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RESEARCH ARTICLE

OPTIMIZATION OF *IN VITRO* ADVENTITIOUS SHOOT INDUCTION IN *JATROPHA CURCAS* BY RESPONSE SURFACE METHODOLOGY.

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Abstract

Jatropha curcas is a second generation biofuel crop that is also an important biological resource. Meeting increasing biofuel demand requires wider cultivation of specialized varieties, highlighting the need for massive culture techniques such as *in vitro* micropropagation. Key stages in plant tissue culture processes, such as adventitious shoot induction, can be optimized to increase micropropagation protocol efficiency. Response surface methodology, a statistical tool for this purpose, was applied to optimize *in vitro* induction of adventitious shoots in the *J. curcas* micropropagation process. A preliminary assay was done in which 6-(γ , γ -dimethylallylamino) purine and indolacetic acid were selected as the factors to optimize. A 2^2 central composite design with four central points was then applied to fit it to a second grade model and a rotatable design applied to produce the final model and surface graphics. Focused on maximum point, the surface model design identified maximum adventitious shoot induction to be 21 shoots per explant in Murashige and Skoog medium at 75% ionic strength and containing 83.3 μ M 6-(γ , γ -dimethylallylamino) purine and 1.3 μ M indolacetic acid. This is the first report in which response surface methodology was found to be an effective statistical technique for optimizing adventitious shoot induction in *Jatropha curcas*.

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Introduction:-

A member of the *Euphorbiaceae* plant family, *Jatropha curcas* is native to Mexico and Central America (Navarro et al., 2016). Its seeds can contain up to 40% oil which has made it a preferred source for producing biodiesel by transesterification (Li et al., 2016; Soares et al., 2016). Increasing demand for biodiesel has driven efforts to expand its cultivation. Asexual propagation through stakes and sexual propagation through seeds have been used to broaden cultivation, although production remains challenging due to the species low drought resistance, disease susceptibility and weak radicular system (Sujatha et al., 2005; Datta et al., 2007). Growing demand for *J. curcas* genetic material has led to use of massive plant multiplication techniques (Panghal et al., 2012). *In vitro* plant tissue culture of *J. curcas* has been studied and shown to be viable for adventitious shoot induction by indirect organogenesis,

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producing up to thirteen shoots per explant (Liu et al., 2016). There are other reports of adventitious shoot morphogenesis producing seven, six and eleven shoots from petiole, stem and leaf explants, respectively (Verma and Verma, 2015). In other studies, direct organogenesis of petiole resulted in seven adventitious shoots (Liu et al., 2015), and mature leaves produced up to 2.98 shoots per explant (Zhang et al., 2013).

Plant growth regulators (PGR) are vital to developing an efficient micropropagation protocol. The PGR 6-(γ , γ -dimethylallylamino) purine (2ip) cytokinin is involved in regulation of cell proliferation, cell cycle progression and division (Werner et al., 2003); shoot growth and root growth inhibition (Miller et al., 1956); and developmental processes in plants, including organ formation, leaf senescence, shoot meristem formation and maintenance (Monk, 1994). Another PGR used in combination with cytokinin for *in vitro* development of adventitious shoots is indolacetic acid (IAA) auxin, an indispensable phytohormone that regulates many aspects of plant development. It has been implicated in embryonic and post-embryonic development, tropisms such as movement in relation to light and gravity, and it influences aspects of cell division, cell elongation and cell differentiation (Teale et al., 2006).

