TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2020) 44: 312-321

doi:10.3906/tar-1905-43

Screening of factors affecting somatic callusing and embryo induction in *Allium cepa* L. through Plackett–Burman methodology

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Received: 13.05.2019 • **Accepted/Published Online:** 26.11.2019 • **Final Version:** 01.06.2019

Abstract: Induction of embryonic callus through available asexual explant culture is applied in the protection and maintenance of cytoplasmic-male sterility and in the maintenance of other lines of onion (*Allium cepa* L.). Usage of the Plackett–Burman (PB) experimental design has been suggested as a screening method for many variables that influence the desired induction. In order to overcome limitations of standard experimental designs and prioritization of media culture components, a systematic investigation was carried out using the PB design. In this investigation, the effects of different factors such as 2,4-D, putrescine, carbohydrate sources (maltose, sorbitol, and sucrose), silver nitrate, glutamine, and glycine concentrations were assayed on callus and embryo induction through mature zygotic embryo explant culture. Out of the eight medium components screened, the four components of putrescine, glutamine, 2,4-D, and glycine were found to contribute positively to the callus production with a maximum production rate of 97.14%. The normal plot and linear regression equation with positive largest coefficient showed that putrescine had the highest significant positive effect with 200 mg/L and three elements comprising 2,4-D and glycine showing a significant reducing effect on callus induction. In embryonic callus induction, a wide range of responses were observed from 0% to 100% in the 21 trials. Results of linear regression data showed that among the eight elements, 2,4-D, glutamine, and glycine had significant effects on embryo production. Glycine and 2,4-D had the highest (26.34%) and the lowest (8.33%) contribution to explaining embryonic callus induction, respectively. A wide range of responses (0 to 44.1 mg) were observed in the Plackett–Burman design with respect to the fresh weight. The regression analysis for the Plackett–Burman design (PBD) demonstrated that glycine, sorbitol, and sucrose were of high influence on the fresh weight ($P \leq 0.05$). Glycine (31.4%) and sucrose (6.7%) were found to have the highest and lowest contribution to the fresh weight of callus, respectively. Thus, this practical article seeks to show some helpful statistical approaches to typical problems in data analyses of tissue culture research.

Key words: Design of experiment, onion, tissue culture, explant number

1. Introduction

The genus *Allium*, belonging to the family Liliaceae, is cultivated worldwide as a condiment, vegetable, and medicinal herb with various applications. In traditional and ancient medicine, onion (*Allium cepa* L.) has been used as an herbal drug for the treatment of various ailments (Nagata et al., 2007; Benitez et al., 2011; Ramakrishnan et al., 2013).

Micropropagation of onion is usually applied in 1) preservation of the plant and its germplasm, 2) hybrid production: a process of seed production through cytoplasmic-male sterility, 3) breeding programs, and 4) application as a specific tool for the achievement of different biotechnological aspects. Some of these methods ensure stability of genetic material, high propagation rate, and protection of explant stems as explained by many advantages of the procedure introduced by Marinangeli (2012). Tissue culture medium screening studies traditionally focus on a few factors studied simultaneously based on simple ANOVA or classical factorial designs, (i.e. nature and concentration of additive components in the media). They are in turn influenced differently by plant species response, growth medium salts, plant growth regulators, and temperature and lighting that affect the success of micropropagation (Akin et al., 2017a; Akin et al., 2017b).

Tissue culture medium screening is a complex process because of the effects and interactions of many factors, and effective nutrient composition screening requires a careful experimental design and statistical analysis (Kovalchuk

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et al., 2017). Most of plant tissue culture studies focus on factorial trials using the three standard designs: 1) completely randomized design, 2) randomized complete block design, and 3) split-plot design that require high number of treatment combinations (Compton, 1994; Compton and Mize, 1999; Nuri et al., 2005). The number of treatments could be reduced through the design of experiment (DOE) methodology (Akin et al., 2017b). It is a statistical tool deployed in various types of system, process, and product optimization designs. It is a multipurpose tool that can be used in applications, such as comparison, screening, transfer, identification, and optimization (Durakovic, 2017).

However, not all categories of experiments may be suitable for all tissue culture studies. Typically, various causes such as limited number of available explants, loss of explants as well as the great number of goals may limit the application of factorial trials in the foregoing designs besides, preventing optimization of factors. Hence, at first goals should be prioritized and then elements under evaluation should be selected in consideration of the budget and time available (Compton, 1994; Compton and Mize, 1999; Nuri et al., 2005). The kind of data obtained in micropropagation studies is often problematic, since they do not follow an even distribution. Additionally, difficulties in the observation of culture vessels further complicate the measurements involving the response of the influencing factors. Therefore, the use of conventional standard analyses often leads to misinterpretation (Ibanez et al., 2003).

