

IN VITRO PROPAGATION OF VACHELLIA MACRACANTHA, AN IMPORTANT SPECIES OF THE SEASONALLY DRY TROPICAL FOREST IN NORTHERN PERU



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ABSTRACT

Vachellia macracantha (ex *Acacia macracantha*) is one of the most important tree species in the Seasonally Dry Tropical Forest (SDTR) of northern Peru. The study aims to develop an efficient callus induction and micropropagation protocol from microcuttings of adult trees of this mentioned species, distributed across the North of Peru to the South of Ecuador. Shoot and uninodal microcuttings from three to five-year-old plants were investigated respecting their vegetative propagation and rooting capacity *in vitro*. High levels of NAA (2.5 to 7.5 mg L⁻¹) were most efficient for shoot elongation and axillary bud development, although the basal callus formation and defoliation occurrence was also very high. In this case, the NAA 0.02 mg L⁻¹, BAP 0.05, and GA₃ 0.05 mg L⁻¹ interaction has been produced a better multiplication rate after 30 days without both basal callus formation and defoliation. Our findings suggest that other treatments tested such as NAA-BAP, NAA-KIN and NAA-2iP interaction, not were significantly satisfactory. Rooting response and consequently shoot elongation were always better with IBA (0.5 to 2.0 mg L⁻¹) than NAA, under the same growth regulators concentrations.

Abbreviations: BAP, 6-benzylaminopurine; NAA, α -naphthaleneacetic acid; IBA, 4-[3-indolyl] butyric acid; GA₃, gibberellic acid; KIN, kinetin; 2iP, N⁶-[2-Isopentenyl] adenine.

1. INTRODUCTION

The genus *Acacia* belongs to the family Fabaceae (Leguminosae), comprising more than 1.350 species, distributed throughout tropical and warm temperate areas around the world [1]. *Vachellia macracantha* (Humb. &

In Vitro Propagation of *Vachellia Macracantha*, An Important Species of The Seasonally Dry Tropical Forest in Northern Peru

Bonpl. ex Willd.) Seigler & Ebinger (ex *Acacia macracantha*) is a forest tree species well adapted to water stress conditions and like most species of the genus *Acacia* have the natural ability to fix atmospheric nitrogen [2]. It grows in the seasonally dry tropical forest of northern coast of Peru, especially at the Pampas de Olmos, Lambayeque and Piura (Lat. 3° and 8° South) with precipitation between 100 and 600 mm and average temperature of 20 to 25 °C, on flat or rolling terrain, sandy or rocky soil generally derived from eolian or alluvial material from mountain streams, pH 7 to 8.2. *V. macracantha*, is popularly named by locals as “faique”, “espino” or “huarango” [3], and in various pioneering reports it have been considered of greatest importance to reforest dry wastelands, erosion control and stabilization of coastal sand dunes, due to their salt tolerance and nitrogen fixation capacity [4]. Its fruits and leaves are a source of animal fodder and the stems are utilized as fuelwood, timber, among other benefits for humans and animals.

The existence of very heterogeneous stands as a consequence of the use of seeds in artificial plantations seems to be one of the most serious limitations in terms of fruit and woody productivity. In addition, the multiplication of germplasm, selections and massive propagation systems are therefore necessary to attain high numbers of desirable clones [5]. This problem is compounded due to self-incompatibility/outcrossing pollination inherent within several genus of leguminous plants, resulting in numerous interspecific hybrids found in natural populations[6].

Micropropagation, which is often used successfully for the multiplication of herbaceous and woody plants, represents an interesting alternative for this species, while among woody plants, regeneration from adults' subjects is often not successful directly using this method [7]. However, in the last 30 years, the vegetative multiplication of woody species, has been rarely obtained from mature trees of several species of *Vachellia (Acacia)* such as in *A. albida* [8] and in gum arabic tree (*A. senegal*), which findings show that nodal segments of plants produced during four years in a greenhouse induced the highest multiplication rate in MS culture medium with 5.0×10^{-5} M zeatin or BAP[9].

