Original Research Article

Phytotests for assessing phytotoxicity of “Blue moon” liquid detergent: *Lens culinaris* seeds

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The phytotests for phytotoxicity assessment is to analyze the developmental responses of plant seeds to hazardous materials in the environment. The effectiveness of the phytotests in testing the varied contaminants was validated and reported. However, the phytotoxicity of synthetic laundry detergents (SLDs) is poorly understood. Accordingly, “Blue moon” liquid laundry detergents (“Blue moon”-LLD) was targeted as a contaminant in study. Lentil (*Lens culinaris*) tests were used to test the phytotoxicity of “Blue moon”-LLD. Consequences showed percent germination of seed (PGS, ca. 0%-80%) and root length (RL, ca. 0-5 mm) decreased significantly toward the increase in concentration (0.0%, 0.1%, 0.5% and 1.0%) of “Blue moon”-LLD after 72-h. Exposure to the “Blue moon”-LLD throughout 96-h further lowered PGS (ca. 0%-90%) and RL (ca. 0-9 mm). Correlation analysis proved a negative linearity ($k = -155.84$, $R^2 = 0.97$, 72-h; $k = -173.12$, $R^2 = 0.98$, 96-h) between test concentrations and PGS and between the concentrations and RL ($k = -10.4$, $R^2 = 0.91$, 72-h; $k = -15.48$, $R^2 = 0.94$, 96-h). It manifested that the phytotoxicity of “Blue moon”-LLD was a concentration-dependent toxicity. The study is aimed at validating the sensitivity, effectiveness, alternative use of animal toxicity tests and inexpensive phytotesting protocol for phytotoxicity assessment.

Keywords: Phytotests, “Blue moon”, seed germination, root elongation, *Lens culinaris*

INTRODUCTION

The potential application of plant seeds to ecotoxicity tests for hazardous materials catches significant attention of ecotoxicologists to phytotests. The seed germination (SG) and the root elongation (RE) of the seeds were measured in the phytotests (Bellino et al, 2018; Di Salvatore et al, 2008; EPA, 1996; Khan et al, 2019). SG and RE are two significant endpoints for the phytotoxicity assessments of contaminants (Bellino et al, 2018; Di Salvatore et al, 2008; Luo, et al, 2019). The xenobiotic effects and oxidative stress on the SG and RE were well documented at the lethal or sublethal concentration of contaminants (Bengtson Nash et al, 2005; Chang et al, 2015). The xenobiotics might cause a hormetic response at low concentrations. However, concentrated xenobiotic solution could inhibit SG and RE until sterilizing

the seeds (Bengtson Nash et al, 2005; Ostroumov, 1990). On the other hand, the chemical oxidative stress might lead to an irreversible damage to SG and RE because of the reactive oxygen species (ROS) (Gomes et al, 2019; Khan et al, 2019; Mtisi and Gwenzi, 2019). Therefore, SG and RE could be the effective endpoints for toxic contaminants. In addition, dozens of phytotests were carried out by trial and error of various plants to validate the phytotesting sensitivity (Bellino et al, 2018; Bhat et al, 2019). For example, Ostroumov et al. (2014, 2014s and 2017) substantiated that SG and RE were also highly sensitive endpoint measures of phytotoxicity (Ostroumov, 2017; Ostroumov et al, 2014; Ostroumov and Solomonova, 2014). So far, it has been proved that SG and RE are both effective and sensitive

endpoints.

To date, a large quantity of plant species has been reported in the successful cases of phytotoxicity tests. For examples, tomato (*Solanum lycopersicum*) (Bellino et al., 2018, Di Salvatore et al., 2008, Pan and Chu 2016), lettuce (*Lactuca sativa*) (Bagur-González et al., 2010, Di Salvatore et al., 2008; Lago-Vila et al., 2019; Mtisi and Gwenzi, 2019), maize (*Zea mays*) (Bhat et al., 2019; Gomes et al., 2019), wheat (*Triticum aestivum*) (Chang et al., 2015, Lian et al., 2020), Chinese cabbage (*Brassica rapa*) (Luo et al., 2019), carrot (*Daucus carota*) (Pan and Chu, 2016), cucumber (*Cucumis sativus*) (Pan and Chu, 2016), lentil (*Lens culinaris*) (Khan et al., 2019; Ostroumov, 2017), macrophytes (*Elodea canadensis* (Ostroumov and Solomonova, 2014) and *Ceratophyllum demersum* (Ostroumov et al., 2014)), etc. Most of them are recommended by the United States Environmental Protection Agency (USEPA) as model test plants (EPA, 1996). Among them, the lentil (*Lens culinaris*) tests showed a wide range of sensitivity and effectivity toward varied toxic contaminants (Khan et al., 2019; Ostroumov, 2017).

