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RESEARCH ARTICLE

INFLUENCE OF AGNO3 ON ZYGOTIC EMBRYO CULTURE AS AN EFFICIENT TOOL FOR CONSERVATION OF A VULNERABLE MEDICINALLY IMPORTANT FOREST TREE *OROXYLUM INDICUM* (L) KURZ.

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ABSTRACT

The present investigation has been carried out to study the influence of AgNO3 on in vitro zygotic embryoculture in *Oroxylum indicum* (L) Kurz (Bignoniaceae), a vulnerable, medicinal forest tree. The zygotic embryos were cultured on MS medium augmented with different concentrations (1.0-7.0 mg/L) of BAP/KIN/TDZ individually and also in combination with 0.1 mg/L AgNO3. More number of multiple shoots (20±0.82) /explant was observed at 5.0 mg/LBAP. Whereas 0.1 mg/L AgNO3 +3.0 mg/L TDZ had induced the maximum number of shoots (32±0.02)/explant in comparison to all other PGRs used. When the explants with multiple shoots subcultured on the same concentration and combination of PGRs showed the enhancement in multiple shoots proliferation. The micro-shoots developed in vitro were excised, shifted on to MS +1.0 mg/L GA3 for elongation. These elongated micro-shoots were cultured on ½ strength MS + 20 gm/L sucrose+0.5- 3.0 mg/L IAA/IBA/NAA for in vitro rooting. *Ex vitro* rooting was also observed in micro-shoots developed through zygotic embryoculture. After *in vitro* and *ex vitro* rooting, plantlets were successfully acclimatized followed by transfer into research field and were found to be morphologically similar to donor plants. Thus, the protocol developed during the present investigation can be utilized for the mass scale multiplication and conservation of this valuable vulnerable medicinal plant.

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INTRODUCTION

The species *Oroxylum indicum* (L) Kurz (Bignoniaceae) is an indigenous tree commonly known as *Sonapattha* or *Indian trumpet flower tree* which has importance in Traditional medicine. Several medicinal properties have been attributed to the plant not only in *Ayurveda* but also in *Unani* system of medicines (Anonymous, 1972). The tree is known to have wide range of medicinal properties due to possession of bioactive components present in all the parts of the plant (Samatha *et al.,* 2012 and 2013). The plant shows anti-allergic, anti-anorexic, anti-leucodermatic, anti-bronchitic (Hong *et al.,* 2002), anti-ulcer (Maitreyi *et al.,* 2008), anti-cancerous (Roy *et al.,* 2007), anti-inflammatory (Upaganlawar *et al.,* 2009), anti-oxidant (Uttara, 2009; Samatha *et al.,* 2014), anti-rheumatic (Mamatha *et al.,* 2013), analgesic (Zaveri and Sunitha 2010), anti-diabetic (Ashpak *et al.,* 2011), anti bacterial (Samatha *et al.,* 2013), immunomodulatory (Gohil *et al.,* 2008), and hepatoprotective (Bharali *et al.,* 2014) properties. The plant extracts of various parts are also known to be used in the treatment of leprosy and snake bite (Nadkarni, 1982), diarrhoea and dysentery (Mohammed *et al.,* 2010). In addition to its medicinal values stem bark can be used in preparation of a *Khakhi dye*, *Agarbathi* (Jain *et al.,* 2003) and also found to have enhancing effect on silk production of *Bombyx mori* (Samatha *et al.,* 2013) . It is the main ingredient of ayurvedic preparations viz., *Chyavanaprasha, Brahma Rasayana, Dhanawantra Ghrita, Dantydyarista, Awalwha, Misraka Sneha, Dashamula Rasayana, Syonaka Tailam, Pashanabhedadhi gritham, Dasamoola hareethaki, Sidharthakadhi agadam, Mahat-panchamoola thailam, Sathahwadhi dhooma, Pushyanuga chooram, Agastya hareethaki, Masha tailam, Dasamoolarishta, Dasamoola kwatham, Veeratharwadhi kashayam, Syonaka putapakam, Amritarishta, Dantyadyarishta, Dhanwantara Ghrita* and *Narayana Taila*

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(Anonymous, 1998). In view of its medicinal and economic importance it is being over exploited and becoming an endangered species. Due to its demand in pharmaceutical industry it has been pushed into the list of Indian red data book (Ravi Kumar and Ved, 2000). Only 30% of the seeds were found to be viable and frequent failure of germination is due to fungal infection of seeds (Pande *et al.,* 2011; Samatha, 2013). So, there is a need to conserve the species. Hence, we report on the *in vitro* conservation of *O. indicum* through zygotic embryo culture by using PGRs and also in combination with $AgNO_3$.

