AMASYA UNIVERSITY
CENTRAL RESEARCH LABORATORY

International Journal of Science Letters (IJSL)

e-ISSN: 2687-4733
Year: 2020
Volume: 2
Number: 2

Management Office

Amasya University, Central Research Laboratory, 05100, Ipekkoy, Amasya

From The Editor;

Dear Readers and Authors,

As "International Journal of Science Letters (IJSL)", we are pleased and honored to present the second issue of 2020. IJSL, is an international double peer-reviewed open access academic journal published on the basis of research- development and code of practice.

The aims of this journal are to contribute in theoretical and practical applications in relevant researchers of Life Sciences, Biology, Biotechnology, Bioengineering, Agricultural Sciences, Food Biotechnology and Genetics institutions and organizations in Turkey, and to publish solution based papers depending on the principle of impartiality and scientific ethics principles, focusing on innovative and added value work, discussing the current and future.

With these thoughts, we are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in this issue of International Journal of Science Letters.

25.08.2020

Editor in Chief

Prof. Dr. Tuba YILDIRIM

Reviewers of the Issue

Prof. Dr. Cihan DARCAN, Bilecik Şeyh Edebali Üniversitesi, TURKEY.

Assoc. Prof. Dr. Behçet İNAL, Siirt University, TURKEY.

Assoc. Prof. Dr. Satı UZUN, Erciyes University, TURKEY.

Assoc. Prof. Dr. Sevgi MARAKLI, Amasya University, TURKEY.

Assoc. Prof. Dr. Suat ÇOLAK, Erzincan Binali Yıldırım University, TURKEY.

Assist Prof. Dr. Deniz ALTUN ÇOLAK, Erzincan Binali Yıldırım University, TURKEY.

Assist Prof. Dr. Feyza TUFAN DÜLGER, Halic University, TURKEY.

Assist Prof. Dr. Hasan Hüseyin ÖKTEN, Amasya University, TURKEY.

Assist Prof. Dr. Oğuzhan YANAR, Ondokuz Mayis University, TURKEY.

Assist Prof. Dr. Yılmaz DEMİRCİ, Abdullah Gul University, TURKEY.

Assist Prof. Dr. Yılmaz KAYA, Ondokuz Mayis University, TURKEY.

Dr. Jibrin MOHAMMED NDEJİKO, Ibrahim Badamasi Babangida University, NIGERIA.

Dr. Abdulkarim ALİ DEBA, Abubakar Tafawa Balewa University Bauchi, NIGERIA.

Contents

Isolation and molecular phylogeny of *RFT1* gene from upland rice leaves cultivar 52

Sulaiman Mohammed* 52

An easy and reliable method for establishment and maintenance of tissue cultures of *Nicotiana tabacum* cv. TAPM 26.....62

Nurliyana binti Moh Hussein, Fahrul Huyop, Yilmaz Kaya* 62

An *in vivo* study on *Drosophila melanogaster*, *Artemia salina*, and *Daphnia magna*: Is activated carbon used as a food additive reliable? 79

Mehmet Fidan*, Arif Ayar 79

Coatomic refinable modules 92

Burcu Nişancı Türkmen, Ergül Türkmen* 92

Application of different molecular markers in biotechnology……..98

Berna Kocaman, Sevim Toy, Sevgi Marakli* 98

Mohammed, S. International Journal of Science Letters (IJSL). 2020. 2(2): 52-61.
Research Article

Isolation and molecular phylogeny of *RFT1* gene from upland rice leaves cultivar

Sulaiman Mohammed*

[1]Department of Biological Sciences, Faculty of Science, Gombe State University, Gombe/Nigeria

Abstract

Rice is a modern short-day plant but equally flower during non-inductive long-day condition due to the possession of second florigen, *Rice Flowering Locus T1* (*RFT1*). *RFT1* is an ortholog of *Arabidopsis FT* gene which triggers flowering induction under long-day (LD) condition. The gene isolation from Malaysian upland rice *cv.* Wai that induces flowering were reported. Nucleic acids isolation and amplification of the *RFT1* were observed, then phylogenetic relationship among other 5 *indica* and 1 *japonica* rice were inferred by comparing the gene amino acids sequence data sets using multiple sequence alignment databases. The amplicon quality result indicated that the gene was fully amplified at above 0.5kb, whereas the sequence alignment revealed that the *RFT1* gene was partially amplified based on the gaps identified. Among the 6 *indica* cultivars sequence aligned, they were almost all conserved except on only 12 amino acids. While the cladogram tree result classified the cultivars into three major groups and include Wai under clade that contains both *indica* and *japonica* cultivars. This finding concluded that the cultivars are of high evolutionary relationship and well established their molecular relationship. The cultivars relationship provides necessary information for better understanding of molecular evolution and designing scientific breeding system for generation of new rice cultivar.

Article History

Received 10.03.2020
Accepted 28.04.2020

Keywords

Amplification,
Flower,
Florigen,
Phylogeny,
RFT1,
Upland rice

1. Introduction

Rice or its scientific name *Oryza sativa* L.; the second major cereal crop cultivated across the globe, is a suitable source of food and model plant for studies of genome organization, gene expression as well as transgenes behaviuor (Bajaj and Mohanty, 2005; Manimaran et al., 2013). It is a facultative short-day (SD) plant as it shows early flowering at SD environment, but

[1]Correspondence: sumulsu@yahoo.com

equally flower during non-inductive long-day (LD) condition due to the possession of second florigen, *Rice Flowering Locus T1* (*RFT1*), as described by Komiya et al. (2008). Previous analyses point-out the correlation between early flowering under LD condition and high *RFT1* mRNA level, which suggest that *RFT1* is the main activator to induce flowering under LD condition in *japonica* rice sub-species (Mohammed et al., 2019).

RFT1 is a homolog of rice *Hd3a* and ortholog of *Arabidopsis FT* gene which triggers floral induction processes under LD environment. *RFT1* regulates flowering period through a complex genetic network by translocation from leaf to shoot apical meristem (SAM) (Komiya et al., 2008; Osugi et al., 2011; Tsuji et al., 2011; Itoh and Izawa, 2013). The rice flower normally evolved from vegetative structures i.e. SAM and then later transit to reproductive stage that produces flower and fruits/seeds (Komiya et al., 2008). Flowering time in *Oryza sativa* is intensively regulated by both florigen and range of environmental signals (photoperiod) which provides suitable sign for heading season. Such signals must integrate into single decision-to flower (Komiya et al., 2008; Albani and Coupland, 2010; Xiang et al., 2013).

However, to date, there is no report on the *RFT1* gene isolation, molecular activity and transformation from Malaysian upland rice, sub-species *indica*. Understanding of the gene function in such rice cultivars grown under LD condition countries is still unclear. Though Malaysia has many upland rice cultivars as reported by Sohrabi et al. (2012), Sohrabi et al. (2013); generally upland rice cultivars comprises almost 80% of the world cultivated rice, but contribute only 12% of the global rice production. Because of such shortages in production; molecular isolation, characterization and transformation of *RFT1* gene responsible for promoting flowering under LD condition is of significant interest. It will necessitate the development of transgenic rice with early flower production. Here we reported on *RFT1* gene isolation from Malaysian upland rice cultivar Wai which could later be used for genetic transformation to induce early flowering.

2. Materials and Methods

2.1. Genomic DNA Extraction and PCR Amplification of RFT1 Gene from Malaysian Upland Rice Cultivar Wai

Genomic DNA (gDNA) was extracted as described by Edwards et al. (1991) with little modification from leaves of upland Malaysian rice (*indica* subspecies) cultivar Wai between 8 11 weeks old of growing period. The gDNA quantity was analysed using NanoDrop1000 spectrophotometer and later stored at -20^{o}C prior to further analysis. *RFT1* gene was amplified from the gDNA using different gene-specific primers: *EX1*-F (TGGCTAGCTTAACCTTCCTG , *EX1*- GTCTACCATCACCTGTAGGT , *EX4*- CGGAGGGAGTATCTATTTTG , *EX4*-R CACACTTAAGAGCCTGCATG), *RS1*-F GCTCGTGAAGGCAGGAGATA and *RS1*- TTTTTACATGGCGAGGCCG G .

