Journal of Developmental Biology and Tissue Engineering Vol. 3(8), pp. 92-98, August 2011
Available online http://www.academicjournals.org/jdbte
ISSN 2141-2251 ©2011 Academic Journals

Full Length Research Paper

Micropropagation of *Maerua oblongifolia*: A rare ornamental from semi arid regions of Rajasthan, India

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Accepted 15 July, 2011

A method for *in vitro* regeneration of *Maerua oblongifolia* (Capparaceae) from nodal shoot explants is outlined. Percent shoot response with multiplication rate (21.1± 2.33) shoots per explant (30 mm length) was achieved when cultured on semisolid Murashige and Skoog (MS) medium containing 3% sucrose and supplemented with 2.0 mgl^{-1} of (Benzylaminopurine) BAP + additives (25.0 mgl^{-1} adenine sulphate + 25.0 mgl^{-1} citric acid + 50.0 mgl^{-1} ascorbic acid). Further amplification of shoots was achieved when concentration of BAP was lowered (0.25 mgl $^{-1}$) and Kinetin (0.25 mgl $^{-1}$) along with 0.1 mgl $^{-1}$ IAA was incorporated in the MS medium. A maximum of 58.1 ± 3.88 shoots of length 4-5 cm were obtained. The *in vitro* regenerated shoots rooted *in vitro* on half-strength MS medium containing 3.0 mgl^{-1} of IBA. About 85% of shoot rooted (4.04 ± 0.96 roots per shoot) on this medium. Other auxins such as NOA also promoted rooting but, the response in terms of percentage of rooting (75%) and shoot number (2.9 ± 1.59 roots per shoot) was low as compared to IBA. *In vitro* regenerated shoots of length 4 to 5 cm having 1-2 nodes were excise individually and pulse treated with 200.0 mgl^{-1} of IBA for 3.0 min for *ex vitro* rooting. After an initial acclimatization period of 2-3 months in a green house, about 80% plants were successfully hardened and were then transferred to earthen pots in nursery. Protocol developed is highly reproducible and economical as commercial agar and sugar cubes has been used. Multiplication rate is very high *in vitro* reported so far for this plant species. This standard protocol of mass propagation of *M. oblongifolia* eliminates the dependence on natural stands for seed production and will also serve for conservation of this threatened species.

Key words: Acclimatization, *ex vitro* rooting, *in vitro,* micropropagation, *Maerua oblongifolia,* soilrite.

INTRODUCTION

Maerua oblongifolia (Forsk) A. Rich in Guill and Perr. (Capparaceae) is locally known as *Orapa* (Bhandari, 1990). It is rare liana of the Thar Desert, and is a large, scabrous, unarmed, woody climbing shrub with pale-brown smooth bark. This plant bears greenish-white flowers, in corymbs on short lateral or terminal shoots. Fruits are pale-brown, 8 to 12 cm long, constricted between the seeds, forming an elongated, twisted and knotted berry; each knot is one-seeded. This plant exhibits wide variation in fruit size and morphology (Rathore et al., 2005). *M. oblongifolia* is a threatened plant in the area. Plant is woody climber and climbs over *Prosopis cineraria, Maytenus emarginata, Tecomella undulata, Salvadora* spp. and resembles *Cocculus* species. Plants produce aromatic flowers during summers. The ripe fruits are sugar rich, sweet with high calorific value and are rarely seen as these are eaten by squirrels and birds. *M. oblongifolia* due to its attractive aromatic flowers can be developed as garden and ornamental plant. *M. oblongifolia* is highly drought and high temperature resistant therefore it can be a potential target for gene prospecting. Plant provides shelter and

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Abbreviations: BAP, Benzylaminopurine; **IAA,** indole-3- acetic acid; **IBA,** indole-3-butyric acid; **Kn,** kinetin; **MS,** Murashige and Skoog (1962) medium; **NOA,** naphthoxyacetic acid; **PGR,** plant growth regulator; **RH,** relative humidity; **SFP,** spectral flux photon.