MATERIALS AND METHODS

Germplasm collection

The pods of *O. indicum* were collected from Mallur Medicinal Forest reserve of Warangal district, Telangana State, India. The plants were grown and maintained in the research field, Department of Biotechnology, Kakatiya University, Warangal, TS, India. The fruits were washed thoroughly under running tap water to remove the surface contaminants, after proper drying, the healthy fruits were split open and collected the seeds (Fig.1 a-b).

Culture Media and Culture Conditions

The seeds were washed thoroughly under running tap water, distilled water and followed by surface sterilization with 0.1% (w/v) Mercuric Chloride solution for 10 min, rinsed 4 to 5 times with sterile distilled water and soaked for 24 hrs. The seed coat was removed mechanically and the zygotic embryo was separated from cotyledons under aseptic conditions. These excised embryos were inoculated on MS (Murashige a& Skoog, 1962) basal medium solidified with 0.8% (w/v) agar-agar and augmented with different concentrations (1.0-7.0 mg/L) of BAP/ KIN/ TDZ individually and also in combination with 0.1 mg/L $AgNO_3$. The pH of the medium was adjusted to 5.8±0.2 by using either 0.1 N HCl or 0.1 N NaOH prior to autoclaving. The medium was sterilized under 15lbs pressure at 121°C for 15 min. All the cultures were incubated at 25±2ºC under 16 hrs photoperiod with photon flux density of 50-60 µ mol m^{-2} S^{-1} provided with white fluorescent lights.

Multiple shoots production

The explants with multiple shoots were subcultured on fresh MS medium supplemented with 3.0 mg/L TDZ in combination with 0.1 mg/L $AgNO_3$ for every 4 weeks for further proliferation.

Shoot elongation

The micro-shoots were excised and cultured on MS medium augmented with 1.0 mg/L GA_3 for elongation. The fully elongated micro-shoots were used for *in vitro* rooting.

In vitro rooting and plantlet establishment

The healthy elongated micro-shoots (4.0 cm) were transferred on to half- strength MS medium supplemented with 0.5-3.0 mg/L IAA/IBA/NAA for *in vitro* rooting. After *in vitro* rooting the plantlets were taken out from the culture vessels and washed with sterile distilled water to remove the traces of agar and transferred to plastic cups containing sterile soil rite. These cups were covered with polythene bags to maintain the relative humidity (85-90%) and kept in culture room. After 4 weeks of acclimatization these plantlets were shifted to earthenware pots containing garden soil and maintained under shady conditions.

***Ex vitro* rooting**: 30% of the *in vitro* developed micro-shoots were directly transferred to earthenware pots containing garden soil and kept in the research field under shady place for *ex vitro* rooting.

Data analysis

The time taken for the germination of zygotic embryo, number of multiple shoots per explant, number of roots/microshoot and average root lengths was recorded periodically. Maximum of 30 replicates were maintained for each experiment and each experiment was repeated at least thrice. The data were analysed statistically following the method of Pillai and Sinha, 1968.

