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pH exudate test as a method to estimate viability and vigor of *Citrus limonia* Osbeck seeds[1]

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ABSTRACT - The pH exudate is a quick test used to check the seed membranes integrity; however, its performance is limited due to the singularity of each specie requiring adjustments about its methodology. In this sense, the aim of this study was to perform a quick and accurate physiological potential estimation of *Citrus limonia* Osb. ('Rangpur Lime') seeds through pH exudate test. First, there was carried out the characterization of five seedlots of 'Rangpur Lime' by determination of the thousand seed mass, water content, germination and emergence. Then, a complete randomized experimental design was installed in a factorial scheme: 5 × 3 × 2 (five seedlots, three indicator solutions, and two immersion periods), to evaluate the pH exudate. The seeds were individually distributed in polypropylene trays, and after the immersion periods, there were taken pictures and analyzed by a numeric scale. Despite the similarity, the emergence test showed to be more efficient for 'Rangpur Lime' seeds comparing to the germination test, because it promoted higher percentage of seedlings. Regarding the pH exudate test, there were not observed differences between the seedlots for both analysis, visual and image, not being efficient to discriminate lots of 'Rangpur Lime'.

Index terms: 'Rangpur Lime', propagation, quick tests, seed quality.

Teste do pH do exsudato como método para estimar a viabilidade e o vigor de sementes de *Citrus limonia* Osbeck

RESUMO - O pH do exsudato é um teste rápido utilizado para verificar a integridade das membranas de sementes, porém seu desempenho é limitado devido à singularidade de cada espécie necessitando de ajustes sobre sua metodologia. O objetivo deste estudo foi realizar uma estimativa rápida e precisa do potencial fisiológico de sementes de *Citrus limonia* Osb. ('Limão Cravo') pelo teste pH do exsudato. Inicialmente, realizou-se a caracterização de cinco lotes de sementes de 'Limão Cravo' pela determinação da massa de mil sementes, teor de água, germinação e emergência. Posteriormente, foi instalado um delineamento inteiramente casualizado em esquema fatorial: 5 × 3 × 2 (cinco lotes de sementes, três concentrações de solução indicadora e dois períodos de imersão), para avaliar o pH do exudato. As sementes foram distribuídas individualmente em bandejas de polipropileno e, após os períodos de imersão, foram fotografadas e analisadas por meio de escala numérica. Apesar da similaridade, o teste de emergência mostrou ser mais eficiente para sementes de 'Limão Cravo' comparado ao teste de germinação, pois promoveu maior percentual de plântulas. Em relação ao teste pH do exsudato, não foi possível observar diferenças entre lotes de semente para ambas análises, visual e de imagem, não sendo um teste eficiente para discriminar lotes de 'Limão Cravo'.

Termos para indexação: 'Limão Cravo', propagação, testes rápidos, qualidade de semente.

Introduction

The 'Rangpur Lime' (*Citrus limonia* Osbeck) is the most used rootstock in the Brazilian citrus orchards due to its compatibility to most scion cultivars, satisfactory yield, induction of early-bearing and adequate performance under water deficit condition, presenting substantial importance for the rain-fed *Citrus* growing area (Mourão Filho et al., 2007;

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Oliveira et al., 2015; Banhos et al., 2016).

For commercial production of rootstock, the propagation by seeds is the main way to obtain new plants to carry out the grafting method. Therefore, the knowledge of the physiologic potential of citrus seeds is fundamental, because it allows information about the potential number of plants produced and establishment of adequate planning, both spatially and temporally (Carvalho et al., 2002a,b; Nakano et al., 2013; Santos et al., 2015).

In this sense, the seed germination is the main characteristic applied to determine the maximum potential of seed lots reach normal seedlings, under optimal development conditions (Brasil, 2009). However, depending on the specie, as citrus, the test can be extended from 34 to 60 days hindering the decision-making about seedlots management in terms of sowing or storage (Carvalho and Silva, 2013; Souza et al., 2015).

