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OPTIMISATION OF TETRAZOLIUM CONCENTRATION AND IMMERSION TIME IN THE VIABILITY TEST OF *SWIETENIA MACROPHYLLA* SEEDS BY USING RESPONSE SURFACE METHODOLOGY

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Abstract.The aim of this study was to optimise tetrazolium concentration and immersion time in the viability test of *S. macrophylla* seeds by using Response Surface Methodology (RSM). For this, a RSM Central Composite Design (CCD), type 2^2 was applied. The quantification of viable and non-viable seed germs was performed using the interpretation of topological patterns. The viability of the seed expressed as a percentage was selected as a response variable whilst the tetrazolium concentration and immersion time were independent factors. The quadratic polynomial model of the four evaluated aspects was best adjusted with 0.99 and 0.93 for the coefficients R^2 and Predicted-R^2, respectively. Using ANOVA, it was demonstrated that only immersion had a significant effect. The optimisation study showed that it is possible to achieve values of viability above 90% at low tetrazolium concentrations (0.05%) using immersion times between 75 and 90 minutes.

Keywords: embryo, response surface methodology; prediction, forest.

1 Introduction

Swietenia macrophylla King, also known as big leaf mahogany, is a tropical tree species native to Central and South America. *S. macrophylla* has a wide natural distribution, however it is nearly extinct in Ecuador, Colombia, Panama and Costa Rica. The depletion of this species has led to concern for its future (Krisnawati et al. 2011; Lugo & Alayón, 2003). This species has a high commercial value due to its multiple uses. Its heartwood is moderately resistant to dry-wood termites and of unsurpassed natural beauty (Wadsworth & González, 2008). Also, it has great potential for reforestation and afforestation, particularly for improving soil (Krisnawati et al. 2011). For this reason, the production and use of seeds from this species plays an important role.

Amongst the various methods of seed quality control, the tetrazolium test has been noted for its speed, accuracy and the large amount of information that can be obtained through it (Lakon, 1942). This test is based on the activity of dehydrogenase enzymes in respiring tissue that reduces the 2,3,5-triphenyl tetrazolium chloride to stable and insoluble red formazan (Marin et al. 2017). Recently, several papers have been published on the application of the TZ test to evaluate the viability and vigor of seeds in a wide variety of species. Amongst those we can find: *Carapa guianensis* Aubl. and *Carapa surinamensis* Miq. (Amoedo & Ferraz, 2017), S*imira gardneriana* (Oliveira et al. 2016), *Cucumis anguria L* (Paiva et al. 2017), *Actinidia deliciosa* (Windauer et al. 2016) and *Vaccinium ashei* (de Azevedo Pasqualini et al. 2016). In addition, Marin et al. (2017) studied eight European native species (*Centaurea nigra, Cyanus segetum, Knautia arvensis, Papaver rhoeas, Prunella vulgaris, Rhinanthus minor, Silene vulgaris* and *Valeriana officinalis*). The authors confirmed that there is a significant relationship between TZ test results and maximum final germination, with a correlation coefficient of 96% between the two tests.

However, increasing the efficiency of the germination processes or reducing the costs and time for seed germination are determining factors in order to be able to obtain a greater number of plants. In this sense, one can find a study to improve the efficiency and reliability of the tetrazolium test for peanut seeds (Santos et al. 2012). However, it does not consider the impact of variables or

their interactions. In addition, the reviewed studies consider values of TZ concentrations up to 0.5% or 0.5% to 1%. No investigation was found that considered a wide range of concentrations. To carry out a study with these characteristics, statistical tools such as Response Surface Methodology (RSM) can be applied. This multivariate statistics technique allows for a significant reduction in the number of experiments and the description of the impact of the independent variables (individually or in combination) in the process (Amini & Younesi, 2009; Witek-Krowiak et al. 2014).

Optimisation is a method for determining the best solution on the basis of certain criteria. It allows for the analysis of the variables using a system where the mathematical relationship of the factors and the independent variable is unknown. It is a powerful data modelling tool, which is able to capture and represent complex nonlinear relationships between dependent and independent variables. It almost always improves the process yield and reduces costs (Witek-Krowiak et al. 2014).

The aim of this study was to optimise of tetrazolium concentration and immersion time in the viability test of *S. macrophylla* seeds by using Response Surface Methodology.