RESULTS

The zygotic embryos (Fig. 1c) of *O. indicum* were cultured on MS medium fortified with different concentrations of BAP/KIN/TDZ individually (Table. 1) and also in combination with 0.1 mg/L $AgNO_3$ (Table. 2). Emergence of healthy embryonal axis with shoot and root within 2-18 days was observed on all the concentrations of BAP/KIN/TDZ alone used (Fig. 1d). Whereas zygotic embryos were germinated on MSO without PGRs after 15-18 days of incubation. But an early germination was noted at 3.0 mg/L TDZ in combination with 0.1 $AgNO_3$ and also at 5.0 mg/L BAP/KIN (5 & 2 days). While in zygotic embryos cultured on MS medium supplemented with 3.0, 4.0 and 5.0 mg/L TDZ in combination with $AgNO_3$, the emergence of healthy embryonal axis with shoot and root within 2, 3 & 5 days with 100%, 91% and 79% of germination was observed on MS medium respectively. Whereas MS supplemented with 5.0 mg/L BAP/KIN in combination with $AgNO_3$ resulted in 92% & 89% of germination and the time taken for the germination was found to be 6-8 days respectively. Of all the PGRs used 4.0-5.0 mg/L BAP were found to be effective for shoot initiation as the time required for appearance of embryonal axis was ranging between 2-3 days.

The percentage of germination was enhanced gradually from lower concentration upto 5.0 mg/L BAP/KIN/TDZ individually and also in combination with 0.1 mg/L $AgNO_3$ but gradually decreasing effect was found at higher concentrations. Absolute percentage of germination was found at 5.0 mg/L BAP/KIN (Samatha *et al.,* 2013) alone whereas 3.0 mg/L TDZ and 4.0-5.0 mg/L BAP/KIN in combination with 0.1 mg/L $AgNO_3$ had shown 90-92% of germination. The zygotic embryos cultured on MS medium fortified with 1.0-7.0 mg/L BAP/KIN/TDZ induced multiple shoots within two weeks of incubation. Maximum number (20±0.82) of multiple shoots/explant was produced at 5.0 mg/L BAP alone. Whereas the Zygotic embryos cultured on MS medium supplemented with BAP/KIN/ TDZ in combination with 0.1mg/L $AgNO_3$ showed enhancement in the number of shoots/explant (32±0.02; 28±0.62) at 3.0 - 4.0 mg/L TDZ respectively.

Table 1. Effect of BAP, KIN and TDZ on zygotic embryo culture in *O. indicum*[*]

Concentration of PGR (mg/L)	% of germination	Time taken for germination (days)	Average no. of shoots/explants (±SE)[a]
MSO	38	18	2±0.47
1.0 BAP	40	7	4±0.67
KIN	(30)	(10)	(3±0.45)
TDZ	[56]	[8]	[3±0.47]
2.0	64	9	7±0.27
	(72)	(8)	(7±0.27)
	[39]	[10]	[4±0.58]
3.0	82	5	9±0.75
	(80)	(6)	(8±0.72)
	[45]	[11]	[6±1.02]
4.0	90	3	16±1.02
	(84)	(4)	(12±0.42)
	[51]	[14]	[7±0.52]
5.0	100	2	20±0.82
	(100)	(2)	(10±1.07)
	[65]	[18]	[8±0.42]
6.0	80	9	6±0.45
	(74)	(9)	(9±0.75)
	[43]	[13]	[6±0.25]
7.0	76	10	3±0.54
	(65)	(11)	(4±0.67)
	[24]	[9]	[4±0.34]

[a] Mean ± Standard Error BAP; ( ) = KIN; [ ] = TDZ. *Samatha *et al*. (2013)

Table 2. Effect of $AgNO_3$+ BAP/KIN/ TDZ on zygotic embryo culture in *O. indicum*

Concentration of PGR (mg/L)	% of germination	Time taken for germination (days)	Average no. of shoots/explants (±SE)[a]
1.0 BAP	48	6	5±0.23
KIN	(40)	(9)	(3±0.36)
TDZ	[56]	[5]	[6±0.82]
2.0	52	8	7±0.62
	(66)	(6)	(5±0.28)
	[73]	[7]	[9±0.85]
3.0	72	7	11±0.57
	(78)	(5)	(9±0.24)
	[100]	[2]	[32±0.02]
4.0	90	5	18±0.32
	(86)	(6)	(14±0.34)
	[91]	[3]	[28±0.62]
5.0	92	6	24±0.29
	(89)	(8)	(18±0.07)
	[79]	[5]	[21±0.52]
6.0	80	9	6±0.52
	(74)	(11)	(10±0.36)
	[61]	[13]	[10±0.54]
	76	11	5±0.24
7.0	(65)	(13)	(7±0.47)
	[53]	[15]	[8±0.56]

[a] Mean ± Standard Error BAP; ( ) = KIN; [ ] = TDZ.