Thus, the use of quick and accurate test to estimate the potential of germination and vigor is desired to generate information in short period, allowing appropriate seeds destination. In this way, several quick tests can be cited, such as tetrazolium, electrical conductivity and pH exudate via phenolphthalein (Dode et al., 2013).

The pH exudate is a quick test used to check the seed membranes integrity, assuming that during the imbibition phase, non-vigorous seeds reorganize the membrane system more slowly than vigorous seeds, resulting in high metabolites release such as sugars, organic acids, and H^+ ions, which acidify the medium and decrease the water pH of seed imbibition (Rech et al., 1999; Carvalho et al., 2002a; Santos et al., 2011). Through the data obtained by pH-measuring devices or acid-base indicator solutions, it is possible to evaluate the physiological condition of each seed and, consequently, the physiological potential of lots (Araldi and Coelho, 2015; Alves et al., 2016).

Despite the pH exudate be a well-known test in the seed technology, it is limited due to the singularity of each specie, which need adjustments about the methodology regarding the imbibition period, the indicator solution concentration, and the evaluation technique. The subjectivity is the most important limitation when performed by acid-base indicator solution, because the results are based on empirical interpretation of the analyst as the tonalities that the exudate solution assumes according to the pH, after addition of the indicator solution.

In this sense, the image analysis is a promising alternative to eliminate this subjectivity, because it allows a quantitative reading of different shades for the same color (pink), giving credibility to the test. Thereby, the aim of this study was to perform a quick and accurate physiological potential estimation of *C. limonia* seeds through pH exudate test, using both visual and image analysis of the immersion solution.

Material and Methods

The 'Rangpur Lime' seeds (*C. limonia*), represented by five seedlots, were obtained at the Seed Germplasm Bank of the Instituto Agronômico do Paraná, Londrina, Brazil, and stored in a refrigerated chamber at 10 ºC until to analysis. Initially, there was carried out the seeds characterization regarding thousand seed mass, water content, germination and emergence in greenhouse according to the following methodologies:

Thousand seed mass: performed by weighing eight replicates of 100 seeds from each lot according to Brasil (2009).

Water content: determined from two samples of 10 seeds, which were weighed and remained in an greenhouse at 105 ± 3 °C for 24 hours (Brasil, 2009). The results were expressed in percentage, according to the humidity degree at wet base.

Germination test: 200 seeds from each lot, divided into four samples in a complete randomized experimental design, were distributed on germitest paper, moistened with distilled water equivalent to 2.5 times the weight of the non-hydrated substrate. The experimental set was maintained in a germinator model Mangelsdorf regulated at 25 ± 1 °C in constant white lighting. The evaluations were carried out each three days, counting the primary root protrusion and the number of normal seedlings according to Brasil (2009). Through the counting data, there were calculated the germination results, expressed in percentage of primary root protrusion, germination (normal seedlings) and germination speed index, calculated according to Maguire (1962).

Seedling emergence in greenhouse: the seeds from each lot, divided into four samples of 50 seeds in a complete randomized experimental design, were sown twice depth of the seed size into sand boxes (44 × 30 × 7 cm) and kept in a greenhouse during 46 days (until complete emergence). There was recorded the number of emerged seedlings weekly, the results were calculated as percentage of total seedlings and emergence speed index, by formula proposed by Maguire (1962). At the end of the test, there was established the fresh and dry matter of normal seedlings by greenhouse method (70 ± 2 °C) until constant mass (Nakagawa, 1999).

After initial characterization of the lots, a complete randomized experimental design was installed in a factorial scheme: 5 × 3 × 2, using five seedlots of 'Rangpur Lime' combined with three concentration of indicator solutions and two immersion periods, to evaluate the pH exudate.