The thermo-cycling conditions were; initial denaturation for 4 min at 94^{o}C, followed by 30 cycles of denaturation for 30 sec at 94^{o}C, annealing for 40 sec at 53^{o}C and extension for 1 min 20 sec at 72^{o}C, then final extension for 5 min at 72^{o}C and cooling at 4^{o}C. The quality of the amplicon was analyzed by electrophoresis (74 V, 425 A and 45 min) on 1 % (w/v) agarose gel stained with SYBR safe. The gel bands were excised, purified using Wizard SV gel and PCR clean-
to Medigene Sdn Bhd and 1st Base Sdn Bhd Company.

2.2. RNA Extraction, cDNA Synthesis and PCR Amplification of RFT1 Gene

Total RNA was also isolated from the leaves at same age for gDNA extraction using Trizol reagent (Sigma-
treated with DNase prior to reverse transcriptase PCR (RT-PCR) for cDNA synthesis. cDNA was synthesized from the DNase-treated RNA using Superscript II Reverse transcriptase kit (Invitrogen) as described by the manufacturer. PCR amplification of *RFT1* was performed using cDNA as template and *EX1* primers at the same thermo-cycling condition as above. The amplicon was run on 1 % (w/v) agarose. The gel bands were excised, purified and sent for sequencing as described above.

2.3. Bioinformatics Analysis of RFT1 Gene Sequences

RFT1 amino acids sequences were analyzed using protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification. Meanwhile, the *RFT1* sequences from five different *indica* rice cultivars which include Basmati 370 (BAH30236), Pokkali (BAO03221), Bleiyo (BAJ53916), Muha (BAJ53912) and Kemasin (AB838579.1) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) for multiple sequence alignment (MSA) with the current sequence. All the sequences (from Wai and 5 other cultivars) were aligned in FASTA format and analyzed using clustalX and T-Coffee softwares. Phylogenic tree analysis of the six cultivars sequence plus Nipponbare [(BAB78480) a *japonica* sub-species] were also observed.

3. Results and Discussion

3.1. Molecular Amplification and Sequence Analysis of RFT1 Gene from Malaysian Upland Rice

The study tried to isolate and amplify the gene from Malaysian upland rice cultivar Wai using different primer sets. From all the primers used in this study, *EX1* primer set gave the best amplification result. The agarose gel electrophoresis outcome indicated that the actual expected size of the gene was found using the primer set as shown in Figure 1. The band size on the gel was above 0.5 kb which correspond with the finding of Ebana et al. (2011) which shows that the gene has 178 amino acids, approximately 534 bp nucleotides. *RFT1* gene is the second rice florigen and has been hypothesized as a hormone-like molecule for promoting flowering processes under LD condition (Komiya et al., 2008; Tsuji et al., 2011; Itoh and Izawa, 2013). The gene is normally produced in the leaves and act as mobile signal in the SAM of buds as well as growing tips. Evidences indicated that over-expression of *RFT1* gene molecule with vascular-specific promoter or constitutive promoter results in an early-flowering phenotype under LD condition, while its suppression by RNA-interference (RNAi) procrastinate flowering occurrence as described by Komiya et al. (2008) and Komiya et al. (2009). This implies that flowering under LD condition in rice is basically a result of expression of this highly conserved florigen, *RFT1* (Tamaki et al., 2007; Tsuji et al., 2008).

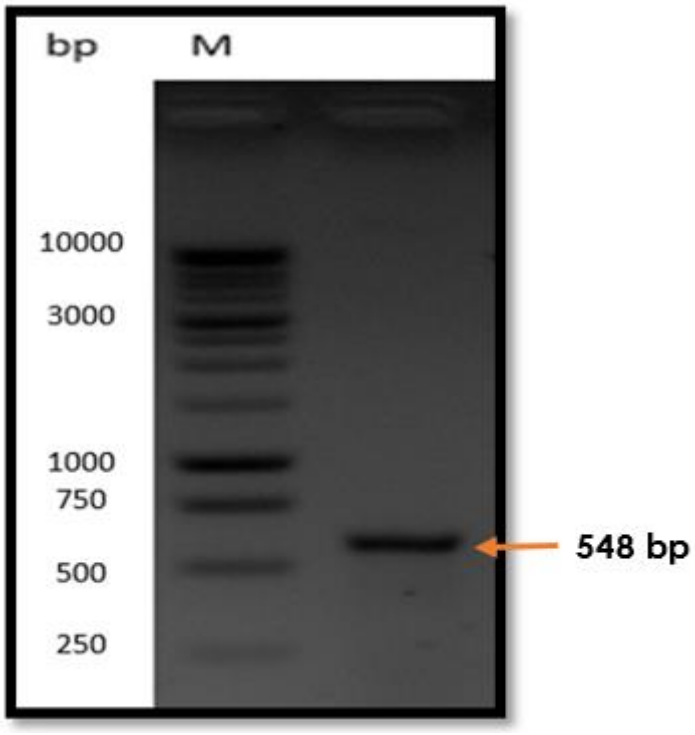

Figure 1. *EX1* primer; separation of PCR product on agarose gel following amplification

The result of the sequencing analysis indicated that all the six sequences are highly conserved from the region beginning at the start of the *RFT1* until amino acid in position 69 with 99 % degree of similarity (Figure 2). The highly conserved region among the different cultivars may be due to their close evolutionary trend (Kojima et al., 2002, Komiya et al., 2008). However, the sequence alignment revealed that *RFT1* gene from the present research was partially amplified due to presence of few gaps after the identified conserved regions. Moreover, the target sequence was 182 amino acid sequence longer than the database template sequences. Uniquely, the target sequence had some amino acids at the beginning of the sequence, which is not in the other *RFT1* sequences.

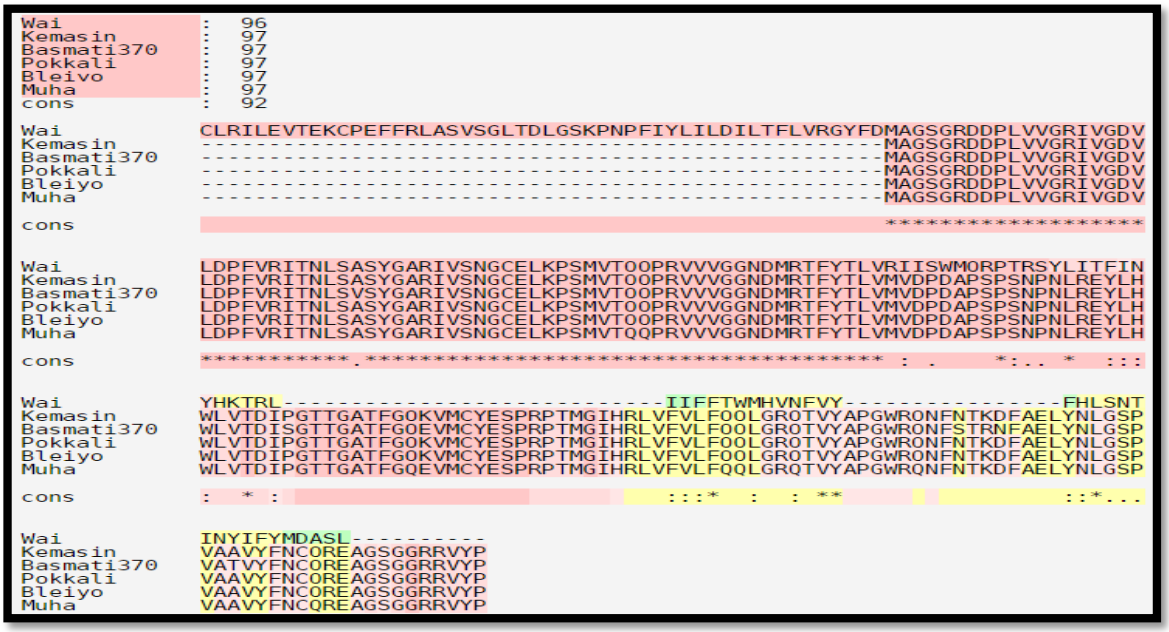

Figure 2. Multiple sequence alignment of six indica rice cultivars. Pink colour indicates highly conserved region (*); Yellow colour indicates average conserved region (:); Green colour indicates less conserved region