Table 3. *In vitro* rooting of shoots developed through zygotic embryo culture in *O. indicum*

Concentration of PGRs (mg/L)	% of response	Mean No. of roots/shoot	Average length of roots (cms) (±SE)[a]
IAA			
0.5	53	7.0±0.18	1.9±0.10
1.0	76	10.5 ± 0.87	2.3±0.07
1.5	62	11.0±0.15	2.1±0.09
2.0	40	8.0±0.85	1.7±0.11
2.5	35	7.2±0.60	2.8±0.13
3.0	23	4.6±0.30	2.4±0.26
IBA			
0.5	68	8.0±0.50	4.0±0.62
1.0	86	13±0.03	3.6±0.48
1.5	78	8.3±0.80	3.2±0.20
2.0	57	7.9±0.12	3.0±0.21
2.5	51	7.0±0.09	3.4±0.08
3.0	28	5.4±0.92	2.9±0.13
NAA			
0.5	45	5.4±0.60	3.2±0.06
1.0	58	7.0±0.90	2.9±0.52
1.5	78	10.0±0.52	3.6±0.08
2.0	51	8.6±0.05	3.3±0.48
2.5	45	6.2±0.12	3.9±0.07
3.0	25	5.1±0.42	3.5±0.32

[a] Mean ± Standard Error

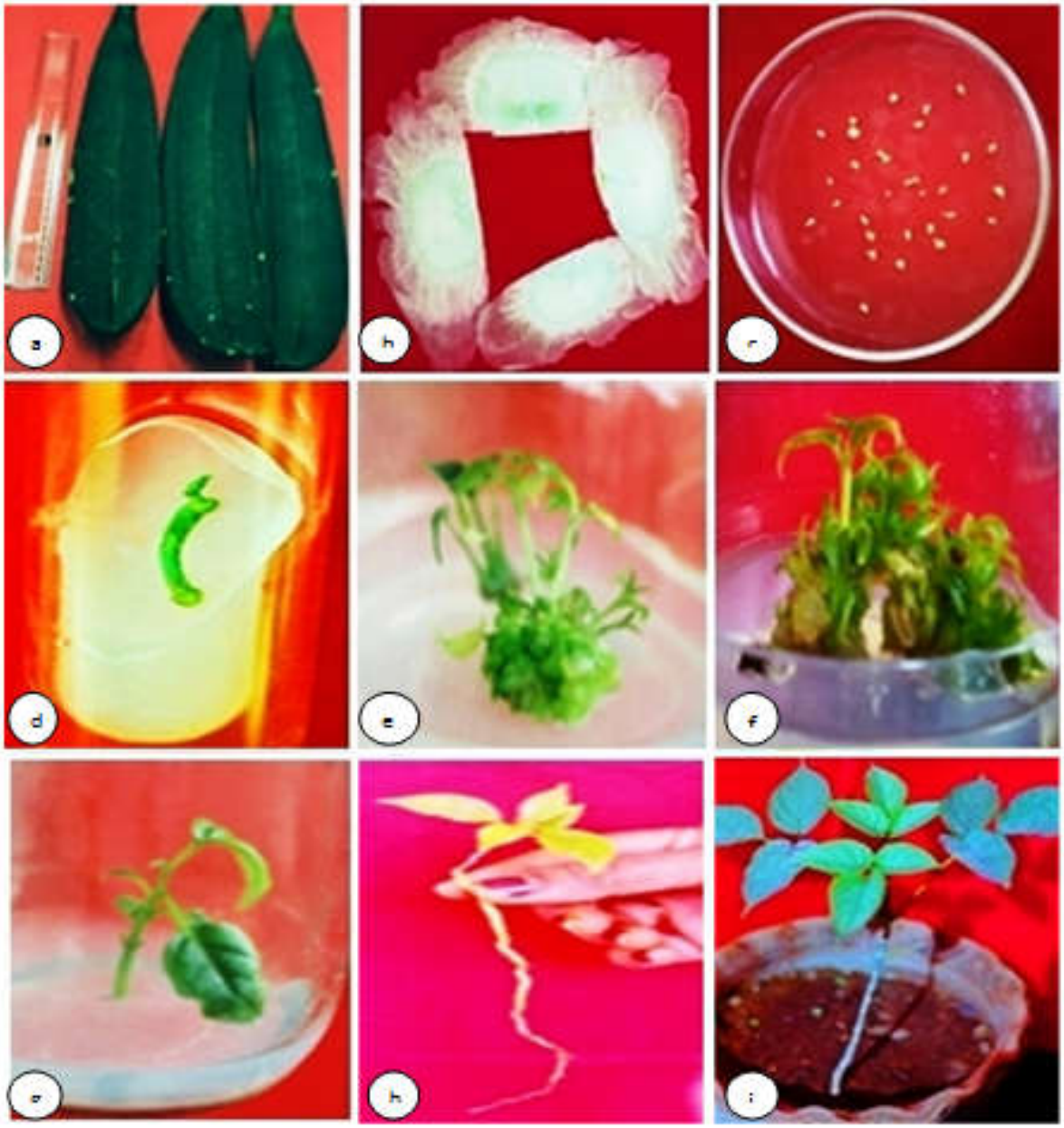

Fig. 1. ***In vitro*** **zygotic embryo culture in** ***O. indicum***

a) Fruits ; b) seeds; c) Isolated zygotic embryos; d) Multiple shoots formation on MS+3.0 mg/L TDZ after 6 weeks; e & f) further proliferation of individual micro-shoots; g) Elongation of individual shoots on MS+1.0mg/L GA_3; h) *In vitro* raised plant with well developed root system; i) Acclimatized plantlet in an earthenware pot containing garden soil

MS medium fortified with BAP alone showed superiority over Kn and TDZ for the induction of multiple shoots, whereas 3.0 mg/L TDZ along with 0.1 mg/L $AgNO_3$ was found to be the best PGR for the production of maximum number of multiple shoots per explant.

Multiple shoots induction