A great number of experimental factors influence the induction and production of callus and embryo. Conventional methods for screening and optimizing the experimental factors could lead to unreliable and wrong conclusions and thus waste of time and cost (Zeinab et al., 2015). Use of suitable experimental and statistical designs in in vitro investigations is essential for unbiased precise evaluation of the effects and to ensure appropriate interpretation of the results (Nuri et al., 2005). Statistical analysis is an essential part of biological research. Statistical methods available to biological researchers range from very simple to extremely complex. Therefore, caution should be made in the choice of a statistical method. Where necessary, it is better to avoid complicated statistical procedures because they are difficult to interpret, and may mislead comparison of treatments (Compton, 1994). Advanced methods involving experimental design and statistical analysis provide a more precise way of probing into the issue of mineral nutrient optimization (Kovalchuk et al., 2017). Tissue culture data is generally nonlinear as revealed by the decision tree algorithms. The classification and regression tree (CART) algorithm was used to predict optimal outputs from mixture-design factors. The CART algorithm was found superior to RSM, chi-squared automatic interaction detector (CHAID), and exhaustive CHAID; however, these three models may constitute a better alternative for analyzing other types of tissue culture data sets. The response surface methodology mixture-component design and a data mining algorithm were applied to assess and optimize ionic nitrogen proportions to improve micropropagation (Akin et al. 2017c; Kovalchuk et al. 2018). The Plackett–Burman methodology and fractional factorial design can be used more effectively with only a limited number of explants to test the effects of many factors and to prioritize important ones (Nuri et al., 2005).

The Plackett–Burman design is applied to define the significance of various known and unknown factors and growth conditions for the investigation of the traits being studied (Zeinab et al., 2015). Assuming that interactional effects have been considered negligible, and that the saturated factorial fractional design takes place at the two levels of k+1 treatments, it is possible to estimate the main effects of k factors independently using the Plackett–Burman methodology (Plackett and Burman, 1946). Saturated designs are applied in the early phases of experimentation to define and screen out insignificant factors among a large number of possible components. In full factorial designs, the number of elements increases exponentially leading to an uncontrollable number of trials (Hunter, 1985). Therefore, among fractional factorial methods, Plackett–Burman design is an important method for primary screening of important media elements (Dean at al., 2017). In order to save time and materials, the methodology has switched to statistical analyses that offer superior techniques over conventional designs in being quick and valid. It contributes to understanding the interactions among the materials at various concentration levels, and to lower as much as possible the total number of trials (Zeinab et al., 2015).

The current report is an attempt to screen factors that lead to callus induction and embryo development using the Plackett–Burman design. The main aim of this study is to develop directions for trial designs and data analyses of in vitro experiments.

2. Materials and methods

2.1. Explant preparation and surface sterilization

This investigation was carried out in the tissue culture laboratory of Plant Improvement and Seed Production Research Center (PISPRC), Esfahan (Khorasgan) Branch of Islamic Azad University. The mature seeds of Azar Shahr landraces were obtained from the Seed and Plant Improvement Institute. Seeds were thoroughly washed in sterile distilled water for 5 min. They were then sterilized in 70% ethanol for 90 s before they were immersed

in sterilized water (3 times), and then rinsed in 20% sodium hypochlorite for 5 min. The process continued with washing the explants in sterilized distilled water for 3–5 times. The disinfection continued with 2% sodium hypochlorite treatment for 20 min followed by washing 3–5 times.

The treated seeds were refrigerated at 5 °C for 20–24 h. Under aseptic conditions, the seeds were opened and embryos were isolated with a scalpel blade (Figure 1). The cotyledons were removed and the initial explants were cultured on the different treatments.

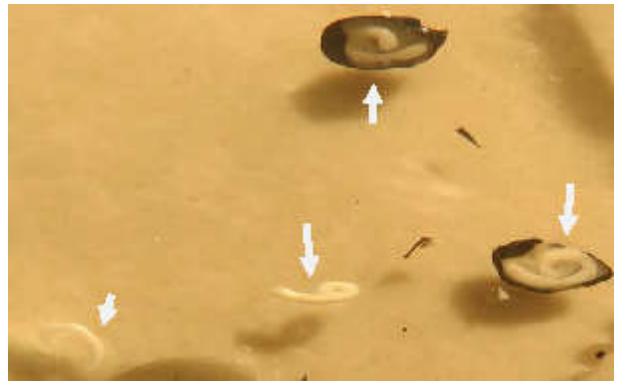

Figure 1. Overall view of the dissected onion seeds and isolated normal embryos.

