Use of Response Surface Methodology on Medium Components Optimization for Embryogenic Callus Induction in Sugarcane (Saccharum Sp.) Cv. Cp 72-2086

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Abstract

The importance of sugarcane culture has in the agriculture and industrial market, demand the development of efficient *in vitro* propagation systems; in this sense embryogenic callus has a great impact in basic research and industrial applications, due to this, the development of protocols for embryogenic callus induction is of great importance. In this work, an optimized protocol for embryogenic callus induction was developed; to do this, the balance of the phytohormones 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-butyric acid (IBA) in the culture media composition was optimized using response surface methodology (RSM) in order to maximize the percentage of samples with embryogenic callus (%SEC) and the weight gain on callus produced (ΔCW) in *Saccharum* sp. cv. CP72-2086. Best results in the %SEC were obtained as the 2,4-D concentration decreased; meanwhile, in the case of IBA the best results were obtained in the concentration range from 2.5 to 5.5 mg L^{-1}. Regarding ΔCW, maximum values were obtained at 2,4-D concentrations in the range of 0.5 mg L^{-1} to 1.89 mg L^{-1} in combination with IBA in the range of 4.55 mg L^{-1} to 5.25 mg L^{-1}. The best predicted response was obtained at a combination of 0.5 mg L^{-1} 2,4-D + 3.53 mg L^{-1} IBA, from which the predicted values of the model were 100.0 of %SEC and 1.4323 g of ΔCW. The experimental values were very close to those predicted by RSM, which suggests that a computer-based experimental design it is very useful to optimize protocols for successful embryogenic callus induction in sugarcane.

Introduction

Sugarcane (*Saccharum officinarum*) is a tropical, semitropical and subtropical perennial grass belonging to the family of the poaceae. It is cultured in over 120 countries, primarily for its ability to store high concentrations of sucrose in the stem, in addition to its multiple uses (Bomfim de Alcantara et al. 2014; Yao et al. 2017; Salokhe 2021). Near up to 74% of the sugar supply in the world is obtained from sugarcane, which makes it a very important commercial crop in agricultural and industrial market. Although the principal industries are found in Brazil, India, Pakistan, United States and China, sugarcane is also commercialized in other countries including Mexico (Dal Bianco et al. 2012; OECD/FAO 2016).

In spite of the estimated reduction in the production of some cane-producing countries in the next seasons, it is expected that the demand should increase throughout the decade. Over the ten-year period, the increase in production is foreseen to average 2.1% annual (OECD/FAO 2016). This crop can be reproduced by asexual or sexual modes, but for its commercial production is propagated mainly by vegetative method; this technique involves the planting of stakes obtained from mature stems, stem cuttings, or premature cane about 8 to 12 months old grown. However, this system presents some disadvantages, such as the spread of pests and diseases, vigor loss over time, and a low multiplication coefficient (Getnet 2017; Tolera and Shimelis 2017; Salokhe 2021).

Due to this is necessary the development of alternative techniques to allow the effective propagation, diseases reduction and quality enhancement of sugarcane materials in short time (Basnayake et al. 2011; Dal Bianco et al. 2011; Basso et al. 2017). For this purpose, some tissue culture techniques have been

used to develop and improve protocols for *in vitro* propagation of some sugarcane varieties, either through embryogenesis and/or organogenesis (Ramanand et al. 2006; Kumar and Sahoo 2009; Kavita et al. 2016; Salokhe 2021); nevertheless, previous reports indicate that some sugarcane genotypes are more prone to *in vitro* instability than others, possibly due to the interaction between genotype and culture medium. Thus, the establishment of a suitable culture medium is critical for *in vitro* propagation of sugarcane, especially when it is desirable that the protocol is based on somatic embryogenesis (Snyman et al. 2011; Maulidiya et al. 2020).

Induction of somatic embryogenesis has a great impact on the micropropagation of sugarcane, so it is widely used to generate somatic embryos with high regenerative frequency, allowing for obtaining a massive production of *in vitro* seedlings in short times (Ikeuchi et al. 2013; Bomfim de Alcantara et al. 2014; Dong-Mei and Hai-Long 2014; Passamani et al. 2019). Also, embryogenic callus is considered the ideal biological material for induction of stable genetic modifications and somaclonal variants, which subsequently could be used for crop improvement, obtaining homogenous seedlings with high quality, both from a genetic and physiological point of view (Arjun and Rao 2015; Dinesh et al. 2017; Naz and Hayat 2017; Passamani et al. 2019).

