)	No. of calli inoculation for shoot	Number of shoot induction	Percentage of shoot induction
BAP	*MS + 0.0 mg/L Kn	15	3.00	20.00
	*MS + 2.0 mg/L Kn	15	8.00	53.33
	*MS + 4.0 mg/L Kn	15	10.00	66.67
	*MS + 6.0 mg/L Kn	15	12.00	80.00
Kinetin	*MS + 0.0 mg/L BAP	15	1.00	6.67
	*MS + 2.0 mg/L BAP	15	2.00	13.33
	*MS + 4.0 mg/L BAP	15	7.00	46.67
BAP & Kinetin	*MS + 4.0 mg/L BAP+0.5 mg Kn	15	11.00	73.33
	*MS + 4.0 mg/L BAP+ 1.0 mg Kn	15	14.00	93.33
	*MS + 4.0 mg/L BAP+ 1.5 mg Kn	15	9.00	60.00
	*MS + 4.0 mg/L BAP+ 2.0 mg Kn	15	4.00	26.67

*Basal half MS medium used in all experiments for shoot regeneration

Table 4: Effect of different concentration of auxins 2,4-D, NAA and 2,4-D with NAA on root regeneration (After 3 weeks of culture) from shoots**.**

Name of the hormone	Hormone concentration(mg/L)	No. of explants	Number of callus induction	Percentage of shoot induction
2,4-D	*MS + 0.0 mg/L 2,4 D	15	0.00	0.00
	*MS + 2.0 mg/L 2,4 D	15	3.00	20.00
	*MS + 4.0 mg/L 2,4 D	15	8.00	53.33
	*MS + 6.0 mg/L 2,4 D	15	8.00	53.33
NAA	*MS + 0.0 mg/L NAA	15	0.00	0.00
	*MS + 2.0 mg/L NAA	15	0.00	0.00
	*MS + 4.0 mg/L NAA	15	3.00	20.00
2,4-D & NAA	*MS + 3.0 mg/L 2,4 D+ 1.0 mg NAA	15	7.00	46.67
	*MS + 3.0 mg/L 2,4 D+ 2.0 mg NAA	15	10.00	66.67
	*MS + 3.0 mg/L 2,4 D+ 3.0 mg NAA	15	7.00	46.67
	*MS + 3.0 mg/L 2,4 D+ 4.0 mg NAA	15	15.00	100.00

