Research Article

Screening of significant medium components for enhanced elicitor production from Fusarium oxysporum cubense using Plackett-Burman Design

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Received: 30/03/2017
Revised: 08/07/2017
Accepted: 04/08/2017

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Abstract:

The Plackett-Burman design, a statistical screening method, was used to screen eleven media such as glucose, sucrose, fructose, sorbitol, glycerol, malt extract, yeast extract, urea, NaNO3, NH4NO3 and (NH4)2SO4 for biomass production of Fusarium oxysporum f.sp . cubense (Foc), a causative agent of Panama disease of bananas. Malt extract, yeast extract and fructose have been found to have a significant positive effect on the production of elicitors, while glycerol, NaNO3, NH4NO3 and (NH4)2SO4 exhibited a significant negative effect on the production of the elicitor. In addition, a 3-fold increase in dry mycelial weight (DMW), which served as a source of elicitor, was achieved in the medium combination 3 in only six days compared to the biomass of Foc achieved in 21 days in potato dextrose broth. Therefore, medium components with significant positive and negative effects were identified that could be increased or decreased, respectively for higher elicitor production and further optimization.

Keywords: Elicitor, Fusarium oxysporum f.sp. cubense, media component, Dry mycelial weight

INTRODUCTION

World production of banana in 2016 was about 103 million tons, among which about 16.8 million tons was contributed by India as the largest banana producer. Many commercial varieties of banana succumb to a destructive pathogen *Fusarium oxysporum* f.sp. *cubense* (Foc) causing Panama disease (fusarial wilt). For countering the wilt caused by Foc, several reports support the use of pathogen derived elicitor or synthetic elicitors, mimicking the process of vaccination, to induce complex defense against pathogen (Lakshmanan and Selvaraj, 1986; Mandeel and Baker 1991; Kothari et al. 2008; Bektas and Eulgem 2015). Upon subsequent infection by pathogen in these primed plants resulted in more rapidly activated and robust defense responses (Bektas and Eulgem 2015). Although this concept is very aged, its molecular basis is still only partly understood. Recently, Khan et al. (2016) reported that they characterized the role of *Magnaporthe oryzae* hypersensitive protein 2 (MoHrip2) as an elicitor protein from blast pathogen *M. oryzae*. MoHrip2-treated rice seedlings exhibited induced rice resistance to blast. However, the majority of the mode of action is still unknown. In our previous study, the effectiveness of crude extract prepared from biomass of pathogenic Foc (elicitor) was investigated for the control of Panama disease in tissue cultured banana plantlets (Thakker et al. 2007). Defense related

enzymes induced upon application of elicitors were purified, and their direct antifungal activity was also reported (Thakker et al. 2009). Growth of Foc is slow on conventional growth medium like potato dextrose broth convoyed by a low biomass production (Patel et al. 2004) demands the development of a new medium to support rapid growth with a commendable biomass production.

Present study focuses on the screening of medium components for mass production of the biomass which can be used to prepare elicitor using statistical experiment design. Media composition as well as the growth conditions influence the culture growth, therefore, a fermentation improvement program begins with measurement of product yield as a response to test variables. Changing one independent variable at a time (OVAT) while keeping others at fixed concentration used conventionally or applying statistical designs is used to screen large number of variables. The rapid, and reliable results, shortlisting of significant nutrients, comprehend the interactions among the nutrients at various concentrations, and reduction in number of experiments which saves time, cost, chemicals, and manpower are the advantages offered by statistical methods over conventional methods (Srinivas et al. 1994; El-sersy and Abou-Elela, 2006). The Plackett-Burman design is an efficient way to screen a large number of variables rapidly to identify the most important variables (Deshmukh and Puranik, 2010). The crude biomass of the Foc was sucessfully tested as an elicitor in *in vitro* (Thakker et al. 2007) and *in vivo* (Thakker et al. 2011). Therefore, the aim of the present study was to evaluate the media components significantly affecting the biomass production of Foc using Plackett-Burman design.

MATERIALS AND METHODS

Microorganism

Previously isolated culture of Foc from infected banana plant was maintained on potato dextrose agar (PDA) as described by Thakker et al. (2009).

Chemicals and Solvents

All the dehydrated media components and solvents (99.9%) were purchased from Hi-Media (Mumbai, India) and Qualigens (Mumbai, India) respectively.