On the other hand, media composition is one of the most important factors within plant tissue culture protocols, in such a way that each medium should be formulated considering the specific requirements for every crop and even the aim of the experiments (Larraburu et al. 2012; Bomfim de Alcantara et al. 2014; Dong-Mei and Hai-Long 2014; Benmahioul 2017). In this sense, one of the most important factors affecting the development and differentiation of plant tissues under *in vitro* conditions is the use of growth regulators, showing the peculiarity that even the same combination of these, offers different physiological responses depending on the plant species, the explant type, and even the concentration used (Benmahioul 2017). Within the options used for this purpose stand out the use of auxins like indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA); and cytokinins like kinetin (KIN) and 6-benzylaminopurine (BAP) (Chengalrayan and Gallo-Meagher 2001; Razdan 2003; Bomfim de Alcantara et al. 2014; Dong-Mei and Hai-Long 2014; Passamani et al. 2019).

During the formulation of a certain culture medium, the combination possibilities between different doses of nutrients and compounds to be used can be immense; thus, one of the options to formulate efficient culture media is the application of experimental design tools such as the response surface methodology (RSM), which is a collection of computer-based experimental designs to develop and adjust mathematical models using statistical techniques, determining the factor or combination of factors required to establish the optimal conditions for obtaining the best results, minimizing the number of experimental trials, saving time, labor and cost (Ibañez et al. 2003; Gutiérrez-Miceli et al. 2010; Aghayeh et al. 2020). RSM has been successfully applied in media components optimization such as for micropropagation protocols of *Dianthus caryophyllus* L. (Gutiérrez-Miceli et al. 2010) and *Handroanthus impetiginosus* (Larraburu et al. 2012); shoot regeneration protocols in *Basilicum polystachyon* (Chakraborty et al. 2010) and Chinese jujube (*Ziziphus jujube* Mill) (Hou et al. 2018); callus induction and

shoot regeneration of soybean (*Glycine max* L. Merr.) (Abbasi et al. 2016); and the establishment and proliferation of seedless barberry (Aghayeh et al. 2020).

The aim of this study was the determination of optimum amounts of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-butyric acid (IBA), for embryogenic callus induction of *Saccharum* sp. CP 72-2086 by using response surface approach.

Materials And Methods

Plant Materials

For the experiments, meristems from sugarcane plants (*Saccharum* sp.) of the most important cultivar in Mexico (cv. CP72-2086), with growth time between 6 to 7 months were used. Plants with no visible signs of pests or diseases were selected (Fig. 1A). Cuttings of 50 ± 1 cm were obtained; these segments were washed with distilled water and commercial detergent before being stored in refrigeration at 4°C for 24 h. The outer leaves were removed under aseptically conditions, and then immersed in an antioxidant solution for 1 h (0.3 g L^{-1} of ascorbic acid). The apical meristems were obtained and placed in 1% Benzal solution for 10 min with constant movement; then, they were disinfected by immersion in 10% (w/v) NaOCl solution for 20 min adding two drops of Tween 80 per 100 mL, and washed three times with sterile distilled water (Fig. 1B). Later, cuttings were summited to a hydrothermal treatment at 50–55°C for 8 min, and finally rinsed three times with sterile distilled water (Parreño, 2012). Finally, disc meristems of 2 mm thick and 1.5 cm in diameter were excised and placed in the different callus inducing media (CIM) (Fig. 1C-D), consisting of distilled water with 4.43 g L^{-1} MS, 30 g L^{-1} sucrose, and 7 g L^{-1} agar, added with different amounts and combinations of the auxins 2,4-D and IBA for each treatment, according with the experimental design. All the culture media were adjusted to pH = 5.8 and sterilized by autoclaving at 121°C for 15 min at a pressure of 100 kPa (15 psi). Phytohormones were sterilized by filtration.