Auxin and cytokinin are important to producing cellular response in plant tissue culture. However, achieving the most efficient cellular response in a single or multiple-step micropropagation protocol requires an extensive preliminary experimental process to identify the most appropriate conditions considering the possible interactions that can occur between different factors. Statistical tools are crucial in this stage. These can include preliminary assays, factorial designs and surface response methodology (SRM) (Anderson and Whitcomb, 2016). Introduced by Box and Wilson (1992), SRM consists of a group of mathematical and statistical techniques based on the fit of empirical models to experimental data collected on an experimental design. Linear or square polynomial functions are applied to describe the studied system and then modeling and displacement are used to explore the experimental conditions until they are optimized (Bezerra et al., 2008). After the initial experimental stage, the experiment generally needs to be displaced to an adequate region or a more detailed exploration made of the region to increase the desired response variable (Humberto and de la Vara, 2003; Zhao et al., 2014; Zhu et al., 2014). SRM can be used to evaluate effects in many factors and their interactions in one or more response variables (Myers et al., 2004). One of its advantages is the ability to consider interactions between different variables, consequently reducing the number of experiments needed to produce a mathematical model that can help to predict the behavior of the dependent variables with respect to the independent variables (Guan and Yao, 2008; Karacabey and Mazza, 2010; Prasad et al., 2011; Wang et al., 2012). It is often applied to optimize efficiency in plant tissue culture processes and responses; for example, to optimize callus production and shoot regeneration in *Glycine max* (Abbasi et al., 2016). In other studies, RSM has been used to optimize the interaction between pH and sucrose in *Mucuna pruriens* callus culture (Sundaram et al., 2013), or to define the required mineral nutrients for micropropagation of *C. avellana* cultivars (Hand et al., 2014). Its methodology has also been shown to be very useful in optimizing PGR to improve shoot multiplication in *B. polystachyon* (Chakraborty et al., 2010) and *Lactuca sativa* (Gómez-Montes et al., 2015). A RSM system was also applied to improve efficiency for *in vitro* propagation and regeneration of *Ziziphus jujuba* Mill (Hou et al., 2018).

The present study objective was to optimize *in vitro* adventitious shoot induction in *J. curcas* by using RSM as a function of the factors 2ip and IAA for use in rapid and massive propagation, genetic transformation studies, polyploidy induction and/or *in vitro* germplasm conservation.

Materials and Methods:-

Protocol for plant production from adventitious shoot organogenesis of *J. curcas*

Explant establishment

Jatropha curcas seeds were collected from a plantation in Sierra Papacal, Yucatan, Mexico. These were superficially sterilized with 0.5% benzalkonium chloride (Degasa, México, D.F.), submerged in 1% Extran[®] (Hyclin-Plus, México, D.F.) for 15 minutes and dried on absorbent paper for 30 minutes. The seed cover was removed to extract the embryos which were disinfected in a laminar flow cabinet (ESCO, Singapur). Embryos disinfection was done by submerging them in a 5% Extran[®] solution for 5 minutes, transferring them to 70% ethanol for 1 minute, placing them in 30% sodium hypochlorite (Reactivos CIVEQ, Iztapalapa, México) for 15 minutes and finally rinsing them three times with sterile distilled water. The disinfected embryos were transferred to a magenta GA-7 Vessel (Sigma-Aldrich, Saint Louis, Missouri, United States) containing 40 mL MS medium at 100% ionic strength, and incubating them at room temperature (23±2 °C) and 60% relative humidity (RH) in darkness for fifteen days. Germinated embryos were transferred to a 16/8 h (light/dark) photoperiod with light provided by light-emitting diode (LED) lamps (Manufacturer, Trading Company, China) (60 $\mu\text{mol}^{-2}\text{s}^{-1}$ luminous intensity).

Callogenesis and adventitious shoot induction from explants

After 15 days of in vitro plant development, 1 cm² portions of cotyledonal leaves were cut to provide an explant resource. A previous assay was run to identify the factors that have the greatest influence on variable response. These were done in MS medium (Sigma-Aldrich, Saint Louis, Missouri, United State) at 75% ionic strength and containing 282.34 µM AdS, 22.5% sucrose (w/v) and 0.2% Gelrite (w/v); different concentrations of 2ip, IAA and Adenine sulfate (AdS) were tested.

Adventitious shoot elongation and rooting

Adventitious shoots cultured for 45 days (1 cm long) were transferred to MS medium at 100% ionic strength containing 543 µM AdS, 4.4 µM benzylaminopurine (BAP), 1 µM IAA, 22.5% sucrose (w/v) and 0.3% Gelrite (w/v). For rooting, elongated shoots up to 2 cm long length were cultured in MS medium at 50% ionic strength containing 20.7 µM indolbutiric acid (IBA) (Sigma-Aldrich, Saint Louis, Missouri, United State), 22.5% sucrose (w/v) (Sigma-Aldrich, Saint Louis, Missouri, United State) and 0.3% Gelrite (w/v) (Sigma-Aldrich, Saint Louis, Missouri, United State). Culture conditions for elongation and rooting were 23±2 °C, 60% RH and a 16/8 h (light/dark) photoperiod; light was provided by light-emitting diode (LED) lamps (60 µmol⁻²s⁻¹ luminous intensity).