2.2. Culture media and conditions

MS basal medium (Murashige and Skoog, 1962) was used with some modifications supplemented with plant growth regulators and other additives as needed by the designed experiment in PB methodology. In order to obtain the desired callus and embryonic callus induction, the media components and composition were arranged during the screening study of PBD (Tables 1 and 2). The pH of the media was set to 5.77–5.83. These media were then solidified with 8.0 g/L agar. They were sterilized by autoclaving (at 121 °C, 15 psi, for 20 min). All culture media were incubated at 25 ± 1 °C in dark. After 40 days induction in medium, the amount of callus induction, embryonic callus, and fresh weight of calli were calculated.

2.3. Identification of the significant factors by the Plackett–Burman design (PBD)

The purpose of the current study was to screen effective components (factors) of the culture medium through PB design. The experimental design for testing components of media such as plant growth regulators, carbon and nitrogen sources, and silver nitrate was used for the PB design. Plackett–Burman factorial design with twenty-one different combinations was used. To distinguish the significant influence of the media ingredients, each variable was set at two levels, low, high and center point run as a midway of levels, as shown in Table 1. As mentioned earlier in this section, center points enter the experimental setting runs for two purposes: A) to provide for a measure of process stability and inherent variability, B) to check for the response curvature. The center points help to detect significant inputs and thus estimating variability.

2.4. Statistical analysis using MINITAB 18

All experiments were performed in duplicates and the data consisted of means of independent measurements. The absolute value for the effects was displayed on Pareto chart and a standardized reference line was also included at a 95% confidence level. The effects' values crossing the

Table 1. Different levels of experimental variables as used in the production of calli and embryonic callus using the Plackett–Burman design.

Variables	Media components	Codes	– values (mg/L)	0 values (mg/L)	+ values (mg/L)
X_1	2,4-D	2,4-D	2	3	4
X_2	Putrescine	TDZ	100	150	200
X_3	Silver nitrate	SN	5	7.5	10
X_4	Glutamine	GLU	400	600	800
X_5	Glycine	GLY	2	3	4
X_6	Maltose	M	0	15,000	30,000
X_7	Sorbitol	SO	0	15,000	30,000
X_8	Sucrose	SU	0	15,000	30,000

X1-X7 represents different assigned variables; the sign '+' stands for high concentration of variables, the sign '-' stands for low concentration of variables, and '0' stands for mid-levels of concentration.

Table 2. Plackett–Burman experimental design consisting of 21 combinations of eight variables with coded values along the observed results of screening of significant factors affecting calli, embryonic callusing, and fresh weight.

Run	Variables								Response mean		
	2,4-D (mg/L)	PU (mg/L)	SN (mg/L)	GLU (mg/L)	GLY (mg/L)	M (mg/L)	SO (mg/L)	SU (mg/L)	CI (%)	ECI (%)	FW (g)
1	+1	-1	-1	+1	+1	-1	+1	+1	45.71	59.33	0.044
2	+1	-1	+1	+1	+1	+1	-1	-1	67.14	55.00	0.027
3	–1	+1	–1	+1	+1	+1	+1	–1	55.62	67.50	0.016
4	–1	+1	+1	+1	+1	–1	–1	+1	42.85	95.00	0.021
5	–1	–1	+1	+1	–1	+1	+1	–1	19.89	50.00	0.013
6	+1	–1	+1	+1	–1	–1	–1	–1	0.00	0.00	0.000
7	+1	+1	+1	+1	–1	–1	+1	+1	44.21	47.89	0.016
8	+1	+1	+1	–1	–1	+1	+1	–1	46.38	72.58	0.009
9	–1	+1	–1	+1	–1	+1	+1	+1	59.44	100.00	0.006
10	+1	+1	–1	–1	–1	–1	+1	–1	0.00	0.00	0.000
11	–1	–1	+1	–1	+1	–1	+1	+1	97.14	68.61	0.011
12	+1	+1	–1	+1	+1	–1	–1	–1	0.00	0.00	0.000
13	–1	–1	–1	+1	–1	+1	–1	+1	80.00	91.67	0.005
14	–1	+1	+1	–1	–1	–1	–1	+1	0.00	0.00	0.000
15	+1	+1	–1	–1	+1	+1	–1	+1	49.32	90.20	0.034
16	–1	–1	–1	–1	+1	–1	+1	–1	0.00	0.00	0.000
17	0	0	0	0	0	0	0	0	10.79	100.00	0.034
18	–1	+1	+1	–1	+1	+1	–1	–1	57.14	50.00	0.022
19	+1	–1	+1	–1	+1	+1	+1	+1	77.86	87.7	0.019
20	+1	–1	–1	–1	–1	+1	–1	+1	31.43	75.00	0.0441
21	–1	–1	–1	–1	–1	–1	–1	–1	43.06	0.00	0.005

Abbreviation: PU: Putrescine, SN: Silver nitrate, GLU: Glutamine, GLY: Glycine, M: Maltose, SO: Sorbitol, SU: Sucrose, CI: Callus Induction, ECI: Embryonic Callus Induction, FW: Fresh weight.

reference line were considered statistically significant ($P \leq 0.05$). Additionally, the influence of the parameters, either positive or negative, was recognized with regression coefficient effects. This model does not describe the interaction among the factors, but only evaluates and selects important factors that influence the response (Abdel Mawgoud and Dawoud, 2013).