The phytotesting protocol that prioritized *Lens culinaris* possessed the following advantages. Compared to the chemical tests, the phytotests were not only able to measure the results but also show the dynamical biotic response to toxicants (Bengtson Nash et al., 2005; Bhat et al., 2019). Unlike the microbial tests, the phytotests could play critical role *in vitro* and *in vivo* tests like animal tests (Bhat et al., 2019). Apart from animal tests, however, the phytotests concern the vascular terrestrial plants rather than living animals. It has therefore never challenged bioethics (Ostroumov, 2017). Besides, the phytotests cost less than chemical, microbial and animal tests (Bhat et al., 2019, Pan and Chu, 2016). All of these advantageous illustrations determined that the phytotests are potentially alternative and inexpensive biotests for toxicity assessments.

The phytotesting protocol is used for testing a broad variety of contaminants. It can be to test some single-components of a detergent, such as antibiotics (Bellino et al., 2018; Gomes et al., 2019; Luo et al., 2019; Pan and Chu, 2016), herbicides (Bengtson Nash et al., 2005), sodium dodecyl sulfate (SDS) (Chang et al., 2015, Lazareva and Ostroumov, 2009), the nanomaterials (Khan et al., 2019; Lago-Vila et al., 2019; Lian et al., 2020; Ostroumov et al., 2014), various heavy metals (Bagur-González et al., 2010; Di Salvatore et al., 2008), etc. Also, it is applicable for toxicity tests of contaminant mixtures like coal ash (Mtisi and Gwenzi, 2019), synthetic detergents (Ostroumov and Solomonova, 2014; Warne and Schiffko, 1999), industrial effluents (Bhat et al., 2019), etc. The phytotoxicity of the synthetic multicomponent detergent (e.g., SLDs), however, remains unclear, except that toxicity of the single-components (e.g., alkaline agents, surfactants, builders, enzymes, etc.) of detergents were reported. (Bajpai and Tyagi, 2007; Ostroumov and Solomonova, 2014; Warne and Schiffko, 1999).

Lens culinaris is a widespread plant species in the terrestrial ecosystems. It shows a favorable performance of phytotoxicity assessment. Hence, *Lens culinaris* is a model

plant for environmental risk assessment (Khan et al., 2019). Furthermore, *Lens culinaris* seeds are the elementary vegetable foodstuffs through the trophic webs to affect humans. Thus, *Lens culinaris* phytotests can account for human health assessment as a bioindicator (Lago-Vila et al., 2019, Pan and Chu, 2016).

The study carried out an array of *Lens culinaris* tests that subcategorized SG tests and RL tests, dividedly. Seed germination index (SGI) and root length index (RLI) worked out for the qualitative assessment of phytotoxicity. The objective of study is to build a validated phytotesting system for liquid detergent ecotoxicity tests, in which 1) SGI and REI are calculated for ecotoxicological assessment; 2) correlation analysis can show relationship between test concentrations and SG or RE. Finally, a sensitive, effective, alternative and inexpensive phytotesting protocol of using *Lens culinaris* seeds was validated.

MATERIALS AND METHODS

“Blue moon” detergent and *Lens culinaris* seeds

“Blue moon”-LLD are supplied by Blue Moon group Co., Ltd, (Guangzhou, China). The formulation of “Blue moon”-LLD is composed of anionic surfactant (8%-18%), preservatives (2%-6%), stabilizers (< 1%), enzymes (0.3%-0.8%), conditioners (< 1%), colorant (< 1%) and fragrances (< 1%), etc. The 10 g liquid detergent dissolved in 3 L water ($\approx$ 0.3%) is best ratio for laundry cleaning. Hence, the test concentrations were determined at 0.0%, 0.1%, 0.5% and 1.0%. *Lens culinaris* seeds were purchased from Ailimeng Seed Sci-Tech Co., Ltd (Shanghai, China). All the solutions used in this work were prepared with ultrapure water.

