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Optimization of media for micro-propagation of *Aloe barbadensis* Mill.

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Abstract
An efficient protocol for rapid *in vitro* propagation of valuable medicinal plant, *Aloe barbadensis* Mill. (var. Gujrat 20) by using shoot tip as explants were done. *Aloe barbadensis* is likely to become a major source of a number of medicinal products of high value in the coming future. Therefore, efficient and fast mechanized production need to be established for *Aloe barbadensis* to address the need of the present time. Our works deal with the establishment of a viable protocol for decontamination with $HgCl_2$, *in vitro* shooting and rooting of this valued medicinal plant. Out of nine different hormonal regime tried for induction of multiplication on shoot explants, MS media supplemented with 6.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l $AdSO_4$ was found best to induce maximum number of plantlets per explants. For rooting, increase in auxin (IAA) and decrease in cytokinin (BAP) and adenine sulphate was found effective.

Keywords: Micro-propagation, cytokinin, *Aloe barbadensis, in-vitro* and auxin

Introduction
The name *Aloe vera* derives from the Arabic word "Alloeh" meaning "shining bitter substance," while "vera" in Latin means "true" (Surjushe *et al*, 2008) [15]. *Aloe barbadensis* Mill. (Syn. *Aloe vera*) has been used for medicinal purposes in several cultures for millennia: Greece, Egypt, India, Mexico, Japan and China. Egyptian queens Nefertiti and Cleopatra used it as part of their regular beauty regimes. Alexander the Great, and Christopher Columbus used it to treat soldier's wounds. The first reference to Aloe vera in English was a translation by John Goodyew in A.D. 1655 of Dioscorides' Medical treatise De Materia Medica. By the early 1800s, *Aloe vera* was in use as a laxative in the United States, but in the mid-1930s, a turning point occurred when it was successfully used to treat chronic and severe radiation dermatitis. *Aloe* is a perennial herb with a stem less or very short-stemmed plant growing to 60-100 cm (24-39 inch) tall, spreading by offsets and shallow root system (Scala Di *et al*. 2013) [13]. It grows wild in tropical climates around the world and is cultivated for agricultural and medicinal uses. *Aloe* is also used for decorative purposes and grows successfully indoors as a potted plant. It is also called the *burn* plant, first aid plant and miracle plant. The *Aloe* leaf contains over 75 nutrients and 200 active compounds, including 20 minerals, 20 amino acids, and 12 vitamins makes for a high quality *Aloe* drink (Josias H. Hamman, 2008) [5]. Micro propagation via shoot culture, often utilized to maintain clonal fidelity, would be especially appropriate in this respect. Micro propagation is an effective approach to conserve important germplasms through true-to-type propagation of selected genotypes. It is one of the most successful methodology for rapid multiplication and production of quality planting materials free of any disease and pest which ensure maximum production of potential varieties. Marfori *et al*. (2005) [6] taken nodal explants of *A. barbadensis* and they placed in Murashige and Skoog (MS) medium containing different levels of kinetin or 6-benzylaminopurine (BAP) to induce multiple shoot formation. The best treatment for multiple shoot induction was 1 mg/L BAP, which produced an average of 11 shoots per explant in 1 month. Individual shoots from the multiple shoot clumps were taken and transferred in MS medium containing different levels of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) for 11 rooting. The best auxin for root formation was NAA, with an optimum concentration of 0.10 mgL^{-1}. Taking the idea from their research we established the protocol with different quantity of regimes to micro-propagate the qualitative *Aloe vera* plant of variety Gujrat 20.

Materials and Methods
Explants surface sterilization: The mother plants used were 25 to 30 cm long, 1-2 cm thick and 2-3 months old of variety

Gujrat 20, growing in Faculty of Forestry campus, B.A.U., Ranchi. The explant were washed under tap water for 30 minutes and then treated with 0.10% (w/v) Bavistin solution for 45 minutes followed by washing with distilled water 3 times. It was then surface sterilized with 0.05%, 0.10% and 0.20% (w/v) $HgCl_2$ for 10, 15 and 20 minutes under aseptic condition followed by rinsing with autoclaved distilled water for 5 times for removal of traces of mercuric chloride. Each experiment for the *Aloe vera* was set up with 5 replications and repeated twice.

