Use of cytological and molecular biological method for water pollution monitoring

A Farizan¹, M Y Norfatimah¹, Z N Aili¹, W Z A Lyena¹², and M A Indah²

¹School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
²Nonclinical Sciences, Faculty of Dentistry, Universiti Teknologi MARA 40450 Shah Alam, Selangor, Malaysia
*Corresponding author: lyenawatty@uitm.edu.my.

Abstract. Allium cepa test is widely used to evaluate the effects of water pollution based on dividing cells since it is a very sensitive tool for prediction and recognition of environmental stresses. This study aimed to evaluate the potential use of A. cepa as a molecular biological indicator to detect the presence of water pollution. A. cepa roots were exposed to water samples at 24 and 48 hours with tap water and hydrogen peroxide solution as controls. The effects of water sample exposure on A. cepa were analysed based on the plant cytotoxicity, genotoxicity, and expression of stress gene between test samples and control sets. The findings showed no significant changes observed in mitotic index of A. cepa exposed to water samples compared to negative control. There is also no expression of alliinase gene was detected. However, there is chromosomal abnormalities observed in A. cepa exposed at 24 and 48 hours. The chromosomal abnormalities detected include lagging chromosome, c-mitosis, disrupted anaphase, disrupted metaphase, spindle disturbance, and stickiness. Our study shows that molecular biological method could be a potential method to serve as an effective, sensitive, and useful marker for water pollution determination.

1. Introduction
For chemical or physical analysis, the water quality is determined by analysing the presence of hazardous substances or chemical composition in the water. Whereas the biological monitoring method involves detection of changes in the quality of water by examining the fish, plant and other creatures living in the water [1]. By the use of biological indicators, the natural state of a certain degree of contamination can be predicted [2]. Water contamination is due to discharges of industrial, agricultural and domestical effluents containing various kinds of chemical compounds [3]. The formation of complex mixtures arises from these chemical compounds can cause problems to human health and organisms that live and use the source [4, 5]. The chemical compounds that may cause water toxicity include heavy metals such as chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn) and lead (Pb). The water quality of the Shah Alam lake was fell in below 2B, close to 3B making it unsuitable for recreation and considered polluted water [6]. Toxicity is a harmful effect produced by continuous exposure to the toxic wastes. This test analysis is usually performed on plants, particularly among the higher plant species such as Allium cepa, Zea mays, Nicotiana tabacum, Crepis capillaris Tradescantia pallida, Vicia faba and Hordeum vulgare [7]. Higher plants are important biological materials that have large size chromosomes and immediate chemical responses with other biological systems thus, use of plant is therefore a good choice for an environmental sample analysis of cytotoxicity and genotoxicity detection [8-10].
Allium cepa assay is an effective test system used to evaluate the genotoxic potential of chemical substances in the environment due to its sensitivity and good correlation with mammalian test systems [11]. Allium test has been used in monitoring potential synergistic effects of a mixture of pollutants, including heavy metals as well as hydrophilic and lipophilic chemicals [12]. A variety of morphological and cytogenetic parameters that may serve as toxicity indicators, including the induction of micronuclei and chromosomal aberrations using root-tip cells [13] could be analysed by A. cepa test. The genetic categories of parameters analyzed by this test system have been performed on the plant roots [14]. Roots could exhibit the highest sensitivity, even at a lower concentration of the test chemical, with significant effects. The decreased of root growth of more than 45% [15] indicates the presence of toxic nature of substances with sub lethal effects on plants. The Allium cepa test is easy to perform, quick and very sensitive to detect environmental genotoxicity or antigenotoxicity of chemicals or natural plant products [14]. The cytotoxic level of a test compound can be determined based on the change in total number of chromosomes or chromosome structures because of the exposure of chemical toxicant [16].