Acclimatization

Pots (90 x 80 x 70 mm) (MATEC, Morelia, México) were filled with expanded perlite rock (Accimin, Tlalnepantla, México) moistened with tap water. Thirty plantlets with roots were selected and rinsed with tap water to remove excess medium from the roots. Before transplanting, any leaves were removed from the plantlets to avoid dehydration and death. Once transplanted into the pots the plantlets were fumigated with 1 mg·L⁻¹ copper oxychloride (w/v) (VITA, México, D.F., México), fertilized with bat manure (NPK-10:3:1) (Carmich, Michoacán, México) and covered with transparent nylon bags (Productos de polietileno, Mérida, México) with small holes. Plantlets were cultured in a greenhouse at 30 – 20 ± 2 °C max-min temperature, 70% HR and 11/13 h (light/dark) average photoperiod. After seven days the nylon cover was removed, the plantlets watered with distilled water, and bat manure (NPK-10:3:1) added. For the following month and a half, watering was done every other day, fertilizing once a week, and fumigating (1 mg·L⁻¹ copper oxychloride) once a week. Total greenhouse culture period was 60 days.

Optimization of adventitious shoot induction

Central composite design

Previous assays of adventitious shoot induction found a combination of cytokinin and auxin to produce the best response, so the central composite design was established using these phytohormones. Embryo establishment was repeated to produce cotyledonal leaves as an explant resource. A 2² factorial design with four central points was implemented in which the plant growth regulators 2ip and IAA were the factors to be optimized. The explants were cultured under different treatments (Table 1) in MS medium at 75% ionic strength containing 282.34 µM Adenine sulfate (AdS) (Sigma-Aldrich, Saint Louis, Missouri, United State), 22.5% sucrose (w/v) and 0.2% Gelrite (w/v).

Table 1:- Variables for central composite design in induction of *J. curcas* adventitious shoots.

Design	Treatment number	Encoded variable		Real variable	
		X ₁	X ₂	2ip (µM)	IAA (µM)
First	1	-	-	050	1.2
	2	+	-	100	1.2
	3	-	+	050	1.4
	4	+	+	100	1.4
	5	0	0	075	1.3
	6	0	0	075	1.3
	7	0	0	075	1.3
	8	0	0	075	1.3

1-4 factorial treatments, 5-8 central point treatments; 2ip: 6-(γ, γ-Dimethylallylamino) purine; IAA: indolacetic acid.

Rotatable design

The rotatable design experiments were done after analyzing the central composite design experiment and proving that it fit a second grade model. In this second experiment, the amounts of 2ip and IAA to be used were calculated with the formula:

$$\sqrt{X_1} * = \frac{(X'_1 - CP)}{\Delta}$$

Isolating the variable X'_1 :

$$X'_1 = CP + \Delta (\sqrt{X_1} *)$$

Where X'_1 = real value to attain in each variable; $\sqrt{X_1} *$ = coded variable replaced by $-\sqrt{2}$ or $\sqrt{2}$; CP= central point of the variable range; and Δ = increase of range to the central point.

For this experiment (Table 2), cotyledonal leaf explants were placed in MS medium at 75% ionic strength containing 282.34 μ M AdS, 22.5% sucrose (w/v) and 0.2% Gelrite (w/v).

Table 2:- Second experimental design for optimization of induction of *J. curcas* adventitious shoots.

	Treatment number	Encoded variable		Real variable	
				2ip	IAA
DESIGN		X1	X2	μ M	μ M
Second	9	$+\sqrt{2}$	0	110.4	1.3
	10	$-\sqrt{2}$	0	39.65	1.3
	11	0	$+\sqrt{2}$	75.00	1.4
	12	0	$-\sqrt{2}$	75.00	1.1

9-12 rotatable treatments

Results validation

Once the second order model and optimum values were obtained, an experiment based on those results were established and analyzed in order to verify and calculate the error percentage of the model.

Experimental development

The first experimental stage was the central composite design using a 2^2 factorial design with four central points and five replicates. The second stage was the rotatable treatments with four treatments and five replicates. The results validation was a treatment with ten replicates realized three times. Average shoot number per treatment was evaluated in all experiments. The experimental unit was one explant from *J. curcas* cotyledonal leaves per flask. All data were processed to fit them for quadratic behavior, produce the response surface and then graph the contours. Processing was done with the Statgraphics Centurion XVI software (Statgraphics Technologies, Inc. The Plains, Virginia, URL: <http://www.statgraphics.com/>).

Results and Discussion:-

J. curcas Micropropagation

The *J. curcas* cotyledonal leaf explants exhibited a 100% response rate in the formulated medium (Figure 1). At 45 days the shoots had elongated to approximately 2 cm in length with an average of 15 ± 3 roots developed per plant. Survival rate of acclimated plants was 90%.