Phytotesting preparations

Phytotesting preparations include test solution and test seed preparation. To prepare the stock solution (1.0% test solution), the commercial “Blue moon”-LLD mixture was pipetted and diluted at the ratio of 1:100 (v/v). Other test solutions (0.1% and 0.5% test solutions) were prepared on the basis of the stock solution. The ultrapure water was set as blank control (0.0% test solution) for the guarantee of *Lens culinaris* seed viability. All the test solutions were prepared freshly for the phytotests. The rest of stock solution was stored in a fridge (4 °C) for subsequent use.

To prepare test seeds, the handpicked seeds were immersed (5 min) and rinsed (3 times) using ultrapure water before the phytotests. A sheet of round filter paper (110 mm Whatman No. 1) was laid on the bottom of each glass Petri dish (100 × 15 mm). Finally, the sanitary seeds were divided evenly into 12 dishes at random. Each Petri dish therefore contained 30 seeds.

Phytotesting procedures

The phytotests were carried out by incubating *Lens culinaris*

Figure 1 : Morphology of *Lens culinaris* seeds imaged after 96-h exposure to “Blue moon”-LLD. The SG tests after 96-h incubation in (a) 0.0% (control), (b) 0.1%, (c) 0.5% and (d) 1.0% “Blue moon”-LLD (20 mL) at 20.0 ± 1.5 °C. Moreover, the magnified (3 times as large) images focused on *Lens culinaris* RE after 96-h testing in (e) 0.0% (control), (f) 0.1%, (g) 0.5% and (h) 1.0% “Blue moon”-LLD.

seeds for 72-h or 96-h of exposure to “Blue moon”-LLD solution at various concentrations (0.0% (control), 0.1%, 0.5% and 1.0%). Each Petri dish contained 30 test seeds. Each group encompassed test dishes in triplicate (i.e., 3 of the total 12 dishes tested at same concentration) at 0.0% (control), 0.1%, 0.5% and 1.0% of “Blue moon”-LLD solution. Consequently, the samples in triplicate were moistened with a test solution (20 mL, 0.0%, 0.1%, 0.5% or 1.0% concentration). All the test samples were incubated in the dark at 20.0 ± 1.5 °C. After 72-h and 96-h, the number of germinated seeds was counted manually, while the length

of elongated root was measured with twitter and ruler. Notably, 1-plus mm of RL was valid for the germinated seeds in this study (Di Salvatore et al, 2008; Gomes, et al, 2019).

Morphological analysis

A high-resolution digital camera (Nikon, Tokyo, Japan) was employed to characterize morphology of each test sample for 96-h exposure to the concentrations of 0.0% (control), 0.1%, 0.5% and 1.0%. The most characteristic images were selected and combined together as shown in Figure 1 (a), (b), (c) and (d). The magnified images (Figure 1 (e), (f), (g) and (h)) were listed on the right side, while original ones (Figure 1 (a), (b), (c) and (d)) are on the left.

Data analysis

Microsoft Excel 2019 was used to calculate PGS (%) and RL (mm). The results of PGS and RL were expressed in forms of mean $\pm$ standard error. The Excel worksheet offers *t*-test to examine the statistical difference ($p < 0.05$, significance; $p > 0.05$, insignificance) between control (0.0%) and test groups (0.1%, 0.5% and 1.0%).

To assess the phytotoxicity, the raw data were further converted to SGI by Eq. (1) and RLI by Eq. (2) as follows,

$$SGI = \frac{N_T(i) - N_C}{N_C} \quad (1)$$

$$RLI = \frac{L_T(i) - L_C}{L_C} \quad (2)$$

where $N_T(i)$ and N_C represent the number of germinated seeds in test (i) and in control, respectively. $L_T(i)$ and L_C denote the mean root length in test (i) and in control. Based on SGI and RLI values, four empirical assessments of phytotoxicity were reported, such as (1) slight ($-0.25 \leq SGI$ or $RLI < 0$), (2) moderate ($-0.5 \leq SGI$ or $RLI < -0.25$), (3) high ($-0.75 \leq SGI$ or $RLI < -0.5$), and (4) extreme toxicity ($-1 \leq SGI$ or $RLI < -0.75$) (Bagur-González et al, 2010; Mtisi and Gwenzi, 2019).