The Allium test is also one of the popular test systems used as a standard for environmental monitoring. A summary of the advantages of A. cepa test system has been provided in the work of Leme & Marin-Morales [13]. The advantages listed include short term, easy to handle, low-cost, high sensitivity and good correlation compared with other test systems, like mammals. This is supported by previous work which published that the results of the Allium test would be rapid, reproducible as well as easy to perform as a standard toxicity test [17]. Furthermore, the Allium test can be utilised in analysing various types of chemical mutagens. Herrero and co-workers have proven this fact by establishing significant differences in mitotic index and root length of the onion upon incubation with several types of chemical agents [18]. The samples to be tested with A. cepa for environmental monitoring can be taken from various origins. Mazzeo et al. (2015) has used sewage sludge as test solutions in Allium test to assess its toxicity on the onion. The results showed damage to DNA in A. cepa upon exposure to the test solutions [19]. Conversely, Radić et al. (2010) has utilised surface and wastewater in their study. The polluted water samples give positive results in both macroscopic and microscopic analysis of A. cepa [20]. This study also proved that the Allium test is simple, requires minimum facilities, and can give consistent results even in long monitoring periods. Several available literatures relate and compare the effectiveness of the Allium test with other test systems. For instance, Nunes et al. (2011) found a positive correlation between the results of Allium test and the V79 test system, which utilised Chinese hamster lung fibroblasts, in examining the genotoxicity of river water [21]. Besides, a study reported that the Allium test could be used complementarily with Nile tilapia (Oreochromis niloticus) erythrocyte bioassays in the cytotoxic screening of source water, wastewater and treated water [22].

The biological screenings of polluted sewage water show that the P. adspersus, L. sativa and A. cepa bioassays are slightly advantageous since no cultures were involved, unlike the bioassays of D. magna and H. attenuate, which require cultures leading them to be time-consuming [23]. Another study found [24], that the seedlings of L. sativa has established a faster initial development and higher mitotic index compared to A. cepa. However, A. cepa has presented greater sensitivity in determining the toxicity of test solutions in terms of DNA fragmentation and cell death. Besides that, Ma et al. (1995) also proves that the A. cepa testing system is more sensitive and efficient than Vicia faba [25].

2. Methodology

2.1. Incubation of A. cepa with water samples
Water samples were obtained from the sampling location at Taman Tasik Shah Alam. Water samples were collected at least 15-20 cm (6-8 inches) of lake surface deep for each sampling site. The water sample was stored at 4°C in a dark condition [26]. Equal sized bulbs of Allium cepa (1.5-2.0 cm diameter) and weight (about 30 g) were selected. Next, the scales of A. cepa were removed and the root primordia kept left intact. The bulbs were cleaned and the outer scales along with brownish bottom plates of the bulbs were removed. After that, the bulbs were placed on top of a beaker containing tap
water for 24 hours at room temperature in darkness, so that the rootlets could emerge. When the roots had reached the length of at least 2 cm, treatments were performed. Bulbs were treated with water samples at 24 and 48 hours of incubation time. The bulbs were incubated away from direct sunlight, at room temperature. The negative control was performed by using tap water. Three replicates (5 bulbs each) were performed in this study.

2.2. Measurement of macroscopic and microscopic parameters of *A. cepa*
After the completion of the designated incubation period for each treatment, the rootlets were cut. The length of selected roots was measured with a ruler and recorded. The changes in root length were identified by calculating the differences between the root length after treatment and the root length before treatment. About 2 cm long of each bulb was immediately fixed in Carnoy’s fixative (3 ethanol: 1 acetic acid) for ten minutes at room temperature. The roots were immersed in iced water for five minutes and then dried with filter papers. For microscope slides preparation, the roots were immersed for 5 minutes in 1 M hydrochloric acid that was already preheated at 60°C to soften the tissue. The roots were placed again into iced water for minutes and then dried with filter papers. The root tips were transferred to clean glass slides with mounted needles and were cut into about 2 mm length by a scalpel. For staining, two drops of 2% aceto orcein were added onto the root tips and left for two minutes. The slides were observed under compound light microscope at 400x magnification. The mitotic index (MI) and chromosomal aberration were analysed using equation 1 and 2, respectively.

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\text{Mitotic index: } MI = \frac{\text{Prophase} + \text{Metaphase} + \text{Anaphase} + \text{Telophase}}{\text{Total number of cells}} \times 100
\]

\[
\text{Chromosomal aberration: } \% \text{ of abnormal cells} = \frac{\text{Number of aberrant cells}}{\text{Number of dividing cells}} \times 100
\]

2.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)
Qiagen RNeasy Plant Mini Kit was used to extract total RNA from *Allium cepa* according to manufacturer’s procedure [27]. After one week of growth, the onion roots were collected by forceps and grind in liquid nitrogen to become a fine powder using a pestle and mortar. The ground powder was placed in a 2ml micro tube. The eluted total RNA solution was stored at -20°C. Qiagen QuantiTect Reverse Transcription Kit was used to synthesizing cDNA from RNA template. Genomic DNA elimination reaction was prepared on ice and incubate for 2 minutes at 42°C. The PCR procedure was carried out according to the handbook provided by Qiagen, (2015).

