Article

Low frequency ultrasound as an enhancer for the germination process of *Stizolobium pruriens*

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Abstract

Mucuna (*Stizolobium pruriens*) is widely used in agriculture as a green allowance and in crop rotation, due to its ability to fix nitrogen and recover degraded areas; without embargo, there is a slow and uneven germination. This study used some classical methods, together with the use of low-frequency ultrasound to accelerate and homogenize the germination and emergence of the seeds. The experiment was carried out at the Plant Tissue Cultivation Laboratory of the Ilha Solteira *Campus*, São Paulo, Brazil. The design used was a completely randomized one, with five replications, in a 3x6 factorial scheme, the factors being: three pre-treatments for latency break: mechanical scarification, thermal scarification, and without scarification with six levels of ultrasound exposure: 0, 1, 2, 3, 5, 8 min, totaling 18 treatments. For eight days the germination and the initial stages of the seedlings were controlled. The method without scarification subjected to 4.5 min of ultrasound can become an excellent alternative, since it presented greater germination vigor, while 3.14 min of exposure to ultrasound were enough to improve the emergence speed, regardless of the method used in the preparation of seeds. In conclusion, only with the use of low-frequency ultrasound, it is possible to improve both the germination speed index and the germination vigor, without the need for additional treatments.

Keywords: mechanical scarification, thermal scarification, ultrasonic.

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Introduction

The *S. pruriens* (Black mucuna) is native from the West Indies, and it is adapted to tropical and subtropical climates (Cruz *et al.*, 2011). It is characterized as an annual legume, with climbing branches and low habit. Its growth is undetermined, with a life cycle lasting over 150 days (Ambrosano *et al.*, 2016; Angeletti *et al*., 2016). The specie's development has differential physiology that provides for rapid overlapping of vegetation, hindering growth and performance of other plant forms (Ramos *et al.*, 2018). Black mucuna is commonly used as green manure, in the recovery of degraded areas, with active performance in crop rotation processes and soil decompaction. It also has a direct effect on nitrogen levels through biological fixation, suppressing some species of nematodes, it also acts as a weed controller, functioning mainly in grain production systems without direct seeding (Castro *et al*., 2011; Ragassi and Melo, 2017).

The species is classified in the the Fabaceae family, one of the largest families of Angiosperms with more than 650 genera (Mello *et al.*, 2015), including species that stimulate the mineralization of some herbicides with phytoremediation effects. The *Stizolobium pruriens* is efficient in the processes of decontamination, showing some tolerance to herbicides (Silva *et al*., 2012). Widespread in Cerrado areas, due to the availability of seeds, *S. pruriens* is well adapted to water hortfall conditions, withstanding high temperatures, with no photoperiod restriction (Teodoro *et al.*, 2015). However, even with all its potential, difficulties are still being faced due to the low and uneven seed germination (Oliveira *et al.*, 2017).

Many plant species with viable seeds, do not absorb enough water to germinate, even under favorable conditions due to hardness and impermeability of tegument (Ramos *et al*., 2019). Several treatments are available to overcome this type of seed dormancy, such as immersion in acids, hot or cold water, alcohol, removal of the caruncle, mechanical scarification, among others. The set of treatments that will produce greater efficiency in germination depend on the particularities of each species (Oliveira *et al.*, 2017; Pereira *et al.*, 2020).

The use of low-frequency ultrasound in a liquid environment has a high potential to stimulate germination, as it contributes to water imbibition by seeds (Gordon, 1963; Yaldagard *et al*., 2008; Venâncio *et al*., 2016), and promotes development of living tissue (Venâncio and Martins, 2019). This work aims to study the behavior of *S. pruriens* seeds concerning the germination speed index and seedling emergence. Therefore, low-frequency ultrasound is associated with classical methods of dormancy breaking (wet thermal scarification and manual mechanical scarification).

Material and methods

The experiment took place in the Plant Tissue Culture Laboratory at UNESP campus of Ilha Solteira, São Paulo, Brazil, under controlled conditions. The seeds of *S. pruriens* used are from 2017 harvest under field cultivation, of those 94% are pure and 82% are viable, values informed by the seed producing company.