Additionally, the sequence alignment between Wai cultivar and other five cultivars from NCBI revealed that the amino acids diversity towards the end of the sequences is quite enormous. This corresponds with the finding of Hagiwara et al. (2009) and Ogiso-Tanaka et al. (2013) which indicated that defective *RFT1* gene is found in *indica* cultivars and higher diversity of nucleotides. They reported that about 16 amino acids changes in *RFT1* was found and this implies that functional constraint was relaxed in *RFT1* after gene duplication. The authors also demonstrated that the haplotype diversity of *RFT1* and *Hd3a* was similar in cultivated rice. Even though the *RFT1* haplotype number is larger than that of *Hd3a* in the entire gene region but smaller in the coding region.

There were almost 12 amino acid differences observed at closely loci as specified by regions with blank or single dot below (cons) (Figure 2). These amino acid differences seem to be lesser than the one reported by Ogiso-Tanaka et al. (2013) which communicated that about 16 amino acid changes in *RFT1*, but still they shared higher uniformity. Equally, all the *indica* cultivars with the exception of Basmati 370 were virtually 100 % conserved, while amino acid substitution observed in Basmati 370 may contribute to the diversity of the cultivar.

3.3. Molecular Phylogeny of RFT1 Gene from Malaysian Upland Rice

In evolutionary study, cladogram tree construction is of paramount importance as it functions in inferring and clarifying the evolutionary relationship among species being studied (Mohammed et al., 2019). As for this study, the relationship of amino acid sequences from different rice cultivars (five *indica* and one *japonica*) obtained from the NCBI database and with the one obtained from this study were analyzed with all positions. The phylogenetic relationship of all rice cultivars was presented in a cladogram (Figure 3). The cladogram shows the consensus phylogenetic tree of the 6 *indica* varieties and single *japonica* variety with a consistency index (CI) of 1 and retention index (RI) of 1 which indicates that there is no homoplasy and the character is totally steady with phylogeny (Drummond and Strimmer, 2001; Norulaini et al., 2001). As shown in the consensus tree, the cultivars were classified into three major groups and group one was categorized into sub-clades. The first group comprises of Wai, Basmati370, Nipponbare, Muha and Kemasin. The second group consists of only Pokkali, while third group has only Bleiyo.

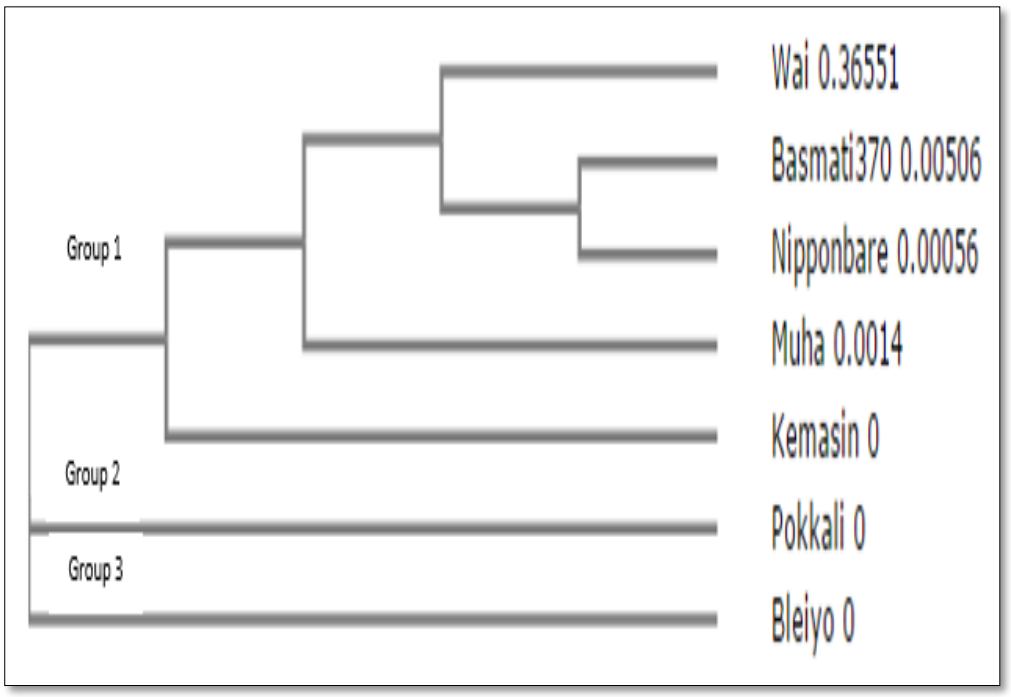

Figure 3. The consensus tree derived from the multiple sequence analysis of *RFT1* gene of *indica* rice varieties

From the cladogram tree, Basmati 370 had some nucleotides as well as amino acids differences from the other *indica* cultivars but showed close relation with Nipponbare

(*japonica*). These correspond to the previous finding of Kovach et al. (2009), which revealed that Basmati 370 has close evolutionary relationship with *japonica* varieties that is based on its fragrance characteristic. Similarly, this analysis inferred classification of diverse *indica* into three major groups and suggested that single *indica* cultivar from the five obtained from database and Wai had some amino acids difference and relationship with *japonica*, even though they originate from different areas. This actually gave better insight with regards to the relationship among the aligned cultivars. However, upon construction of cladogram, Wai cultivar indicated similarity to the *japonica* close related cultivar and *japonica* cultivar. Though it forms the same sub-clade with Muha, a typical *indica* rice.

As well, these findings were supported by distinct phenotypic characteristic of the rice cultivars. For example; *indica* varieties in terms of appearance and size they possess slender and long grains, while *japonica* possess stumpy and short grains (Khush, 2000). Additionally, most *indica* cultivars originated from many countries like India, Thailand and Indonesia, whereas *japonica* cultivars originated from countries like China, Japan, Laos, Taiwan and Vietnam (Sasaki et al., 2010). Therefore, there is a degree of similarity and diversity of *RFT1* gene sequence among the verified cultivars from these two rice subspecies and thus, suggest that *RFT1* have high variability in Asia, which was recommended based on the *RFT1* gene phylogenetic analysis as described by Hagiwara et al. (2009).

4. Conclusion

The sequence alignment outcome showed that *RFT1* was partially isolated, even though EX1 primer amplified the gene-based on its expected size. The analysis indicated that some amino acids are missing at the middle or not amplified by the primers, but shown some amino acids at the beginning. Similarly, the phylogenetic analysis inferred classification of diverse *indica* into three major groups and, then, suggested that Wai and Basmati had some amino acids differences and high similarity even though they originate from different areas. This actually gave better insight with regards to the relationship among the aligned cultivars. Hence, this implies that *RFT1* gene could function as a potential biomarker for molecular characterization of rice cultivars. Also, provides important information for better understanding of molecular evolution as well as developing good breeding programs that would lead to development of new cultivar with early flower production and good grain quality. Furthermore, better understanding of *RFT1*

gene function and regulation will make rice a powerful model plant for understanding the flower development at molecular level.

References

Albani, M.C., Coupland, G. 2010. Chapter eleven-comparative analysis of flowering in annual and perennial plants, Current Topics in Developmental Biology, 91(10): 323-348.

Bajaj, S., Mohanty, A. 2005. Recent advances in rice biotechnology—towards genetically superior transgenic rice, Plant Biotechnology Journal, 3(3): 275-307.