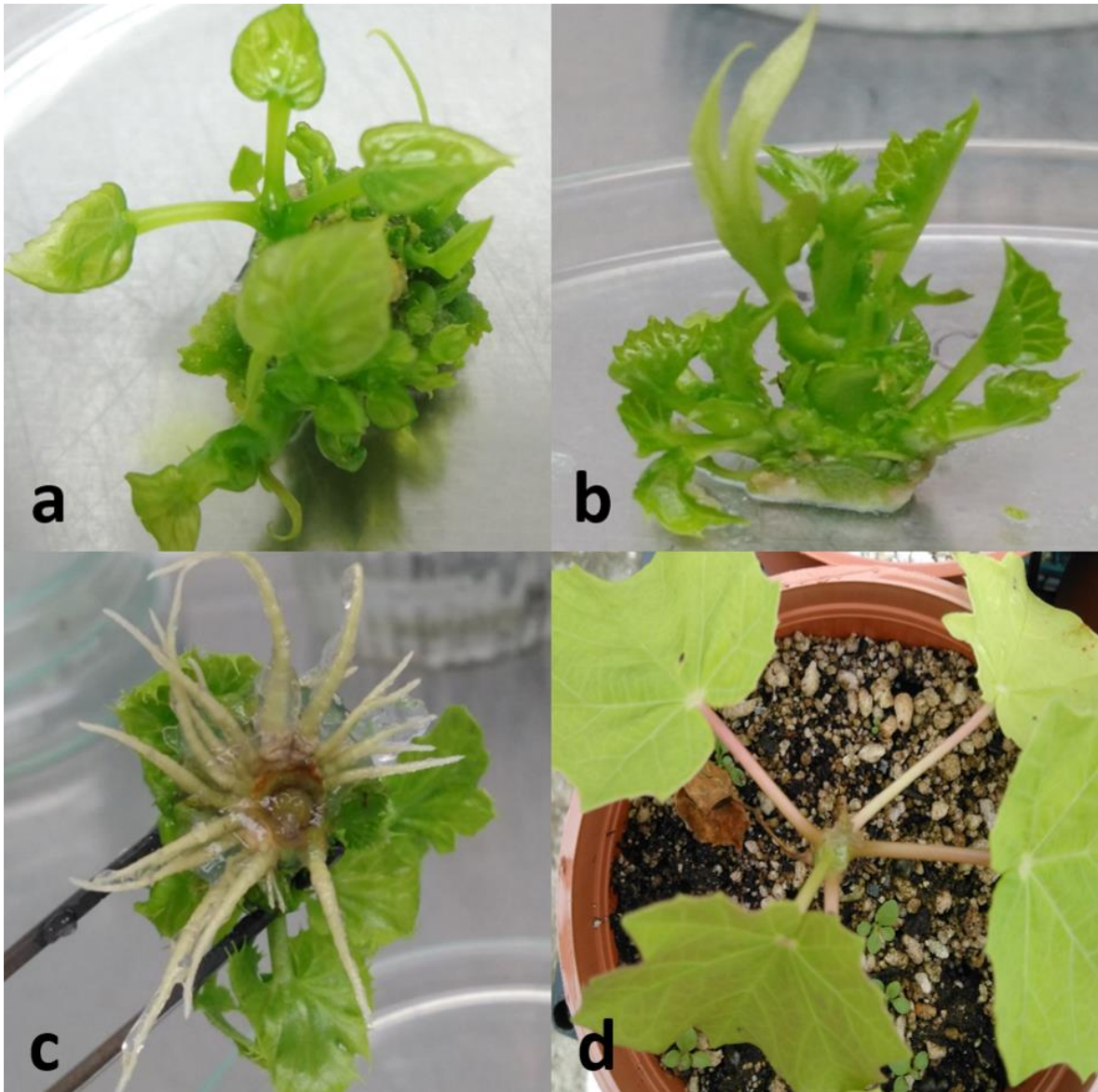


Figure 1:- Micropropagation of *J. curcas*. a) Adventitious shoots from cotyledonal leaf explants at 45 days; b) elongated adventitious shoots (2 ± 0.5 cm long); c) plantlet roots; and d) acclimated plantlets in greenhouse at 60 days.