Linear analysis

The linear analysis was to understand the concentration-response correlation. Origin Program 2018 software was used to plot the decrease in PGS and RL along with the increasing concentration in Figure 4. The mean values of PGS (Figure 4 (a)) and RL (Figure 4 (b)) were expressed with scatterplots. The error bars denoted standard errors. The software also established linear analysis of the scattering points versus test concentrations. The linear regression equations showed the correlation coefficients (k) and the determination coefficient (R^2).

RESULTS AND DISCUSSION

Morphological images

The morphological characteristics of *Lens culinaris* SG and

Table 1. Summary of *Lens culinaris* SG phytotests for “Blue moon”-LLD. The tests were carried out at each concentration (0.0% (control), 0.1%, 0.5% and 1.0%) kept at 20.0±1.5 °C in the dark for 72 h and 96 h, respectively.

Concentration (%)	72 h			96 h		
	PGS (%)	SGI	PC	PGS (%)	SGI	PC
0.0 (control)	83.33 ± 3.93	0.00	-	88.89 ± 2.27	0.00	-
0.1	41.67 ± 6.80	-0.50	High	41.67 ± 3.41	-0.53	High
0.5	0.00 ± 0.00	-1.00	Extreme	0.00 ± 0.00	-1.00	Extreme
1.0	0.00 ± 0.00	-1.00	Extreme	0.00 ± 0.00	-1.00	Extreme

SG: seed germination, “Blue moon”-LLD: “Blue moon” liquid laundry detergent, PGS: percent germination of seed (mean ± standard error), SGI: seed germination index, PC: phytotoxicity class.

Table 2. Summary of *Lens culinaris* RE phytotests for “Blue moon”-LLD. The tests were carried out at each concentration (0.0% (control), 0.1%, 0.5% and 1.0%) kept at 20.0±1.5 °C in the dark for 72 h and 96 h, respectively.

Concentration (%)	72 h			96 h		
	RL (mm)	RLI	PC	RL (mm)	RLI	PC
0.0 (control)	5.2 ± 0.5	0.00	-	9.0 ± 1.2	0.00	-
0.1	3.2 ± 0.5	-0.57	High	5.6 ± 0.5	-0.71	High
0.5	0.0 ± 0.0	-1.00	Extreme	0.0 ± 0.0	-1.00	Extreme
1.0	0.0 ± 0.0	-1.00	Extreme	0.0 ± 0.0	-1.00	Extreme

RE: root elongation, “Blue moon”-LLD: “Blue moon” liquid laundry detergent, RL: root length (mean ± standard error), RLI: root length index, PC: phytotoxicity class.

RE over 96-h incubation were imaged in Figure 1. Figure 1 (a) showed the morphology of *Lens culinaris* SG tested at 0.0% (control), (b) at 0.1%, (c) at 0.5% and (d) at 1.0%. Most (ca. 90%) of germinated seeds were observed in Figure 1 (a), which validated the viability of *Lens culinaris* seeds (Chang et al., 2015; Gomes, et al., 2019). As the concentration increased, the number of SG plummeted at 0.1% concentration (Figure 1 (b)). All of the seeds unfortunately perished at 0.5% (Figure (c)) and 1.0% concentrations (Figure (d)). The observation was consistent with the PGS (%) calculation presented in Table 1 and Figure 2. To better observe RE morphology, Figure 1 (e) was magnified (3 times as large) from Figure 1 (a). In turn, Figure 1 (f), (g) and (h) were from (b), (c) and (d), accordingly. The blooming RE appeared in the control sample (Figure 1 (e)), while undeveloped RE could be found in 0.1% (Figure 1 (f)) and 0.5% (Figure 1 (g)) test group, respectively. The magnified image (Figure 1 (h)) of 1.0% test group showed the ungerminated seeds, and no RE was measurable. In summary, the morphological RE (shown as RL (mm) parameter in Table 2 and Figure 3) decreased in proportion to SG (shown as PGS (%) in Table 1 and Figure 2).