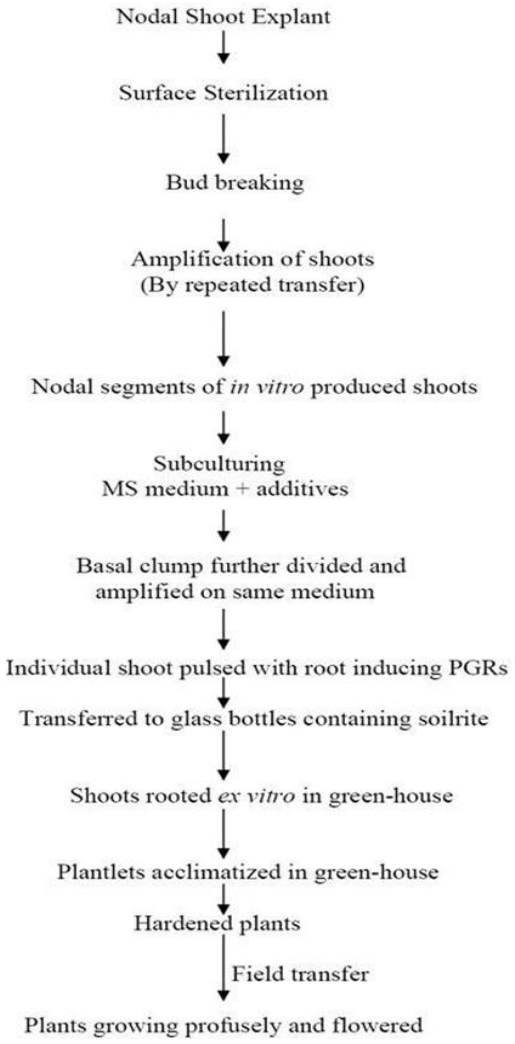

Figure 1. Schematic diagram of different steps performed for micropropagation.

food to birds and animals (Bhandari, 1990). This plant always grow in association with some specific tree species, as the population of these trees is decreasing day by day due to their over exploitation for fodder, food, timber and for medicinal purposes the plant of *M. oblongifolia* is also facing threat of extinction. Moreover, the plant propagates in nature through seeds. The viability of these seeds is less and most of the seeds are eaten up by the rodents. So, there is an urgent need for development of non conventional methods for mass propagation of *M. oblongifolia*. Plant tissue culture as means of non-conventional method of propagation is being applied for conservation and propagation of plant germplasm (Deora and Shekhawat, 1995; Rathore et al., 1993; 2007). We report protocol for mass scale clonal propagation of *M. oblongifolia.* The protocol developed is highly reproducible and the multiplication rate is very high as compared to previously reported studies (Rathore et al., 2005).

MATERIAL AND METHODS

Surface sterilization and inoculation of explant

Explants of *Maerua oblongifolia* were harvested from mature plant growing at the campus of Central Arid Zone Research Institute (CAZRI), Jodhpur, India. Explants were harvested in all quarters of the year 2005. Nodal shoot segments were used for culture initiation. Nodal shoot segments with 1-2 nodes (4 to 5 cm in length) were surface sterilized with aqueous solution of 0.1% w/v $HgCl_2$ (Mercuric chloride) solution for 4 to 6 min. The surface-sterilized explants were washed thoroughly 6 to 8 times with sterile water. These were then kept in chilled aqueous solution of ascorbic acid (0.1% w/v) and citric acid (0.05% w/v) for 5.0 min to prevent leaching of phenolics.

The surface sterilized explants were inoculated vertically on MS semisolid medium (Murashige and Skoog, 1962) containing a range of concentrations (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mgl^{-1}) of BAP and Kn separately for the activation of axillary meristem. Initiation medium was supplemented with 25.0 mgl^{-1} adenine sulphate + 25.0 mgl^{-1} citric acid + 50.0 mgl^{-1} ascorbic acid as additives (Rathore et al., 2009). Cultures were incubated in culture rooms with constant temperature (28±2°C), 60% Relative Humidity and 25 to 30 µmol m^{-2} s^{-1} SFP for 12 h photoperiod day night cycle.

Multiplication of shoots

The cultures were multiplied by (i) Repeated transfer of mother explants along with regenerated shoots on fresh medium, and (ii) Subculture of *in vitro* produced shoots, were cut into nodal shoot segments with 1-2 nodes and cultured on fresh media. Different concentrations (0.10, 0.25, 0.50, 1.0 and 2.0 mgl^{-1}) of BAP and Kinetin were used separately. BAP (1.0 mgl^{-1}) in combination with varying concentrations of Kn (0.10, 0.25, 0.50, 1.0 mgl^{-1}) and 0.1 mgl^{-1} IAA was also incorporated in the MS medium (both liquid and semisolid) along with additives in order to optimize culture conditions for shoot amplification.