To avoid contamination, seeds were disinfected soaking them for 1 minute in a 50% water solution of Lysoform® (0.45% de Cocobenzyl Alkyl Dimethyl Ammonium Chloride/Didecyl Dimethyl Ammonium Chloride); after they were washed with distilled water.

Seeds were pre-treated, before applying the ultrasound, as follows: 1) wet thermal scarification, soaking in hot water until reaching 60 °C, drained, and cooling at room temperature for 24 hours; 2) manual mechanical scarification, which consists of sanding the seeds with 150 mm sandpaper; 3) and non-scarified seeds (control), and in each method, ten seeds were used.

After the process carried out with the three methods of scarification (pre-treated), seeds were exposed to the ultrasound treatment (application of sound waves via ultrasonic probe with frequency of 3 mHz, 120 volts ~ 50/60 Hz) for different periods of time (0, 1, 2, 3, 5 and 8 min). For ultrasound exposure, 10 seeds (replications) were placed in a 50 ml glass container with 20 ml of distilled water, where the ultrasonic probe was introduced, and the treatments (Table 1).

Table 1. Description of the treatments covered in the study.

Treatments	Descriptions
WS	Control (seeds without scarification and no ultrasound exposure);
TS	Wet thermal scarification and no ultrasound exposure;
MS	Manual mechanical scarification with sandpaper and no ultrasound exposure
WSU1	WS + 1 minute of ultrasound exposure
WSU2	WS + 2 minutes of ultrasound exposure
WSU3	WS + 3 minutes of ultrasound exposure
WSU5	WS + 5 minutes of ultrasound exposure
WSU3	WS + 8 minutes of ultrasound exposure
TSU1	TS + 1 minute of ultrasound exposure
TSU2	TS + 2 minutes of ultrasound exposure
TSU3	TS + 3 minutes of ultrasound exposure
TSU5	TS + 5 minutes of ultrasound exposure
TSU8	TS + 8 minutes of ultrasound exposure
MSU1	MS + 1 minute of ultrasound exposure
MSU2	MS + 2 minutes of ultrasound exposure
MSU3	MS + 3 minutes of ultrasound exposure
MSU5	MS + 5 minutes of ultrasound exposure
MSU8	MS + 8 minutes of ultrasound exposure

Treated seeds were introduced in containers with water and 20 g L^{-1} Phytagel, in a horizontal laminar flow chamber and placed in a growth room, with a constant temperature of 22 ºC and 18 h of photoperiod. Germination (G, in%) was evaluated daily, for 15 days, to obtain an average germination time (AGT) in equation 1, emergency speed index (ESI) in equation 2 (Maguire, 1962) and germination vigor (GV).

$AGT=\frac{\sum n_i.t_i}{\sum n_i}$ 1); $ESI=\sum n_i .t_i$ 2); where: n= number of seeds germinated on the evaluation day; t= number of days after sowing on which the evaluation was carried out. Germination vigor (GV) was assessed via a grade ranging from 0 to 6 (Table 2, Figure 1).

Table 2. Description of the phases observed in the study.

Range	Descriptions
0	Did not germinate
1	Rupture of the integument
2	Radicle elongation
3	Differentiation of radicle, colleto and hypocotyl
4	Detachment of cotyledons from the seed coat, and beginning to opening
5	Open of cotyledons and emergence of the apical bud
6	Epicotyl and first pair of leaves opened