2 Materials and methods

2.1 Seed material

In the period between February and March 2017, a total of approximately 400 seeds were collected from *S. macrophylla* trees located in the Amazon rainforest in the Nushino Ishpingo community, in the Arajuno parish, Pastaza province, Ecuador (see figure 1). This zone is characterised by having an infratropical and lower thermotropical rainy bioclimate with an average temperature of 24ºC, an altitudinal variation of <350 masl and an annual rainfall of between 2.346 and 3.723mm. This ecosystem includes forest communities with great compositional variation, as it is one of the most floristically diverse areas of the Amazon (Iglesias et al. 2013).

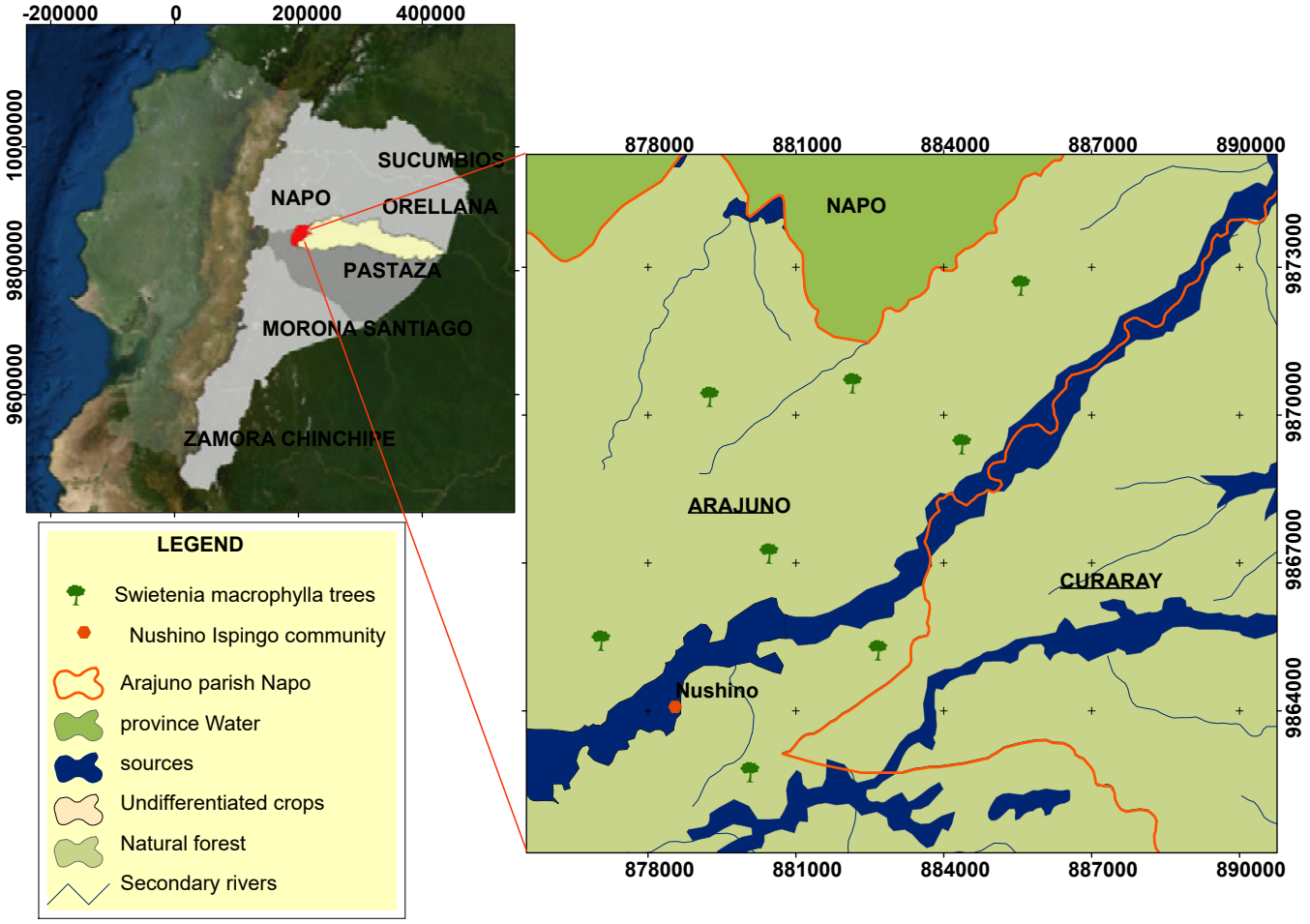

Figure 1: Geographical distribution of the *S. macrophylla* trees recollected.