To perform the pH exudate test, a phenolphthalein ($C_{20}H_{14}O_4$) indicator solution was formulated using 1 g of phenolphthalein dissolved into 100 mL of absolute alcohol and 100 mL of distilled water. Also, three base solutions were formulated by 0.2 g, 0.6 g and 1.0 g Na_2CO_3 dissolved in 100 mL of distilled

Table 1. Concentration, pH, and volume of the standard solutions used in the pH exudate test of *Citrus limonia* Osbeck seeds at different immersion periods.

Standard solution	Concentration ($g.L^{-1}$)		pH solution	Volume (µL)	Immersion period (minutes)
	$C_{20}H_{14}O_4$	Na_2CO_3			
1.0	5.0	10.0	11.4	200	20 and 40
0.6	5.0	6.0	11.1	1.000	60 and 80
0.2	5.0	2.0	10.5	1.200	20 and 40

water. After formulations, the base solutions (Na_2CO_3) and the phenolphthalein indicator were mixed in a 1:1 ratio to compose three standard solutions.

The seeds were individually distributed in polypropylene trays (24 x 12 cm) of 40 alveoli (10 mL) filled with 5 mL of distilled water, there was left one alveoli without seed addition taken as control. After preliminary tests, the standard solutions were added in different periods, according to Table 1.

After the solutions addition and immersion periods, there were taken pictures using a Nikon® D3100 camera and 18-55 mm lens, at the following settings: ISO 800, 1/30 and f/3.5. The images were analyzed by the Image J® program to quantify the colors through the numeric scale (Figure 1). At first, there was proposed an index, wich was based on the seed and the control mean (index = seed mean – control mean / control mean) varying from zero to 1 according to the color intensity, when closer to zero more pinkish (darker) and when closer to 1 more colorless (lightness), i.e. the vigorous seed did not release exudates (zero) as much as the non-vigorous seed released (1). Next, the indexes were distributed in the following scale, divided by three points: <0.33 (index minor than 0.33), considered as vigorous seeds; 0.33 – 0.66 (index between 0.33 and 0.66), representing those with medium vigor; and 0.66 (index greater than 0.66), considered as non-vigorous seeds.

Similarly, there was established a parameter to evaluate the seed performance via visual analysis, which the dark pink color represented those seeds with high performance, vigorous seeds; when presented light pink color, it was considered as medium vigor; and when was not observed the pinkish color (colorless), there was considered as non-vigorous seeds. For both, image and visual analysis, the results were expressed as percentage.

All data were submitted to the R program for analysis of variance and the means were compared by Tukey's test at 5% of probability. In parallel, there was performed the Pearson correlation.