3. Results and discussion

3.1. Effects of water samples on root length of *A. cepa*
Results of *A. cepa* root length after treatment with controls and water samples at 24 and 48 hours showed the mean of root length for water samples from the lake was 0.67 ± 0.13 cm, which is not significantly different to root length of the control treatment (0.8 ± 0.16 cm). Meanwhile, there was no statistically significant reduction in 48 hours of treatment, as the length of the roots treated in lake water was 0.65 ± 0.08 cm and the control was 0.71 ± 0.08 cm. This may suggest that the water samples did not contain substances that could inhibit or decrease the root length following the treatment. In contrast to this study, it was found that inhibition of root growth occurred in *A. cepa* when treated with environmental pollutants [28].

3.2. Mitotic index of *A. cepa* after 24 and 48 hours of treatment
After 24 hours of incubation, the *A. cepa* treated with lake water samples was slightly lower than the negative control. The mean mitotic index for negative control was 9.22 ± 1.48 and 8.55 ± 1.24 for water samples. However, the reduction was not significant enough to deduce that the water samples caused a decrease in the number of dividing cells. The data obtained did not show any statistically significant different between the group means of *A. cepa* treated with water samples from the lake and tap water (p value=0.734), as demonstrated by an independent T-test. It shown that the mitotic index of *A. cepa* did
not differ much when treated with water samples from the lake or tap water. This could mean that the lake water did not have a significant effect on the division of root cells of \textit{A. cepa}.

Data from this study showed that the mean mitotic index for onion bulbs treated with water samples from study site was higher than that of treatment with tap water, as negative control. The mean mitotic index for negative control was \(10.53 \pm 0.57\) and for the treatment with lake water was \(12.34 \pm 1.22\). Therefore, this indicated an increase in the number of dividing cells. Nevertheless, the slightly higher mitotic index was not statistically significant (p value=0.197). This indicated that the lake water samples did not significantly increase the number of dividing cells over time.

3.3. Percentage of chromosome abnormalities

There were several types of chromosome abnormalities found in this study which included lagging chromosome, c-mitosis, disturbed anaphase, disturbed metaphase, stickiness and spindle disturbance. Levan (1938) described c-mitosis or colchicine mitosis as a random scattering of chromosomes over the cell due to spindle inactivation [29]. Besides, chromosome stickiness was also a condition in which chromosomes attached together and the chromosome fibres intermingled with each other due to improper folding of fiber into single chromatid and chromosomes [30]. Lagging chromosome was the result of chromosomes failure to separate and move to either pole. Disturbed anaphase and metaphase are characterised by the improper separation of chromosomes during anaphase and improper arrangement of chromosomes on the metaphase plate during metaphase stage. There was a significant increase in the percentage of chromosomal abnormalities of \textit{A. cepa} for 24 hours treatment. Statistical analysis using Two-way ANOVA between the overall means of each type of chromosome abnormalities observed for lake water treatment and negative control showed p<0.05. For 24 hours of incubation, spindle disturbance was significantly higher (p value= 0.009). Data showed in Table 1.

For the 48 hours treatment with water samples from the study site, there was also a significant increase in the percentage of abnormal cells when compared to the negative control. The overall mean of each type of chromosome abnormalities observed for lake water treatment and negative control were analysed using Two-way ANOVA as demonstrated by p<0.05. Lagging chromosomes and disturbed anaphase showed a significant increase in percentage for \textit{A. cepa} treated with lake water samples. The p values for lagging chromosome and disturbed anaphase were 0.007 and 0.02, respectively. Data is shown in Table 2.

| Table 1: Percentage of different types of chromosome abnormalities for 24 hours treatment. |
|--------------------------------------------------|
| Type of cell abnormalities, (mean ±se)          |
| L       | C-mitosis | DA       | DM       | SD       | S        |
| Control | 3.272 ±0.700\(^a\) | 0.000    | 2.999±0.700\(^a\) | 0.469±0.700\(^a\) | 0.752±0.700\(^a\) | 0.485±0.700\(^a\) |
| Lake water | 4.878±0.700\(^a\) | 0.920±0.700 | 2.918±0.700 \(^a\) | 1.602±0.700\(^a\) | 3.380±0.700 \(^a\) | 0.688±0.700\(^a\) |

Note: Mean values with different letters within a column are significantly different at p<0.05. (L-lagging, DA-disturbed anaphase, DM-disturbed metaphase, SD-spindle disturbance, S-stickness)

| Table 2: Percentage of different types of chromosome abnormalities for 48 hours treatment. |
|--------------------------------------------------|
| Type of cell abnormalities (mean ±se)            |
| L       | C-mitosis | DA       | DM       | SD       | S        |
| Control | 3.374±0.444\(^a\) | 0.496±0.444\(^a\) | 2.969±0.444\(^a\) | 0.672±0.444\(^a\) | 1.386±0.444\(^a\) | 0.287±0.444\(^a\) |
| Lake water | 5.114±0.444\(^b\) | 0.489±0.444\(^a\) | 4.456±0.444\(^c\) | 1.664±0.444\(^c\) | 1.466±0.444\(^c\) | 0.499±0.444\(^a\) |

Note: Mean values with different letters within a column are significantly different at p<0.05. (L-lagging, DA-disturbed anaphase, DM-disturbed metaphase, SD-spindle disturbance, S-stickness)

The data collected for percentage of chromosome abnormalities showed that the water samples taken from Taman Tasik Shah Alam caused an increase in the number of abnormal dividing cells. This indicated that the water samples were significantly genotoxic to the dividing cells of \textit{A. cepa} albeit the cytotoxicity is not statistically significant. A study by Buschini and the team [31] on lake water purified with sodium hypochlorite and chloride dioxide as disinfectants, demonstrated the generation of DNA
damage within cells. The presence of chemicals from pesticides also induced chromosome abnormalities [32]. Hence, there might be substances within the Shah Alam Lake water that have caused alterations within dividing cells, such as chlorine used in disinfectants and chemicals present in pesticides. There was no strong correlation between cytotoxicity and genotoxicity in this study, which differs from most previous studies. A study showed a positive correlation between cytotoxicity and genotoxicity. Even so, a research [33] found that one of the plant extracts tested did produce genotoxic effects, although not cytotoxic to the bacterial cells. A study on the cytotoxicity and genotoxicity of untreated hospital effluents using bacterial assays, did not detect any cytotoxic effects of the effluent samples, but the sample did cause weak genotoxicity [34]. This study found a remarkably high percentage of lagging chromosomes, disturbed anaphase and spindle disturbance among the types of chromosome abnormalities observed. On the other hand, other study [28] found that the chromosome stickiness was amongst the highest types of abnormalities observed in the study of environmental pollutants in river water. A research on chlorinated river water by [35] using Allium test recorded a high percentage of chromosome breakages in the onion cells. Aneuploidy had also been identified in a study of polluted wastewater discharges [36]. In order to further evaluated the potential use of the molecular method to detect water pollution, alliinase gene was detected as a pollution marker. Alliinase enzyme situated in the vacuoles, as a result of biotic or abiotic stress [37]. Alliinase has been found in many plants of the genus Allium such as garlic (A. sativum), onion (A. cepa), which is an important enzyme involved in Allium sp. defense against pest. Alliinase is present in A. cepa bulbs at high levels, representing up to 6% by weight of the soluble protein [39].

As no studies of alliinase transcript abundance in A. cepa root tissues have been performed, it is expected that possible difficulties in amplifying an alliinase encoding sequence from cDNA may be accounted for by a low abundance of alliinase transcripts within root tip RNA. Based on the result obtained, the alliinase gene was expressed only on the A. cepa root that had been exposed to positive control. The PCR product showed the target alliinase gene fragment with a clear and sharp band of expected size 180bp. No expression of the alliinase gene detected in the water samples proposed that no water pollution present. The data from the molecular method identification is in lined with the cytological analysis, whereby no significant different on the root length and mitotic index were found in the water samples as compared to controls.

4. Conclusion
In conclusion, the water samples taken from Taman Tasik Shah Alam did not show any statistically significant differences in the changes of root length and mitotic index of A. cepa. In addition, the water samples showed no detectable expression of the alliinase gene at the molecular level thus, indicating that the water samples were not significantly cytotoxic to the cells. However, the percentage of chromosome abnormalities increased when treated with water samples may suggest that the water samples were genotoxic to the cells. In this study, it was concluded that cytotoxicity was not strictly correlated to genotoxicity.

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References
[1] Cassanego M and Droste A 2017 Assessing the spatial pattern of a river water quality in southern Brazil by multivariate analysis of biological and chemical indicators Brazilian Journal of Biology 77 118-126
[2] Khatri N and Tyagi S 2015 Influences of natural and anthropogenic factors on surface and groundwater quality in rural and urban areas Frontiers in Life Science 8 23-39
[3] Batista N J C, Cavalcante A A d C M, de Oliveira M G, Medeiros E C N, Machado J L, Evangelista S R, Dias J F, dos Santos C E, Duarte A and da Silva F R 2016 Genotoxic and
mutagenic evaluation of water samples from a river under the influence of different anthropogenic activities. Chemosphere 164 134-141

[4] Hering D, Carvalho L, Argillier C, Beklioglu M, Borja A, Cardoso A C, Duel H, Ferreira T, Globevnik L and Hangarau J 2015 Managing aquatic ecosystems and water resources under multiple stress—An introduction to the MARS project Science of the total environment 503 10-21

[5] Lorente C, Causape J, Glud R N, Hancke K, Merchan D, Muniz S, Val J and Navarro E 2015 Impacts of agricultural irrigation on nearby freshwater ecosystems: The seasonal influence of triazine herbicides in benthic algal communities Science of the total environment 503 151-158

[6] Michael K 2015 Shah Alam park and lake need upgrade 16 November

[7] Grant W F 1994 The present status of higher plant bioassays for the detection of environmental mutagens Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 310 175-185

[8] Liman R, Akyil D, Eren Y and Konuk M 2010 Testing of the mutagenicity and genotoxicity of metolcarb by using both Ames/Salmonella and Allium test Chemosphere 80 1056-1061

[9] Yi H and Meng Z 2003 Genotoxicity of hydrated sulfur dioxide on root tips of Allium sativum and Vicia faba Mutation Research/Genetic Toxicology and Environmental Mutagenesis 537 109-114

[10] Grant W F 1999 Higher plant assays for the detection of chromosomal aberrations and gene mutations—a brief historical background on their use for screening and monitoring environmental chemicals Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 426 107-112

[11] Pastori T, Flores F C, Boligon A A, Athayde M L, da Silva C d B, do Canto-Dorow T S and Tedesco S B 2013 Genotoxic effects of Campomanesia xanthocarpa extracts on Allium cepa vegetal system Pharmaceutical Biology 51 1249-1255

[12] Amal H M A E-M, Shaaban A A-W S A and Abdel-Raahman D H M 2009 Resumption of Mutagenicity In Vicia After Secondary Treatment of Sewage Water The Egyptian Journal of Experimental Biology (Botany) 5 189-198

[13] Leme D M and Marin-Morales M A 2009 Allium cepa test in environmental monitoring: a review on its application Mutation Research 682 71-81

[14] Khanna N and Sharma S 2013 Allium cepa root chromosomal aberration assay: a review Indian journal of pharmaceutical and biological research 1 2320-9267

[15] Wierzbicka M 1999 The effect of lead on the cell cycle in the root meristem ofAllium cepa L Protoplasma 207 186-194

[16] Smaka-Kincl V, Stegnar P, Lovka M and Toman M J 1996 The evaluation of waste, surface and ground water quality using the Allium test procedure Mutation Research/Genetic Toxicology 368 171-179

[17] Fiskešjö G 1985 The Allium test as a standard in environmental monitoring Hereditas 102 99-112

[18] Herrero O, Martín J P, Freire P F, López L C, Peropadre A and Hazen M 2012 Toxicological evaluation of three contaminants of emerging concern by use of the Allium cepa test Mutation Research/Genetic Toxicology and Environmental Mutagenesis 743 20-24

[19] Mazzeo D E C, Fernandes T C C, Levy C E, Fontanetti C S and Marin-Morales M A 2015 Monitoring the natural attenuation of a sewage sludge toxicity using the Allium cepa test Ecological Indicators 56 60-69

[20] Radić S, Stipaničev D, Vujčić V, Rajčić M M, Širac S and Pevalek-Kozlina B 2010 The evaluation of surface and wastewater genotoxicity using the Allium cepa test Science of the Total Environment 408 1228-1233

[21] Nunes E A, de Lemos C T, Gavronski L, Moreira T N, Oliveira N C and da Silva J 2011 Genotoxic assessment on river water using different biological systems Chemosphere 84 47-53

[22] Hemachandra C K and Pathiratne A 2017 Cytogenotoxicity screening of source water, wastewater
and treated water of drinking water treatment plants using two in vivo test systems: Allium cepa root based and Nile tilapia erythrocyte based tests Water Research 108 320-329

[23] Oberholster P, Botha A-M and Cloete T 2008 Biological and chemical evaluation of sewage water pollution in the Rietvlei nature reserve wetland area, South Africa Environmental Pollution 156 184-192

[24] Silveira G L, Lima M G F, dos Reis G B, Palmieri M J and Andrade-Vieria L F 2017 Toxic effects of environmental pollutants: Comparative investigation using Allium cepa L. and Lactuca sativa L Chemosphere 178 359-367

[25] Ma T-H, Xu Z, Xu C, McConnell H, Rabago E V, Arreola G A and Zhang H 1995 The improved Allium/Vicia root tip micronucleus assay for clastogenicity of environmental pollutants Mutation Research/Environmental Mutagenesis and Related Subjects 334 185-195

[26] Musselman R 2012 Sampling procedure for lake or stream surface water chemistry Res. Note RMRS-RN-49. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station 11 49

[27] Qiagen R N easy 2012 Mini Handbook Qiagen Hilden (Germany)

[28] Fiskeşjö G 1988 The Allium test—an alternative in environmental studies: the relative toxicity of metal ions Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 197 243-260

[29] Levan A 1938 The effect of colchicine on root mitoses in Allium Hereditas 24 471-486

[30] Klášterská I, Natarajan A and Ramel C 1976 An interpretation of the origin of subchromatid aberrations and chromosome stickiness as a category of chromatid aberrations Hereditas 83 153-162

[31] Buschini A, Martino A, Gustavino B, Monfrinotti M, Poli P, Rossi C, Santoro M, Dörr A and Rizzoni M 2004 Comet assay and micronucleus test in circulating erythrocytes of Cyprinus carpio specimens exposed in situ to lake waters treated with disinfectants for potabilization Mutation Research/Genetic Toxicology and Environmental Mutagenesis 557 119-129

[32] Aleem A and Malik A 2005 Genotoxicity of the Yamuna river water at Okhla (Delhi), India Ecotoxicology and environmental safety 61 404-412

[33] Chichioco-Hernandez C, Wudarski J, Gevaert L and Verschaeve L 2011 Evaluation of cytotoxicity and genotoxicity of some Philippine medicinal plants Pharmacognosy magazine 7 171

[34] Ortolan M d G S and Ayub M A Z 2007 Cytotoxicity and genotoxicity of untreated hospital effluents Brazilian Archives of Biology and Technology 50 637-643

[35] Al-Sabti K and Kurelec B 1985 Chromosomal aberrations in onion (Allium cepa) induced by water chlorination by-products Bulletin of environmental contamination and toxicology 34 80-88

[36] Choi I 2017 Does wastewater discharge have relations with increase of Turner syndrome and Down syndrome? Environmental health and toxicology 32

[37] Kato M, Masamura N, Shono J, Okamoto D, Abe T and Imai S 2016 Production and characterization of tearless and non-pungent onion Scientific Reports 6 1-9

[38] Ovesná J, Mitrová K and Kučera L 2015 Garlic (A. sativum L.) alliinase gene family polymorphism reflects bolting types and cysteine sulfoxides content BMC Genetics 16 53

[39] Lancaster J E, Shaw M L and Walton E F 2000 S-Alk (en) yl-L-cysteine sulfoxides, alliinase and aroma in Leucocoryne Phytochemistry 55 127-130