*Basal half MS medium used in all experiments for shoot regeneration

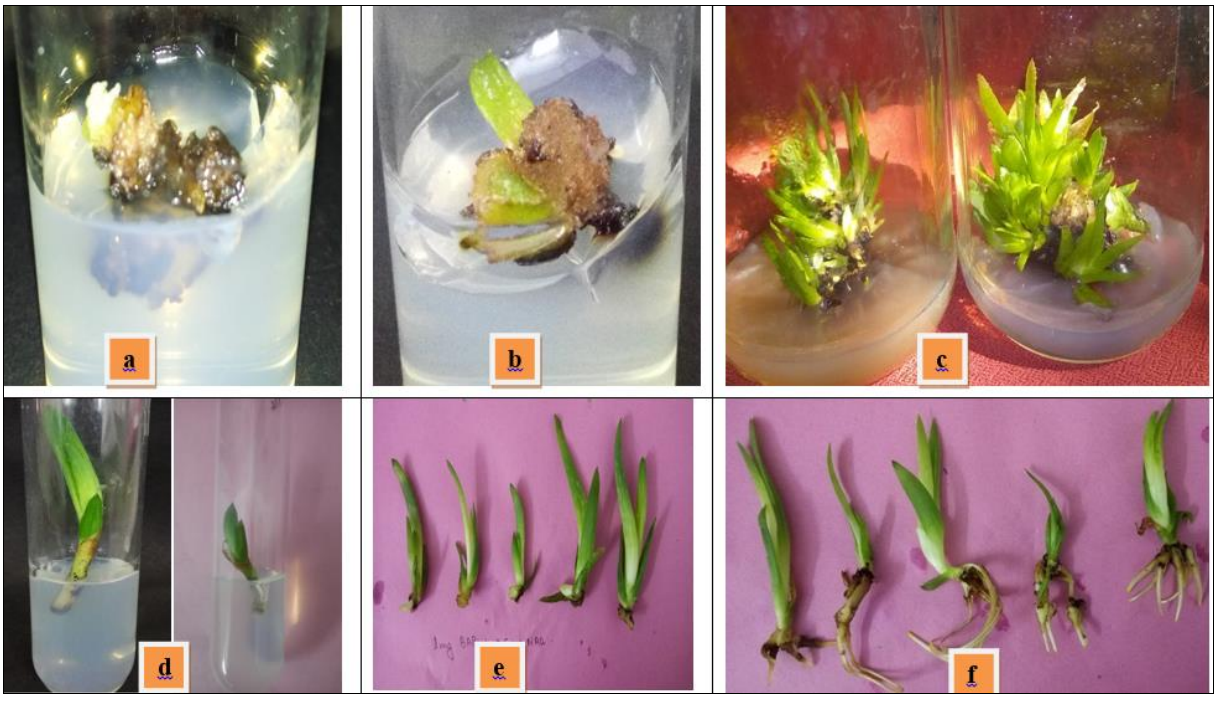
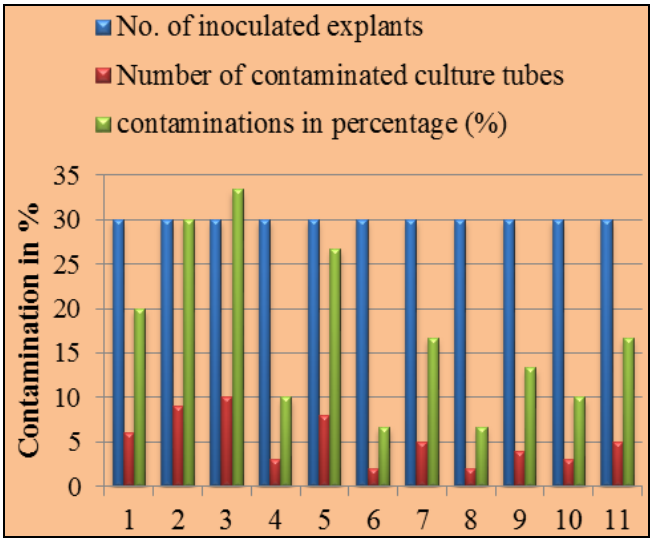
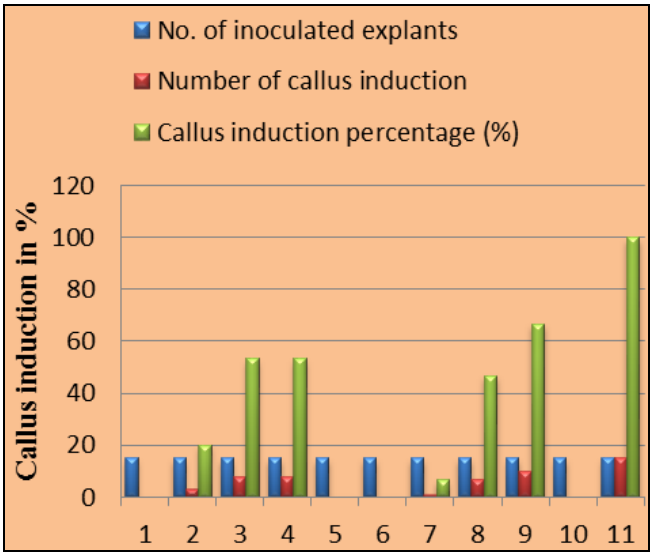