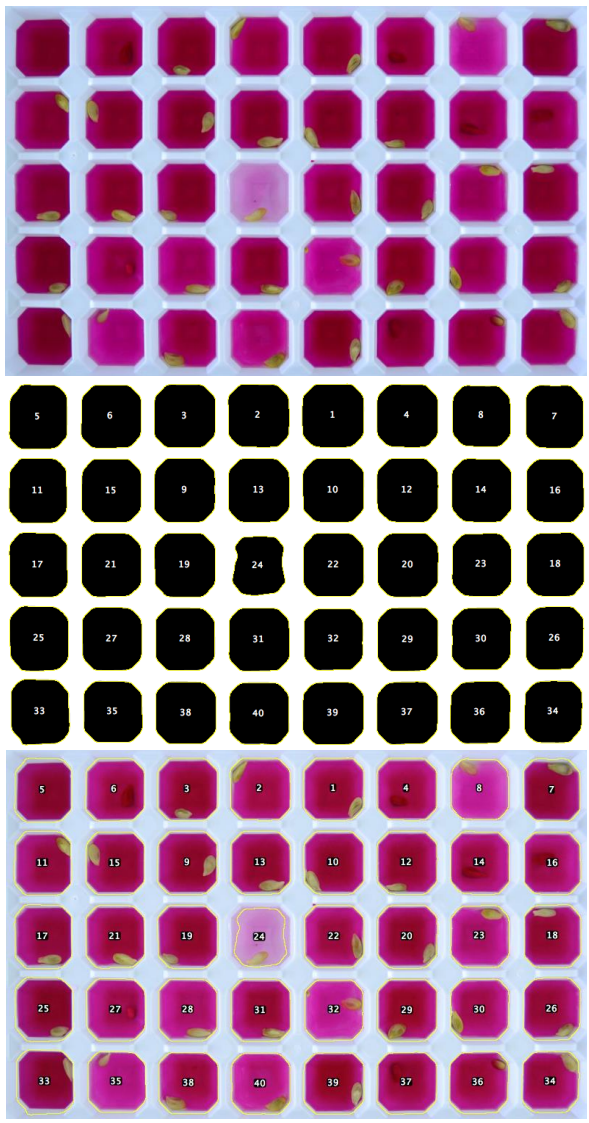

Figure 1. (A) Polypropylene trays containing 'Rangpur Lime' seeds after immersion period of 60 minutes at 0.6 ($C_{20}H_{14}O_4 + Na_2CO_3$); (B) alveolus delimited by the outlines; (C) alveolus area used to analyze the color intensity, (C-5) alveoli without seed addition, taken as control.

Results and Discussion

Regarding seed characteristics, it was observed similar values for most of the lots, except to lot E that presented the highest mean for thousand seeds mass (Table 2). It must indicate that this lot was

Table 2. Thousand seed mass (TSM), percentage of water content (WC), primary root protrusion (PRP), germination (Germ), and germination speed index (GSI) in different lots of *Citrus limonia* seeds.

Lots	TSM (g)	WC (%)	PRP (%)*	Germ (%)*	GSI*
A	53.63	13.1	82 a	72 a	1.62 a
B	54.02	14.8	78 ab	65 ab	1.57 ab
C	54.29	13.5	69 b	53 bc	1.34 b
D	53.87	11.5	52 c	43 cd	1.02 c
E	67.56	14.3	43 c	33 d	0.83 c
CV (%)	-	-	7.76	12.43	9.78

*Means followed by the same letters in the column do not differ statistically by Tukey's test at 0.05 probability level.

compounded for seeds of large size making the lot heavier, which can influence the seed physiological potential. The water content means were analogous, ranging from 11 to 14% (3.3 points percent for maximum point) showing that seeds from all lots were in the same metabolic level, which did not interfere to the experimental results.

The germination test variables are also presented at the Table 2, which can be observed a significant difference among the seed lots. The lots A and B did not differ for the variables analyzed, attaining the highest means for the percentage of primary root protrusion (over than 78%), germination (greater than 65% of seedlings), and germination speed index (over than 1.57). However, the seedlots D and E differed from all, for the percentage of primary root protrusion and germination speed index, reaching just 52 and 43% for the first variable, 1.02 and 0.83 for the second, respectively. These lots also presented the lowest percentage of germination.

There was noticed corresponding results for the emergence test, wich can be seen at the Table 3. The lots A and B showed to be more vigorous presenting higher means for all characteristics, contrasting to lot E that attained low means for these variables, especially for the emergence speed index that was almost three times lower than lot A. Consequently, these seedlings may have become more vulnerable to the adverse environmental conditions, because it emerged slowly and spent more time in the initial growth stage (Martins et al., 1999).

Despite the similarity, this test showed to be more efficient for 'Rangpur Lime' seeds comparing to the germination test, because it promoted higher percentage for most of the lots. In this sense, the microorganisms such as fungi and bacteria observed during the germination test, may have interfered to the results as observed by Carvalho et al. (2002a) for citrumelo 'Swingle', since the seeds were maintained in germinator during 46 days at favorable temperature and humidity for mostly microorganism (25 ^{o}C and 95%).

With reference to pH exudate test, the results are presented at the following tables (Tables 4, 5 and 6). Most of all, there were not observed differences between the seedlots for both analysis, visual and image, just for some specific concentrations and periods.