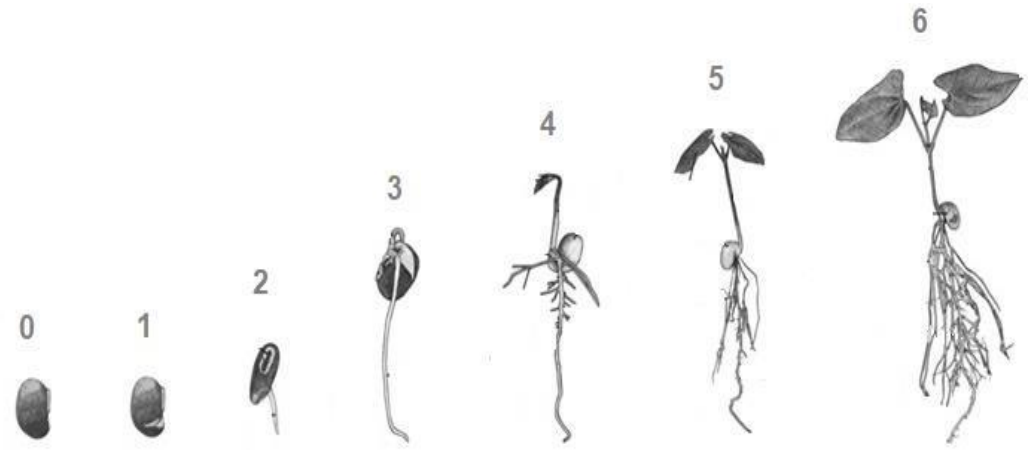

Figure 1. Notes regarding the germination vigor phases. Adapted from Abud *et al*. (2009).

A completely randomized designed was used, in a 3 x 6 factorial scheme, (without scarification - WS, wet thermal scarification -TS, mechanical scarification with sandpaper -MS) and six period of time of ultrasound incidence (0, 1, 2, 3, 5 and 8 minutes), producing 18 treatments with 5 replications each. The statistical analyses were performed using the SISVAR software. In the statistical analysis, the hypothesis of normality was initial tested using the Shapiro-Wilk test, after which an analysis of variance (Anova) was performed by F test at 5% probability to detect differences between factors and interactions.

The scarification factor effect was decomposed to verify the effect of the ultrasound time factor separately for each scarification method. In the presence of significant differences, the Tukey test ($p< 0.05$) was used to compare methods and the regression for ultrasound times. The regression model was verified via p-value of the regression deviation. The polynomial regression models with the higher determination coefficients (R^2), were selected among the significant regressions by the F test.

Results and discussion

Seed germination differed ($p< 0.05$) among the methods used for breaking dormancy (Table 3), indicating that heating the seed is better than mechanical scarification, but both do not differ from the control without scarification. Ultrasound times alone and their interaction with dormancy-breaking methods did not show significant effects ($p< 0.05$) on seed germination (Table 3).

Table 3. Analysis of variance for germination (G) in percentage, average germination time (AGT) emergence speed index (ESI) and germination vigor (GV).

Source of Variation	G		AGT		ESI		GV	
	MSq[1]	*P*-value	MSq	*P*-value	MSq[1]	*P*-value	MSq	*P*-value
Methods (M)	4.44	0.012*	4.39	0.01*	4.55	0.139^{ns}	16.1	0.004**
Ultrasound (U)	1.81	0.104^{ns}	0.4	0.812^{ns}	6.33	0.022*	36.42	0**
M x U	0.84	0.552^{ns}	1.81	0.046*	4.23	0.062^{ns}	32.84	0**
CV (%)	56		54		1		6	
Overall average	0.55		1.78		1.01		2.88	
Methods							Average	
WS	0.51 ab		1.413 ab		1.014		2.873 ab	
MS	0.68 a		1.75 a		1.018		3.08 a	
TS	0.45 b		2.176 b		1.01		2.702 b	
Ultrasound time (min)								
0	0.49		2.073		1.011		2.773	
1	0.609		1.733		1.019		2.706	
2	0.706		1.746		1.018		3.035	
3	0.615		1.566		1.023		3.444	
5	0.423		1.806		1.014		3.088	
8	0.458		1.753		1.004		2.262	
Regression					MSq	*p*-value	MSq	*p*-value
Linear	-		-		0.124	0.059^{ns}	24.625	0.003**
Quadratic	-		-		0.212	0.014*	128.981	0**
Cubic	-		-		0.006	0.665^{ns}	2.083	0.392^{ns}
Deviation	-		-		0.116	0.068^{ns}	13.205	0.01*

**, *, ^{ns}= significant at 1%, 5% and non-significant. By F test. Averages followed by the same letter in each column do not differ by Tukey test at $p< 0.05$.