SG phytotests

The PGS values obtained in SG phytotests were listed in Table 1 and graphed in Figure 2. Table 1 showed the highest PGS value (83.33% ± 3.93%, $p < 0.05$, 72-h) in control, the intermediate PGS value (41.67% ± 6.80%, $p < 0.05$, 72-h) in 0.1% test group. However, no obvious PGS (0.0%, $p < 0.05$) in both 0.5% and 1.0% test groups were observed. The

similar PGS values (Table 1) could be obtained in control (88.89% ± 2.27%, $p < 0.05$), 0.1% (41.67% ± 3.41%, $p < 0.05$), 0.5% and 1.0% (0.0%, $p < 0.05$) test groups over 96-h of exposure. The statistical difference (p -test, $p < 0.05$) was significant. It manifested that *Lens culinaris* SG was sensitive to “Blue moon”-LLD (Bagur-González et al., 2010). However, the statistical difference (p -test, $p > 0.05$) between 72-h and 96-h testing was insignificant. It revealed that 72-h phytotests had already been able to satisfy the phytotoxicity assessment (Ostroumov, 2017; Ostroumov, et al., 2014). The results of SG phytotests were graphed in Figure 2. All the SG results were consistent with the morphological images (Figure 1) and the following RE results (Table 2 and Figure 3).

RE phytotests

The RL values in RE phytotests were given in Table 2 and depicted in Figure 3. Table 2 showed the RL value (5.2 ± 0.5 mm, $p < 0.05$) in 0.0%, the RL value (3.2 ± 0.5 mm, $p < 0.05$) in 0.1%, and no RL (0.0 mm, $p < 0.05$) in 0.5% and 1.0% test groups. Table 2 also presented a decreasing trend towards RL values (9.0 ± 1.2 mm in controls, 5.6 ± 0.5 mm in 0.1%, 0.0 mm in 0.5% and 1.0% test groups, all $p < 0.05$) after 96-h period of RE phytotests. Compared to SG, RE was a more dynamical and quantitative endpoint of “Blue moon”-LLD toxicity assessment. For example, RE results in control after 72-h differed significantly from the counterparts after 96-h in statistical analysis, which implied that seed roots could sustain the elongation in healthy environment with time going on. (Chang et al., 2015; Di Salvatore et al., 2008). This is largely due to the direct exposure of the roots to the “Blue

Figure 2: The PGS variations of *Lens culinaris* seeds after 72-h and 96-h testing for “Blue moon”-LLD at different concentrations. The SG phytotests (72-h and 96-h testing) of “Blue moon”-LLD at each concentration (0.0% (control), 0.1%, 0.5% and 1.0%) were measured as PGS (mean $\pm$ standard error, %). The column and error bars represent the mean values of PGS and their standard errors, respectively. The statistical analysis shows the significant difference ($p < 0.05$) between every test and control

Figure 3: The RL variations of *Lens culinaris* seeds after 72-h and 96-h testing for “Blue moon”-LLD at different concentrations. The RE phytotests (72-h and 96-h testing) of “Blue moon”-LLD at each concentration (0.0% (control), 0.1%, 0.5% and 1.0%) were measured as RL (mean $\pm$ standard error, %). The column and error bars represent the mean values of RL and their standard errors, respectively. The statistical analysis shows the significant difference ($p < 0.05$) between every test and control.

moon”-LLD solution (Figure 1). This demonstrated *Lens culinaris* RE phytotests were more sensitive than SG phytotests for testing phytotoxicity (Bagur-González et al,

2010; Bellino et al, 2018; Mtisi and Gwenzi, 2019; Pan and Chu, 2016). For better comparisons, all the RE phytotesting results were illustrated in Figure 3.

Figure 4: Correlation analysis of the PGS (%) and the RE (mm). (a) The PGS (mean $\pm$ standard error), and the (b) RE (mean $\pm$ standard error) as the function of "Blue moon"-LLD concentrations over 72-h and 96-h testing were analyzed, respectively. Error bars represent the standard errors.

Figure 5: Schematic diagram of *Lens culinaris* phytotests.