Inoculation for shooting

For the preparation of media all the glassware were properly cleaned with detergent and finally washed with distilled water and dried for further use. As listed below protocol, BAP (mg/l), $AdSO_4$ (mg/l) and IAA (mg/l) were added of different combinations in MS basal medium and final volume was made up to 1 litre. The pH was adjusted to 5.7 (using 1N HCl or 1 N NaOH). Agar 0.75% (w/v) was added as gelling agent to the medium. To dissolve agar properly, medium was boiled. The medium with dissolved agar were poured into 300ml capacity of sterilized culture bottles (50 ml) and capped tightly. The MS Basal (Hi-media) (Murashige and Skoog, 1962) [9], supplemented with hormones were prepared in nine different compositions as- 1. MS + BAP 2.0 mg/l + IAA 0.2 mg/l, 2. MS + BAP 4.0 mg/l + IAA 0.2 mg/l, 3. MS + BAP 6.0 mg/l + IAA 0.2 mg/l, 4. MS + BAP 2.0 mg/l + IAA 0.2 mg/l + AdSO450 mg/l, 5. MS + BAP 2.0 mg/l + IAA 0.2 mg/l + AdSO4 100 mg/l, 6. MS + BAP 4.0 mg/l + IAA 0.2 mg/l + AdSO4 50 mg/l, 7. MS + BAP 4.0 mg/l + IAA 0.2 mg/l + AdSO4 100 mg/l, 8. MS + BAP 6.0 mg/l + IAA 0.2 mg/l + AdSO4 50 mg/l, and 9. MS + BAP 6.0 mg/l + IAA 0.2 mg/l + AdSO4 100 mg/l. The explants were inoculated inside the UV treated laminar air flow and grown under 3000 lux light from Phillips fluorescent day tube for 16 hours light and 8 hours dark period. The ambient temperature was maintained at 25 ± 2^0C and the relative humidity was adjusted to approximately 55%. To ensure proper supply of nutrients to tissue cultured plants and to overcome excessive phenolic exudation of *Aloe barbadensis* for their growth and maintenance, subculturing was done periodically after 15 days on the same medium.

Inoculation for rooting

The fully developed plantlets having 2-4 leaves were transferred for the initiation and development of roots in three different rooting medium mentioned as- 1. MS + BAP 2.0 mg/l + IAA 2.0 mg/l + AdSO4 50 mg/l, 2. MS + BAP 2.0 mg/l + IAA 4.0 mg/l + AdSO4 50 mg/l and 3. MS + BAP 2.0 mg/l + IAA 6.0 mg/l + AdSO4 50 mg/l.

Results and Discussion

Standardization of surface sterilization of explants

Mercuric chloride is a widely used surface sterilants which has been used by several authors (Haque *et al*. 2009; Usha *et al*. 2010; Rani and Kumar, 2011; Garima and Shruthi; 2012, Rout and Sahoo, 2013; Shukla *et al*. 2016) [3, 4, 11, 12, 14, 16]. In the present study mercuric chloride in different concentrations was used as surface sterilant. To optimize surface sterilization 0.05%, 0.10% and 0.20% mercuric chloride was used to sterilize shoot tip explants of *Aloe barbadensis* var. Gujarat 20 for 10 min., 15 min. and 20 min. duration. Maximum percentage of decontamination was observed 88.89% when treated with 0.10% mercuric chloride for 20 minutes (Table 1). A gradual decrease in decontamination percentage was observed with the increase of mercuric chloride concentration as well as duration.

Table 1: Effect of different concentration of $HgCl_2$ on percentage of decontamination of explants of *Aloe barbadensis* var. Gujarat 20

Concentration of $HgCl_2$	Mean percentage of decontamination after 7 days		
	10 mins.	15 mins.	20 mins.
0.05%	42.23	55.56	73.34
0.10%	57.78	68.89	88.89
0.20%	60.00	53.33	44.44