Cultural technique

The basal medium which contained (g L^{-1}) KH_2PO_4, 2.0; KCl, 0.3; $ZnSO_4$ $7H_2O$, 0.03; $CuSO_4$ $7H_2O$, 0.003; and Na_2MoO_4 $2H_2O$, 0.003 was used as base throughout the study. The levels and concentrations of other medium components viz glucose, sucrose, fructose, sorbitol, glycerol, malt extract, yeast extract, urea, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, and their combination were as per given in Table 1, and 2, respectively. The nutrient components required to be screened were added to the base as per required concentration. The pH was maintained 5.5 throughout the experiment. Using a sterile cork borer of eight mm diameter, an agar plug was cut from three days old fungal mat on PDA. One plug was used for inoculation of 100 ml broth in 250 ml Erlenmeyer flask and incubated for six days at 27°C in static condition.

Table 1. Medium components, their variables design codes and respective concentration coded by high (+) and low (-) used in Plackett-Burman design

Variable	Medium Components	+ Value (g L^{-1})	- Value (g L^{-1})
X1	Glucose	20	2
X2	Sucrose	20	2
X3	Fructose	20	2
X4	Sorbitol	20	2
X5	Glycerol	20	2
X6	Malt Extract	20	2
X7	Yeast Extract	10	1
X8	Urea	10	1
X9	$NaNO_3$	10	1
X10	$(NH_4)_2SO_4$	10	1
X11	NH_4NO_3	10	1

Plackett-Burman experimental design

Each independent variable was tested at two level of concentrations represented by high (+), and low (-), for all eleven medium components selected for the study. Each variable with a code, variable name and their respective concentrations corresponding to high and low level values are shown in Table 1. The design space was formed with a two level design with only minima, and maxima of input variables. Eleven input parameters resulted in twelve experiments with design v+1 where, v is a number of variables. The resulting twelve experimental cases are shown in Table 2 with the number of positive signs for high concentration, and negative signs for low concentration of the respective variable. Each column contains the same number of positive and negative characters. Each row then represents a test batch and each column represents an independent variable. The effect of each variable was determined by Eq (1):

$$E_{(xi)}=(\mathrm{S}DMW_i^{+}-DMW_i^{-})/N \quad (1)$$

Where $E_{(xi)}$ is the concentration effect of the tested variable. $DMWi^{+}$ and $DMWi^{-}$ are DMW test productions where the measured variable (*xi*) was present at high and low concentrations; N is the number of trials divided by two. Experimental error was determined by calculating variance between six times replicated variable as Eq (2):

$$V_{eff}=\mathrm{S}(E_{spr})^2/n \quad (2)$$

Where *Veff* is the varience of the concentration effect, *Espr* is the concentration effect for the replicated variable with a single point and n is the number of replicats of variable.

The standard error (SE) of the concentration effect was calculated by square root of the variance of the effect, and the significance level (p-value) of each concentration effect was determined using the student's t-test (Eq 3):

$$t(xi)=E(xi)/SE \quad (3)$$

Where, $E_{(xi)}$ is the effect of variable *xi* (Ahuja et al. 2004; Shi and Zhu, 2006).

Measurement of DMW

The fungal growth was determined by DMW measurement. After 6 days of growth, the mycelia mat was separated from the broth by filtration through a pre-weighed filter paper and dried in an oven at 60 ° C until three consecutive constant values were obtained.

Statistical analysis of data

The DMW yields obtained in the experiments were calculated to determine the variability of significance using the Microsoft Excel regression coefficient and the statistical t-values for equal unpaired samples.

RESULTS AND DISCUSSION

Choosing the right carbon source, nitrogen source and other nutrients is one of the most important stage for developing an efficient and economical fermentation process. Plackett-Burman is recommended if more than five factors are to be investigated (Reddy et al. 1999).

These designs are very useful for the economic detection of large effects of variables, provided that all interactions are negligible compared to a few important main individual effect of variables (Siva Kiran et al. 2010). The two-level Plackett-Burman design was selected because it only screens the variable in the experiment "v + 1" (Plackett and Burman, 1946; Bie et al. 2005).