Drummond, A., Strimmer, K. 2001. PAL: an object-oriented programming library for molecular evolution and phylogenetics, Bioinformatics, 17(7): 662-663.

Ebana, K., Shibaya, T., Wu, J., Matsubara, K., Kanamori, H. et al. 2011. Uncovering of major genetic factors generating naturally occurring variation in heading date among Asian rice cultivars, Theoretical and Applied Genetics, 122(6): 1199-1210.

Edwards, K., Johnstone, C., Thompson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis, Nucleic Acids Research, 19(6): 1349.

Hagiwara, W.E., Uwatoko, N., Sasaki, A., Matsubara, K., Nagano, H. et al. 2009. Diversification in flowering time due to tandem FT-like gene duplication, generating novel Mendelian factors in wild and cultivated rice, Molecular Ecology, 18(7): 1537-1549.

Itoh, H., Izawa, T. 2013. The coincidence of critical day length recognition for florigen gene expression and floral transition under long-day conditions in rice, Molecular Plant, 6(3): 635-649.

Khush, G. 2000. Taxonomy and origin of rice, Aromatic Rices, 5-13.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T. et al. 2002. *Hd3a*, a rice ortholog of the Arabidopsis *FT* gene, promotes transition to flowering downstream of Hd1 under short-day conditions, Plant and Cell Physiology, 43(10): 1096-1105.

Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S., Shimamoto, K. 2008. *Hd3a* and *RFT1* are essential for flowering in rice, Development, 135(4): 767-774.

Komiya, R., Yokoi, S., Shimamoto, K. 2009. A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice, Development, 136(20): 3443-3450.

Kovach, M.J., Calingacion, M.N., Fitzgerald, M.A., Mccouch, S.R. 2009. The origin and evolution of fragrance in rice (*Oryza sativa* L.), Proceedings of the National Academy of Sciences, 106(34): 14444-14449.

Manimaran, P., Kumar, G.R., Reddy, M.R., Jain, S., Rao, T.B. et al. 2013. Infection of early and young callus tissues of indica rice BPT 5204 enhances regeneration and transformation efficiency, Rice Science, 20: 415-426.

Mohammed, S., Abd Samad, A., Rahmat, Z. 2019. Isolation, cloning, and sequence analysis of the full-length *RFT1* gene from Malaysian upland rice (*Oryza sativa* subsp. indica, Cultivar Wai), Jordan Journal of Biological Sciences, 12(3): 259-265.

Norulaini, N., Zuhair, A., Hakimi, M., Omar, M. 2001. Chemical coagulation of setileable solid-free palm oil mill effluent (Pome) for organic load reduction, Journal of Industrial Technology, 10(1): 55-72.

Ogiso-Tanaka, E., Matsubara, K., Yamamoto, S-I., Nonoue, Y., Wu, J. et al. 2013. Natural variation of the rice flowering locus T1 contributes to flowering time divergence in rice, PLoS One, 8(10): e75959.

Osugi, A., Itoh, H., Ikeda-Kawakatsu, K., Takano, M., Izawa, T. 2011. Molecular dissection of the roles of phytochrome in photoperiodic flowering in rice, Plant Physiology, 157(3): 1128-1137.

Sasaki, K., Ikeda, S., Eda, S., Mitsui, H., Hanzawa, E. et al. 2010. Impact of plant genotype and nitrogen level on rice growth response to inoculation with *Azospirillum* sp. strain B510 under paddy field conditions, Soil Science & Plant Nutrition, 56(4): 636-644.

Sohrabi, M., Rafii, M., Hanafi, M., Siti Nor Akmar, A., Latif, M. 2012. Genetic diversity of upland rice germplasm in Malaysia based on quantitative traits, The Scientific World Journal, 2012, 1-9.

Sohrabi, M., Rafii, M.Y., Hanafi, M.M., Latif, M.A. 2013. Genetic divergence of Malaysian upland rices revealed by microsatellite markers, Plant Omics, 6(3): 175

Tamaki, S., Matsuo, S., Wong, H. L., Yokoi, S., Shimamoto, K. 2007. *Hd3a* protein is a mobile flowering signal in rice, Science, 316(5827): 1033-1036.

Tsuji, H., Tamaki, S., Komiya, R., Shimamoto, K. 2008. Florigen and the photoperiodic control of flowering in rice, Rice, 1(1): 25-35.

Tsuji, H., Taoka, K-I., Shimamoto, K. 2011. Regulation of flowering in rice: two florigen genes, a complex gene network, and natural variation, Current Opinion in Plant Biology, 14(1): 45-52.

Xiang, C., Qu, L.-J., Gao, Y.-M., Shi, Y.-Y. 2013. Flower development and photoperiodic control of flowering in rice, Rice Science, 20(2): 79-87.

Hussein, NBM, Huyop, F, Kaya, Y. International Journal of Science Letters (IJSL). 2020. 2(2): 62-71.
Research Article

An easy and reliable method for establishment and maintenance of tissue cultures of *Nicotiana tabacum* cv. TAPM 26

Nurliyana binti Moh Hussein[1,2], Fahrul Huyop[1], Yilmaz Kaya[1,3*]

[1]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, Johor/Malaysia
[2]Faculty of Engineering Technology, Universiti Malaysia Pahang, Pahang/Malaysia
[3]Department of Agricultural Biotechnology, Faculty of Agriculture, Ondokuz Mayis University, Samsun/Turkey

Abstract

In vitro regeneration of *Nicotiana tabacum* was efficiently achieved using growth regulators combination supplemented into semi-solid MS medium using leaves from 1-month-old plant as explants. The growth regulators used were NAA for auxin (0.0, 1.0, 2.0, and 3.0 mgL^{-1}) and BAP (0.0, 0.5, 1.0, 2.5, and 5.0 mgL^{-1}). The effects of growth regulator combination were assessed based on a number of callus formation, shoots formation and fresh callus weight. The maximum number of callus formation was 100 % at five hormone combination, observed at the fourth week after culture. Maximum number of shoots produced per explant was 21.4 shoots at 1.0 mgL^{-1} BAP + 3.0 mgL^{-1} NAA, after 6 weeks of culture. The maximum callus fresh weights were obtained at 0.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA after 6 weeks (9.92 g). The best combination for shoots regeneration of *Nicotiana tabacum* was 1.0 mgL^{-1} BAP + 3.0 mgL^{-1} NAA. And the best combination for heaviest callus production was 0.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA.

Article History

Received 06.07.2020
Accepted 16.08.2020

Keywords

Nicotiana tabacum TAPM 26,
Callus,
Tissue culture,
Maintenance tissue culture,
Callus formation

Abbreviations BAP, 6-benzyl-aminopurine; IAA, indole-3-acetic acid; MS, Murashige and Skoog medium (1962); NAA, napthaleneacetic acid

1. Introduction

Plant tissue culture covers the development of selected plant tissue and grown aseptically for indefinite duration on a nutrient medium under controlled conditions (Mohammed, 2020). Tissue culture usually applied as a medium for micropropagation, creation of virus-free plants (Arvas et al., 2018), genetics transformation (Kutty et al., 2011; Kaya et al., 2013) and it is also demonstrated more effective in the creation of secondary metabolites,

[1]Correspondence: yilmaz.kaya@omu.edu.tr

for examples, phenolics (Ozyigit, 2008), Juglone (Kocacaliskan et al., 2018) and Anthocyanins (Marchev et al., 2020) etc. The most regular technique in tissue culture is micropropagation which defined as a technique to procreate genetically clonal plantlets by using tissue culture methods and it supports in creating pathogen-free stock plants (Arvas et al., 2018) or genetically superior clones that cannot be propagated by seeds or plant that with low propagation efficiency in conventional vegetative propagation (Kutty et al., 2010). Moreover, plant cell exhibits its totipotency that every single cell can regenerate into a whole new plant.