Callus growth

Embryogenic callus were induced from each sample consisting of a disc of apical meristem; each one was weighed and cultured in an assay tube containing a CIM correspondent to each treatment. Samples were kept in controlled conditions in the darkness at 25 ºC for 60 days. After this, the percentage of samples with embryogenic callus (% SEC) and the weight gain on callus produced (ΔCW) (Eq. 1) per treatment were evaluated.

$$\Delta CW = W_F - W_0 \qquad (1)$$

Where W_F is the final weight and W_0 is the initial weight of the explant.

Experimental design

An experimental factorial design with two process variables was used to optimize the balance of the phytohormones 2,4-D and IBA in the culture media composition for the maximum %SEC and ΔCW in sugarcane. The experimental design was determined by introducing the conditions in the software Design Expert 7.0 from which thirteen treatments were obtained, eight corresponded to factorial combinations of the independent variables and five to central point's corresponding to the replicates (Table 1). Twenty samples for each treatment were evaluated. Furthermore, a central composite factorial design was used to find the relationship between the independent variables (2,4-D and IBA), and the response variables (%SEC and ΔCW), through a second-order polynomial. Literature data and preliminary experiments were taken into account to determine the different amounts and combinations of the phytohormones. The model below shows the relationship between two independent variables X_1 = 2,4-D, X_2 = IBA; and two response or dependent variables Y_1= %SEC, Y_2 = ΔCW, through a second-order polynomial (Eq. 2)

Table 1
Experimental design and results for the embryogenic callus induction obtained with different concentrations of 2,4-D and IBA.

Treatment	2,4-D (mg L^{-1})	IBA (mg L^{-1})	%SEC	ΔCW (g)
1	1.89	1.89	94.11	1.095
2	8.61	1.89	10.00	0.698
3	1.89	8.61	85.71	0.950
4	8.61	8.61	35.71	1.026
5	0.5	5.25	100.0	1.510
6	10.0	5.25	16.00	1.086
7	5.25	0.5	54.54	0.700
8	5.25	10.0	40.00	0.950
9	5.25	5.25	60.00	0.889
10	5.25	5.25	87.50	0.997
11	5.25	5.25	50.00	0.940
12	5.25	5.25	78.57	1.053
13	5.25	5.25	70.00	0.923

%SEC: percentage of samples with embryogenic callus; ΔCW: weight gain of callus produced.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{\substack{i,j \\ i<j}} \beta_{ij} X_i X_j + \varepsilon \qquad (2)$$

Where β_0 is the compensatory term; β_i is the dependent term of input factor X_i; β_{ii} is the quadratic effect of the input factor X_i; and β_{ij} is the linear-linear interaction effect between the input factor X_i and X_j.

Regression analysis was applied to the values of the response variables obtained experimentally, the non-significant terms ($p > 0.1$) were eliminated and a new polynomial (prediction model) was recalculated for each response variable (Khuri and Cornell 1987). From each prediction model, response and contour surface graphs were constructed to study the effect of the process variables on each of the response variables analyzed, and those which presented the greatest significant effect on the % SEC and ΔCW were selected.

Optimization analysis

To obtain the optimum conditions for callus induction (searching the maximization of %SEC and the highest ΔCW), the Derringer function or desirability (D) was used. This methodology consists in adjusting the response variables (dependent) at the same time; which is necessary to find optimal comprises between the total numbers of responses. The global D value was analyzed based on individual desirabilities. Statistical analyses were performed operating the Design-Expert software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA).

Results And Discussion

Induction of embryogenic callus

Embryogenic callus was triggered in all tested treatments, with %SEC ranging between 10 and 100%. After 60 days of explant cultivation, the weight gain on callus produced varied from 0.698 to 1.510 g. Despite in all treatments there was induction of callus, the best result was obtained using a combination of 0.5 mg L^{-1} 2,4-D + 5.25 mg L^{-1} IBA, reaching values of %SEC = 100.0 and ΔCW = 1.510 g (Table 1).