Most studies regarding micropropagation of *Acacia* species have been started from nodal segments of seedlings of various ages. In *A. albida*, cotyledonary nodes obtained from seedlings induced multiple shoots in MS culture medium supplemented with 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} BAP [10]. In *A. auriculiformis*, a native species of Papua New Guinea and northern Australia, the *in vitro* micropropagation was observed in nodal segments from 5-months-old seedlings in MS culture medium supplemented with $0.44 \mu\text{M}$ (0.5 mg L^{-1}) BA, yielding 10 shoots/explant, and the investigations also recorded that MS medium produced a higher number of shoots compared to both WPM [11] and B5[12] culture medium [13]. Despite that these authors carried out the most recent work, there are other botanical case reports on the successful micropropagation of *A. auriculiformis* [14],[16]. In black wattle (*A. mearnsii*), native plant from Australian, *in vitro* propagation was observed in nodal segments from 9-months-old seedlings in half-strength MS mineral salts supplemented with 0.05 or 0.1 mg L^{-1} BA and/or 0.05 mg L^{-1} IBA [17]. In *A. nilotica* subsp. *indica*, cotyledonary nodes differentiated multiple shoots in B5 medium [12] supplemented with the cytokinins BA, 2iP, KIN or ZEA, [18]. In *A. nilotica* subsp. *hemispherica*, an endangered and endemic species from Southern Pakistan, a micropropagation system was developed from seedlings in MS or B5 culture medium with various levels of BAP (0.5 to 4.0 mg L^{-1}) along with 0.5 mg L^{-1} NAA, and MS medium produced the highest number of shoots[19]. In *A. tortilis* subsp. *raddiana* and *A. nilotica*, the micropropagation was observed only in nodal segments from 4-weeks-old seedlings of *A. nilotica* in MS culture medium with 2.5 mg L^{-1} BA, and rooting with 4.0 mg L^{-1} IBA [20]. Correspondingly, in *A. chundra*, the bud sprout was obtained from shoot tip and nodal segment derived from seedlings on MS culture medium with 1.0 mg L^{-1} BA and 20 mg L^{-1} adenine sulfate [21].

On the other hand, studies and informations on *Acacia* micropropagation, using explants obtained from juvenile plants under greenhouse conditions or from field plants, are scarce. In *A. mangium*, one of the most important forest species due to the quality of its wood, nodal segments of three-month-old plants were used under greenhouse conditions, observing that explants cultured in MS culture medium supplemented with $2.22 \mu\text{M}$ BAP and $2.69 \mu\text{M}$ NAA induced the highest number of shoots and roots, respectively [22]. In another study conducted on *A. mangium* and *A. mangium* x *A. auriculiformis*, an interspecific hybrid of natural origin, micropropagation was started either from seeds or from explants collected from outdoors on MS culture medium with $4.4 \mu\text{M}$ BA [23]. In the last decade, some reviews articles have been published on *in vitro* work done on the *Acacia* species, such as the one carried out by Beck and Dunlop [24], highlighting the excellent review carried out by Gantait et al.[25].

In other context, there are studies on phytochemical aspects and biological activity of numerous *Acacia* species. In *A. nilotica*, methanolic leaf and bark extracts showed some biological activity against *Escherichia coli* and *Candida glabrata*, respectively [26]. In *A. arabica (A. nilotica)*, phytochemical analysis showed the occurrence of several secondary compounds such as tannins, terpenoids, anthraquinones, alkaloids, and others, and acetone bark extract

was found to be most potent against select MDR pathogenic bacteria as *Pseudomonas aeruginosa*, *E. coli*, *Streptococcus pyogenes*, and others [27]. However, in a more complete study conducted on *A. nilotica*, the raw extract of leaves using two methods (cold and hot) and three solvents (butanol, ethanol and methanol), revealed the presence of alkaloids, flavonoids, tannins, terpenoids, and others compounds, as long as the result of antibacterial activity revealed more susceptibility toward Gram-positive bacteria such as *Streptococcus pyogenes* when it is compared to Gram-negative bacteria as *Pseudomonas aeruginosa* [28]. In another species of *Acacia* such as *A. saligna* (*A. cyanophylla*), leaves ethanol extracts showed antimicrobial activity against *Klebsiella pneumoniae* (Gram-negative) and *Micrococcus* (Gram-positive), and pathogenic fungi *Aspergillus fumigatus* and *C. albicans*, as long as the major phenol compounds were gallic and p-Coumaric acids [29]. In this same species, water extract of flowers showed antifungal and antibacterial activities against *Penicillium chrysogenum* (3%) and *Pectobacterium caratovorum* subsp. *caratovorum* (MIC=100 µg/mL), respectively, and the most abundant phenolic and flavonoid compounds were benzoic acid and naringenin, respectively [30]. On the other hand, in *Acacia implexa* and *A. falcata*, native species of New South Wales, Australia, 70% aqueous ethanol extracts showed antibacterial activity against sensitive and MDR strains of *Staphylococcus aureus*, and *A. implexa* possessed high TPC (Total Phenolic Contents) and strong DPPH radical scavenging abilities [31].