Shoot regeneration for tobacco from an explant has achieved attention in the past and has promising application in the area of plant biotechnology (Deo et al., 2010). In addition, Kaya (2010) showed that adventitious regeneration of tobacco plant has a higher regeneration potential via embryogenesis when explants derived from cotyledons were used. Thus, the aim of the research is to establish an easy and reliable technique for maintenance of tissue cultures of *Nicotiana tabacum* cv. TAPM 26.

2. Materials and Methods

2.1. Culture Conditions

The seed of Nicotiana tabacum cv. TAPM 26 was provided by National Tobacco Board Kota Bharu Kelantan. The study was achieved in the Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia. In vitro plant regeneration was pursued at 25 ± 2°C in either dark or light with a 16 h photoperiod and 6 h dark distributed by cool white fluorescent tubes. The tissue culture medium was heated to melting solutes and then distributed in tissue culture tubes for seed germination of tobacco plant and tubes for regeneration.

2.2. Basic Media Preparation

Basic media was prepared based on Murashige and Skoog (1962) containing mineral salts, micronutrients and vitamins, supplied with 20 gL^{-1} sucrose, 0.1 gL^{-1} myo-inositol and 3.2 gL^{-1} phytagel. The pH of the plant culture media was adjusted to 5.7 with either 1N NaOH (sodium hydroxide) before sterilization. The tissue culture media were sterilized at 121°C

for 21 min in an autoclave. After autoclaving, the medium was left to cool down to 40 to 45°C before combination of growth hormone (BAP and NAA) were added.

2.3. Preparation of Explants Material

Approximately 30 seeds of *Nicotiana tabacum* was placed in a test tube supplied with 2 mL of sterilizing solution (70 % ethyl alcohol). It was mixed gently for 20 min. Then the sterilising solution removed and washed all the seeds with double sterile water for 3 times. The seeds were put onto a sterile petri dish for drying and then the seeds were transferred to germination medium. Only 5 seeds were put onto each petri dishes. After a few days the germinated seeds will be ready to be used as a young plantlet that will be the source of explants for plant tissue culture work. The leaves of plantlet were sterile as they grow in the tissue culture systems under controlled parameters. In 4 weeks, the old plantlets were used as sources of explant. Callus induction and shoots regeneration from callus was obtained by using MS medium modified with auxin and cytokinin. The callus was induced from leaf explant on media contained MS (Murashige and Skoog, 1962) salts, vitamins, 30 gL^{-1} sucrose, 8 gL^{-1} agar powder, 6-benzylaminopurine (0.0, 0.5, 1.0, 2.5 or 5.0) and 1-naphthalene acetic acid (0.0, 1.0, 2.0 or 3.0 mgL^{-1}).

The pH was adjusted during each experiment to 5.7 ± 1 with 1 N NaOH or 1 N HCl using an electronic pH indicator. All the operations and inoculations were carried out under aseptic conditions in laminar airflow cabinet.

2.4. Data Analysis

All the assays were carried out in triplicates and the data were subjected to statistical analysis of one-way ANOVA using SPSS 15.0 for Windows. A value of $P<0.05$ was considered to be significant.

3. Results and Discussion

The explants produced callus on the one month after cultivation on all combination of NAA and BAP at room temperature and 16 h light under plant growth chamber. The results achieved in the aspects of callus weight and percentage of callus from hypocotyl explants.

The first step in the start of plant tissue culture represents the disinfection of the explant or seed. The efficiency of the surficial sterilization technique has an important effect on the development and growth of the plant for tissue culture. Standard surficial sterilization technique for starting a tissue culture of members of the tobacco plants comprise the application of alcohol, sodium hypochlorite (Steyn et al., 1996; Ganapathi et al., 2004).

3.1. Effect of BAP/NAA on Callus Fresh Weight

In Figure 1, the maximum average weights of callus form were detected at 0.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA The maximum mean weight produces was 9.92 g while the minimum mean weight was 0.127 g observed at the presence of zero growth regulator. The product of experiments was analyzed using one-way ANOVA and the results were significant for a value $P<0.05$. From the previous researches, the maximum mean weight produced was 1.81 g at 0.2 mgL^{-1} BAP and 3.0 mgL^{-1} NAA (Ali et al., 2007). This showed that lower concentration of BAP and NAA created heavier and bulkier callus but too low or too high concentration may retard the growth of the callus. Figure 2 showed the best callus formed in terms of fresh callus weight as described in Figure 1.

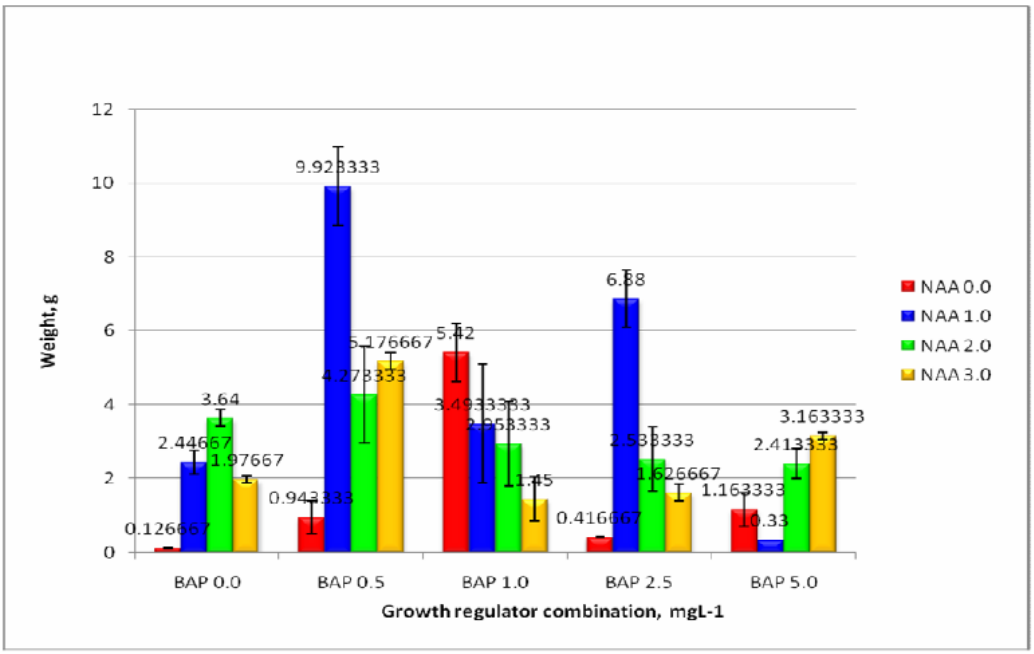

Figure 1. The average weight of callus on different combination of growth regulator

Figure 2. Fresh callus at MS+0.5 mgL^{-1}BAP+1.0 mgL^{-1}NAA. (Scale bar = 1.0 cm)

3.2. Effect of BAP/NAA on Callus Formation

At 3.0 mgL^{-1} NAA concentration; the callus formation was lower than others (Table 1). The same result was observed for the maximum concentration BAP which only formed 20 % of callus compared to other BAP and NAA combinations. This showed that explants need both growth regulators combination to form callus.

Table 1. Percentage of callus formed for each growth regulator combinations

Hormone combination mgL^{-1}		BAP				
		0.0%	0.5	1.0	2.5	5.0
NAA	0.0	33.3% callus formation	86.7% callus formation	80.0% callus formation	60.0% callus formation	20.0% callus formation
	1.0	53.3% callus formation	86.7% callus formation	86.7% callus formation	93.3% callus formation	33.3% callus formation
	2.0	53.3% callus formation	100% callus formation	100% callus formation	100% callus formation	73.3% callus formation
	3.0	13.3% callus formation	100% callus formation	100% callus formation	73.3% callus formation	66.7% callus formation

In Figure 3, the maximum number of callus induction percentage was 100 % at MS

media containing 0.5 mgL^{-1} BAP + 2.0 mgL^{-1} NAA, 1.0 mgL^{-1} BAP + 2.0 mgL^{-1}, 2.5 mgL^{-1} BAP + 2.0 mgL^{1} NAA, 0.5 mgL^{-1} BAP + 3.0 mgL^{-1} NAA and 1.0 mgL^{-1} BAP + 3.0 mgL^{-1} mgL^{-1} NAA. Maximum concentration of hormone combination gives a medium percentage of calli formation, about 66.7 %. This indicated that too low or too high concentration of growth regulator may inhibit the formation of calluses.