In vitro rooting of *in vitro* produced shoots

The cloned shoots rejuvenated *in vitro* were excised individually and transferred on to root induction media. The media evaluated for this purpose was half-strength MS semisolid medium containing NAA, NOA or IBA in concentration range of 0.5 to 4.0 mgl^{-1} along with 0.1% activated charcoal. These individual inoculated shoots were then placed under diffused light.

Ex vitro rooting and hardening

Healthy and strong *in vitro* produced shoots were harvested and washed with autoclaved water to remove adhered nutrient medium. Individual shoot was then pulse treated with various concentrations (50.0, 100, 200, 300, 400 and 500.0 mgl^{-1}) of either IBA or NOA for different time durations (1 to 10 min). These were inoculated on soilrite moistened with MS macro salts dissolved in distilled water. The bottles containing pulse treated shoots inoculated on soilrite were then capped with polycarbonate and placed near pad section of green house. After 7 to 8 weeks the plantlets were transferred to black polybags and were placed near fan section of green house for gradual acclimatization and hardening. Hardened plants were then transferred to earthen pots containing mixture of garden soil, organic manure, sand, and vermicompost in 3:1:1:1 ratio and were placed in nursery. The outline of whole process of *invitro* production of cloned plants is represented diagrammatically in Figure 1.

Figure 2A. *In vitro* buds break from nodal segment of M. oblongifolia on MS with 2.0 mgl-1 BAP + additives.

Data analysis

All experiments were set up in completely randomized block design (RBD) for single factor experiments (Compton and Mize, 1999) and repeated three times. Each treatment had minimum ten replicates. The observations on number of shoots, height of shoots and percentage of rooted shoots were scored after a regular time interval of 15 days. The data were subjected to the standard deviation of the mean and single factor ANOVA (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

In vitro culture initiation

The explants collected during the months of July-August (rainy seasons) responded the best *in vitro* as compared to explants harvested in any other months of the year. It was also recorded that mature nodal explants selected from the adult plants showed poor response as compared to nodal stem segments prepared from fresh shoots sprouts. It was recorded that (i) these mature/old stem segments carried recalcitrant contamination in cultures, and (ii) the response of explants in terms of bud breaking was very low even if these were harvested during rainy seasons. Among all these explants tested, juvenile shoot segment of length 4 to 5 cm with 2-3 nodes were found to be most suitable for culture initiation. These shoots when surface sterilized with 0.1% $HgCl_2$ for 5.0 min did not showed any kind of contamination in cultures. A 100% bud break occurred after 10 days of inoculation on MS semisolid medium. On MS medium supplemented with 2.0 mgl^{-1} BAP + additives, a maximum of 21.1±2.33 shoots of length 3.85±0.80 cm were obtained (Figure 2A and Table 1). This rate of shoot regeneration is very high as compared to previous report (Rathore et al., 2005). Fewer shoots (4.2±1.03) of length 2.81±0.82 cm were differentiated on MS medium with higher concentration of Kinetin along with additives. Thus, rejuvenation of instant meristem was achieved in *M. oblongifolia* by selection of explant type, and season for explant harvest and by treatment of cytokinins. Such treatments were recommended for micropropagation of woody trees (Aitken-Christie and Connett, 1992; Rathore et al., 1993; Shekhawat et al., 1993). Higher light conditions (25 to 30 µmol m^{-2} s^{-1}) favored early bud breaking which has not been reported by any of the previous workers for this plant.