A positive regression coefficient in tabular columns indicated a synergistic effect on improving medium components, whereas a negative coefficient showed an antagonistic effect. The effect of medium improvement of the components was analyzed using the percentage of calli, embryonic calli induction, and their fresh weight as responses. The data were subjected to the normality and homoscedasticity tests prior to the analysis of variance using MINITAB 18. The results obtained were subjected to regression analysis, and the analysis of variance (ANOVA) was performed using the MINITAB 18.

3. Results and discussion

The current study was performed to develop an efficient protocol for calli and embryonic callus induction of onion. Induction potential systems of the Iranian onion landrace were assayed and investigated using the Plackett–Burman design. A total of 294 mature zygotic embryo explants from Azar Shahr landrace seeds were aseptically removed and incubated under 21 different treatments (Table 2).

3.1. Callus observation

Various callus formations could be observed after 6–8 days of incubation. Anatomically, after a few days, the explants grew two to three times of their original size. Zygotic embryo explants were evaluated for calli production potential, and this induction has been shown to result in responsive explants. Among the important morphological signs in callogenesis are cases such as primary elongation, swelling of the tissue, and callus formations in the edges of each responding explant, as well as unorganized cell division.

Confirming the current report, Ikeuchi et al. (2013) declared that presence or absence of biotic and abiotic stimuli around the plants lead to the production of calli and tumors as a group of undifferentiated cells. In some cases, the development of calli in plants emerges after exposure to various natural or artificial harsh growth situations. In the current study, a wide range of morphological variations were also found in different systems of callus production among the investigated experiments. Based on morphological observations, three different callus types and embryo structures could be easily distinguished: 1. compact, white or green nodular types, 2. watery, transparent types, and 3. friable types with no apparent structures were seen from such cases (Figures 2).

Ikeuchi et al. (2013) concluded that these plants have powerful systems to prevent undesirable callus induction for the maintenance of tissue structures and in some cases, these systems lead to lack of desired induction. The calli could be classified into subgroups such as friable, compact, rooty, shooty, or embryonic calli. This nomenclature comes from the apparent regeneration.

3.2. Plackett–Burman analysis

3.2.1. Screening of vital medium ingredients

The first step of screening for optimal conditions is recognizing the variables that significantly affect the tested responses. It also seeks to define appropriate ranges of variables. In the current study, the elements involving the culture medium were set for 8 different factors (Table 1). In evaluating the DOE-history, its concept, and its relevance to the in vitro culture, Niedz and Evens (2016) stated that DOE is a large and well-developed methodology for understanding and improving the performance of complex systems. Because in vitro culture systems are complex and could be easily manipulated under controlled conditions, they are particularly well-suited for the application of DOE principles and techniques.

3.2.2. Callus induction

The results demonstrated that a wide range of responses to callus induction (0 to 97.14%) were achieved in this experiment. Maximum induction of calli formation was observed in the 11th trial (+ve concentration of silver nitrate, glycine, sorbitol, and sucrose, as well as – ve concentration of 2,4-D, putrescine, glutamine, and maltose). No responses were observed in the 6th, 10th, 12th, 14th, and 16th trials (Table 2). The contribution percentage, adjusted mean-square (Adj MS) and P-values on traits under investigation were found appropriate (Table 3). Statistical analyses indicated that there were distinct differences in the calli induction frequencies among all trials when different levels of studied factors used in zygotic embryo explants were involved. The results demonstrated that Putrescine had the highest (36.94%) and glycine had the lowest (8.67%) contribution in explaining callus induction frequency (Table 3).

Pawar et al. (2015) stated that sorbitol with osmotic characteristics not only improved the quantity, but also the quality of calli and embryos produced (Hassan et al., 2009). In the current study, 2,4-D, putrescine, glutamine, and glycine had the following 10.21% + 36.94% + 13.28% + 8.67% individual contributions, respectively out of the total 72.14% model contribution on data explanation (Table 3). The effects of proline and glutamine on improvement of calli growth induction in rice were assayed by Pawar et al. (2015) and the results showed that the highest callus formation (85.3%) was obtained from mature embryo explants tested on Murashige and Skoog (MS) media fortified with 2.0 mg/L 2,4-dichlorophenoxyacetic acid, 500 mg/L proline, and 500 mg/L glutamine. They mentioned that media supplemented with amino acids like glutamine and proline provided superior results over the media without them.