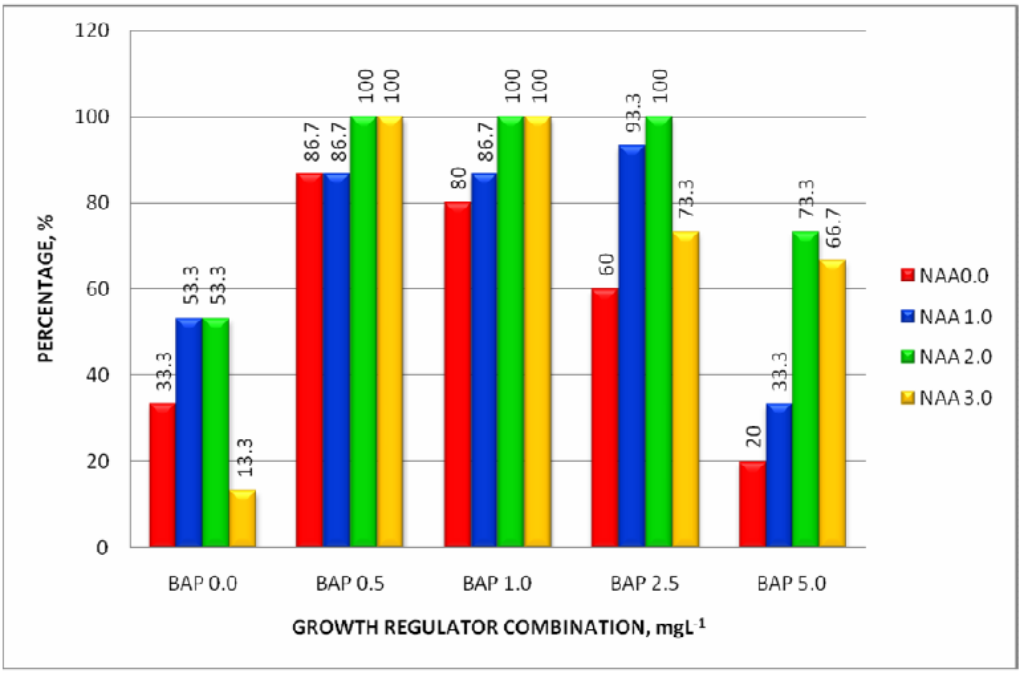

Figure 3. The percentage of callus formed in different growth regulators combination

3.3. Effect of BAP/NAA on Shoots Induction

In Figure 4, it demonstrationed that shoots need 2.0 mgL^{-1} and 3.0 mgL^{-1} NAA concentrations. The highest average number of shoots was 21.4 ± 2.13 per explant. At 0.0 mgL^{-1} and 1.0 mgL^{-1} of NAA concentration, there was no shoot formed. At all BAP concentrations, there were shoots formed and it varied upon the combination. The highest average number of shoots per explant was obtained from the combination of 1.0 mgL^{-1} BAP + 3.0 mgL^{-1} NAA. The results were analysed using one-way ANOVA and the results were significant for a value $P<0.05$. The lowest number of shoots for per explants was observed on MS medium supplemented with 2.0 mgL^{-1} NA (1.067 shoots per explants). Roots initiation was not detected on all explants after 6 weeks culture. This showed that the NAA concentration should be higher to achieve rooting or it takes longer time to regenerate. Figure

5 shows the shoot callus for the best combination growth regulator used as described in Figure 4.

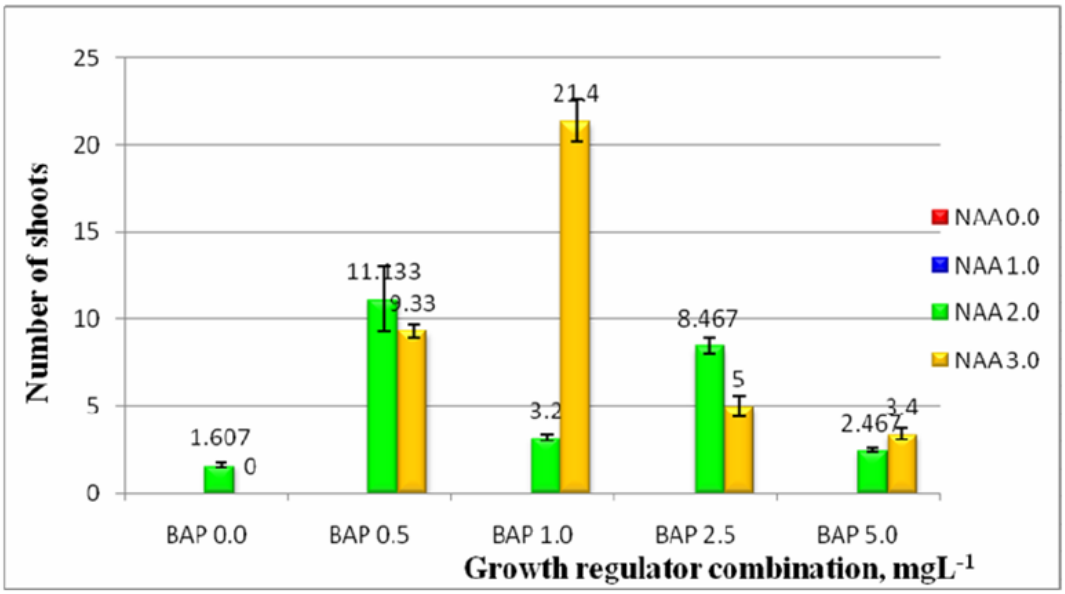

Figure 4. The average number of shoots formed

Figure 5. Shoots regenerated at 1.0mgL-1 BAP+ 3.0mgL-1 NAA. (Scale bar = 0.5cm)

From Table 2, it was indicated that different sizes of callus formed at different combination of growth regulator. From the observation, the combination of 0.5 mgL^{-1} BAP and 2.0 mgL^{-1} NAA showed the best biggest callus size formed.

Table 2. Different sizes of callus formed regenerates based on varies growth regulator concentration after treatment for 6 weeks

Hormone combination		BAP concentration in mgL^{-1}				
		0.0	0.5	1.0	2.5	5.0
NAA concentration in mgL^{-1}	0.0					
	1.0					
	2.0					
	3.0					

The tobacco plant is created of several repetitive modules in its vegetative part, successively generated by meristems. Tissue culture methods via calli have become an essential tool for plant biotechnology such as *Gossypium hirsutum* L. (Ozyigit et al., 2007), *Impatiens balsamina* (Taha et al., 2009), *Lycopersicon esculentum* Mill. (Jawad et al., 2020), *Oryza sativa* (Kaya and Karakutuk, 2018) *Tagetes minuta* (Latifian et al., 2018), *Citrullus lanatus* cv. Round Dragon (Ganasan and Huyop, 2010) and many others. The explant type and hormones are also significant influences for *in vitro* plant regeneration (Kumar and Reddy, 2010). Explant was a critical parameter when optimizing tissue culture methods (Kumar et al., 2011). Therefore, choice of appropriate explants is an important factor of tissue culture of *Nicotiana tabacum* (Kaya, 2010). Growing the explant of *Nicotiana tabacum* under different concentrations of plant growth hormones such as BAP, NAA as very essential (Table 1). The combination of plant growth hormone was crucial in determining the tissue culture performance resulting in plant regenaration seen in shoot cultures. The present study (Figures 3 and 4) established the good combination of hormone in the media preparation where the shoot formation from regeneration of callus were established.