In vitro multiplication of cultures

Shoots initiated from activation of axillary meristem were further amplified by two ways (i) repeated transfer of mother explant (Boulay, 1987; Franclet and Boulay, 1989; Deora and Shekhawat, 1995) or (ii) subculturing of *in vitro* rejuvenated shoots. Repeated transfer was carried out on MS semisolid medium supplemented with 0.25 mgl^{-1} of BAP and 0.1 mgl^{-1} IAA + additives. On this medium 17-21 shoots of length 2.0 to 3.0cm were obtained after 15-20 days (Figure 2B). This medium was supportive for shoot development up to fourth culture cycle. For subculturing of *in vitro* rejuvenated shoots liquid MS medium was also tried for further amplification of shoots but, that was not found suitable for multiplication and maintenance of cultures. Multiplication of cultures was achieved by subculturing shoots on MS semisolid medium supplemented with 1.0 mgl^{-1} of BAP + 0.25 of Kinetin + 0.1 mgl^{-1} IAA and additives proved to appropriate medium for mass multiplication of shoots. About 58.1 ± 3.38 shoots of length 4.19 ± 0.29 cm were obtained after 20-22 days of inoculation (Figure 2C and Table 2). Increase in IAA resulted in callus formation at the base of shoots. Among the cytokinins BAP was found superior to Kinetin (Bonga and Von-Aderkas, 1992) when taken along with additives and placed in 12 h photoperiod. This treatment was not reported by other workers (Rathore et al., 2005). It was also observed during the investigation that culture medium containing more than the average cytokinin, number and length of shoots was

Table 1. Effects of cytokinins on multiple shoots induction from nodal explant of *Maerua oblongifolia* on MS medium + additives.

	PGR concentration (mg/l)	Response (%)	Shoot number ± SD	Shoot length ± SD (cm)
Control		0	0.1±0.31	0.15±0.47
BAP	0.5	65	11.8±0.63	1.31±0.16
	1.0	85	13.8±0.78	2.12±0.62
	2.0	100	21.1±2.33	3.85±0.80
	3.0	100	17.7±1.56	2.49±0.58
	4.0	100	14.6±0.69	2.01±0.31
	5.0	95	13.9±0.99	1.78±0.41
Kinetin	0.5	45	0.5±0.70	0.61±0.80
	1.0	65	1.9±0.99	1.37±0.31
	2.0	85	3.2±1.13	1.98±0.48
	3.0	90	4.2±1.03	2.81±0.82
	4.0	95	2.1±0.56	1.82±0.42
	5.0	95	1.5±0.70	1.47±0.35
Computed F				
BAP	Replication		1.198265^{ns}	1.745911_{ns}
	Treatment		92.6342**	51.51383**
	CD		1.079681	0.4438736
Kinetin	Replication		1.451614_{ns}	2.02361^{ns}
	Treatment		32.2407**	26.90421**
	CD		0.7153642	0.4673683

^{ns} Non-significant; * significant ($p \leq 0.05$); ** highly significant ($p \leq 0.01$).

Figure 2B. Repeated transfer of explant on MS supplemented with 0.25 mgl-1 BAP and 0.1 mgl-1 IAA + additives.

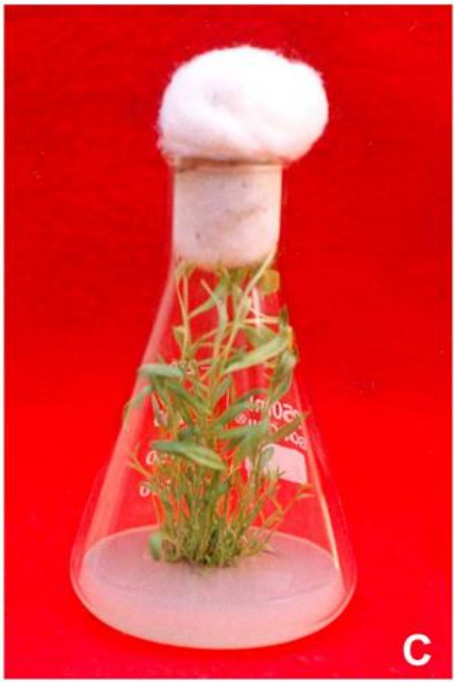

Figure 2C. Subculturing of *in vitro* produced shoots on MS supplemented with 1.0 mgl-1 of BAP + 0.25 of Kinetin + 0.1 mgl-1 IAA + additives.

Table 2. Amplification of shoots on MS medium supplemented with different concentrations of cytokinins + 0.1mgl $^{-1}$ IAA and additives.