The independent and dependent variables were analyzed; the regression representations were adjusted for %SEC and ΔCW obtaining the following uncoded models:

$$\%SEC = +92.99201 - 9.41237*(2,4\text{-}D) + 8.65026*(IBA) - 0.83538*(IBA)^2 \quad (3)$$

$$\Delta CW = +1.43078 - 0.22065*(2,4\text{-}D) + 0.056268*(IBA) + 0.010482*(2,4\text{-}D)*(IBA) + 0.012465*(2,4\text{-}D)^2 - 0.00869*(IBA) \quad (4)$$

The equations 3 and 4 predict the percentage of samples with embryogenic callus and the weight gain on callus produced respectively, based on the values of 2,4-D and IBA; furthermore, the models used a good significance level ($p = 0.0005$ and 0.004, respectively). The multiple determination coefficients (R^2)

obtained were 0.8472 and 0.8772 respectively, indicating a satisfactory fitting on the models predicted to the experimental data.

According to Myer and Montgomery (2002), a good prediction model must have a determination coefficient (R^2) of at least 0.80 and a significance level (p) lower of 0.05. All these parameters have been used to decide the level of satisfaction of the models.

Induction of sugarcane embryogenic callus has been already reported mainly using 2,4-D, IBA and NAA in a wide range of concentrations and combinations (Bomfim de Alcantara et al. 2014; Arjun and Rao 2015; Solangi et al. 2016; Naz and Hayat 2017; Jamil et al. 2017; Passamani et al. 2020). In this study, a clear tendency to increase the %SEC as the 2,4-D concentration decreased was observed. Meanwhile, in the case of IBA the best results for %SEC were obtained in the concentration range from 2.5 to 5.5 mg L^{-1} (Fig. 2A-B); thus, the best treatment to embryogenic callus induction (reaching a value of %SEC = 100.0), confirmed by largest red region in the surface response, was the combination of 0.5 mg L^{-1} 2,4-D + 5.25 mg L^{-1} IBA.

Razdan (2003) suggested the use of 2,4-D in the ranged of 0.5 to 2.5 mg L^{-1} for somatic embryogenesis induction in monocotyledons as *S. officinarum*, because higher doses could inhibit the embryogenic callus induction. Similar results have been reported in sugarcane cultivar CP-841198, where the percentage of embryogenic callus induction gradually decreased as the concentration of 2,4-D was increased (Chengalrayan and Gallo-Meagher 2001; Zamir et al. 2014). Passamani et al. (2019), demonstrated that long-term culture of sugarcane callus with 2.2 mg L^{-1} 2,4-D decreased gradually the embryogenic competence, through affecting the polyamine metabolism, the regulation of late embryogenesis proteins and the accumulation of putrescine and spermidine as well. Orłowska and Kępczyńska (2020), in a study conducted with *Medicago truncatula*, reported that higher concentrations of 2,4-D causes an increase in O_2 accumulation and antioxidant enzyme activity, altering formation of callus and somatic embryos. In this work, the lowest SEC percentages (10 to 35.7%) were obtained in treatments in which high concentrations of 2,4-D (8.61 and 10.0 mg L^{-1}) were used (Table 1).

On the other hand, for the ΔCW variable it was observed that combinations of 2,4-D and IBA in the range of 5.25-10.0 mg L^{-1} and 0.5–5.25 mg L^{-1}, respectively, were not adequate for callus growth, corresponding to the lowest values shown in the blue area (Fig. 2C-D). Similar results were reported by Naz and Hayat (2017), obtaining decreases in the weight of induced callus up to 86% less as the concentrations of 2,4-D increased above 3 mg L^{-1} in two varieties of sugarcane.

Although there is not a clear effect of the growth regulators in ΔCW, the region of maximum response values was obtained through combinations of 2,4-D in the range of 0.5 mg L^{-1} to 1.89 mg L^{-1} along with IBA in the range between 4.55 mg L^{-1} to 5.25 mg L^{-1} (Fig. 2C-D). Van der Vyver (2010) obtained a high rate of embryogenic callus from leaf disks of sugarcane (Black Cheribon genotype) in medium containing 0.5 mg L^{-1} 2,4-D. Khan et al. (2004) reported that 2.0 mg L^{-1} 2,4-D, proved to be an excellent dose for

embryogenic callus induction and rapid proliferation in ten sugarcane genotypes. Solangi et al. (2016) evaluated the effect of different doses of the auxins 2,4-D, NAA and picloram on young meristems of three varieties of sugarcane, reported that 2,4-D at 3.0 mg L^{-1} concentration proved to be the most effective auxin for callus mass induction of all the varieties tested. Moreover, Naz and Hayat (2017) evaluated the effect of several doses of 2,4-D (0.5 to 4 mg L^{-1}) on three sugarcane varieties, finding that the concentrations of 1.0 and 2.0 mg L^{-1} were the best conditions for greatest callus induction, and also reported that variations in 2,4-D concentration could adversely influenced the ratio of callus development and growth.