Considering this mentioned scenario, in this paper we report the establishment of culture conditions for plant propagation of microcuttings from adult trees of *Vachellia macracantha* (ex *Acacia macracantha*), an important woody species of the seasonally dry tropical forest of northern Peru.

2. MATERIALS AND METHODS

2.1. PLANTING STOCK

Three to five-old plants, on natural field conditions, were selected from located near Motupe–Olmos (Lambayeque, Peru) (Figure 1a, b and c). The shoot tip and nodal segment explants 50 mm were taken from the non-lignified part of young branches. All the leaves were removed, then the explants were dipped for 10 min and agitated in detergent solution and then in 0.2% Orthocide for 30 min. Subsequently the explants were surface-sterilized in 70% ethanol for 1 min and 5% commercial sodium hypochlorite solution (Clorox®) for 5 min before being rinsed three times in sterillized distilled water. The shoot and node segments were then cut in uninodal microcuttings of 10 to 15 mm in lenght and aseptically introduced into test tubes (18x150 mm) containing the culture medium. The same method was used on the micropropagation process.



Figure 1: Plant propagation and germplasm conservation of *Vachellia macracantha* (faique). a. Adult plants used as a living fence, b. Branches with flowers, c. Branches with fruits

2.2. CULTURE MEDIUM AND GROWING CONDITIONS

For shoots elongation was used a medium consisting of the MS salts [32] and vitamins 1.0 mg L⁻¹ thiamine.HCl and 100 mg L⁻¹ myo-inositol. This basal medium was supplemented with NAA (0.0, 2.5, 5.0 and 7.5 mg L⁻¹) in high concentration. In indeed, we have tested NAA (0.5 mg L⁻¹) – BAP or KIN or 2iP (0.05, 0.5, 2.5, 5.0 and 10.0 mg L⁻¹) interactions, respectively.

For shoots elongation the uninodal segments were cultured en MS medium supplemented with 0.02 mg L⁻¹ NAA – 0.05, 0.5 or 2.5 mg L⁻¹ BAP – 0.05 mg L⁻¹ GA₃, interaction. Rooting was tested in culture medium supplemented with NAA (0.05 to 2.0 mg L⁻¹) or IBA (0.05 to 2.0 mg L⁻¹).

Both culture medium, shoots elongation and rooting plants, containing 30 g L⁻¹ sucrose was solidified with 7 g L⁻¹ agar-agar and sterilized at 120 °C for 15 min. The pH was adjusted to 5.8±0.1 before autoclaving process. Explants were cultivated in pyrex tubes (18x150 mm) each containing 15 mL of culture medium. Cultures were maintained at 26±2 °C, under light (16 h day photoperiod; 35 µE.m⁻². s⁻¹) provided by cool fluorescent lamps (Grolux Sylvania).

After rooting, the micro-plants were carefully extracted from the growing tubes and were directly transplanted in pots on a substratum consisting of vermiculite mixed equally with river sand.

2.3. STATISTICAL ANALYSIS

Each experiment was repeated two times with 25 explants per replicate. All data were subjected to one-way analysis of variance test (ANOVA) and performed with version 3.4.0 R software. Tuckey's test was used to compare the mean values at the 5% level.

3. RESULTS

3.1. SHOOT ELONGATION

Growth responses of *V. macracantha* are summarized in tables 1, 2 and 3. Table 1 illustrates the effect of different concentrations of NAA on the shoot elongation and development of nodal segment explants. After 45 days of growth, nodal explants showed profuse callus formation on the basal region in contact with nutrient solution. Callus was observed in all treatments and the tissue turned brown after a few days.