The explants with multiple shoots were cultured on MS medium fortified with 3.0 mg/L TDZ in combination with 0.1 mg/L $AgNO_3$ for further proliferation. Multiple shoots number per explant was enhanced after 4 weeks of incubation (Fig.1.e-f). After the passage of third subculture, individual shoot was excised and cultured on medium supplemented with 1.0 mg/L GA_3 for further elongation (Fig.1g).

In vitro **rooting and plantlet establishment**

The fully elongated micro-shoots were then transferred onto MS medium supplemented with 0.5-3.0 mg/L IAA/IBA/NAA for *in vitro* rooting. Well developed roots were observed after 15 days of inoculation. More percentage of *in vitro* rooting was recorded at 1.0 mg/L IAA/IBA and 1.5 mg/L NAA (Table. 3). Maximum frequency number of roots was found at 1.0mg/L IBA followed by 1.5 mg/L IAA and NAA compared to other concentrations of auxins tested (Fig. 1h). The plantlets were

taken out of the culture vessels, washed with sterile distilled water and transferred to sterile soil rite for acclimatization followed by transfer into research field.

Ex vitro rooting

The *in vitro* derived micro-shoots were rooted in the pots containing garden soil within 20-25 days. The achievement of *ex vitro* rooting in *O. indicum* is important as it reduces the cost of micropropagation and also within short duration the regenerated plantlets can be transferred into field. *In vitro* and *ex vitro* rooted plantlets of *O. indicum* were found to be identical to their mother plant in morphology with 79% and 85% of survival rate respectively (Fig.1i).