Bud breaking and shoot multiplication

The sterilized explants were inoculated in Murashige and Skoog (1962) [9] basal media supplemented with 9 different combination and concentrations of exogenous phytohormones. Bud breaking was observed after 7 days in all combinations of media while those were not equally responsive for initiation of multiplication (Fig. 3). Out of 9 different hormonal regime used no multiplication was observed in MS supplemented with a combination of 2.0 and 4.0 mg/l BAP and 0.2 mg/l IAA. With the increase in BAP concentration to 6.0 mg/l along with 0.2 mg/l IAA a slight increase in multiplication rate to 1.93 shoots/explants was observed after 30 days in culture. However, addition of adenine sulphate with BAP and IAA was found effective to enhance the multiplication rate. When 50mg/l adenine sulphate was combined with 2.0 mg/l BAP and 0.2 mg/l IAA it was observed that the rate of multiplication was 2.0 and 2.93 after 15 and 30 days respectively (Fig. 4). It was further increased to 4.0 when concentration of adenine sulphate was increased to 100.0 mg/l (Table 2, Fig. 1, Fig. 5). A gradual increase in rate of multiplication was observed with the increase in BAP as well as adenine sulphate concentration (Fig. 6). Maximum number of shoots was observed 10.00 after 30 and 45 days in 6.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l adenine sulphate (Table 2, Fig 1, Fig. 7 and 8). In the present study it was observed that adenine sulphate plays a significant role in enhancing the rate of multiplication. Earlier studies indicated also that addition of adenine sulphate to BAP containing medium enhances both bud breaking as well as shoot multiplication (Eeswara *et al*. 1998; Parveen *et al*. 2005) [2, 10]. Shukla *et al*. (2016) [14] used adenine sulphate as an antioxidant agent for establishment of callus culture of *Aloe vera* to overcome the problem of phenolic exudation. Molasaghi *et al*. (2014) reported highest rate of multiplication in *Aloe vera* while inoculated in MS supplemented with 1.0 mg/l IAA and 4.0 mg/l BAP as well as 0.2 mg/l IAA and 0.8 mg/l BAP. Chowhan (2017) [1] used IBA instead of IAA along with BAP and found it effective in inducing maximum shoot proliferation in *Aloe vera*.

Table 2: Effect of different combinations and concentration of phytohormones on rate of multiplication of shoot explants of *Aloe barbadensis* var. Gujarat 20

MS + Hormonal regime	Number of days in culture		
	15 d	30 d	45 d
1. BAP 2.0 mg/l + IAA 0.2 mg/l	-	-	-
2. BAP 4.0 mg/l + IAA 0.2 mg/l	-	-	-

3. BAP 6.0 mg/l + IAA 0.2 mg/l	1.00	1.93	1.93
4. BAP 2.0 mg/l + IAA 0.2 mg/l + AdSO4 50.0 mg/l	2.00	2.93	2.93
5. BAP 2.0 mg/l + IAA 0.2 mg/l + AdSO4 100.0 mg/l	4.00	4.00	4.00
6. BAP 4.0 mg/l + IAA 0.2 mg/l + AdSO4 50.0 mg/l	4.00	5.00	5.00
7. BAP 4.0 mg/l + IAA 0.2 mg/l + AdSO4 100.0 mg/l	4.00	6.00	6.00
8. BAP 6.0 mg/l + IAA 0.2 mg/l + AdSO4 50.0 mg/l	5.00	6.93	6.93
9. BAP 6.0 mg/l + IAA 0.2 mg/l + AdSO4 100.0 mg/l	7.93	10.00	10.00

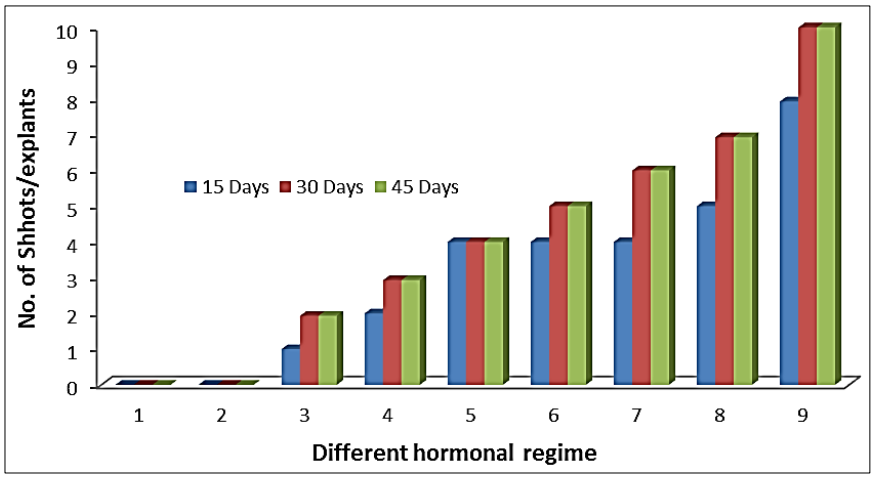

Fig 1: Effect of different phytohormones on rate of multiplication in *Aloe barbadensis* var. Gujarat 20

Induction of root on excised shootlets

For induction of rooting, the excised shootlets were inoculated in MS media supplemented with 3 different hormonal regime. Addition of increased amount of IAA, 2.0, 4.0 and 6.0 mg/l along with 2.0 mg/l BAP and 50.0 mg/l adenine sulphate was found effective for root induction on excised shootlets (Table.3, Fig 2). However, maximum 8.13 roots were observed after 21 days in MS with 2.0 mg/l BAP + 50.0 mg/l adenine sulphate and 2.0 mg/l IAA (Fig. 9 and 10). A gradual decrease in number of roots per shootlets was observed with the increase of IAA concentration to 4.0 and 6.0 mg/l (Table 3, Fig 2).