Respecting to visual analysis, the lot E presented significantly higher mean for medium vigor seeds at 0.2% and 20 minutes, around 42%. The lot E also showed higher percentage of vigorous seeds at 0.6% in both periods, 60 and 80 minutes. This performance was not observed at 1.0% and 40 minutes where lot E reached elevate percentage for non-vigorous seeds, since all the lots had presented low percentage for vigorous and medium vigor seeds. Therefore, it was observed dissimilarity among the characterization results and pH exudate test for 'Rangpur Lime' and high coefficient of variance, which did not allow the recommendation of any method.

For the image analysis, there was just observed difference

Table 3. Percentage of emergence in greenhouse (EG), emergence speed index (ESI), shoot fresh matter (SFM), and shoot dry matter (SDM) of different lots of *Citrus limonia* seeds.

Lots	EG (%)*	ESI	SFM (g) (g.seedling^{-1})*	SDM (g) (g.seedling^{-1})*
A	88 a	1.49 a	5.01 a	1.64 a
B	80 ab	1.34 ab	4.57 a	1.39 a
C	62 bc	1.14 bc	4.11 ab	1.28 ab
D	58 c	0.93 c	2.90 b	0.94 b
E	32 d	0.49 d	1.65 c	0.49 c
CV (%)	12.63	12.49	15.28	17.20

*Means followed by the same letters in the column do not differ statistically by Tukey's test at 0.05 probability level.

Table 4. Visual analysis, expressed in percentage, of pH exudate at different concentrations and periods in different lots of *Citrus limonia* seeds.

Lots	A	B	C	D	E	CV (%)
0.2% - 20 minutes						
VIG	38.1 ^{ns}	46.2	43.6	44.9	32.1	31.5
MDV*	16.7 b	19.2 b	21.8 b	23.1 b	42.3 a	13.2
NVIG	44.9 ^{ns}	34.6	34.6	32.0	25.6	35.7
0.2% - 40 minutes						
VIG	24.4 ^{ns}	25.6	34.6	26.9	12.8	36.2
MDV	17.9 ^{ns}	21.8	19.2	23.1	30.8	39.0
NVIG	57.7 ^{ns}	52.6	46.2	50.0	56.4	27.4
0.6% - 60 minutes						
VIG*	76.9 ab	73.0 c	83.4 ab	76.9 ab	97.4 a	6.7
MDV*	16.7 ab	24.4 a	12.8 ab	19.3 a	2.6 b	25.7
NVIG	6.4 ^{ns}	2.6	3.8	3.8	0.0	79.9
0.6% - 80 minutes						
VIG*	62.8 b	65.4 b	69.2 b	67.9 b	87.2 a	4.5
MDV*	26.9 a	29.5 a	25.6 a	27.0 a	12.8 b	8.8
NVIG	10.3 ^{ns}	5.1	5.2	5.1	0.0	70.9
1.0% - 20 minutes						
VIG	23.1 ^{ns}	32.1	24.4	25.6	19.2	14.2
MDV	9.0 ^{ns}	14.1	6.4	10.3	14.1	60.1
NVIG	67.9 ^{ns}	53.8	69.2	64.1	66.7	12.9
1.0% - 40 minutes						
VIG*	2.6 b	16.7 a	6.4 ab	9.0 ab	2.6 b	42.3
MDV*	3.8 ab	11.5 a	5.1 ab	9.0 a	0.0 b	36.0
NVIG*	93.6 ab	71.8 c	88.5 ab	82.0 bc	97.4 a	4.4

VIG – Vigorous; MDV – Medium vigor; NVIG – Non-vigorous. *Means followed by the same letters in the row do not differ statistically by Tukey's test at 0.05 probability level. ^{ns} – non-significative.

Table 5. Image analysis of pH exudate at different concentration and periods in different lots of *Citrus limonia* seeds, expressed in percentage.