Table 1: Effect of NAA on shoot development from three to five-year-old *Vachellia macracantha* plants, after 45 days of culture.

NAA (mg L ⁻¹)	Survival rate (%)	Shoot size (mm)	Nodes formed (No)	Leaves formed (No)	Lateral branched (No)	Defoliation (%)	Rotting (%)
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.5	100.0	35.0±16.9 ^a	12.3	8.0	0.7	100.0	25.0
5.0	100.0	29.0±7.1 ^c	8.6	5.2	1.8	100.0	17.0
7.5	100.0	31.0±10.8 ^b	7.6	8.0	1.6	100.0	0.0

Data were the mean ± SE.

Means within each column followed by similar letters are not significantly different at 0.05 probability level.

Table 2: Effect of several cytokinins on shoot development from three to five-year-old *Vachellia macracantha* plants, after 45 days of culture.

Plant growth regulators (mg L ⁻¹)				Response		
NAA	BAP	KIN	2iP	Callus induction (%)	Shoot size (mm)	Survival rate (%)
0.5	0.05			100 (+)	10.0 - 15.0	20.0
	0.5			100 (+)	10.0 - 15.0	20.0
	2.5			100 (++)	5.0 - 10.0	10.0

	5.0			100 (++)	5.0 - 10.0	0.0
	10.0			100 (+++)	< 5.0	0.0
0.5		0.05		100 (+)	10.0 - 15.0	15.0
		0.5		100 (+)	10.0 - 15.0	15.0
		2.5		100 (++)	5.0 - 10.0	10.0
		5.0		100 (++)	5.0 - 10.0	10.0
		10.0		100 (+++)	< 5.0	0.0
0.5			0.05	100 (+)	10.0 - 15.0	20.0
			0.5	100 (+)	10.0 - 15.0	15.0
			2.5	100 (++)	5.0 - 10.0	10.0
			5.0	100 (++)	5.0 - 10.0	10.0
			10.0	100 (+++)	< 5.0	0.0

Callus induction: +, callus < 5 mm Ø; ++, callus between 6-10 mm Ø; +++, callus > 10 mm Ø

Table 3: Effect of NAA-BAP-GA₃ on shoot developmen from three to five-year-old *Vachellia macracantha* plants, after 45 days of culture.

Plant growth regulators (mg L ⁻¹)			Response					
NAA	BAP	GA ₃	Survival rate (%)	Shoot size (mm)	Nodes formed (No)	Leaves formed (No)	Defoliation (%)	Rooting (%)
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.02	0.05	0.05	100.0	31.5±13.5 ^a	5.8	11.6	0.0	20.0
0.02	0.5	0.05	100.0	25.3±6.3 ^b	3.2	5.3	0.0	0.0
0.02	2.5	0.05	100.0	23.2±4.1 ^c	2.8	4.7	0.0	0.0

Data were the mean ± SE.

Means within each column followed by similar letters are not significantly different at 0.05 probability level

The treatment 2.5 mg L⁻¹ NAA induced better shoot development than all of the other treatments and resulted in the formation of a shoot which average 35 mm in length. The shoot showed twelve nodes and produce three to four explants suitable to continue micropropagation. In this same treatment defoliation was often observed in all plantlets but not all the leaves fall. The root formation was only 25%. In the other treatments conducted, 5.0 and 7.5 mg L⁻¹ NAA, at the base of the nodal segments, callus developed which had a greater volumen, specially when using a concentration of 7.5 mg L⁻¹ NAA. However, the proliferation of basal callus did not seem to affect the development of the axillary bud. In these treatments, most of the explants developed a shoot approximately 30 mm in length. The lateral branched was increased from 0.7 to 1.8, and the defoliation was also 100% but with a dramatic leaves fall. Rooted plants was observed only 5.0 mg L⁻¹ NAA treatment with a rate estimated in 17%.

The NAA-BAP, NAA-KIN and NAA-2iP treatments, also were tested on the shoot initiation an elongation (Table 2), but the results not were satisfactory. In all cases studied, after 45 days of the culture, shoots reached a mean length of < 5 to 15 mm as compared to 35.0 mm (NAA 2.5 mg L⁻¹) or 31.5 mm (NAA-0.05 mg L⁻¹ BAP-GA₃ interaction). The percentage of explants with shoot greater than 10 mm in length was only 20%, in some treatments, reaching the same survival rate.