Correlation analysis

The PGS versus "Blue moon"-LLD concentration were well-fitted with negative linear correlation ($k = -155.84$, $R^2 = 0.97$, 72-h; $k = -173.12$, $R^2 = 0.98$, 96-h) shown in Figure 4 (a). Likewise, a negative linearity ($k = -10.4$, $R^2 = 0.91$, 72-h; $k = -15.48$, $R^2 = 0.94$, 96-h) between RL and "Blue moon"-LLD concentrations was given in Figure 4 (b). The linear models estimated that SG and RE diminished linearly until no detection. This suggested the phytotoxicity was a concentration-response effect on *Lens culinaris* SG and RE (Chang et al, 2015; Mtisi and Gwenzi, 2019). Threshold value is also a key parameter to assess environmental risks and human health in toxicological researches (Mtisi and Gwenzi, 2019). Up to now, many articles encouraged the use

of the half maximal effect concentration (EC_{50}) as threshold value for toxicity assessments (Mtisi and Gwenzi, 2019; Pan and Chu, 2016; Warne and Schiffko, 1999). Finally, the linear regression equations (Figure 4 (a) and (b)) could estimate EC_{50} of SG at 0.25% of PGS and EC_{50} of RE at 0.25% of RL, respectively. The threshold EC_{50} could be regarded as the Chinese national standard of detergent discharge.

Phytotoxicity assessment

To assess the phytotoxicity of "Blue moon"-LLD, the PGS were converted to SGI by Eq. (1) and listed in Table 1. RLI were converted from RL by Eq. (2) and listed in Table 2. If the control was regarded as no toxicity (0.00), SGI (-0.50, 72-h; -0.53, 96-h) in 0.1% test group indicated that the

phytotoxicity class (PC) was high (Bagur-González et al, 2010; Mtisi and Gwenzi, 2019). According to this, SGI values in 0.5% and 1.0% test samples (Table 1 and Figure 1 (c), (d), (g) and (h)) equated to -1.00. it suggested “Blue moon”-LLD at 0.5% and 1.0% concentration was extremely phytotoxic. Similarly, RLI (-0.57, 72-h; -0.71, 96-h) in 0.1% showed high phytotoxicity. Also, extreme phytotoxicity was exhibited by RLI (-1.00, 72-h; -1.00, 96-h) in both 0.5% and 1.0% test samples. 0.1% test sample attested that RLI (-0.57, 72-h; -0.71, 96-h) were both less than SGI (-0.50, 72-h; -0.53, 96-h). In summary, the lower the value of SGI and RLI was, the higher phytotoxicity would be. Thus, RLI was the more restricted phytotoxic index than SGI due to the higher sensitivity of RE than SG (Mtisi and Gwenzi, 2019).

Future perspective and outlook

The procedure of *Lens culinaris* phytotesting protocol was described through a schematic diagram in Figure 5. The *Lens culinaris* phytotesting method systematically include two parts, SG and RE phytotests. All PGS and RL data (Table 1 and 2) supported that SG and RE were sensitive and effective endpoints to phytotoxicity assessment. In consequence, both SG and RE phytotests ensure the technical qualification for future application. The diagrammatic structure of phytotests (Figure 5) explains why the method is convenient and applicable for phytotoxicity assessments (Bhat et al, 2019). Comparing to animal tests, the phytotests will never challenge bioethics. Moreover, phytotests can keep the results in conformity with animal tests (Ostroumov, 2017). In the future perspective, the sustainable, ecofriendly and non-animal tests can facilitate the technical progress and application. In addition, cost-efficiency, low risk, and data reproducibility also shed the light on the applicable prospect of environmental risks and human health assessments.

CONCLUSION

Lens culinaris phytotests for phytotoxicity assessment of “Blue moon”-LLD were validated in the study. In the phytotests, the *Lens culinaris* seeds were used to screen for phytotoxicity at various concentrations. SG and RE decreased linearly until no observable measurements as the concentration increased. Linear correlation analysis showed that either PGS or RL versus concentration was a concentration-response relationship. EC_{50} could be the threshold value of “Blue moon”-LLD effluent in wastewater dischargement. SGI and RLI showed that no toxicity in control, high toxicity in 0.1%, and extreme toxicity in 0.5% and 1.0% test samples. In conclusion, *Lens culinaris* test is a sensitive, effective, inexpensive and alternative phytotest for environmental risks and human health assessments.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the paper.

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