	PGR concentration (mgl $^{-1}$)	Shoot number ± SD	Shoot length ± SD (cm)
Control	0.00	4.9±1.19	1.36±0.22
BAP	0.10	14.3±1.15	2.41±0.50
	0.25	21.1±2.76	3.61±0.66
	0.50	17.2±1.98	3.24±0.51
	1.00	13.8±1.75	2.97±0.28
	2.00	12.5±1.58	2.78±0.31
Kinetin	0.10	06.1±1.28	1.83±0.35
	0.25	06.8±1.13	2.06±0.30
	0.50	10.2±1.69	2.91±0.54
	1.00	13.2±1.54	3.23±0.35
	2.00	10.1±0.87	2.89±0.36
BAP 1.0 + Kinetin	0.10	48.2±3.70	3.57±0.29
	0.20	51.2±3.61	3.96±0.35
	0.25	58.1±3.38	4.19±0.29
	0.50	54.4±4.83	3.85±0.54
	1.00	51.5±4.19	3.64±0.17
Computed F			
BAP	Replication	0.7687483^{ns}	1.582865^{ns}
	Treatment	84.31517**	34.03079**
	CD	1.681016	0.3841529
Kinetin			
	Replication	0.8292583^{ns}	0.7227636_{ns}
	Treatment	56.34505**	37.20142**
	CD	1.193614	0.3406382
BAP 1.0 + Kinetin	Replication	0.5734175^{ns}	0.5811815^{ns}
	Treatment	96.61724**	142.802**
	CD	3.438248	0.2490872

^{ns}- Non-significant; *- significant ($p \leq 0.05$); **- highly significant ($p \leq 0.01$).

reduced (Rathore et al., 1993; Shekhawat et al., 1993).

Rooting and hardening

In vitro rooting of individual shoots was achieved with ½ strength semisolid MS medium containing 3.0 mgl^{-1} of IBA + 0.1% activated charcoal. A 80% response was recorded in terms of *in vitro* rooting. About 2-3 roots of length 2 to 3 cm were obtained after 20-25 days of inoculation. The *in vitro* root induction was low on medium supplemented with NOA (1-2 roots per shoot). Activated charcoal is said to promote *in vitro* rooting as it provides darkness and adsorbs PGRs (Thomas, 2008). Diffused light (10 -20 µmolm^{-2}s^{-1} SFP) also favored *in vitro* root induction. Delayed rooting was observed under high light intensity (30 to 50 µmol m^{-2} s^{-1} SFP). *In vitro* produced shoots also rooted under *ex vitro* conditions. Shoots of length 4 to 5 cm having 2-3 nodes were harvested and when pulse treated with 200.0 mgl^{-1} of IBA for 3.0 min, 85% of the shoots rooted after 20-25 days of this treatment. About 4.9 ± 0.87 roots of length 4.03 ± 0.49 cm were obtained. Delayed and poor rooting was recorded if shoots were pulse treated with NOA. IBA was found more suitable for *ex vitro* rooting of shoots (Rathore et al., 2010) (Table 3). Induction of rooting is affected by several intrinsic and extrinsic factors (Schiefelbein and Benfey, 1991; Shimizu-Sato et al., 2009; Wilson and Van Staden, 1990). The concentration of IBA and way of its treatment influenced the root induction (Van der Krieken et al., 1993). Higher concentrations (400 to 500 mgl^{-1}) of each PGR (IBA and NOA) produce a decrease in root number and reduce the root length. These rooted plantlets were then subjected to

Table 3. Effect of treatments of various root inducing auxins on *ex vitro* root induction from *in vitro* produced shoots of *Maerua oblongifolia*.

	PGR concentration (mgl^{-1})	Response (%)	Root number ± SD	Root length ± SD (cm)
Control		0	0.00±0.00	0.00±0.00
IBA				
	50	40	2.5±0.84	2.33±0.78
	100	55	2.9±0.73	2.93±0.43
	200	85	4.9±0.87	4.03±0.49
	300	80	4.4±0.96	3.96±0.57
	400	80	3.6±0.69	3.16±0.55
	500	75	3.2±0.63	2.74±0.71
NOA				
	50	20	1.4±0.51	0.86±0.35
	100	35	1.9±0.73	1.58±0.34
	200	55	2.5±0.84	2.47±0.33
	300	75	2.9±1.59	3.23±0.68
	400	80	1.9±0.87	2.61±0.36
	500	75	1.4±0.51	2.13±0.33
Computed F				
IBA				
	Replication		2.169284^{ns}	0.9480646^{ns}
	Treatment		53.65695**	58.07076**
	CD		0.6144642	0.5034854
NOA				
	Replication		0.5714286^{ns}	1.260765^{ns}
	Treatment		11.20408**	83.54815**
	CD		0.7888107	0.3441053

^{ns} Non-significant; * significant ($p \leq 0.05$); ** highly significant ($p \leq 0.01$).