Differences on embryogenic callus induction through the application of different doses of 2,4-D in sugarcane could be due to several factors such as genotype, explant type, and/or the combination with other phytohormones into the medium (Khan et al. 2004; Dinesh et al. 2017; Getnet 2017; Naz and Hayat 2017).

On the other hand, desirability function-based method was applied for the optimization of embryogenic callus induction, considering the maximum response values for %SEC and ΔCW (Fig. 3). To optimize the concentration of auxins needed to induce embryogenic callus, the desirability value obtained was 0.945, which is close to the maximum possible global desirability (value = 1); being the best predicted response corresponding to 2,4-D (0.5 mg L^{-1}) and IBA (3.53 mg L^{-1}); this values correspond to independent variables associated with the maximum overall desirability, where the predicted values of the model were 100.0% of SEC and 1.4323 g of ΔCW (Table 2).

Table 2
Regression coefficients and analyses of variance of the experimental prediction models showing in coded factors the relationship among process variables (X) and response variables (Y).

Coefficient	%SEC	ΔCW
Intercept		
$ß_0$	65.97	0.96
Linear		
$ß_1$	-31.61	-0.12
$ß_2$	-0.41	0.67
Quadratic		
$ß_{11}$		0.14
$ß_{22}$	-9.42	-0.098
Interaction		
$ß_{12}$		0.12
Lack of fit	0.6965	0.1308
R^2	0.8472	0.8775
R^2adj	0.7962	0.7900
P	0.0005	0.0043
C.V.	21.85	9.43

To corroborate the optimized conditions for the embryogenic callus induction, a validation assay using 0.5 mg L^{-1} 2,4-D + 3.53 mg L^{-1} IBA was performed, from which a value of 94.0% was obtained for %SEC, against 100% predicted by numerical optimization. Meanwhile, for ΔCW, the average weight of the samples was of 1.510 g, against the predicted value for the model of 1.4323 g. The experimental values obtained with the validation assay developed experimentally, were very close to the predicted ones generated with the RSM optimization. About this topic, Andressen et al. (2009) indicates that the RSM allows evaluating the substances concentrations which can modify the growth and development of different organs and analyze their effects. Also, Abbasi et al. (2016) and Aghayeh et al. (2020), suggested that this statistical modeling can be applied to estimate the best combinations of the phytohormones needed for successful *in vitro* plant regeneration, maximizing the responses and minimizing the

application of hormonal treatments. In agreement with those previous reports, the results obtained in the present work indicate that the use of this statistical tool can be applied with a high degree of certainty in the development of tissue culture tools, such as embryogenic callus induction of elite cultivars of sugarcane.

Related to the induced callus type, through the optimization tests and even under optimized conditions, different types of callus were obtained; thus, fluffy and soft non-embryogenic callus were obtained from 8.61 mg L^{-1} 2,4-D + 8.61 mg L^{-1} IBA (Fig. 4A); compact non-friable callus were induced from 5.25 mg L^{-1} 2,4-D + 0.5 mg L^{-1} IBA (Fig. 4B); and combinations of non-embryogenic and embryogenic callus induced over the same apical meristem were also obtained (Fig. 4C). Finally, under the optimized conditions (0.5 mg L^{-1} 2,4-D + 3.53 mg L^{-1} IBA) embryogenic callus was mainly obtained (Fig. 4D-F). It has been reported that as the formulation of the medium is modified, different types of callus are induced, which are classified based on their macroscopic characteristics (Ikeuchi et al. 2013). As starting point, the type of induced callus could be originated by specific and distinct gene expression profile; which in turn, could be related to the interaction between growth regulators present in the culture medium and those produced by the explant itself (Iwase et al. 2011; Maulidiya et al. 2020).