In conclusion, the results obtained showing the success of tissue culture of *Nicotiana tobacum* with the best hormone combination would be MS + 1.0 mgL^{-1} BAP + 3.0 mgL^{-1}

NAA for production of maximum number of callus and also the highest shoots formation. The callus started to form at fourth weeks after cultivation and the shoots started to initiate two weeks later. Suitable growth regulator combination promote the optimum growth explants and also induced the callus and shoots formation. It also helps to shorten the time taken to initiate callus and shoots. The present method demonstrated that *Nicotiana tabacum* TAPM 26 was successfully propagated by organogenesis and can be applied for further research such as transgenic *Nicotiana tabacum* TAPM 26.

References

Ali, G., Hadi, F., Ali, Z., Tariq, M., Khan, M.A.(2007). Callus induction and *in vitro* complete plant regeneration of different cultivars of tobacco (*Nicotiana tabacum* L.) on media of different hormonal concentration, Journal of Biotechnology, 6 (4): 561-566.

Arvas, Y.E., Aksoy, H.M., Kaya, Y., 2018. Patates bitkisinde biyoteknolojik çalışmalar, International Journal of Life Sciences and Biotechnology, 1(1): 37-47.

Deo, P.C., Tyagi, A.P., Taylor, M., Harding, R., Becker, D. 2010. Factors affecting somatic embryogenesis and transformation in modern plant breeding, The South Pacific Journal of Natural and Applied Sciences, 28: 27-40.

Ganasan, K., Huyop, F. 2010. *In vitro* regeneration of *Citrullus lanatus* cv. *Round Dragon*, *Journal of Biological Sciences*, 10(2): 131-137.

Ganapathi, T.R., Suprasanna, P., Rao, P.S., Bapat, V.A. 2004. Tobacco (*Nicotiana tabacum* L.) - A model system for tissue culture interventions and genetic engineering. Indian Journal of Biotechnology, 3: 171-184.

Jawad, Z.A., Turker, M., Ozdemir, F.A. 2020. Effect of different plant growth regulator on in vitro propagation of endangered plant; yellow tomato (*Lycopersicon esculentum* Mill.), International Journal of Agriculture Forestry and Life Sciences, 4(1): 92-98.

Kaya, Y. 2010. *In vitro* Plant Regeneration of Tobacco. PhD Thesis. Universiti Teknologi, Malaysia..

Kaya, Y., Yilmaz, S., Marakli, S., Gozukirmizi, N., Huyop, F. 2013. Transformation of *Nicotiana tabacum* with *dehE* gene, Journal of Food Agriculture and Environment, 11(3-4): 777-780.

Kaya, Y., Karakütük, S. 2018. Farklı büyüme düzenleyicilerin Türk kır çeltiği rejenerasyonuna etkisi, Anadolu Tarım Bilimleri Dergisi, 33(3): 226-231.

Kocacaliskan, İ., Akgul, T., Erisen, S. 2019. Effect of juglone on seed germination and seedling growth of four common vegetables, International Journal of Life Sciences and Biotechnology, 2(1): 43-49.

Kutty P.C., Parveez, G.K.A., Huyop, F. 2010. An easy method for *Agrobacterium tumefaciens*-mediated gene transfer to *Nicotiana tabacum* cv. TAPM26, Journal of Biological Sciences, 10(6): 480-489.

Kutty, P.C., Parveez, G.K.A., Huyop F. 2011. *Agrobacterium tumefaciens*-infection strategies for greater transgenic recovery in *Nicotiana tabacum* cv. TAPM26, International Journal of Agricultural Research, 6(2): 119-113.

Kumar, N., Reddy, M.P. 2010. Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant, *Annals of Applied Biology*, 156: 367-375.

Latifian, E., Arvas, Y.E., Kaya, Y. 2018. *Tagetes minuta* bitkisinin mikro üretimi üzerine farklı BAP ve IAA konsantrasyonlarının etkileri, International Journal of Life Sciences and Biotechnology, 1(2): 96-104.

Marchev, A.S., Georgiev, M.I. 2020. Plant *in vitro* systems as a sustainable source of active ingredients for cosmeceutical application. Molecules, 25(9): 2006.

Mohammed, S. 2020. Effects and quantity ranges of some auxins on embryogenic callus induction from upland rice cultivars: An overview, International Journal of Life Sciences and Biotechnology, 3(2): 197-204

Ozyigit, I.I., Kahraman, M.V., Ercan, O. 2007. Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.), African Journal of Biotechnology, 6(1): 3-8.

Ozyigit, I.I. 2008. Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips, African Journal of Biotechnology, 7(8): 1145-1150.

Steyn Anita, E. , Brink, J.A., Coetzer, L.A. 1997. A comparison of culture methods for tobacco (*Nicotiana tabacum*) anthers, South African Journal of Plant and Soil, 14: 27-30.

Taha, A.M., Wagiran, A., Ghazali, H., Huyop, F., Parveez, G.K.A. 2009. *In vitro* regeneration of garden balsam, *Impatiens balsamina* using cotyledons derived from seedlings, Biotechnology, 8(1): 44-52.

Konar, V, İdil, Ö, Çelikoğlu, E, Çelikoğlu, U. International Journal of Science Letters (IJSL). 2020. 2(2): 72-78.

Research Article

Ferro-chelatase enzyme activity of blue green algae from Yeşilırmak

Vahit Konar[1]*, Önder İdil[2], Emine Çelikoğlu[1], Umut Çelikoğlu[3]

[1]Department of Biology, Faculty of Arts and Science, Amasya University, Amasya/Turkey
[2]Faculty of Education, Amasya University, Amasya/Turkey
[3]Central Research Laboratory, Amasya University, Amasya/Turkey

Abstract

Blue green algae are microscopic photosynthetic bacteria, naturally in ponds, rivers, lakes and streams. Tetra pyrroles can be classified based on the presence, position and substituents of a chelated metal in the pyrrole ring. Heme and chlorophyll, which are the most common tetra pyrolle in nature, are synthesized by blue green algae. Heme is an essential cofactor for virtually all forms of life and the last step of heme biosynthesis is catalyzed by ferrochelatase enzyme. In this study, blue green algae, photosynthetic bacteria, isolated from Yeşilırmak were used. Eight morphologically different isolates were obtained. The highest specific activity belongs to isolate 8 as 0.217 Umg^{-1}.

Article History

Received 16.07.2020
Accepted 16.08.2020

Keywords

Blue green algae,
Ferro-chelatase,
Tetra pyrrole,
Yeşilırmak

1. Introduction

Tetra pyrroles and their derivatives play an important role in all living organisms. They are involved in many metabolic processes such as energy transfer, catalysis and signal transduction.

Ferrochelatase (EC 4.99.1.1, protoHemeferrolyase) enzyme is an enzyme that shows a catalytic effect in the in vivo synthesis of the proto Heme (Heme) structure (Dailey, 1990). Heme, which is formed by the binding of the Fe^{2+} to protoporphyrin IX substrate under the enzyme catalysis of the ferrochetase enzyme, is a compound found in all organisms other than a few bacterial species and extremophile. Protoporphyrin IX is tetrapirol precursors for iron chelation in heme biosynthesis. Although it is involved in oxygen binding and transport, electron transport, anaemia is observed in synthesis disruptions or disorders and protoporphyrins accumulating in the body have a toxic effect It is found in mitochondrial

[1]Correspondence: v.konar@amasya.edu.tr

membrane in mammalian and yeast cells, while in bacteria it is present in the cytoplasmic membrane (Al-Karadaghi et al., 1997; Hardison, 1999).

Blue green algae are versatile tetrapyrrole synthesizers that can produce end products (heme, chlorophyll, phycobilins and cirrhome), which represent all the main branches of the tetrapyrrole biosynthetic pathway. In addition, phylogenetic studies show that tetrapyrrole synthesis genes in plants originate from blue green algae (Suzuki et al., 2002). Although blue green algae are not characterized as much as in plants, recent studies of the biochemistry and molecular genetics of this pathway have begun to take advantage of the oxygenic prokaryotic organisms (Beale, 1994).