The current results of the modeling experiment of components by PBD showed that only 4 out of 8 additive components significantly influenced the callus production (Table 3). Figure 3 shows the normal plot of the standardized effect of the components with significant effects that display the vector nature of their effects. This plot indicated that putrescine had the highest level of significant positive effect on callus production taking place on the right side of the response line. Moreover, Figure 3 shows a significant reducing effect of 2,4-D and glycine with studied concentrations on callus induction as its effect takes place on the left side of the callus production line. The analysis of regression for significant components has been shown in Table 3; it confirms that putrescine has

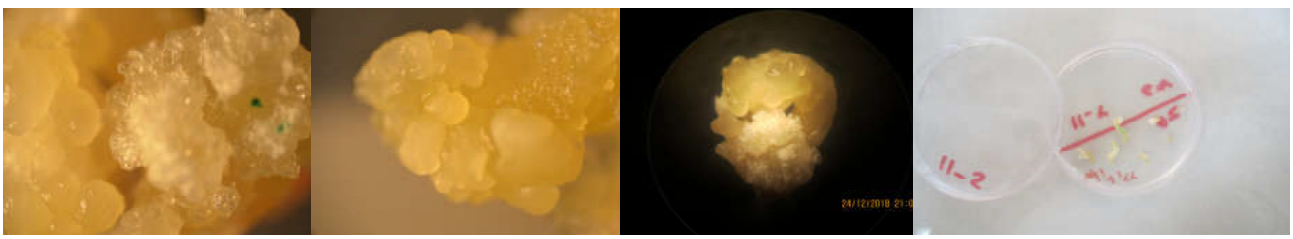

Figure 2. Morphological structure of callus and embryo responding to different culture media.

Table 3. ANOVA of the regression among the eight factors affecting the investigated traits.

Sources	DF	Contribution (%)			Adj. MS and P-value					
		CI	ECI	FW	CI		ECI		FW	
					Adj. MS	P-value	Adj. MS	P-value	Adj. MS	P-value
Model	9	72.14%	61.42%	66.78%	3215.1	0.000	4751.1	0.000	0.00072	0.000
Linear	8	72.07%	52.98%	64.38%	3613.6	0.000	4611	0.000	0.000781	0.000
2,4-D	1	10.21%	8.33%	0.08%	4096.3	0.002	5801.7	0.013	0.000008	0.783
Putrescine	1	36.94%	4.22%	3.52%	14817	0.000	2938.3	0.071	0.000342	0.075
AgNO3	1	0.41%	0.37%	3.79%	166.2	0.495	254.7	0.586	0.000368	0.065
Glutamine	1	13.28%	9.29%	0.3%	5327.6	0.000	6466.9	0.009	0.000029	0.595
Glycine	1	8.67%	26.34%	31.4%	3477.1	0.003	18341.2	0.000	0.003048	0.000
Maltose	1	0.16%	0.02%	2.2%	62.7	0.675	12.6	0.903	0.000213	0.155
Sorbitol	1	0.02%	3.63%	16.4%	9.4	0.871	2527.7	0.092	0.001592	0.000
Sucrose	1	2.37%	0.78%	6.7%	952.3	0.108	544.9	0.426	0.00065	0.016
Curvature	1	0.07%	8.43%	2.4%	27.7	0.780	5872.2	0.013	0.000233	0.138
Error	32	27.86%	38.58%	33.22%	349.2	-	839.4	-	0.000101	-
Lack-of-Fit	11	14.97%	20.53%	17.45%	545.8	0.056	1299.7	0.061	0.000154	0.068
Pure Error	21	12.89%	18.05%	15.77%	246.3	-	598.3	-	0.000073	-
Total	41	100.00%	100.00%	100.00%	-	-	-	-	-	-

Abbreviations of CI, ECI, FW, and Adj. stand for callus induction, embryonic callus induction, fresh weight, and adjusted, respectively.