2.2 Characteristics of *S. macrophylla* seeds

The following characteristics of *S. macrophylla* seeds were determined using guidelines outlined by ISTA (1999) (length 8.42 ±1.2 cm, width 2.54 ±0.4 cm, weight per seed 4.048 ±0.9 g, moisture 11.178 ±0.16 %, purity 95.3 ±2.87 %). The purity analysis was made on many of the 400 seeds collected. To determine the purity, three random samples of approximately 50g apiece were taken, before each sample was separated into pure and impure seeds. The separation was performed manually, and both components were weighed to determine purity based on weight. The weight of the seeds was determined using 10 samples of 10 pure seeds apiece. The moisture content of the seeds was determined in an oven at 105˚C for 24 hours using five 20g seed samples according to the standard (ASTM-E871-82, 2006). Results are expressed as percentages on a fresh weight basis.

2.3 Tetrazolium test

The TZ test was performed according to the procedure outlined by Marin et al. (2017), with some modifications. Twenty-five seeds in each run were used following our experimental planning. Prior to staining, the seeds were preconditioned; this process consisted of keeping the seeds wrapped in germination paper for sixteen hours at 25ºC. To avoid moisture loss, the seed wrappings remained in a dessicator with water instead of silica gel. This allowed for the activation of the enzyme system and softened the seed coat, ensuring the proper development of the stain in the tissue and thus more reliable results.

The prepared seeds were immersed in a 2,3,5-triphenyl tetrazolium chloride solution (concentration of assay) at 35ºC at the time of the experiment. After staining, the seeds were analysed as viable or non-viable on the basis of the staining patterns and the soundness of seed tissue.

Once the ideal colouration was achieved, the seeds were washed in running water and kept submerged in water until the time of evaluation.

For the analysis of the seeds, a longitudinal cut was made on the dorsal side of the seeds following the central axis in order to extract the germ.

Viable and non-viable embryos were quantified according to established topological patterns. The results were expressed as percentages of viable seeds. The interpretation of the topological patterns was as follows:

Viable:

- Embryo with a reddish stain all over.
- Embryo with no stain on top of both cotyledons. The rest of the structures had a reddish stain.
- Embryo with no stain in a cotyledon. The rest of the structures had a reddish stain.
- Absence of staining in the lower half of the hypocotyl and radical meristem. Upper half of the hypocotyl, stem meristem and cotyledons had a reddish stain.
- Absence of staining in the sub-apical radical meristem. Hypocotyl, stem meristem and cotyledons had a reddish stain.

Not viable:

- Absence of staining in most of both cotyledons. The rest of the structures had a reddish stain.
- Absence of staining in both cotyledons. Apical meristem and caulinar hypocotyl had a reddish stain.
- Absence of staining in apical caulinar meristem and cotyledons. Hypocotyl and sub-apical radical meristem had a reddish stain.
- Absence of hypocotyl staining and radical sub-apical meristem. Caulinar apical meristem and cotyledons had a reddish stain.
- Absence of staining in the upper half of the hypocotyl. Caulinar apical meristem, cotyledons and lower half of the hypocotyl and radical meristem had a reddish stain.
- Absence of staining in most of the hypocotyl, caulinar apical meristem and cotyledons. The lower part of the hypocotyl and radical meristem had a reddish stain.

2.4 Statistical analysis

In this research, the tetrazolium concentration and immersion time effects were considered as the independent variable, and the percentage viability was measured as the response variable. The two factors and the central point (low, medium, high) in coded and uncoded independent variables are shown in Table 1.

Table 1: The level of uncoded and coded variables chosen for the RSM-CCD Design type.

Independent Variable	Coded variable level		
	Low	Medium	High
	-1	0	1
Tetrazolium conc. (%)	0.05	0.53	1.00
Immersion time (min)	15.00	52.50	90.00

The experimental runs were carried out according to a set of conditions provided by means of the Design Expert

Table 2: RSM – CCD experimental planning in uncoded and coded forms of the independent and response variable for the *S. macrophylla* seeds.

*Run No.	TZ Conc (%)	Time (min)	Viability (%)
1	0.53 (0)	52.5 (0)	84
2	0.05 (-1)	52.5 (0)	80
3	0.53 (0)	15.0 (-1)	37
4	0.05 (-1)	15.0 (-1)	36
5	1.00 (+1)	52.5 (0)	78
6	0.53 (0)	90.0 (+1)	90
7	0.53 (0)	52.5 (0)	79
8	0.05 (-1)	90.0 (+1)	95
9	0.53 (0)	52.5 (0)	80
10	1.00 (+1)	90.0 (+1)	93
11	1.00 (+1)	15.0 (-1)	47

* Non-randomised

software, version 10.0.3 (Stat Ease, USA), as seen in Table 2.