Conclusions

In order to find a particularly suitable combination for efficient embryogenic callus induction of sugarcane cv. CP 72-2086, in the present work the RSM was applied as a mathematical method. Once the optimized conditions for the embryogenic callus induction were determined, it was possible to achieve results very close to the predicted values obtained by the regression models, with high regression coefficients, R^2 = 0.84 for %SEC and R^2 = 0.88 for ΔCW. Based on these results, we can verify that the RSM can be applied with great precision in biological study models, using minimal amount of resources and reaching good efficiency; in the present work an effective protocol to induce embryogenic callus for massive propagation of *Saccharum* sp. cv. CP 72-2086 was obtained through the use of RSM.

Declarations

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Declarations

The authors have no relevant financial or non-financial interests to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

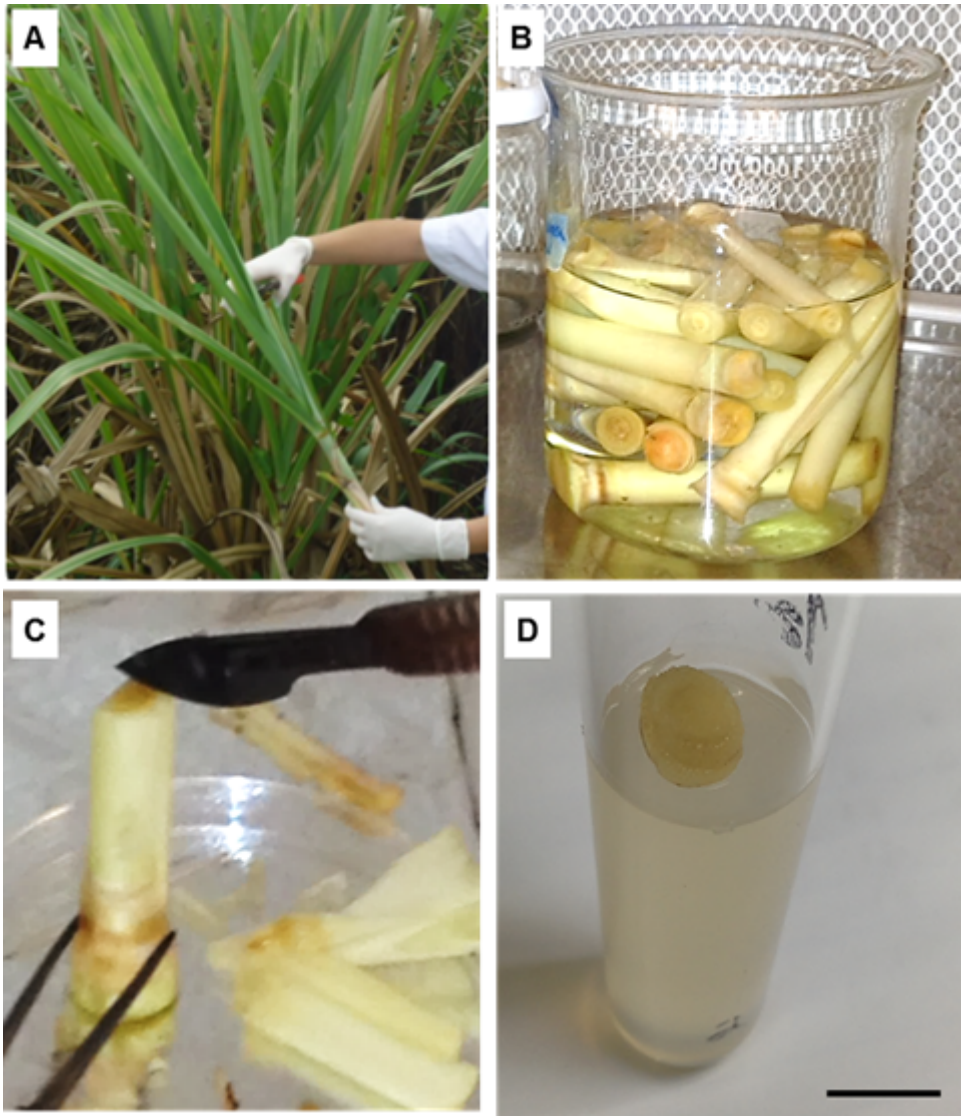

Figure 1

Surface disinfection protocol for sugarcane plants CP 722086. A, apices of 6 to 7 months old plants; B, washing and sterilization of apical meristems; C, apical meristems region, transversally cut into segments of 2 mm thick and 1.5 cm in diameter; D, culture of disc meristems on different embryogenic callus induction media. Bar= 1 cm.