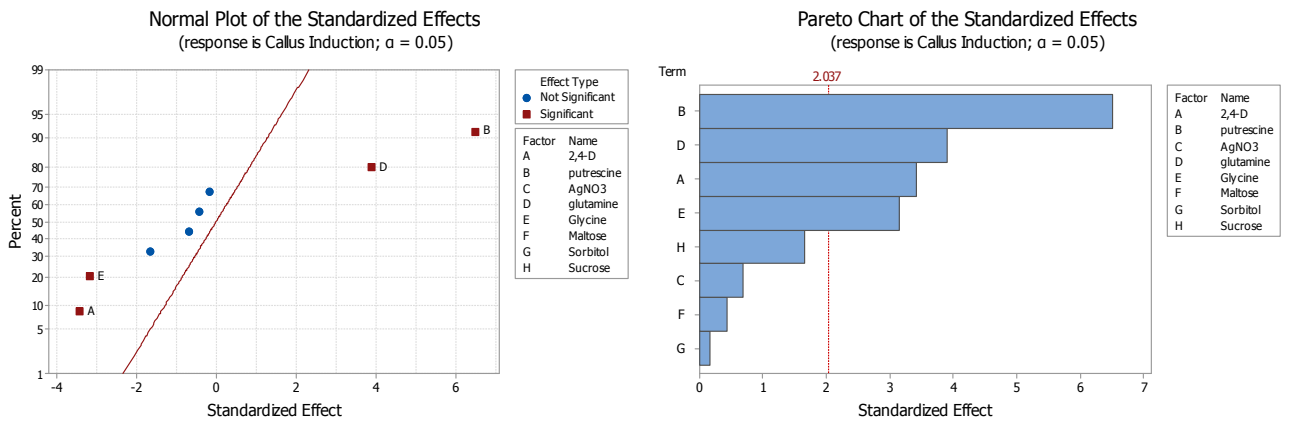

Figure 3. Normal plot and pareto chart of the standardized effects of callus induction in media of different components.

the most significant ($P \leq 0.05$) effect on callus induction. The linear regression coefficient (Table 4) of the adjusted R^2 value of the 72.14% indicated that the uncoded equation of model (Table 5A) was significant and could exhibit variations of the response (72.14%). The equation showed that putrescine produced the largest coefficient, which once again confirms its great developmental effect on callusing.

The Pareto chart analysis was used to identify the key components of callus production based on PBD (Figure 3). This chart showed that the selected four factors, i.e. putrescine, glutamine, 2,4-D, and glycine with t-values above threshold (2.037) and P-values lower than 0.05 had a significant influence on the desired response.

3.2.3. Embryonic callus induction

The regression analysis (Table 3) revealed that the components 2,4-D, glutamine, and glycine had significant effects on the systemic response as their P-values were above the selected criteria for 95% level of confidence. The contribution results showed that glycine had the highest

Table 4. Regression coefficient and corresponding t-value and P-value of the desired traits in the zygote cultures of the Plackett–Burman design.

Terms	Traits								
	Callus induction			Embryonic callus induction			Fresh weight		
	RC	t-value	P-value	RC	t-value	P-value	RC	t-value	P-value
Constant	39.25	13.28	0.000	55.52	12.12	0.000	0.01602	10.09	0.000
2,4-D	–10.12	–3.42	0.002	–12.04	–2.63	0.013	0.00044	0.28	0.783
Putrescine	19.25	6.51	0.000	8.57	1.87	0.071	–0.00292	–1.84	0.075
AgNO3	–2.04	–0.69	0.495	–2.52	–0.55	0.586	–0.00303	–1.91	0.065
Glutamine	11.54	3.91	0.000	12.72	2.78	0.009	0.00085	0.54	0.595
Glycine	–9.32	–3.16	0.003	–21.41	–4.67	0.000	–0.00873	–5.50	0.000
Maltose	–1.25	–0.42	0.675	0.56	0.12	0.903	–0.00231	–1.45	0.155
Sorbitol	–0.48	–0.16	0.871	–7.95	–1.74	0.092	–0.00631	–3.97	0.000
Sucrose	–4.88	–1.65	0.108	3.69	0.81	0.426	–0.00403	–2.54	0.016
Center point	3.8	0.28	0.780	–55.5	–2.64	0.013	–0.01105	–1.52	0.138

Abbreviation: RC: regression coefficient.

Table 5. Regression equations of the fitted models.

No.	Regression equations
A	$Y_1 = 17.9 - 10.12\ X_1 + 0.3849\ X_2 - 0.82\ X_3 + 0.0577\ X_4 - 9.32\ X_5 - 0.083\ X_6 - 0.032\ X_7 - 0.325\ X_8 + 3.8$ Ct Pt
B	$Y_2 = 103.3 - 12.04\ X_1 + 0.1714X_2 - 1.01X_3 + 0.0636X_4 - 21.41X_5 + 0.037X_6 - 0.53X_7 + 0.246X_8 - 55.5$ Ct Pt
C	$Y_3 = 0.0688 + 0.00044\ X_1 - 0.000058\ X_2 - 0.001213\ X_3 + 0.000004\ X_4 - 0.00873\ X_5 - 0.000154\ X_6 - 0.000421\ X_7 - 0.000269\ X_8 - 0.01105$ Ct Pt

Y1: callus induction; Y2: embryonic callus induction; Y3: fresh weight; X_1: 2,4-D; X_2: Putrescine; X_3: Silver Nitrate; X_4: Glutamine; X_5: Glycine; X_6: Maltose, X_7: Sorbitol, X_8: Sucrose.

(26.34%) and 2,4-D had the lowest (8.33%) contribution on explaining ECI frequencies. Briefly, the contributions sum 43.96% of 61.42% modeling contribution on data explaining was observed, where three factors including 2,4-D, glutamine, and glycine with 8.33% + 9.29% + 26.34% individual contributions, respectively, showed significant effects on the desired response.

Pawar et al. (2015) concluded that applying auxins like 2,4-D as a synthesized PGRs in general enhanced embryogenic callus induction, whereas these cells are often capable of synthesizing all amino acids required. In some cases, addition of components such as proline and glutamine to the nutrition media may contribute to cell growth.

The results of the current modeling study (Table 3) showed that the influence of only 3 out of 8 additive components on embryo production were significant. The 38.58% of the total contribution proved erroneous, including lack-of-fit (20.53%) and pure error (18.05%). The results of statistical analysis showed that there were significant differences in the production of embryonic callus among all trials when different levels of investigated components via zygotic embryo explants culture were applied. In embryonic callus induction, wide ranges (0 to 100%) of responses up to the 21st trial were observed. Although an acceptable response was reached in callusing in the 11th trial, the maximum induction of embryos on the produced calli were found to be in the 9th and 17th trials. In the 9th trial, the +ve concentration putrescine, glutamine, and all kinds of carbon sources, together with –ve concentration of 2,4-D, silver nitrate and glycine were applied. The 17th trial was the center point (Table 2). The glutamine had the highest significant positive effect on embryo production since its effect takes place further right on the production line (Figure 4).

Because of the important role of amino acids, as readily available sources of nitrogen in enhancing growth, the callus development by amino acid could be relied on. In this

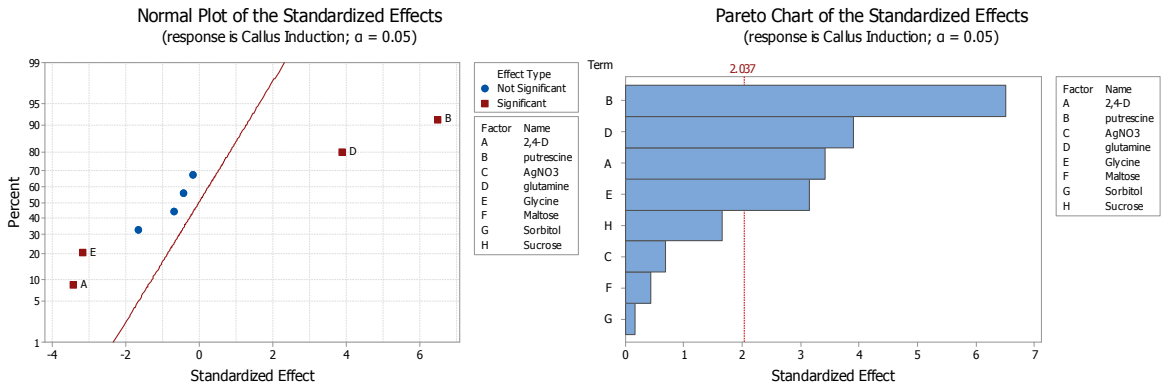

Figure 4. Normal plot and pareto chart of the standardized effects of embryonic callus induction in media of different components.

group, the proline and glutamine as a nontoxic component were effective in the maintenance of a prolonged high growth rate (Pawar et al., 2015). However, the plot showed a significant reducing effect of 2,4-D, sorbitol, and glycine on the desired traits since its effect is positioned to the left of the resulted line (Figure 4). The osmotic stresses are reported to be important for the induction of embryogenic cultures in wheat. As a hygroscopic element, sorbitol creates osmotic stress and plays an important role in improving callus formation (Hassan et al., 2009).

The results showed that glycine had the most significant enhancement effect on embryo induction ($P \leq 0.05$). The linear regression coefficient, adjusted R^2 of 61.42%, showed that the uncoded equation model was significant and could explain 61.42% of the variability in the response data (Table 5B). It showed that glycine had the largest negative coefficient, confirming once again its strong encasing effect on the embryo callus production. In another research, Ibrahim et al. (2014) concluded that both putrescine and salicylic acid may have a positive effect on increasing callus growth and regulation of somatic embryogenesis in *Phoenix dactylifera* L. The number of embryos increased proportionally after the application of putrescine to the medium until its concentration reached a significant level of 2.0 and 3.0 mM and induced 9.1 and 10.3 embryos, respectively. Putrescine is a polyamine with low molecular weight and has been applied in many cellular processes such as cell division, protein synthesis, and DNA replication (Ibrahim et al., 2014).

3.2.4. Fresh weight assessment

The response of the Plackett–Burman design indicated that there are wide ranges of responses to fresh weight (0 to 44.1 mg) in our study. The highest level of fresh weight was obtained in the 20th trial consisting of (+ve concentration of 2,4-D, maltose, and sucrose, as well as –ve concentration of putrescine, silver nitrate, glutamine, glycine, and sorbitol). In the 6th, 10th, 12th, 14th, and 16th trials, no response was achieved (Table 2). The regression analysis of PBD (Table 3) showed that the studied components of glycine, sorbitol, and sucrose had a significant effect on fresh weight because their P-values were above the selected criteria at 95% level of confidence. The response to the application of soluble sugars as carbon sources indicated that date palm callus fresh weight would be promoted with sucrose, fructose, and glucose, and would be declined with mannitol (Abdel-Rahim et al., 1998).