The RSM type CCD was employed for the optimisation of the factors considered in the study. The viability of the seed expressed as a percentage was selected as a response variable whilst the tetrazolium concentration and immersion time were independent factors. Statistical analysis for the determination of significant effects and interactions was used for the analysis of variance (ANOVA). During the first stage, four polynomial models were evaluated (linear, two-factor interactions (2FI), quadratic and cubic) for the selection of the best model. A second-order polynomial equation was established, as follows:

$$Viability\,(\%) = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j \quad (1)$$

where β_0, β_i, $\beta_{ii}$and β_{ij} are the regression cofficients for intercept, linear, quadratic and interaction terms, respectively. The various x_i and $x_j$values are the independent variables affecting the response, $Viability$ and k is the number of variables (Crespo et al. 2017; Kiran et al. 2016).

3 Results and Discussion

RSM is a powerful data modelling tool, which the allows capture and representation of nonlinear relationships between independent variables and responses (Witek-Krowiak et al. 2014). The application of the RSM with a central composite design (CCD), is a commonly used statistical methodology employed to design and evaluate experiments, and optimize specific targets (Mabayo et al. 2018; Mohammadpour et al. 2019; Ocholi et al. 2018; Sulaiman et al. 2019).

The first part of this study consisted of the evaluation of four polynomial models to determine which is more satisfactory for our experimental data. The determination coefficients were used as selection criteria. Regarding the second-order polynomial, all values were above 0.9, which is considered a good agreement. The R-Squared value of 0.989 obtained suggested that the quadratic model had captured most of the variation in the experimental data (˜99%). Furthermore, a difference of less than 0.2 between the values of the adjusted R-squared (0.978) and predicted R-squared (0.9287) was desirable from a statistical viewpoint (Anderson & Whitcomb, 2016). A summary of the results obtained for the four polynomial models is presented in Table 3.

3.1 Optimisation of the tetrazolium concentration and immersion time by using RSM

According to the results, the quadratic polynomial model was applied for the optimisation of the tetrazolium concentration and immersion time based on RSM type CCD. Table 4 outlines the results of ANOVA for a model selected in the study.

From the results of this analysis, it was possible to determine that of the two factors studied, the immersion time (B) had a significant effect, with a p-value <0.0001 and its quadratic coefficient, whilst the effect of the tetrazolium concentration was not significant, with a

Table 3: Statistical summary of the results obtained for the four polynomial models considered in the optimisation of the *S. macrophylla* seeds.

Source	p-value		R-Squared			
	Sequential	Lack of Fit	Estimated	Adjusted	Predicted	
Linear	0.0003	0.0658	0.8699	0.8373	0.7564	
2FI	0.4988	0.0591	0.8787	0.8267	0.6490	
Quadratic	0.0025	0.3698	0.9890	0.9780	0.9287	Suggested
Cubic	0.6261	0.2013	0.9919	0.9731	0.3406	

Table 4: Results of ANOVA for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	4739.71	5	947.94	89.70	< 0.0001	Significant
A-TZ Conc	8.17	1	8.17	0.77	0.4196	
B-Time	4160.67	1	4160.67	393.72	< 0.0001	
AB	42.25	1	42.25	4.00	0.1020	
A2	4.21	1	4.21	0.40	0.5555	
B2	511.58	1	511.58	48.41	0.0009	
Residual	52.84	5	10.57			
Lack of Fit	38.84	3	12.95	1.85	0.3698	Not significant
Pure Error	14.00	2	7.00			
Cor Total	4792.55	10				

p-value>0.05. In addition, the interaction of the factors (AB) and the quadratic term of factor A did not influence the response variable. A non-significant lack of fit is suitable, as it implies that the model is appropriate for representing the data inside the range studied.

Figure 2 illustrates plots of the viability values, computed on the bases of the second-order polynomial model and values determined experimentally. This distribution confirmed that the model is adequate to represent the experimental data. Thus, it is suggested that the model can be employed satisfactorily.