2. Materials and Methods

2.1. Isolation, Purification and Cultivation of Blue-Green Algae

Water samples were taken from Yeşilırmak in Amasya province in 2018. 100 mL of water sample was inoculated in 100 mL of 2X concentrate BG11 medium and incubated for 4-6 week at 28°C in orbital shaker. Subsequent cultures were incubated in the BG11 solid medium (contain 1 % agar). Purified cultures were examined under light microscope (Leica DM500) morphologically.

Purified isolates were incubated in 250 mL volume flasks for 1 month for large volume production, then the flask volume was increased to 1 lt and incubated for 2 months in fresh medium. During incubation, ventilation was provided from the vacuum arm of Nuche flasks with CO_2 and O_2 gases at certain intervals.

2.2. Protein Extraction

The density of the cells was adjusted to 0.5 absorbance at 750 nm. The cells were centrifuged at 10000 g for 10 minutes, the supernatant was removed and the pellet was washed with PBS buffer. After re-centrifugation, the pellet was resuspended in 200 µL PBS. Cells were frozen at -80°C and rapidly thawed at 37°C. Cell wall was broken with ultrasonication probe in ice. Tube walls were washed with 100 µL PBS buffer and collected

by centrifugation at 7000 g for 10 minutes (Ivleva and Golden, 2007). Then the supernatant was used as crude protein extract.

2.3. Determination of Protein Concentration

The protein contents of the extracts were determined according to the Bradford method using bovine serum albumin as standard (Bradford, 1976).

2.4. Determination of Ferro-Chelatase Activity

1 mL of protein extract was incubated at 37°C in a Thunberg tube in a 4.2 mL reaction volume containing 200 µmol porphyrin substrate, 400 µmol $FeSO_4$, 40 µmol GSH and 200 µmol phosphate buffer (pH 7.8). After incubation, the reaction was stopped by adding 1 mL of pyridine, 0.5 mL of 1 N NaOH and 1 mL of water. The reaction mixture was divided into two equal portions. In the first part, 2 mg of solid $Na_2S_20_4$ and in the second part, 0.05 mL of 3 mM $K_3Fe(CN)_6$ were added and analyzed in UV-VIS spectrophotometer (Thermo Genesis 10S) (Porra and Jones, 1963). One unit of ferrochelatase activity is the amount of enzyme that catalyzes the formation of 1 nmol metalloprotoporphyrin at 1 h at 37°C.

3. Results and Discussion

As a result of sampling from Yeşilırmak, blue green algae with 8 different morphologies were isolated. As a result of the light microscopy examination of the isolates; It has been determined that there are filamentous members with heterocyst fixating nitrogen (Figure 1).

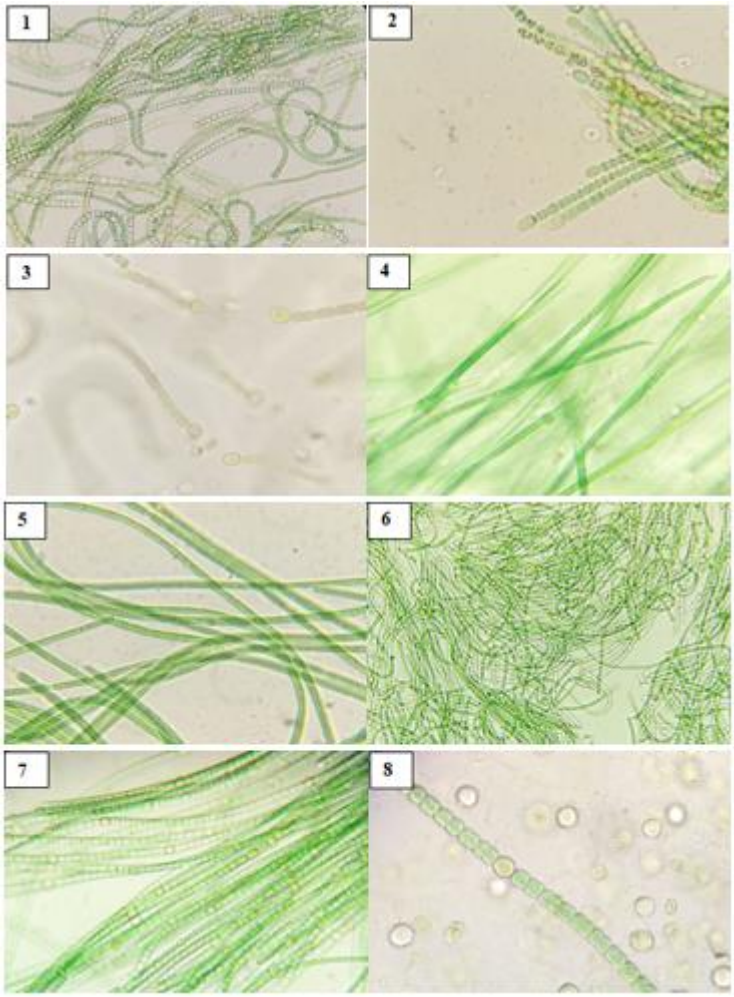

Figure 1. Light microscopy photographies of pure isolates (1-8)

As a result of protein extraction from 8 isolates protein content were found to be 26.1, 24.2, 26.2, 24.3, 23.3, 24.4, 28.1, 24.6 $mgmL^{-1}$, respectively. The highest protein content was found in isolate 7 (28.1 $mgmL^{-1}$) and the lowest protein content was found in isolate 5 (23.3 $mgmL^{-1}$). The ferrochelatase activity of the isolates is 5.12, 4.96, 4.44, 4.82, 4.54, 5.23, 3.91, 5.34 UmL^{-1}, and the specific ferroschetase activity is 0.196, 0.205, 0.169, 0.198, 0.195, 0.214, 0.139, 0.217, respectively (Table 1). It was calculated as Umg^{-1}. The highest specific activity belongs to the isolate 8 as 0.217 Umg^{-1}.

Table 1. Ferro-chelatase activity of isolates

Sample	Protein content ($mgmL^{-1}$)	Activity (UmL^{-1})	Specific activity (Umg^{-1})
Isolate 1	26.1	5.12	0.196
Isolate 2	24.2	4.96	0.205
Isolate 3	26.2	4.44	0.169
Isolate 4	24.3	4.82	0.198
Isolate 5	23.3	4.54	0.195
Isolate 6	24.4	5.23	0.214
Isolate 7	28.1	3.91	0.139
Isolate 8	24.6	5.34	0.217

The ferrochelatase activity of the wild and mutant strains of *Spirillum itersonii* on the crude membrane was found to be 44 Umg^{-1} in the presence of mesoporphyrin substrate (Dailey and Lascelles, 1974).

The specific activity of the ferrochelatase enzyme isolated from the *Saccharomyces cerevisiae* mitochondrial membrane was found to be 15 Umg^{-1} (Camadro and Labbe, 1988).

Ferrochelatase enzyme activity of pig liver tissue homogenate was found as 2.1 Umg^{-1}. As a result of the purification process, the specific activity was calculated as 977 Umg^{-1} (Cánepa and Llambías, 1988).

Myamato et al. (1994) expressed the enzyme ferrochelatase in *Escherichia coli*. The specific activity of the enzyme in the cell was found to be 56.6 Umg^{-1}.

Purification of *Rhodopseudomonas sphaeroides* membrane-bound ferrochelatase, the protein concentration in the crude membrane was found as 21 $mgmL^{-1}$, and the specific activity of the ferrochetase enzyme was found to be 0.98 Umg^{-1}. These results are similar to the protein and specific activity values of our isolates. The specific activity after purification was calculated as 1600 Umg^{-1} and it is thought that we can achieve similar results by applying similar purification steps (Dailey, 1982).

In this study, the ferrochelatase activity of 8 different blue green algae isolates was examined and similar activity results were obtained. The specific enzyme activity in homogenate is low due to its high protein content. Therefore, the enzyme must be purified for

high specific activity. However, there are limited data on the purification of the ferrochelatase enzyme. This is due to the enzyme not being stabilized outside the cell. