Optimizing the Utility of *Allium cepa* L. var. *aggregatum* (sibuyas Tagalog) for the *Allium* Test by Elucidating its Mitotic Periodicity and Rhythmicity Under Varying Light Conditions

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ABSTRACT

The occurrence of pattern of mitotic activity has long been studied in different plants; in the onion *Allium cepa*, determination of its mitotic activity has led to its utilization in the *Allium* test for cytotoxicity and mutagenicity of test substances. In this study, the pattern of mitotic activity of *A. cepa* var. *aggregatum* and the effect of light exposure on mitotic activity were determined to test the utility of *A. cepa* L. var. *aggregatum* as an alternative to the common onion, *A. cepa*, for the *Allium* test. Bulblets of *A. cepa* var. *aggregatum* were allowed to root for three days in tap water under three different set-ups: constant light exposure set-up (Light), constant dark set-up (Dark) and 12 hours light-12 hours dark set-up (Light-Dark). The root tips from the bulblets were then excised and subjected to microscopic observation for the mitotic index (MI) every hour after the third day. The results showed no significant difference observed across the three set-ups. However, MI for the Dark and Light set-ups were periodic, showing peaks or maxima of MI falling between 11 AM and 12 PM, whereas that of Light-Dark set-up was rhythmic, having an hourly fluctuation, but also showed maximum between 11 AM and 12 PM. It is recommended that *A. cepa* var. *aggregatum* root tips be excised between 11 AM and 12 PM for the *Allium* test.

Keywords: Allium cepa var. *aggregatum*, periodicity, rhythmicity, mitotic index, light, *Allium* test

INTRODUCTION

The *Allium* test is widely used as an environmental monitoring tool due to its ease of use and low cost as well as high correlation of its results with animal models (Evandri et al. 2000; Fijesko 1979; Quilang et al. 2008; Rathore et al. 2006; Vidakoviæ et al. 1993; Yi & Meng 2003). The test took its origin from Levan (1938), who studied the effects of colchicine on mitosis using *Allium cepa*. Since then, *A. cepa* has been used as a test organism for cytotoxicity and mutagenicity for environmental monitoring, which involved exposing the roots of *Allium* in a test substance, cutting the root and observing the root cells under the microscope for mitotic activity and chromosomal aberrations (Fijesko 1985).

In the *Allium* test, root tips should be cut during optimal mitotic activity, which is measured in terms of the mitotic index or MI (the ratio of observed dividing and non-dividing cells). Jong (1997) suggested that cutting should be done during midday (12:00-1:00 PM) based on previous studies that determined the optimal mitotic activity of *A. cepa*. However, studies determining optimal mitotic activity show inconsistent results (Lewis 1901; Kellicot 1904; Solomon & Trent 1941). Lewis (1901), using a 4-hour interval set up, showed mitotic maxima at 12 PM and 12 AM, demonstrating periodicity, which is characterized by the occurrence of one or two peaks of high mitotic activity. However, Winter (1929) argued that Lewis' results were uniform rather than showing periodicity as shown by the comparison of the percentage of dividing cells at different times. In a similar work by Kellicot (1904) where the onions were planted in soil and a 2-hour interval was employed, maxima were achieved at 11 AM and 1 PM. Later on, Solomon & Trent (1941) observed mitotic activity under varying light conditions at one-hour intervals. The results showed rhythmicity, or the occurrence of hourly fluctuations in mitotic activity, under normal light condition. However, periodicity was evident in continuous light and continuous dark set-ups.

Effect of light on mitosis is demonstrated in other systems (Yeoman & Davidson 1971; Nemota & Furuya 1985). In *Chattonella antiqua* (red tide flagellate), light can induce cell division, but it can also inhibit division by exposing cells that divide under continuous light (Nemota & Furuya 1985). In callus cultures, light tends to have inhibitory effect on cell division (Yeoman & Davidson 1971; Fraser et al. 1967). This decreased cell division, resulting to decreased callus formation, might be due to reduction of some substance by light (Yeoman & Davidson 1971). It is currently suggested that cell division is affected by circadian clock since cell cycle genes, i.e. cyclins and cyclin-dependent-kinase (CDK), are under circadian control (Moulager et al. 2007). Thus, alteration of exposure to light might change patterns of mitotic activity.

Recent works involving the *Allium* test include those of Evandri et al. (2000), which showed that water in polyethylene bottles could induce cytogenetic aberration; and Quilang et al. (2008), which demonstrated the effects of polychlorinated biphenyls (PCB's) on *Allium* root cells. Both studies made use of the common onion variety of *A. cepa*, though other studies also employed other varieties of *A. cepa* and even other species. For instance, Yi & Meng (2003) observed increased anaphase aberration and micronuclei as well as the presence of pycnotic cells in root tips of *A. sativum* (garlic). In another modified *Allium* test, Vidakovic (1993) used *A. cepa* var. *aggregatum* (*A. ascalonicum* in the literature) as test organism for waste drilling fluid, which promoted cytotoxicity, particularly inhibition of mitotic activity, in the onion roots. *Allium cepa* var. *aggregatum*, also known as the bunching onion or locally as "sibuyas tagalog," has since been synonymized with *A. ascalonicum* (Rabinovitch & Currah 2002). Its utility by Vidakovic (1993) could attest to its suitability, perhaps even more so than the common onion variety of *A. cepa* since *A. cepa* var. *aggregatum* were found to grow faster and in greater numbers than those of the common onions based on authors' personal observations.

Since different species and varieties are being employed for the *Allium* test, there is a need to ascertain the period of optimal mitotic activity for each type of *Allium* other than the common onion variety in order to optimize the time to cut the root tips. This study therefore aimed to investigate the periodicity and rhythmicity of mitotic activity in *A. cepa* var. *aggregatum* root tips. Furthermore, since mitotic

activity is hypothesized to be affected by light, this study also investigated the effects of varying durations of light exposure to the periodicity of mitotic activity in *A. cepa* var. *aggregatum*, by measuring the mitotic index (MI). Other factors that could affect periodicity of mitotic activity such as hormone activity and temperature were not considered in this study.

MATERIALS AND METHODS

Allium cepa var. *aggregatum* were purchased from the local market in San Jose, Nueva Ecija. Scaly leaves were removed until the bulb leaves were exposed. For each bulb, only one bulblet was used. Three set-ups were prepared similar to Solomon & Trent (1941) with modification of light source: 24 hour light exposure (Light), 12-hour-light-exposure then 12-hour-dark exposure (Light-Dark) and no light exposure (Dark). For each set-up, 18 bulblets were prepared as in Quilang et al. (2008) in which each was placed on top of a 100 mL glass bottle filled with tap water to expose the bottom end of the bulblet to water. The Light set up was exposed to continuous light, the Light-Dark set-up was alternately exposed to light and placed in a dark locker every 12 hours (i.e. 7 AM and 7 PM, respectively), and the Dark set-up was placed in a dark locker during the entire duration of the experiment. The Light and Light-Dark set-ups were exposed to light by placing them in front of fluorescent bulbs; light intensity ranged from 70 to 171.5 Fcandle and was measured using an ExTech EA31 Easy View™ light meter. Only 18 bulblets were prepared for each set up due to the limited space on the laboratory bench that allowed uniform exposure of the set-ups to the light apparatus. The roots were allowed to grow for three days.

On the 4th day, roots were excised every hour for 24 hours starting at 7 PM until 6 PM the next day. For every hour, 3 bulblets from each set-up were selected for root excision. Around 3-5 roots were cut from each bulblet using a pair of dissecting scissors. Since there were only 18 bulblets for each set up, each bulblet was sampled for roots for a total of four times to complete the hourly sampling over a 24 hour period. All 18 bulblets for each set up were randomly arranged prior to root excision. The roots were placed into 1.5-ml microfuge tubes containing Farmer's solution (1 part acetic acid, 3 parts ethanol) for fixation, after which they were stored at 4^0 C prior to use.

The root tips were prepared for examination through the squash method following El-Shahaby et al. (2003) with some modifications. Around 1-3 mm of the root tip was cut and placed on a glass slide, then exposed to 1 N HCl for 1-2 minutes, after which the HCl was blotted off using tissue paper. The root tip was then macerated using the cover slip. Aceto-orcein was added onto the macerated tissue; after 5-8 minutes, the slide was passed through a flame to heat-fix the stain on the tissue. A cover slip was placed on top of the tissue, and the excess aceto-orcein was blotted off using tissue paper. The slide was subsequently viewed under a 40x objective. For each root tip sample, 1000 cells were scored, taking note at which stage the cell was in: interphase, prophase, metaphase, anaphase or telophase. Scoring of cells for all the slides began in the upper left side of the slide and proceeded through a convention of moving the slides from left to right until 1000 cells were counted.

The mitotic index (MI) of each root tip sample for every hour (or the number of mitotic cells over 1000) was determined. For the determination of periodicity and rhytmicity, the MI for each hour was plotted. One-way Analysis of Variance (ANOVA) was performed for every hour, comparing the MI of each set-up. For each hour, there were 3 bulblets, and root tips were excised from these bulblets. Each root tip cut from a bublet for every hour was considered as a replicate, yielding three replicates for each hour.

To determine whether there was difference in the MI obtained from different treatments (Light set-up, Dark set-up and Light-Dark set-up), One-way Analysis of Variance (ANOVA) was employed using Excel 2007. Prior to ANOVA, data was transformed using arc sin (Zar 1999). Two trials were done for this experiment.

RESULTS

For Trial 1, the MI obtained from the Dark set-up across different time intervals were very similar with those of the Light-set-up, with the curves of the two set-ups showing a similar pattern (Figure 1). In the Light set-

up, the maxima MI were observed at 1AM, 12 PM and 8 PM, with the highest MI value obtained at 12 PM. In the Dark set-up, 11 AM had the highest MI followed by 12 AM and 8 PM. In both set ups, three maxima MI were observed: (1) 12 AM to 1 AM; (2) 11 AM to 12 PM; and (3) 8 PM; highest maxima was obtained at 11 AM to 12 PM. In the Light-Dark set-up, the maxima MI were at 12 AM, 6 AM and 12 PM; two of these maxima were within the range of the maxima MI in the other two set-ups. Unlike the other two set-ups, however, the MI of Light-Dark set-up appeared to be fluctuating at an hourly interval characterized by an increase in one hour followed by a decrease in the succeeding hour. Furthermore, the MI's of Light-Dark set-up were generally lower than in the other set-ups.

In Trial 2, the Light set-up had maxima MI at 12 AM, 12 PM, and 10 PM with highest maxima at 12 PM; Dark set-up at 12 AM, 12 PM and 11 PM with highest at 12 AM; the Light-Dark at 12 AM, 12 PM and 7 PM, with highest at 12 PM. The Light and Dark set-ups followed a similar pattern in Trial 1 in terms of the presence of maxima MI which were distinctly higher than the other points. In comparison to these two, the Light-Dark set-up had a fluctuating pattern from 12 AM to 12 PM, but continually decreased at 1 PM to 5 PM and increased at 7 PM then followed a fluctuating pattern (Figure 2). Based on the plots, Trial 1 and Trial 2 had almost similar results in terms of the patterns. However, unlike in Trial 1, the MI of Light-Dark set-up in Trial 2 was relatively high making it similar with the other two set-ups at 11 AM to 1 PM; hence the three plots seemed to overlap at this time interval.

In comparing the MI for the three set-ups, a significant difference based on ANOVA was obtained in Trial 1, though this was only observed at two time periods, 1

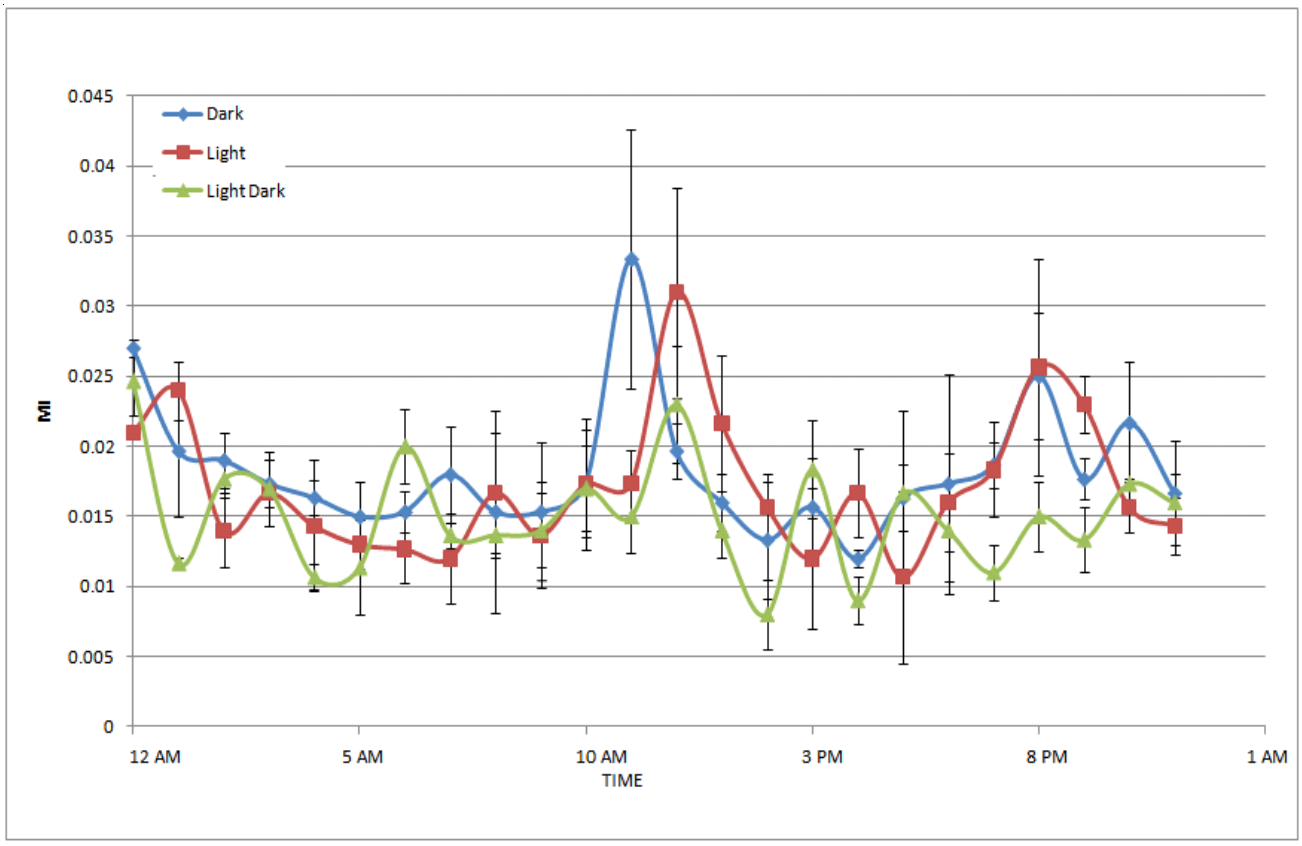

Figure 1. Plot of Mitotic Index for each hour of the day obtained from the first trial showing periodicity for Dark and Light while rhythmicity for Light-Dark set-up. Error bar corresponds to standard error.

AM and 9 PM, (p= 0.063 and p= 0.036, respectively) (Table 1). No significant difference was obtained from Trial 2 based on ANOVA (Table 2).

DISCUSSION

In both trials, the maxima fell in the range of 11 AM to 12 PM and 11 PM to 1 AM, which is in agreement with previous works on the common onion variety of *A. cepa* by Kellicot (1904) and Lewis (1901). These results agree with those of Solomon and Trent (1941); however, the maxima for the continuous light differed slightly, which was observed at 7 AM, 11 AM, 3 PM and 10 PM. The results for the Dark set-up were similar with those of Solomon & Trent (1941) except that the frequency of mitosis in the Dark-set-up in this study was lower than that obtained from the common onion. The Light-Dark set-up appeared to have lower MI than the other two set-ups, particularly in Trial 1. In Trial 2, the Light-Dark set-up appeared to have a similar MI value with the other two set-ups. This result, however, differed with that of Solomon and Trent (1941) in which the day-night set-up (Light-Dark) had the highest MI of all the three set-ups. In all set-ups, the frequency of mitosis was lower than that observed for the common onion variety by Solomon & Trent (1941).

The intensity of light used in the experiment was 70-171.5 Fcandle for both trials. This intensity is low compared to the presumed intensity of light from the sun (720 – 11000 Fcandle), which was used by Solomon & Trent (1941) for their Light-Dark set-up. The disparity in terms of the MI for this study and that of Solomon & Trent (1941) could be attributed to the different light intensities.

By definition of periodicity by Solomon & Trent (1941), which is the occurrence of waves or peaks for the

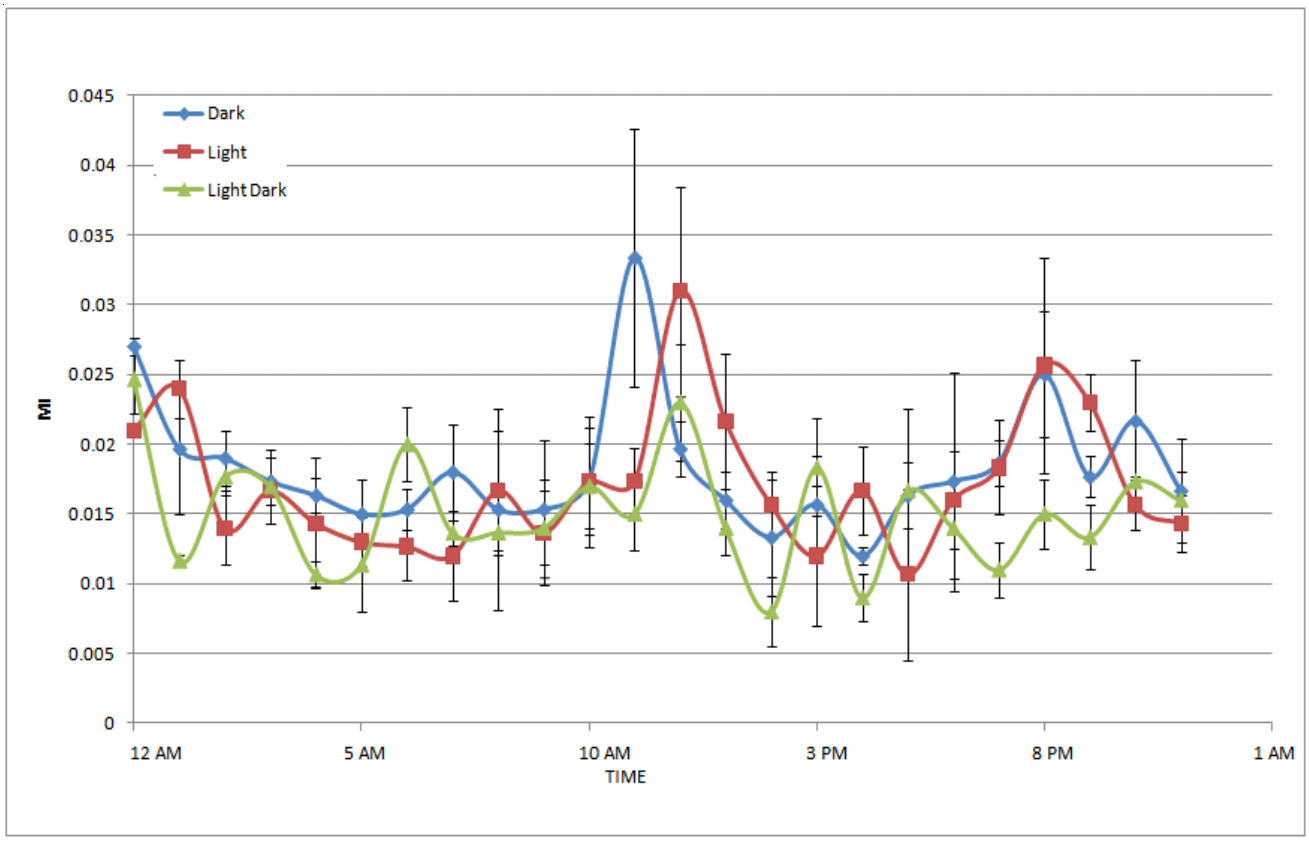

Figure 2. Plot of the Mitotic Index for each hour of the day obtained from the second trial showing periodicity for Dark and Light while rhythmicity for Light-Dark set-up. Error bar corresponds to standard error.

mitotic activity at different times of the day, the MI for *A. cepa* var. *aggregatum* is periodic in both Dark and Light set-ups. The maxima for both were distinct and higher than the other points. On the other hand, the MI of the Light-Dark set-up appeared to be rhythmic, defined by the occurrence of hourly fluctuations in mitosis.

In this study, we tested how light could cause these variations in mitosis. Using ANOVA, it was found that there was no significant difference between each treatment for each hour in Trial 2 (Table 2). Furthermore, the significant difference observed in Trial 1 did not justify the significance of light in defining the pattern for the mitotic activity, particularly as the difference occurred only in two time periods, at 1 AM and at 9 PM (Table 1). The Dark-set up and Light-set up had almost similar pattern (periodic pattern). This suggested that alteration of condition (i.e. from light to dark) affects mitotic activity, thus showing different

Table 1
The average Mitotic Index for each hour and set-up obtained from Trial 1 starting from 12 AM to 11 PM. ANOVA was used to determine the difference between each set-up for each hour. Values expressed as Mean±SD (Standard Deviation)

	Trial 1								
	Dark set-up			Light set-up			Light-Dark set-up		
Time	mean MI		SD	mean MI		SD	mean MI		SD
12 AM	0.0270	±	0.0010	0.0210	±	0.0000	0.0247	±	0.0042
1 AM	0.0197	±	0.0080	0.0240	±	0.0036	0.0117	±	0.0006*
2 AM	0.0190	±	0.0035	0.0140	±	0.0046	0.0177	±	0.0023
3 AM	0.0173	±	0.0029	0.0167	±	0.0040	0.0170	±	0.0046
4 AM	0.0163	±	0.0021	0.0143	±	0.0081	0.0107	±	0.0015
5 AM	0.0150	±	0.0044	0.0130	±	0.0030	0.0113	±	0.0059
6 AM	0.0153	±	0.0025	0.0127	±	0.0042	0.0200	±	0.0046
7 AM	0.0180	±	0.0060	0.0120	±	0.0056	0.0137	±	0.0015
8 AM	0.0153	±	0.0124	0.0167	±	0.0074	0.0137	±	0.0029
9 AM	0.0153	±	0.0085	0.0137	±	0.0065	0.0140	±	0.0046
10 AM	0.0173	±	0.0081	0.0173	±	0.0067	0.0170	±	0.0053
11 AM	0.0333	±	0.0160	0.0173	±	0.0042	0.0150	±	0.0046
12 PM	0.0197	±	0.0035	0.0310	±	0.0130	0.0230	±	0.0072
1 PM	0.0160	±	0.0035	0.0217	±	0.0084	0.0140	±	0.0035
2 PM	0.0133	±	0.0072	0.0157	±	0.0040	0.0080	±	0.0044
3 PM	0.0157	±	0.0060	0.0120	±	0.0087	0.0183	±	0.0060
4 PM	0.0120	±	0.0010	0.0167	±	0.0055	0.0090	±	0.0030
5 PM	0.0163	±	0.0042	0.0107	±	0.0107	0.0167	±	0.0102
6 PM	0.0173	±	0.0136	0.0160	±	0.0061	0.0140	±	0.0062
7 PM	0.0187	±	0.0029	0.0183	±	0.0059	0.0110	±	0.0035
8 PM	0.0250	±	0.0078	0.0257	±	0.0134	0.0150	±	0.0044
9 PM	0.0177	±	0.0025	0.0230	±	0.0035	0.0133	±	0.0040*
10 PM	0.0217	±	0.0076	0.0157	±	0.0031	0.0173	±	0.0006
11 PM	0.0167	±	0.0064	0.0143	±	0.0035	0.0160	±	0.0035

* corresponds to set-up in which there is significant difference (á=0.05)

patterns. Similarly, the effect of light could have been due to the previous light/dark regime in which cells were exposed; exposure to light after cell division could therefore have prevented further divisions (Nemota & Furuya 1985).

In addition to light, cell division is also regulated by cyclin and cyclin-dependent-kinase (CDK) proteins, which increases activity of CDK and release the transcription factor for genes of DNA replications, respectively. Moulager et al. (2007) suggested that cyclins and CDK's are under circadian control. These proteins, in turn, are induced by phytohormones, particularly cytokinin. Cytokinin is said to regulate cell cycle at both the G1/S phase and G2/M phase progressions (Zhang et al. 2004). Cytokinin concentrations also vary across different times of exposure to light (Nova'kova et al. 2005). The role of cylins, CDK's and cytokinins in the rate of cell division should therefore not be ruled out.

Table 2
The average Mitotic Index for each hour and set-up obtained from Trial 2 starting from 12 AM to 11 PM. ANOVA was used to determine the difference between each set-up for each hour. Values expressed as Mean±SD (Standard Deviation)

Trial 2									
	Dark set-up			Light set-up			Light-Dark set-up		
Time	mean MI		SD	mean MI		SD	mean MI		SD
12 AM	0.0267	±	0.0021	0.0203	±	0.0081	0.0210	±	0.0087
1 AM	0.0150	±	0.0035	0.0137	±	0.0046	0.0143	±	0.0045
2 AM	0.0127	±	0.0021	0.0137	±	0.0025	0.0190	±	0.0062
3 AM	0.0147	±	0.0083	0.0127	±	0.0031	0.0107	±	0.0055
4 AM	0.0167	±	0.0029	0.0157	±	0.0116	0.0130	±	0.0053
5 AM	0.0163	±	0.0057	0.0050	±	0.0044	0.0087	±	0.0042
6 AM	0.0123	±	0.0040	0.0137	±	0.0035	0.0190	±	0.0090
7 AM	0.0120	±	0.0072	0.0113	±	0.0055	0.0147	±	0.0015
8 AM	0.0077	±	0.0038	0.0140	±	0.0052	0.0207	±	0.0083
9 AM	0.0083	±	0.0029	0.0117	±	0.0081	0.0127	±	0.0064
10 AM	0.0147	±	0.0050	0.0107	±	0.0059	0.0210	±	0.0010
11 AM	0.0107	±	0.0055	0.0163	±	0.0081	0.0110	±	0.0069
12 PM	0.0230	±	0.0030	0.0243	±	0.0074	0.0233	±	0.0091
1 PM	0.0133	±	0.0093	0.0133	±	0.0025	0.0170	±	0.0082
2 PM	0.0077	±	0.0032	0.0100	±	0.0046	0.0150	±	0.0046
3 PM	0.0150	±	0.0026	0.0080	±	0.0066	0.0117	±	0.0012
4 PM	0.0150	±	0.0035	0.0070	±	0.0026	0.0087	±	0.0035
5 PM	0.0127	±	0.0051	0.0133	±	0.0103	0.0080	±	0.0044
6 PM	0.0113	±	0.0023	0.0087	±	0.0015	0.0117	±	0.0032
7 PM	0.0130	±	0.0026	0.0180	±	0.0026	0.0210	±	0.0104
8 PM	0.0110	±	0.0035	0.0170	±	0.0026	0.0147	±	0.0049
9 PM	0.0143	±	0.0015	0.0170	±	0.0046	0.0177	±	0.0065
10 PM	0.0127	±	0.0015	0.0183	±	0.0040	0.0177	±	0.0015
11 PM	0.0220	±	0.0085	0.0090	±	0.0060	0.0167	±	0.0025

No significant difference in all groups (á=0.05)

CONCLUSION

Even though it was not shown that light affects the pattern of mitotic activity, the periodicity and rhythmicity of mitosis was still evident in the experiment. In this study, the duration of exposure to light at 70-171.5 Fcandle illumination did not affect the mitotic activity in the root tips of *A. cepa var. aggregatum.* However, a pattern of mitotic activity was still seen, and these were periodic for the Light and Dark set-ups and rhythmic for the Light-Dark set-up. The mechanism causing these patterns is still not clear but is being suggested to be controlled by the cyclin and CDK proteins as well as the plant hormone cytokinin.

These observations have practical applications for the *Allium* test, particularly in the usage of *A. cepa. var. aggregatum,* a close relative of *A. cepa*, as model organism. Root excision is suggested to be done around 11 AM – 1PM where MI is highest; this time period also falls within the range given by Jong (1997) for root excision of *A. cepa.*

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