In the Table 3 the effect of the NAA-BAP-GA₃ treatments were demostrated. In this case, the initiation and elongation of the shoot was less than that obtained with NAA treatments, but the plants constitution was more vigorous. A single shoot was formed the axillary bud and it grew to produce three to six nodes suitable to continue micropropagation. With respect to other parameters, basal callus formation and defoliation, not were observed (Figure 1d). Rooted plants were observed only in the treatment that incorporated 0.05 mg L⁻¹ BAP with a rate estimated in 20%.

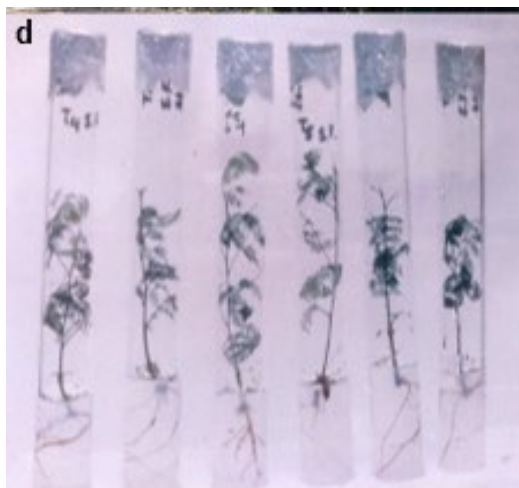


Figure 1: d. In vitro propagated plants

3.2. ROOT INDUCTION AND ELONGATION

Root induction was observed under several concentrations of either NAA or IBA auxins. The Table 4 shows that for the NAA treatments, the root induction was much less than that obtained with IBA. We have registered that in 0.5, 1.0 and 2.0 mg L⁻¹ IBA treatments, after 30 days in culture, the root formed were approximately 30 to 50 mm in length, more or less ramificated and very vigorous (Figure 1e). In this culture medium formulation, although the defoliation rate was high, the germplasm conservation was greater than 9 months. In this size (30 to 50 mm in length) rooted plantlets were transferred to a sterilized substrate consisting of vermiculite and river sand, placed in a growth chamber for one week, and later on transferred to the greenhouse. Under these conditions survival rates were estimated over 80% since *V. macracantha* roots, average 3 to 4 per plant, showed normally rapid growth (Figure 1f).

Table 4: Effect of two auxins, NAA and IBA, on the root formation in *Vachellia macracantha* micropropagated plants, after 45 days of culture.

Plant growth regulators (mg L ⁻¹)		Response		
NAA	IBA	Rooting (%)	Root number	Root size (mm)
0.0		0.0	-	-
0.05		0.0	-	-
0.1		0.0	-	-
0.5		70.0	1-3	< 3.0
1.0		100.0	1-3	< 3.0
2.0		80.0	1-3	< 3.0
	0.0	0.0	-	-
	0.05	0.0	-	-
	0.1	0.0	-	-
	0.5	100.0	2-4	> 5.0
	1.0	100.0	2-4	> 5.0
	2.0	100.0	2-4	> 5.0

Root number: Main roots with numerous lateral roots



Figure 1: e. In vitro plants stored for more than a year, showing strong defoliation, and f. Plants acclimatization

4. DISCUSSION

In the micropropagation of *Acacia* species, studies have been started almost entirely with seedlings of different ages, with the exception of the study carried out in *A. albida* by Gassama and Duhoux [8], similar findings showed in *A. senegal* by Badji et al. [9] and in the present study carried out in *V. macracantha*. Likewise, only in the study carried out in *A. albida* by Duhoux and Davies [10], in the micropropagation process, 0.5 mg L⁻¹ NAA - 3.0 mg L⁻¹ BAP was used. However, in pioneering studies conducted on Fabaceae species, high levels of NAA (3.0 to 10.0 mg L⁻¹) were most efficient for rooting and propagation of plantlets in several species of *Prosopis* (*P. chilensis*, *P. alba* and *P. tamarugo*) [5],[33]. These authors considered that NAA is an only hormone required to trigger root initiation and that the addition of GA₃, although it is inhibitory to some extent, favoured axillary buds growing. This result was considered of extreme importance, since explant normally shown leaf abscission after some days of culture and axillary buds do not initiate growth immediately after root formation [33]. In our system, all concentrations of NAA tested were observed both, axillary bud development and leaf abscission. In others species of *Acacia* as *A. senegal* [9], *A. koa* [34], *A. auriculiformis* [13], *A. nilotica* [20], and others, NAA not was tested in the plant propagation process.