Composite central design

In the Pareto diagrams for the previous assays (Figure 2a), the lowest 2ip level is the factor with the highest standardized effect on adventitious shoot induction. A triple interaction near the standardized effect can be observed. The IAA factor had no effect on the response variable. This contrasts with reports that auxin-cytokinin interactions are indispensable because they form a system that controls many aspects in differentiation and cellular development (Coenen and Lomax, 1997). Neither did AdS have an effect on the response variable. This organic compound is reported to have a synergic effect with other cytokinins (Raha and Roy, 2001), and is also a nitrogen source (Husain et al., 2006). It can therefore stimulate cell growth and improve shoot induction, as demonstrated in a study using $135.7 \mu\text{M}$ AdS in which 10.0 ± 1.30 shoots per *J. curcas* leaf explant were produced (Shrivastava and Banerjee 2008); given this kind of response AdS should not be removed from the central composite design. In the central composite design diagram (Figure 2b), there is a double interaction with 2ip-IAA which surpasses the standardized effect.

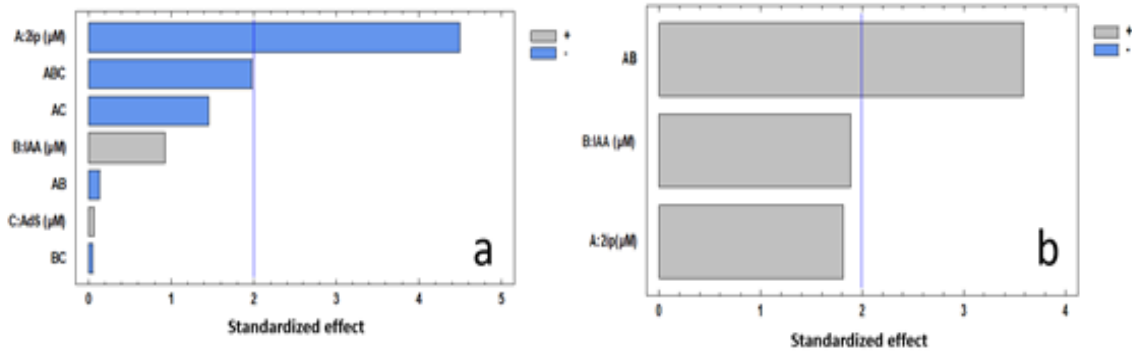


Figure 2:- Pareto diagrams for the 2ip, IAA and AdS factors effect on *J. curcas* adventitious shoot induction. a) Diagram for previous assays; b) diagram for central composite design (2^2 with four central points).

Fit to quadratic model and response surface analysis

When fitted to the quadratic model the 2^2 central composite design with rotatable treatments was shown to fit to a second order model ($P < 0.05$), demonstrating that this model had curvature. The adjusted determination coefficient was 85.1%, indicating that the model explains the data. The resulting equation is:

$$Y = -173.726 + 0.252628X_1 + 274.831X_2 + 0.08X_1X_2 - 0.00216001X_1^2 - 105.001X_2^2$$

The modeled surface for this design was maximum points (Figure 3a); lowest average number of shoots is indicated in blue tones and highest average number in red-orange tones. This surface occurred with the combination of 83 µM 2ip and 1.3 µM IAA. In the maximum points response contour graphic (Figure 3b), the maximum number of shoots obtained was 21. In the same graphic the highest effect factor is IAA, suggesting broad changes in concentrations can easily change the response variable. This agrees with the fact that PGR are organic substances that quickly and effectively influence physiologic processes when applied in low, specific concentrations (Davies, 2010).