The average germination time showed significant differences ($p< 0.05$) for scarification methods and for the interaction between both factors (Table 3). The main effect linked to scarification methods was disregarded, since the interaction effects allowed for the best responses. AGT showed significant differences for the methods and interaction. The variable emergency speed index did

not show significant differences ($p< 0.05$) for methods of breaking dormancy (WS, TS and MS) and interaction. Therefore, there is no need manually to scarify or heat the seed for this purpose. However, ultrasound exposure had significant effects ($p< 0.05$) (Table 3) on this variable, with no significant interaction with the other factor.

It was expected that the TS treatment would accelerate the germination process, as its principle is to soften coat tissues favoring water absorption and gas exchange, accelerating the physiological reactions of seeds linked to germination (Câmara *et al.*, 2015). However, this did not happen with the seeds of *S. pruriens*, which were indifferent to the scarification methods used. For ultrasound times, the levels of significance (*p*-value) of the equations and the deviation of the regression (Table 3) show significance for the quadratic model, with 0.014 of p-value ($p< 0.05$), explaining 86% of the data behavior (Table 3 and Figure 2), the maximum point for this regression model was estimated at 3.14 min of ultrasound exposure.

The variable ESI has statistical significance ($p< 0.05$) for ultrasound times (Table 3). In the quadratic regression model significant difference was found, with 0.014 p-value ($p< 0.05$), explaining 86% of the data. The maximum point for this regression model was estimated at 3.14 min of ultrasound exposure (Table 3 and Figure 2). For other models, the ESI was not significant ($p< 0.05$)

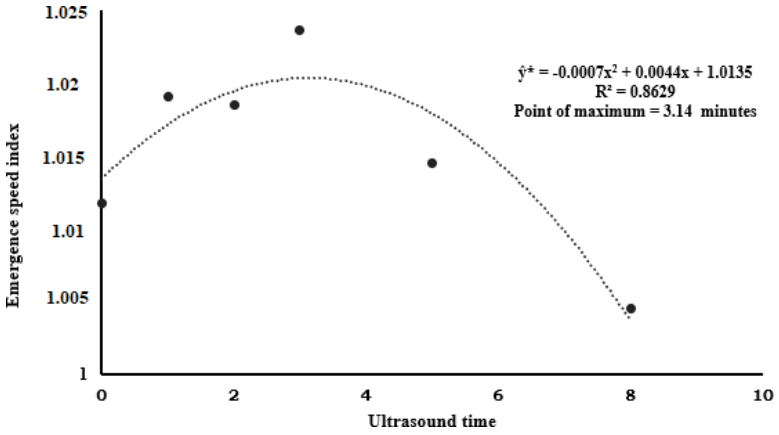

Figure 2. Quadratic regression for ultrasound incidence times regardless of the seed scarification method.

Seed germination vigor showed significant differences ($p< 0.05$) for scarification methods, ultrasound times and their interaction (Table 3). Thus, although the results main effects of individual factors were significant, the analysis of the interaction allowed a better assessment of the effects of these factors on germination vigor. The smallest vigor was obtained with TS, when compared WS and MS treatments (Table 4).

Table 4. Mean values of average germination time for different ultrasound exposure times and seed scarification methods.

Ultrasound times scarification methods times (min)	Control (no scarification) *	Mecanical scarification ns	Termal scarification ns
0*	2.72 b	1.24 a	2.26 ab
1ns	2.28 a	2.72 a	2.92 a
2ns	1.32 b	3.693 a	2.8 b
3ns	1.48 a	3.92 a	2.626 b
5**	0.78 a	1.62 ab	3.02 b
8ns	1.92 a	2.666 a	1.506 b
Regression	MSq	*P*-value		
Linear	2.502	0.101ns	-	-
Quadratic	8.667	0.003**	-	-
Cubic	0.143	0.693ns	-	-
Deviation	0.466	0.601ns	-	-

**, *, ns= significant at 1%, 5% and non-significant. By F test. Averages followed by the same letter in the lines do not differ by Tukey test at 5%.

For average germination time (AGT) a difference was found between the ultrasound incidence time ($p< 0.05$) only in treatments without scarification. The regression deviation was not significant ($p> 0.05$) and the data for adjusted only on the quadratic regression model ($p< 0.05$), with a very high coefficient of determination this 91%. This result suggests an excellent fit in the model.