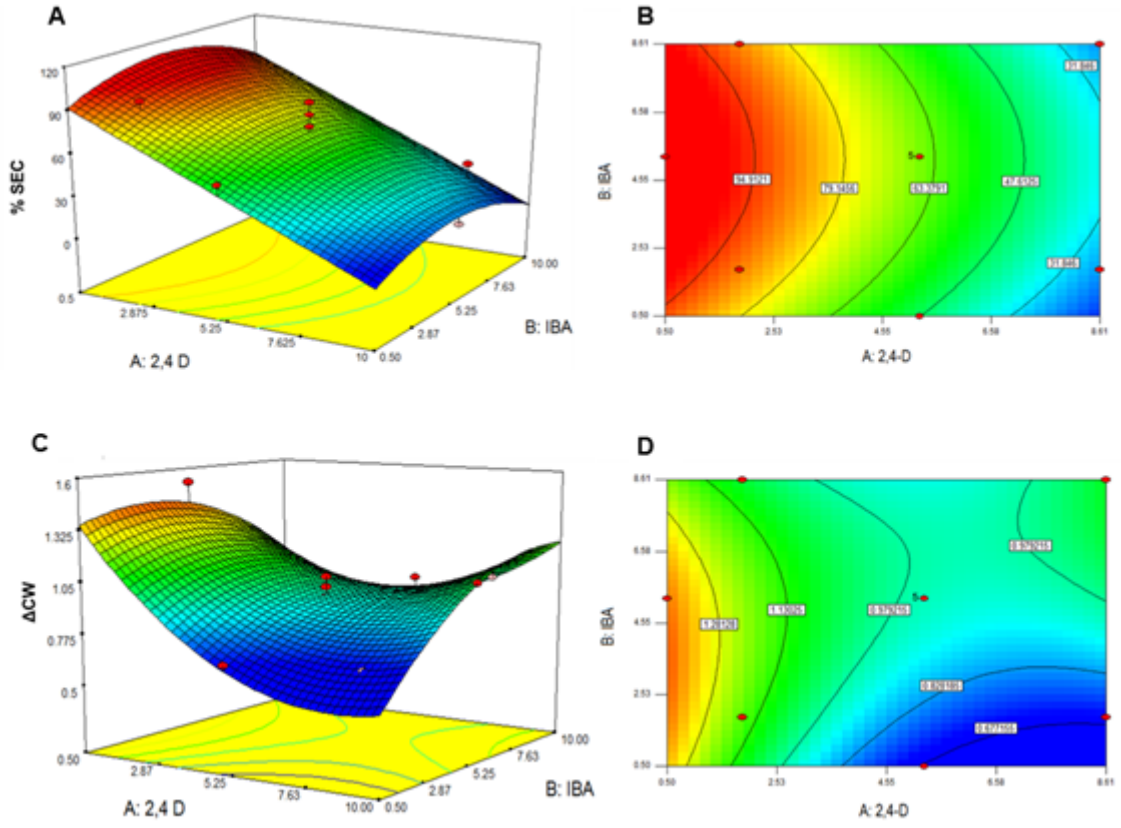

Figure 2

Response surface plot and the corresponding contour plot showing the combined effect of 2,4-D and IBA on %SEC (A and B), and ΔCW (C and D).