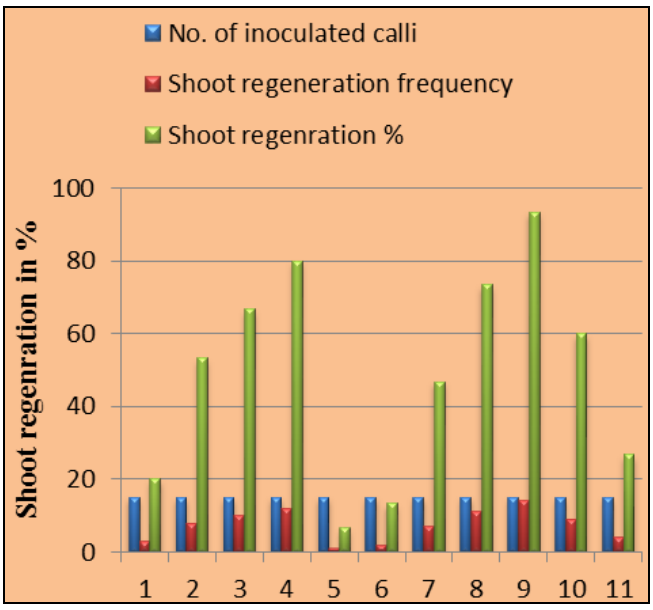
Fig 1: (a) Effects of different treatments on *in vitro* culture of *Aloe vera* for callus induction, callus initiated in MS medium containing NAA alone without any potential for regeneration. (b) The soft, friable, light yellow to greenish and nonorganogenic callus in MS medium containing 2, 4-D (0.2 mg l^{-1}) and NAA. (c) The emergence of large number of shoots from the segment on MS medium supplemented with 4 mg l^{-1} BA and 1.0 mg l^{-1} Kin after 4 weeks. (d) No. of shoots in one piece of callus in MS medium supplemented BAP and with Kinetin 10 days after culture. (e) Single-single shoots from developed by a callus (f) Rooting of a regenerated shoot in MS medium supplemented with 2,4-D and NAA (3.0 mg l^{-1} and 4.0 mg l^{-1}) 15 days after culture.



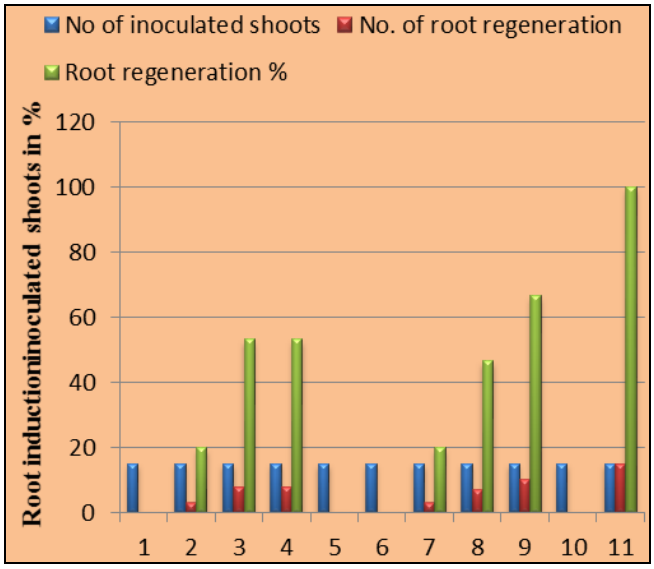
Graph 1: Contamination in callus induction through leaf explants in different combinations of PGRs in percentage



Graph 2: Effect of different plant growth regulators on Callus induction in *Aloe vera's* leaf explants



Graph 3: Effect of different plant growth regulators on shoot regeneration in inoculated calli developed by leaf explants



Graph 4: Effect of different plant growth regulators on root induction in regenerated shoots.

4. Conclusions

On the basis of above study, this is concluded here, that the type and concentration of plant growth regulators (PGRs) played a significant role in plant regeneration during callus induction, shoot initiation, shoot proliferation and plant rooting. Plant regeneration was also depending on the plant genotype and type of explants. This experiment indicated that micropropagation can be a useful tool for proliferation of *A. vera* L. on the basis of present study we can say that *Aloe vera* can be successfully regenerated using in-vitro techniques Mass propagation of *Aloe vera* can be attained by this protocol and it may be very helpful in future study.

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