A systematic experiment was performed by setting the Plackett-Burman independent variables (Plackett and Burman, 1946) at two levels, and the corresponding dry mycelial mass (DMW) was measured in each batch, followed by a statistical analysis to interpret significant media components. Such approach is a useful screening process used to identify the contribution of each media component to a system response that allows reducing the number of variables to be taken into account (Liu et al. 2003). These experimental designs are available in multiples of four and are advantageous over multifactor design which are difficult and include a large number of experiments to screen the variables (2^v where v is the total number of variables). Higher order linear full factorial and quadratic Box-Behnken constructs would require 66 and 52 experimental batches for same number of variable that would be prohibitively uneconomical (Naveena et al. 2005). The aim of this study was to explore the main effects of eleven independent variables in an economical way to increase the production of biomass of Foc. Due to the orthogonal nature of the Plackett-Burman design, it only gives the net effect of every variable that is not preplexed by the interaction between the variables (Dhandhukia and Thakkar, 2007).

Table 1 shows the independent variables and the corresponding high and low concentrations of eleven factors used in the optimization study marked +1 and -1. When adjusting diffrent level, caution is required because a small difference may not have any effect and a large difference for the sensitive component may mask other components (Ahuja et al., 2004). In this study, higher levels of ingredients were selected to correspond to ten times their lower levels (Table 1). The various mono- and disaccharides used in the study were reported as the main source of carbon for various fungi. Various sources of nitrogen in the form of ammonia and nitrate ions were included in the study together with urea. The yeast extract was included as a complex source that provides carbon, nitrogen and a rich source of vitamins (Dhandhukia and Thakkar, 2007). Malt extract contains up to 55% maltose sugar per weight basis as well as provides nitrogen source along with many essential vitamins (Almeida et al. 2005).

Twelve run Plackett-Burman design constructs with two concentration levels for each variable were monitored for screening medium components for their ability to support the DMW production shown in Table 2. The X1-X11 variables represent respective medium components and the replicated variable is denoted by an asterisk. The total output of each batch for

cumulative biomass production followed the order of biomass production as, 3> 2> 9> 6> 7> 1> 8> 5> 12> 11> 10. The highest produced biomass was 15.27 gL^{-1} at run 3 and the lowest biomass production was 1.52 gL^{-1} at run 10 (Table 2).

Table 2. Plackett-Burman design with 12 runs generated by fractional rotation of the full factorial pattern and DMW measured as response where X1 to X11 are independent variables

Run	Components											DMW ($g\ L^{-1}$)
	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	X 11	
1	+	+	-	+	+	+	-	-	-	+	-	3.75
2*	-	+	+	-	+	+	+	-	-	-	+	10.29
3	+	-	+	+	-	+	+	+	-	-	-	15.26
4	-	+	-	+	+	-	+	+	+	-	-	8.51
5	-	-	+	-	+	+	-	+	+	+	-	2.86
6	-	-	-	+	-	+	+	-	+	+	+	7.02
7	+	-	-	-	+	-	+	+	-	+	+	5.36
8	+	+	-	-	-	+	-	+	+	-	+	3.10
9	+	+	+	-	-	-	+	-	+	+	-	9.02
10	-	+	+	+	-	-	-	+	-	+	+	1.52
11	+	-	+	+	+	-	-	-	+	-	+	1.78
12	-	-	-	-	-	-	-	-	-	-	-	2.32

Table 3. Statistical parameters measured in terms of *t*-value, probability, confidence level and ranking of variables for DMW production for each variable tested

Medium Component	E*xi* Absolute	SE	$t_{(xi)}$	Confidence (%)	Ranking
Glucose	11.54**	0.64	48.23	99.99	8
Sucrose	3.13**	0.64	42.90	99.99	11
Fructose	21.35**	0.64	19.97	95.99	6
Sorbitol	9.79**	0.64	67.14	99.99	9
Glycerol	40.0**	0.64	56.08	99.99	2
Malt extract	27.51**	0.64	47.55	99.99	3
Yeast extract	80.24**	0.64	43.19	99.99	1
Urea	4.87	0.64	3.27	99.97	10
$NaNO_3$	12.47	0.64	3.83	82.4	7
NH_4NO_3	23.46	0.64	0.17	16.53	5
$(NH_4)_2SO_4$	25.28*	0.64	4.66	99.14	4