The minimum AGT point occurred at the exposure time of 4.68 minutes; after that time there was an increase in average germination time. The time of ultrasound incidence showed a significant difference ($p< 0.05$) in all applied methods for germination vigor (GV), but this behavior varied within each method (Table 5). In the absence of scarification, the regression deviation was significant ($p< 0.05$), so the adjustment of any model must be accepted with restriction.

Table 5. Averages of the variable germination vigor in the interaction of ultrasound times and methods of breaking dormancy.

Ultrasound times (minutes)	Methods of breaking dormancy		
	WS**	MS**	TS**
0**	2.4 b	2.093 b	3.826 a
1ns	2.48 a	2.72 a	2.92 a
2**	2.613 b	3.693 a	2.8 b
3**	3.786 a	3.92 a	2.626 b
5**	3.346 a	3.386 a	2.533 b
8**	2.613 a	2.666 a	1.506 b

Ultrasound times (minutes)	Methods of breaking dormancy					
	WS**		MS**		TS**	
Regression	MSq	*P*-value	MSq	*P*-value	MSq	*P*-value
Linear	7.82	0.097^{ns}	4.557	0.206^{ns}	182.96	0**
Quadratic	70.841	0**	152.42	0**	1.192	0.517^{ns}
Cubic	3.524	0.266^{ns}	16.496	0.016*	21.941	0.006**
Deviation	17.863	0.002**	5.125	0.165^{ns}	1.385	0.614^{ns}

**, *, ^{ns}= significant at 1%, 5% and non-significant. Respectively. By F Test. Averages followed by the same letter in the lines do not differ by Tukey test at 5%.

In the absence of scarification, the quadratic regression model was the one that best fitted the data ($p < 0.05$), with a high coefficient of determination, but presented regression deviation significative. This result suggests that more studies would be necessary to determine the regression model (linear or non-linear), for this variable it is necessary to look for another model to explain this phenomenon. In the WS treatment, the maximum GV point occurred at the exposure time of 4.44 minutes, after that time there was no increase in germination vigor.

For the WS method, the regression deviation was not significant ($p > 0.5$) and the quadratic regression model was the one that best fitted the data ($p < 0.05$), with an adjustment (R^2) of 85% to the data. In this case, the maximum germination vigor point occurred in 4.25 min of exposure of the seeds to the ultrasound. For both (WS and MS), the exposure time 5 and 8 min caused a decrease in GV. In the TS method, exposure to ultrasound was not beneficial for GV, obtaining decreasing scores as the time of exposure increased (Table 5 and Figure 3).

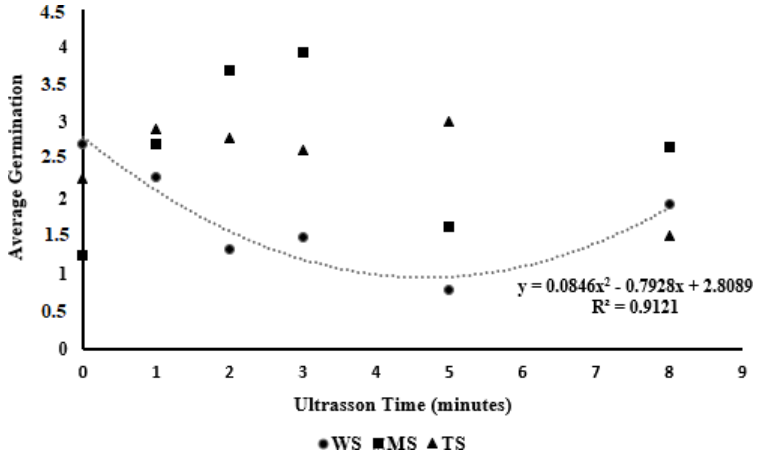

Figure 3. Average germination of *S. pruriens* subjected to different scarification methods (without scarification (WS), mechanical scarification (MS) and thermal scarification (TS)) associated with different ultrasound exposure.