Lots	A	B	C	D	E	CV (%)
0.2% - 20 minutes						
VIG	24.4 ^{ns}	35.9	18.0	15.4	61.5	51.0
MDV	38.5 ^{ns}	35.9	42.3	43.6	38.5	25.6
NVIG	37.1^{ns}	28.2	39.7	41.0	0.0	62.8
0.2% - 40 minutes						
VIG	6.4 ^{ns}	15.4	11.6	5.1	67.9	103.5
MDV	61.5 ^{ns}	51.3	47.4	51.3	32.1	52.5
NVIG	32.1 ^{ns}	33.3	41.0	43.6	0.0	77.3
0.6% - 60 minutes						
VIG	42.3 ^{ns}	84.6	44.9	88.5	96.2	52.4
MDV	38.5 ^{ns}	7.7	41.0	10.2	3.8	121.8
NVIG	19.2 ^{ns}	7.7	14.1	1.3	0.0	155.2
0.6% - 80 minutes						
VIG	67.9 ^{ns}	59.0	87.2	73.1	82.0	17.2
MDV	16.7 ^{ns}	26.9	7.7	23.1	15.4	74.7
NVIG	15.4 ^{ns}	14.1	5.1	3.8	2.6	85.0
1.0% - 20 minutes						
VIG*	14.1 b	75.6 ab	30.8 ab	51.3 ab	100 a	38.8
MDV*	85.9 a	24.4 ab	69.2 ab	48.7 ab	0.0 b	46.2
NVIG	0.0 ^{ns}	0.0	0.0	0.0	0.0	-
1.0% - 40 minutes						
VIG	42.3 ^{ns}	100	80.8	82.1	100	23.8
MDV	57.7 ^{ns}	0.0	19.2	17.9	0.0	101.7
NVIG	0.0 ^{ns}	0.0	0.0	0.0	0.0	-

VIG – Vigorous (<0.33); MDV – Medium vigor (0.33 – 0.66); NVIG – Non-vigorous (>0.66). *Means followed by the same letters in the row do not differ statistically by Tukey's test at 0.05 probability level. ^{ns} – non-significative.

between the lots at 1.0% and 20 minutes. The lot A had most presented medium vigor seeds differently to lot E that presented only vigorous seeds, contradicting to the germination and emergence results. One hypothesis about the image analysis was that it could reduce the subjectivity promoted by visual method by given a quantitative result for each seed, however it was not observed in this research.

These results (Tables 4 and 5) corroborate to Santos et al. (2011) relating that the pH exudate was not efficient to part lots of soybean seeds in different vigor levels just as Barboza et al. (2014), which suggested that this test was not effective to analyze the massive viability and vigor of *Guazuma ulmifolia* seeds because it did not correlate to the emergence test, as seen in this study.

However, Amaral and Peske (2000), Cabrera and Peske (2002), and Alves et al. (2016) found positive correlation between the pH exudate and germination/emergence tests for wheat, corn and cramble seeds. In this way, this test is not recommended at these conditions to analyze the viability and vigor of *C. limonia* seeds, requiring more research about this specie to adjust the best concentration (indicator solution) and immersion periods of the seeds.

According to the Pearson correlation, there were not observed significance at 0.05 of probability for the analyzed characteristics, showing that pH exudate does not correspond to the germination and emergence test (Table 6).

The methodology was conducted correctly during the pH exudate test, but did not achieve the expected results to measure the 'Rangpur Lime' seeds quality, probably because of the seed coat that surrounds the seed. The citrus seed characterizes to present double seed coat, an outer coat derived from the outer integument and an inner seed coat, very compact consisting of the remnants of inner integument, nucellus and endosperm (Koltunow et al., 1995).

This implies that these tissues hinder the seeds to exude quickly, requiring more time to notice their real performance during the pH exudate test. Another possibility would be the extraction of these tissues before to carry the test out.

Table 6. Pearson correlation (Coefficient of simple correlation) between primary root protrusion (PRP), germination (Germ), germination speed index (GSI), emergence in greenhouse (EG), emergence speed index (ESI), shoot fresh matter (SFM), and shoot dry matter (SDM) of vigorous and medium vigor of *Citrus limonia* seeds, according to exudate pH at different concentration and periods at the visual and image analysis.