**p<0.0001, *p<0.05 level of significance

Table 3 shows the effect of each medium component on DMW production, as well as the standard error, t(*xi*), p, the confidence level, and the order of each component. The components were tested at a confidence level of 95% based on their effects. The component with positive effect with confidence level of more than 95% was interpreted to be required at a higher concentration than the indicated high value (+). However, the significantly negative effect showed that the component was effective in DMW production, but the required amount was lower than the indicated low(-) concentration in plackett-Burman design (Dhandhukia and Thakkar, 2007). All factors in this study had an effect on DMW production with a confidence level of 95% or higher, and were considered significant for DMW by Foc (Table 3). The main effects of components in the DMW production medium are shown in Fig. 1

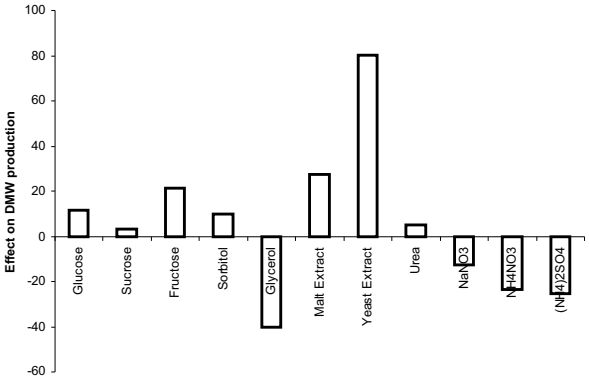

Fig. 1. Main effect of media components on DMW production. The components with positive value increase growth and negative value decrease the growth of Foc.

The yeast extract had the maximum positive effect on DMW production, followed by malt extract, fructose, glucose, sorbitol, urea, and sucrose. The effect of glycerol and other NO_3 salts were negative, indicating that these components are required in the DMW production medium but at a lower concentration than the established low level in the study, or can be omitted from further selection. Contarary to our findings, Gheorghe et al. (2015) reported, the most suitable source of nitrogen for growth of *F. oxysporum f. Sp. glycines*, the pathogen of *Glycine max*, were sodium nitrate, ammonium nitrate, ammonium sulfate, asparagine and peptone. This suggests that different forms of a particular species of *F. oxysporum* have specific requirements.

Yeast extract and malt extract were better components of the medium than any other carbon source tested. Supplementation of one or more "B" group vitamins either as separate or in the form of yeast

extract improved the production of DMW. Yeast extract serves as a source of nitrogen as well as a source of vitamin, but is an expensive element for designing economic media (Dhandhukia and Thakkar, 2007). The results of the Plackett-Burman design indicate that yeast extract is required for growth and should be delivered at a high concentration. Malt extract contains 55% carbohydrates such as maltose (Almeida et al., 2005), it also contains an easily available source of nitrogen, vitamins and minerals that can cause a positive effect on growth. Fructose also had a positive effect on growth.

The ammonical and nitric form of the nitrogen source has a negative effect on the growth of the Foc. In addition, Foc prefers nitrogen from complex sources such as malt extract and yeast extract. Excessive growth can also be due to the presence of vitamins in sufficient quantity together with the readily available essential amino acids present in the yeast extract and malt extract (Almeida et al., 2005).

In previous studies, potato dextrose medium was used to produce elicitors. The maximum achieved DMW of Foc was 5.2 g L^{-1} after 21 days at 27 °C in a static condition (Patel et al., 2004). However, in this study, a three-fold increase in growth was achieved only on a sixth day at 27 °C in a static condition. This suggests that achieving the right combination of medium components by identifying the most significant components, higher biomass of Foc can be achieved in a shorter time. Ahuja et al. (2004) reported the use of the Plackett-Burman design to achieve the exponential growth of aggregated shipworm bacteria. Liu and Wang (2007) have successfully used a statistical approach to biomass optimization and the production of extracellular polysaccharides of *Agaricus blazei*. Similarly, Plackett-Burman design was adopted to determine the media components, ie, magnesium sulphate and ferric ammonium citrate, significantly influencing the antibacterial activity of *Synechocystis aquatilis* (Deshmukh and Puranik 2010). The present study reports several important nutrients useful for increasing the yield of DMW and also identified nutrients that should be used in a lower concentration.

CONCLUSIONS

In contrast to the classical method for optimization of medium components, statistical technique was implemented, where the levels of variables were changed simultaneously to determine their individual impact on biomass production. This study showed that the Plackett-Burman design is a powerful tool in optimizing the composition of the medium used for production of rapid and enhanced biomass of Foc that can be used as an elicitor to combat panama disease.

ACKNOWLEDGEMENT

Authors express their gratitude to Department of Biotechnology, New Delhi for financial support.

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