The polynomial equation in terms of coded factors obtained from a regression analysis can be expressed as follows:

$$\text{Viability} = 79.68 + 1.17A + 26.33B - 3.25AB + \\ + 1.29A^2 - 14.21B^2 \quad (2)$$

This equation may be used for recognising the relative effect of the significant factors by comparing their coefficients, whilst the uncoded equation in terms of actual factors can be used to make predictions about the response variable in the range considered for each factor. The equation in terms of the actual factors:

$$\text{Viability} = 10.22 + 6.034A + 1.859B - 0.182AB + \\ + 5.72A^2 - 0.010B^2 \quad (3)$$

From the surface plot shown in Figure 3, the relationship between tetrazolium concentration and immersion

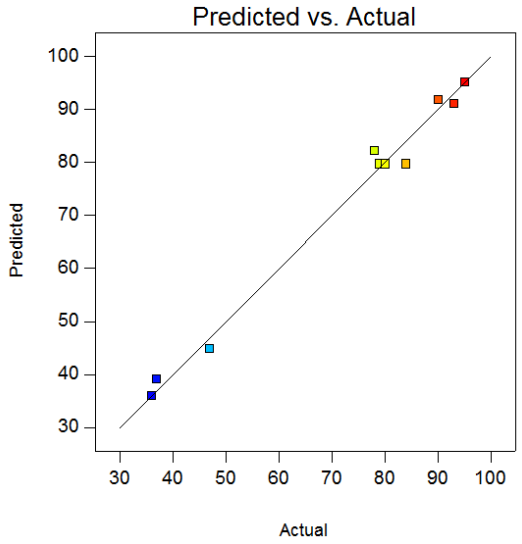

Figure 2: Relationship between actual and predicted data for the viability (%) of *S. macrophylla* seeds.

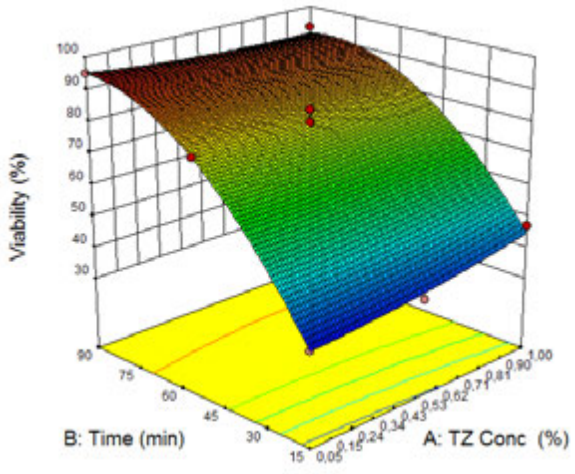

Figure 3: Response Surface Plot of the effects of tetrazolium concentration and immersion time on the viability percentage of *S. macrophylla* seeds.

time on the viability percentage of *S. macrophylla* seeds can be appreciated.

As illustrated in the figure, high staining percentages can be obtained from time-spans greater than 70 min, regardless of the tetrazolium concentration that is used (for the range studied). This is an interesting result from an economic point of view, considering the saving it produces in terms of the reagent, which can be reduced by twenty-fold when working at low concentrations of tetrazolium. However, by employing higher concentrations of tetrazolium, extended times are also required to achieve a high colouring profile. This confirms that the concentration of tetrazolium does not have a significant effect. Paiva et al. (2017) evaluated the viability of *Cucumis anguria* L seeds at three concentrations of TZ (0.1, 0.075 and 0.05%). They concluded that a concentration of 0.05% allows for an adequate colouration and more precise identification of the viable seeds. Similar results were reported by Sarmento, et al. (2013) in the colouration of *Acca sellowiana* seeds under the following experimental conditions: TZ conc. of 0.05% for 2 hours, at 40ºC. Moreover, it is necessary to highlight that testing at a temperature of 40ºC requires lower TZ concentrations. This may be due to the increase in the speed of respiration of germ tissues, the activity of the dehydrogenase enzymes involved in this process and the amount of formazan formed inside the cells, because the increment in temperature accelerates the metabolic reaction of the seeds (Paiva et al. 2017). This is in concordance with the results observed in this research.

4 Conclusion

RSM based on a CCD approach was applied to optimise the immersion time and the concentration of tetrazolium for the viability test for *S. macrophylla* seeds, which is a feasible tool to optimise experimental conditions on a laboratory scale and to guarantee greater reliability. Four polynomial models were evaluated and that which best represented the experimental data was the quadratic second-order model with values of 0.99 and 0.93 for the R^2 and Predicted R^2 coefficients, respectively. From the ANOVA results, it was determined that only time was significant on the viability of the seeds, with a p-value lower than 0.0001, while the concentration of TZ in the range studied was not significant. The response surface plot showed that using low concentrations of TZ (0.05%) and time-spans between 75 and 90 minutes resulted in seed viability greater than 90%.

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