Treat.	Classification	PRP ^{ns}	Germ ^{ns}	GSI ^{ns}	EG ^{ns}	ESI ^{ns}	SFM ^{ns}	SDM ^{ns}
……Visual analysis……								
0.2%	VIG	-0.360	-0.347	-0.524	-0.050	-0.128	-0.307	-0.348
20 min	VIG+MDV	-0.163	-0.349	-0.396	0.116	-0.043	-0.454	-0.489
0.2%	VIG	-0.267	-0.146	-0.241	-0.128	-0.175	-0.222	-0.302
40 min	VIG+MDV	-0.463	-0.273	-0.491	-0.064	-0.128	-0.185	-0.243
0.6%	VIG	-0.055	0.357	0.429	-0.024	-0.030	0.213	0.114
60 min	VIG+MDV	-0.186	0.166	0.206	-0.278	-0.179	0.249	0.194
0.6%	VIG	-0.395	0.141	-0.010	-0.408	-0.254	0.309	0.235
80 min	VIG+MDV	-0.056	0.062	0.244	-0.131	-0.122	0.061	0.019
1.0%	VIG	-0.323	-0.001	-0.375	-0.024	-0.021	0.005	-0.051
20 min	VIG+MDV	-0.163	-0.157	-0.272	0.011	-0.088	-0.328	-0.400
1.0%	VIG	-0.332	-0.102	-0.472	-0.093	-0.077	-0.075	-0.121
40 min	VIG+MDV	0.052	-0.029	-0.052	0.261	0.075	-0.370	-0.444
……Image analysis……								
0.2%	VIG	0.084	-0.097	-0.027	0.264	0.046	-0.472	-0.550
20 min	VIG+MDV	-0.159	-0.292	-0.293	0.095	-0.065	-0.429	-0.482
0.2%	VIG	-0.423	-0.025	-0.307	0.039	0.011	0.133	0.083
40 min	VIG+MDV	-0.093	-0.261	-0.243	0.165	-0.027	-0.467	-0.525
0.6%	VIG	-0.314	0.328	0.139	-0.446	-0.205	0.554	0.504
60 min	VIG+MDV	-0.164	0.347	0.301	-0.338	-0.155	0.470	0.420
0.6%	VIG	0.197	0.405	0.571	0.267	0.144	0.058	-0.035
80 min	VIG+MDV	-0.015	0.125	0.281	-0.117	-0.042	0.276	0.288
1.0%	VIG	0.153	0.327	0.214	0.050	0.118	0.248	0.247
20 min	VIG+MDV	-	-	-	-	-	-	-
1.0%	VIG	-0.075	-0.482	-0.537	0.072	0.019	-0.296	-0.208
40 min	VIG+MDV	-	-	-	-	-	-	-

VIG – Vigorous; MDV – Medium vigor. n.s. – non–significative.

As reported before, this test did not present comparable result to the germination and emergence tests, likely because of the time. In both test, the seeds remained more than 45 days surrounded by germitest paper and sand, for germination and emergence test respectively. Bewley et al. (2013) suggest that microbial action can mediate in the opening of impermeable seed coats. Under these circumstances, the tissues surrounding the embryos, zygotic and nucellar, could have been slowly degraded by microorganisms present into the substrates, increasing the seed permeability to water and gases and consequently allowing its germination and emergence. However, this effect was not observed for the pH exudate test.

In general, there were observed high coefficient of variation in both analysis (visual and image), which indicates a substantial range in the dataset. Also, the membrane permeability test at different concentration solutions and periods did not correlate to the germination and emergence tests in both analysis, not allowing to perform the relation between visual and image analyzes, since these analyses were not correlated to any test.

Conclusions

The 'Rangpur Lime' cannot have their seed physiological potential, quickly and accurately, determined by the pH exudate test under these experimental conditions, through visual method or even when it is used a quantitative image analysis.

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