In regard to the germination vigor variable, taking into account the effect of the methods when using the ultrasound, MS had better result than TS (Table 5), and no significant differences were found between WS and MS for the ultrasound times applied, except for 2 minutes when MS had

greater vigor. At all incidence times to ultrasound, there were differences between the methods studied, except for 1 min. However, the WS + 4.5 minutes of exposure to ultrasound has an advantage, one operation less (without scarification), and the maximum vigor, what is very important if consider use the method as a commercial one (Figure 4).

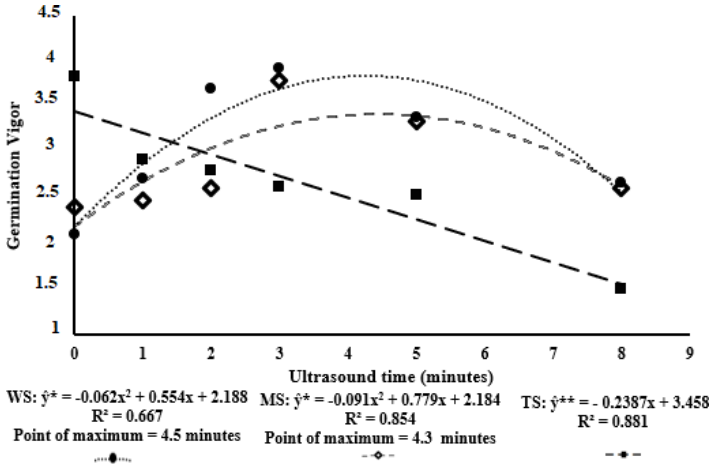

Figure 4. Germination vigor of *S. prupriens* seeds subjected to the different scarification methods (without scarification (WS), mechanical scarification (MS) and thermal scarification (TS)) associated with different ultrasound exposure times.

The use of ultrasound, until a certain time, can be promising to homogenize, accelerate and guarantee vigor in the germination process. The best ultrasound incidence times calculated by the regression equations were 3 (ESI), 4 (GV) and 5 (ATG) minutes (Figures 2, 3 and 4). After 3 min, there is a decrease in the ESI of the seeds of *S. pruriens* and the incidence of ultrasound for more than 3 min caused, probably, a deleterious effect on the seeds. With the incidence of ultrasound in 3 min, 87% of germination was obtained, while the control treatment showed an efficiency of 80%, the germination rate reported by the company producing the seeds used was 82%.

This effect was already expected, since it is known that the ultrasound provides vibrational energy, which can have a positive or negative effect on living tissues depending on the form that is applied, the intensity, the distance of application, frequency and time of exposure (Hebling *et al*., 1995). This effect combined with moist thermal scarification promoted a harmful effect on the seed, regardless of the exposure time.

In general, the dormancy breaking methods show a good increase in *S. pruriens* germination (Wutke *et al*., 1995; Fortes *et al*., 2010; Oliveira *et al*., 2017). However, in this study, the germination did not present significant difference ($p< 0.05$), only vigor was influenced by the scarification methods. The mechanical scarification processes were not promising in relation to the absence of scarification, WS and MS did not produce significant effects on germination (Table 3). For vigor, when the seeds were exposed to ultrasound (Table 5), there was no significant difference among treatments for breaking dormancy, except for 2 min of exposure, when MS had superior results.

Therefore, WS is simpler than WS and TS, which are operationally more expensive (requiring a lot of time; energy and labor), being viable only for small amounts of seeds, about 1 to 10 kg (Bianchetti *et al*., 1997), making it impossible for use in large areas. Also, in MS and TS methods, some care must be taken regarding the intensity, time and form of application, to avoid injuries that reduce vigor, causing seedling abnormality, seed mortality or serving as an entry point for infections by fungi and bacteria (Franke and Baseggio, 1998).

Conclusions

The wet thermal scarification associated with the use of ultrasound is not suitable for seeds of black mucuna, putting at risk the germination vigor. The use of ultrasound had positive effects for both emergence speed index and germination vigor of black mucuna seeds. Without considering seed preparation method (WS, MS or TS) the emergence speed index can be improved by applying ultrasound for 3.14 min. The best results for germination vigor of mucuna seeds was observed when applying ultrasound for 4.5 min, without scarification treatments. One advantage is that there is one less operation.

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