DISCUSSION

The zygotic embryos of *O. indicum* were cultured on MS medium fortified with different concentrations of BAP/KIN/TDZ individually and also in combination with 0.1 mg/L $AgNO_3$. The maximum percentage of germination was found at 5.0 mg/L BAP alone and 3.0 mg/L TDZ in combination with 0.1 mg/L $AgNO_3$. Maximum number (32±0.02) of shoots per explant was developed at 3.0 mg/L TDZ followed by 4.0 mg/L TDZ. MS medium fortified with BAP alone showed superiority over KIN for the induction of maximum frequency number of multiple shoots. While MS medium with lower and higher concentrations of all the cytokinins showed a decreasing effect on percentage of germination, days for germination. These results are similar to Shahnaz Begum (2007) reported in *Ophiorhiza prostrata.* The absolute percentage of zygotic embryo germination (100%) and conversion into seedling was obtained on MS medium supplemented with 3-4.0 mg/L TDZ (32±0.02; 28±0.62) followed by 92 and 89% at 5.0 mg/L BAP and KIN (24±0.29; 18±0.07) respectively in combination with 0.1 mg/L $AgNO_3$; whereas all the other concentrations of PGRs were found to be either inferior or induced abnormalities in the seedlings inducing callus formation from the zygotic embryos. We have observed the enhancement in the induction of number of multiple shoots per explant after addition of $AgNO_3$ along with BAP/KIN/TDZ in *O. indicum*. But it was interesting to note that more pronouncement of multiple shoots induction was recorded in all the concentrations of TDZ tested in combination with 0.1 mg/L $AgNO_3$. Effect of nitrate supplementation in the medium has been well established in tissue culture and found to be enhancing shoot multiplication and somatic embryogenesis. It has also been reported that $AgNO_3$ present in all the media augmented with auxin and cytokinins enhanced the adventitious shoot formation from hypocotyls and cotyledons of *Brassica* sps.

Of all the concentrations of cytokinins tested, 3.0-4.0 mg/L TDZ in combination with $AgNO_3$ and 4.0 mg/L-5.0 mg/L BAP/KIN alone were found to be effective for multiple shoot induction in *O. indicum*. Similar results were also reported in *Wrightia tomentosa* (Purohit *et al.,* 2004) and *Trichodesma indicum* respectively. Similarly it has also been reported that IBA was the best auxin with optimum rooting response in tree species like *Dalbergia sissoo* (Mishra *et al.,* 2002), *Acacia mangium* (Jose *et al.,* 2004) and *Ophiorrhiza mungo* (Jose, 2004). Induction of rooting is an important step in micropropagation and genetic transformation protocols especially in tree species. However, it has been often proved difficult, particularly in the case of woody plants (Ainsley *et al.,* 2001), The beneficial effect of reduced salts and sucrose concentrations during rooting phase was reported in *Garcinia indica* by Chabuskwar (2005) which is also evidenced in our present investigation on ½ MS + 20 mg/L sucrose. In the present investigation, achievement of *ex vitro* rooting in *O. indicum* is also an important because it reduces the cost of micropropagation and also within short duration plants were transferred to field. Similarly *ex vitro* rooting was also reported in *Rotula aquatica* (Martin, 2003), *Ophiorrhiza prostrata* and *Prunus* species (Shahnaz begum, 2007 and Pruski *et al.,* 2005). *Ex vitro* rooting is a promising method by avoiding the *in vitro* rooting, using auxins, reduction in labour and the time of establishment from laboratory to land. Low salt and sucrose concentration enhanced the percentage of *in vitro* rooting in several species of *Garcinia indica* as observed in the present investigations (Chabuskwar, 2005). Liu and Li, 2001, have also noticed a high survival rate of plantlets through *ex vitro* propagation as it is observed in our present investigation (85%) and looks promising for reducing the labor and time of plantlet establishment *Ex vitro* rooting achieved during the investigation is economically advantageous. The culture of zygotic embryos *in vitro* under different conditions defines the factors which regulate embryo germination outside the maternal tissue, and also avoids seed dormancy (Dragana 2008). Thus the protocol, developed during the present investigation can be used for rapid multiplication and conservation of this vulnerable tree species. This study establishes the rapid propagation protocol in order to meet the demand, which could curtail the impact on the natural population and prevent the plant species from becoming endangered. Thus, *In vitro* zygotic embryo culture can be utilized for mass scale multiplication and conservation of *O. indicum* a vulnerable medicinal plant.

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