Supplementing the medium of callus initiation with putrescine showed a significant increase in both fresh and dry weights at 2.0 mM concentration putrescine, while at other levels, although the weights increase, they do not reach a significant level (Ibrahim et al., 2014). In summary, the modeling results indicated that the effects of only 3 out of 8 additive components were significant. The contribution results showed that glycine had the highest (31.4%) and sucrose had the lowest (6.7%) contribution on explaining fresh weight. Glycine, sorbitol, and sucrose with 31.4% + 16.4% + 6.7% individual contributions, respectively summing up to 54.5% out of 66.78% modeling contribution on data explanation indicated significant effects on the fresh weight response (Table 3).

Our statistical analyses indicated that different levels of components studied showed a significant difference among fresh weights in all trials. However, the highest amount of fresh weight was found in the 20th trial and the response to the 1st trial with different components arrays did not show a significant difference in this respect (of +ve concentration 2,4-D, glutamine, glycine, sorbitol, and sucrose, as well as –ve concentration putrescine, silver nitrate, and maltose) (Table 2). Here, glycine had the highest significant negative effect on fresh weight, since its effect was positioned the furthest to the left of the response line. However, this plot showed a non-significant effect of 2,4-D, glutamine, and maltose on the desired trait. It is because its effect was positioned near the result line (Figure 5). Putrescine,

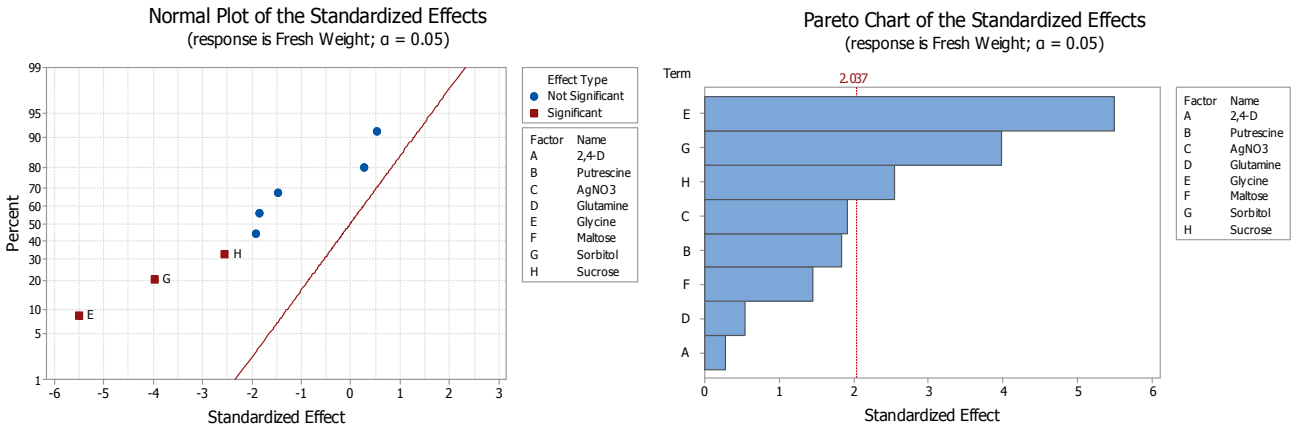

Figure 5. Normal plot and pareto chart of the standardized effects of fresh weight in media of different components.

spermidine, and spermine are the most abundant polyamines among the higher plants, which are involved in the various developmental processes (Tonon et al., 2004).

The results of regression data showed that glycine had the most significant enhancing effect ($P \leq 0.05$) on the fresh weight. The linear regression, adjusted R^2 of 66.78%, showed that the uncoded equation model was significant and could account for the 66.78% of the variability in the response data. The equation indicated that glycine had the largest negative coefficient, confirming once again its strong encasing effect on the desired traits (Table 5C).

In conclusion, we have established an applicable methodology and procedure for the enhancement of the embryonic callus induction of Iranian onion's mature zygotic embryo explants. In tissue culture and new aspect of statistical application, the Plackett–Burman methodology can be used more effectively with only a limited number of explants to evaluate the effects of many factors and to screen important ones. Among the advantages of our trial over other conventional designs is the lowest number of trials needed. Moreover, we can obtain more callus and embryo induction using the lower treatment combinations of factors influencing the tissue culture responses in *Allium cepa*. Using this methodology in future studies, more significant components that affect the production of callus and embryo can be screened and used for media optimization. The development of this methodology is thought to be of vital importance for further studies.

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