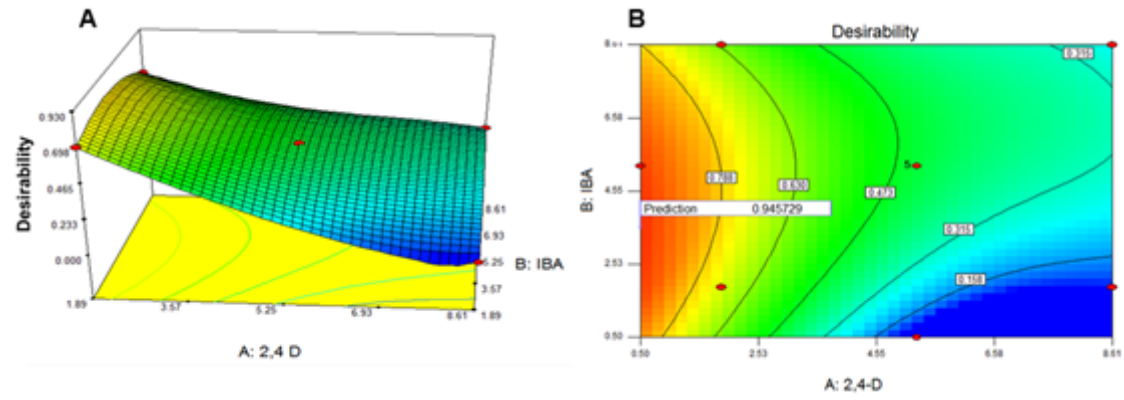

Figure 3

Contour plot of global desirability for the optimization of both variables (A and B).

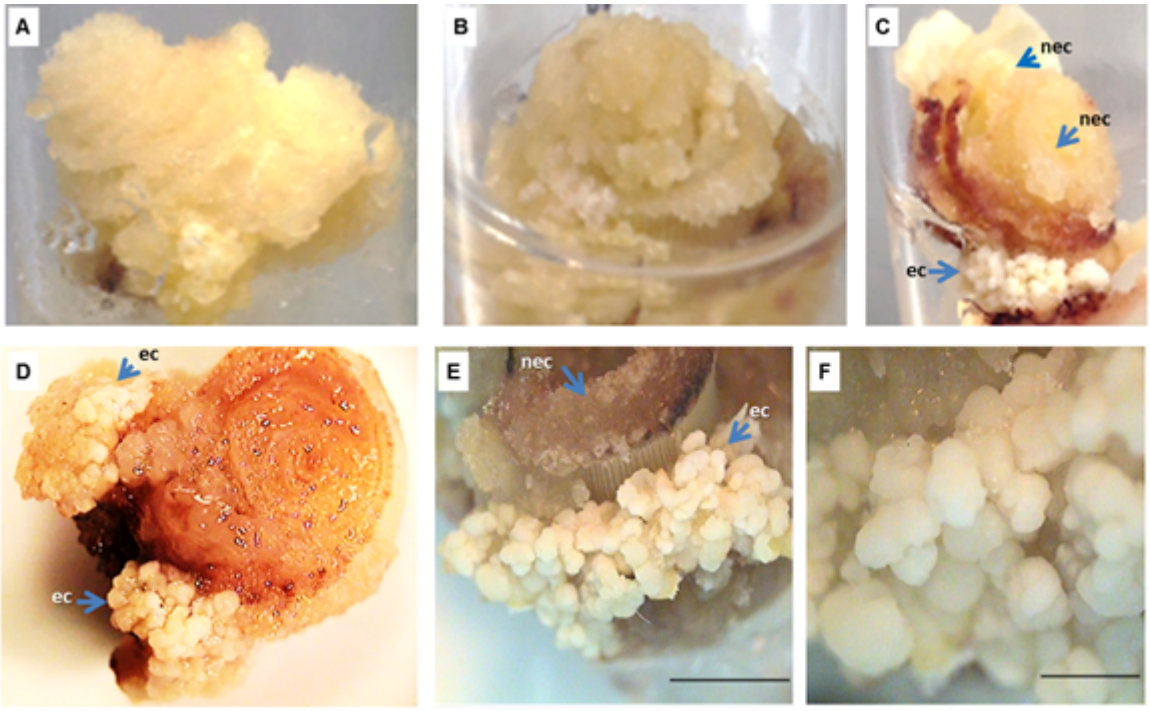

Figure 4

Induced callus through apical meristems of sugarcane cv. CP 72-2086. A, fluffy and soft non-embryogenic callus; 8.61 mg L-1 2,4-D + 8.61 mg L-1 IBA; B, Compact, non-friable callus; 5.25 mg L-1 2,4-D + 0.5 mg L-1 IBA; C, non-embryogenic (nec) and embryogenic callus (ec) induced over the same apical meristem 1.89 mg L-1 2,4-D + 1.89 mg L-1 IBA; D-F, embryogenic callus generated by the optimized media 0.5 mg L-1 2.4-D + 3.53 mg L-1 IBA. Bar in E=5mm, F= 2mm.