In the micropropagation (elongation and shoot proliferation) of numerous *Acacia* species, cytokinins as N⁶-benzyladenine (BA), 6-furfurylamino purine (KIN), N⁶-(2-isopentenyl) adenine (2iP), 4-hydroxy-3-methyl-terms-2-butenyl aminopurine (ZEA) and 1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea (TDZ), have been the main growth regulators used and/or tested in several experiments. In our study, only some interactions of NAA-BAP, NAA-KIN and NAA-2iP, in the lowest concentrations tested, showed little shoot elongation (≤ 15 mm), although with a survival rate not more than 20%. However, in most studies the BA supplement has shown better results than the other cytokinins, as reported in *A. nilotica* subsp. *indica* [18], *A. nilotica* [20], *A. mearnsii* [17], *A. chundra* [21] and *A. auriculiformis* [13]. In another investigations, it has been shown that the culture medium containing BA was much superior to the culture medium containing KIN, for shoot multiplication of *A. mangium* [35],[36], and Badji et al. [9] reported that ZEA produced better induction to multiple shoot formation in *A. senegal*. However, in *A. nilotica* the induction of multiple shoots was observed when 0.6 mg L⁻¹ NAA and it was supplemented to the culture medium, one month after that the explant was cultivated with 1.0 mg L⁻¹ KIN [37]. On the other hand, in *V. macracantha* the best results in the shoot elongation were observed in various combinations of NAA-BAP-GA₃. In this way, there are some studies where the use of a single growth regulator was not as efficient as when combinations of BA-NAA or TDA-NAA were used, as observed in the shoot's proliferation of *A. seyal* [38]. This first observation was ratified with the experiments conducted by Rout et al. [21] regarding in *A. chundra*, who have used the BA-IAA interaction, by Abbas et al. [19] associated *A. nilotica* subsp. *hemispherica*, using the BAP-NAA interaction, Shahinozzaman et al. [36] in *A. mangium*, using the BAA-NAA interaction and in Yadav et al. [39] in *A. auriculiformis*, using the KIN-IAA interaction. In general, in *V. macracantha* BAP, a sintetic cytokinin, in interaction with auxin (NAA), produced a better stimulation of the shoot growth and a vigorous plantlets formation, that using cytokinin alone, as postulated by Gantait et al. [25].

In Vitro Propagation of *Vachellia Macracantha*, An Important Species of The Seasonally Dry Tropical Forest in Northern Peru

In our findings regarding *V. macracantha*, we have showed that various concentrations of NAA and IBA are directly related with the root formation. However, in IBA treatments the percentage of rooted plants reached 100%, which is always more difficult to induce when non-juvenile structures are used as explants [25]. IAA, IBA or NAA auxins have been used in numerous studies on *in vitro* rooting of *Acacia*, in various concentrations and generally individually [13],[40][41]. Likewise, as observed in our study in *V. macracantha*, IBA has been more successful than IAA or NAA [35],[42].

5. CONCLUSIONS

Vachellia macracantha (“faique”), *Prosopis limensis* (“algarrobo”) and *Colicodendron escabridum* (“zapote”), are the most representative forest species of the Seasonally Dry Tropical Forest of northern Peru, as well as those of greater economic importance. That is why this species have been subjected to strong anthropic pressure that the best genotypes have now been exterminated. Given this, the development of biotechnological methods, such as *in vitro* tissue culture, has allowed its propagation from young specimens, achieving elongation of the shoot in culture medium supplemented with NAA or NAA-BAP-GA₃ and plantlets rooting in culture medium supplemented with IBA. These results will enable the spread of *V. macracantha* on a large scale, contributing to the reforestation of the species and the mitigation of climate change in northern Peru.

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CONFLICT OF INTEREST

None.

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In Vitro Propagation of *Vachellia Macracantha*, An Important Species of The Seasonally Dry Tropical Forest in Northern Peru

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