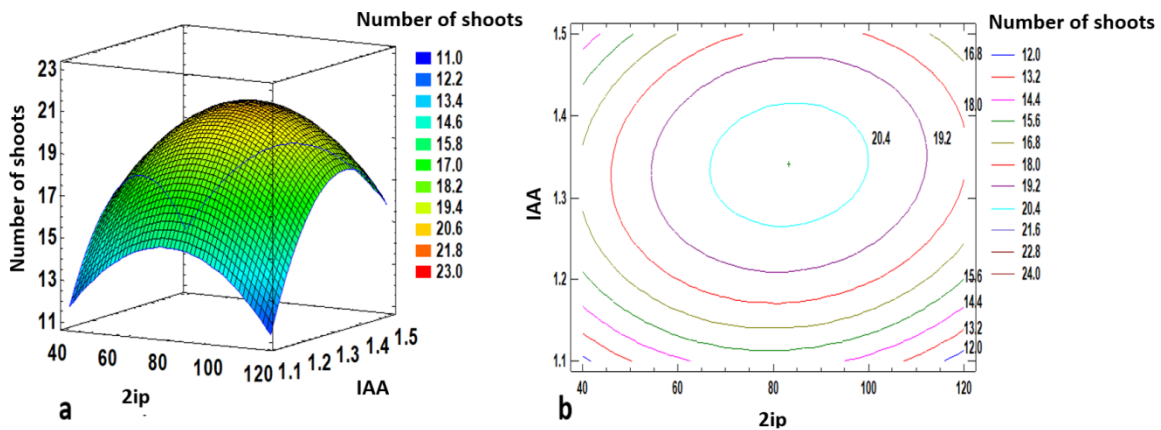


Figure 3:- Response surface generated for optimization of in vitro adventitious shoot induction in *J. curcas*. a) Maximum points surface, b) Contour graphic.

Application of exogenous IAA auxin is generally required to achieve rooting (Diaz-Sala et al., 1996), although 2.85 µM auxin has been used in combination with BAP cytokinin to produce adventitious shoots in *J. curcas* (Rajore and Batra, 2005). Combining an auxin with a cytokinin is indispensable for shoot induction, but depends on which PGR is added to the medium. For example, in a study of induction using 14.7 µM 2ip in combination with different auxins, only white and green compact calluses and light yellowish and soft friable calluses were produced (Kaewpoo and Te-chato, 2009). In the present results, by contrast, IAA auxin in combination with 2ip supported adventitious shoot induction. The 2ip cytokinin is a precursor of zeatin synthesis (Miura and Miller, 1969), and is widely used in plant tissue culture. It is also a PGR with important roles in shoot bud induction and elongation (Shibil et al., 2017),

and development of microshoots and shoot multiplication (Tahtamouni et al., 2017). The average 2ip concentration used for *J. curcas* shoot induction has ranged from 2.5 to 39.4 μM , which produced 1.2 ± 0.05 shoots per nodal explant (Datta et al., 2007), to 1 and 7.5 μM 2ip, which produced 2.7 shoots per explant (Purkayastha et al., 2010). In both cases only 2ip was used for shoot induction. However, addition of IAA auxin can notably increase induction, as shown in the response surface generated here (Figure 3). Indeed, this is the first report on how IAA and 2ip in combination support adventitious shoot induction in *J. curcas*. Most PGR exhibit different modes of action on different species or varieties. For example, in *Juncus effusus* no significant differences were observed between BAP and 2iP in a comparison of regeneration responses, although 2iP did induce the highest number of shoots (Sarma and Rogers, 2000). Continued evaluation of interactions between 2ip and IAA could help to further increase shoot number induction in *J. curcas*.

Previous reports on adventitious shoot induction in *J. curcas* have used different concentrations of PGR such as thidiazuron (TDZ), 6-benzylaminopurine (BAP), 3-indolacetic acid (IAA), gibberellic acid (GA_3), kinetin (Kin), and 2,4-dichlorophenoxyacetic acid (2,4-D). These resulted in induction of up to seven adventitious shoots per explant from petiole leaves (Liu et al., 2015), eleven per leaf explant (Verma and Verma, 2015), and thirteen from cotyledonal leaf explants (Liu et al., 2016). In contrast, application of an RSM in the present study to optimize adventitious shoot induction in *J. curcas* demonstrated that 21 adventitious shoots per explant can be generated using a combination of MS medium at 75% ionic strength, 83.3 μM 2ip and 1.3 μM IAA.

Validation

The validation showed 10.95% of error as can be seen in Table 3 which the optimum obtained was 21 adventitious shoots and validation was 18.7 adventitious shoot buds. From the RSM model and the optimization results, the predicted and measured values are quite close, which

Indicates that the developed model can be effectively used to predict the surface response in adventitious shoot bud induction.

Table 3:- Error percentage obtained accord to theoretical and practical of adventitious shoot buds.

Factors		Number of adventitious shoot buds obtained		Error percentage (%)
2ip (μM)	IAA (μM)	Theoretical	Practical	
83	1.3	21	19	9.5

2ip: 6-(γ , γ -Dimethylallylamino) purine; IAA: indolacetic acid.

Conclusions:-

This is the first report on optimization of adventitious shoot induction in *Jatropha curcas* for use in rapid and massive propagation, genetic improvement or in vitro germplasm conservation. The protocol described here is efficient for indirect organogenesis through cotyledonal leaf explants, and a second order model was produced to predict the effect of 2ip and IAA on adventitious shoot induction in *Jatropha curcas*.

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