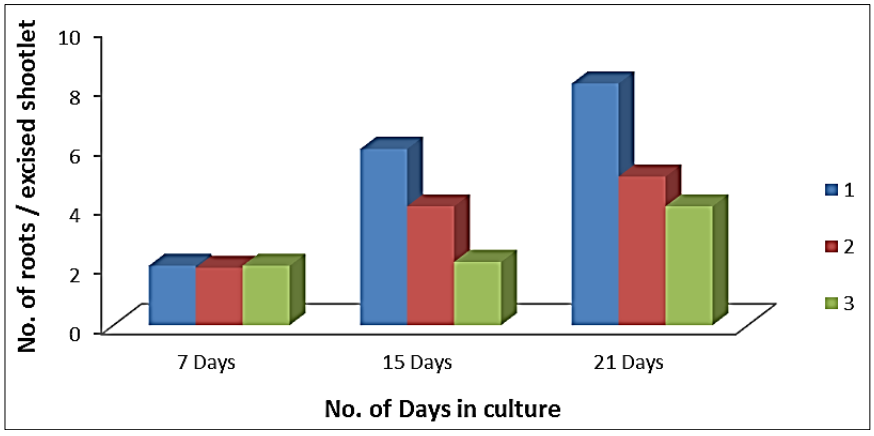

Fig 2: Effect of different phytohormones on root induction of *Aloe barbadensis* var. Gujarat 20

Table 3: Effect of different combinations and concentration of phytohormones on root induction on excised shootlets of *Aloe barbadensis* var. Gujarat 20

MS + Hormonal regime + 2g/l activated charcoal	Number of days in culture		
	7 Days	15 Days	21 days
1. BAP 2.0 mg/l + IAA 2.0 mg/l + AdSO4 50 mg/l	2.00	5.93	8.13
2. BAP 2.0 mg/l + IAA 4.0 mg/l + AdSO4 50 mg/l	1.93	4.00	5.00
3. BAP 2.0 mg/l + IAA 6.0 mg/l + AdSO4 50 mg/l	2.00	2.13	4.00

Acclimatization of rooted plantlets

The rooted *in vitro* grown plantlets of *Aloe barbadensis* var. Gujarat 20 were taken out from the media, washed thoroughly with sterile distilled water followed by a 10 minutes treatment with Bavistin, a systemic fungicide, and finally transferred to pro tray containing coco peat as potting mix. The pro trays were kept within poly tunnel under shade-net to provide high humidity required for primary hardening (Fig. 11). After 15 days of primary hardening the plantlets transferred to poly bags containing soil: sand: FYM in 1:1:1 proportion and kept under shade for another 30 - 45 days before field transfer.

Fig 3: Bud breaking in *Aloe barbadensis* var. Gujarat 20 after 7 days

Fig 4: Initiation of multiplication after 15 and 30 days in culture in MS + 2.0 mg/l BAP + 0.2 mg/l IAA and 50.0 mg/l Adenine sulphate

Fig 5: Multiplication in MS + 2.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l $AdSO_4$ after 30 days

Fig 6: Multiplication in MS + 6.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l $AdSO_4$ after 30 days

Fig 7: Multiplication in MS + 4.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l $AdSO_4$ after 30 days

Fig 8: Multiplication in MS + 6.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l $AdSO_4$ after 45 days

Fig 9: Excised shootlets in rooting media

Fig 10: *In vitro* grown roots plantlets

Fig 11: Primary hardening in coco peat

Conclusion

With the awareness about herbal products the use of this plant is increasing day by day in pharmaceutical as well as cosmetics industry. But the conventional way of getting quality plant materials is quite less so far the demand is concerned. The reason is male sterility in one hand and very less number of vegetatively propagated shootlets on the other. Keeping this in view the present work deals with the establishment of a viable protocol for this valued medicinal plant. Out of nine different hormonal regime tried for induction of mulitiplication on shoot explants, MS supplemented with 6.0 mg/l BAP + 0.2 mg/l IAA + 100.0 mg/l adenine sulphate was found best to induce maximum number of plantlets per explants (Table 2, Fig 2). For rooting, increase in IAA and decrease in BAP and adenine sulphate was found effective.

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