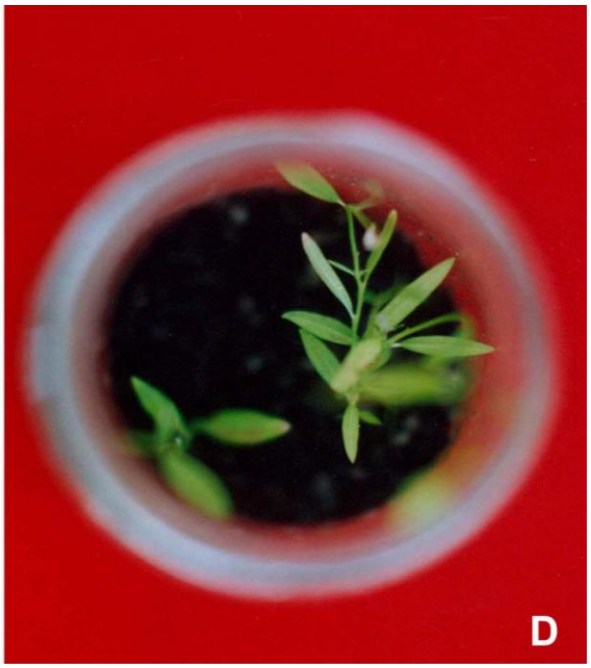

Figure 2D. Plantlets of *M. oblongifolia* under hardening phase in a green house.

different regions of temperature and humidity in a green house (Figure 2D). About 85% plantlets were successfully hardened (Figure 2E).

Significant findings

In micropropagation of *M. oblongifolia* some important attributes were recorded. The culture medium, type of cytokinins used in it, type of explants and harvesting period were found to be critical factors for *in vitro* regeneration of *M. oblongifolia*. The explants harvested during July-August months were found to be most suitable for culture establishment. Our findings hold the suggestion that in woody plants, axillary meristems from shoots of a severely pruned source plant are more amenable to physiological reinvigoration and respond better to tissue culture similar to other workers on tree species (Rathore et al., 2007). Analyses of data reveal that concentration of BAP in the culture initiation medium significantly increased the shoot numbers. On still higher (< 5.0 mgl^{-1}) concentration of this synthetic cytokinin, the difference in shoots length was found to be non-significant. Thus for both subculture and repeated transfer cytokinins

Figure 2E. Hardened and acclimatized plants in polybags.

requirement was significantly low. It was observed that, once the axillary meristem is activated the cytokinin requirement is reduced for proliferation (Rathore et al., 2009). Rate of shoot multiplication achieved in the present study is very high as compared to earlier reports (Rathore et al., 2005). This facilitates high volume shoot production per vessel and thus reducing the cost of production as commercial agar and sugars have been used. The shoots of *M. oblongifolia* rooted the best *in vitro* at diffused light conditions. *Ex vitro* rooting of shoots showed higher response than *in vitro* rooting in terms of root number length and survival rates. *Ex vitro* rooting has been reported for the first time in this plant. *Ex vitro* rooting is more beneficial as it reduces one step of hardening and acclimatization. The high rate of multiplication, successful *ex vitro* rooting, higher survival of cloned plantlets makes this protocol suitable for cloning and multiplication of selected germplasm of *M. oblongifolia*.

ACKNOWLEDGEMENTS

Research programmes in the Department of Botany are sponsored by the University Grants Commission (UGC), New Delhi under SAP-DSA Phase-II (1997-2001) and Phase-III (2002 to 2007). Micropropagation and Hardening facilities are established by the financial support from the Department of Biotechnology (DBT), Govt. of India. The Department of Science and Technology (DST), Govt. of India provided funds for infrastructure development in the Department of Botany under FIST programme.

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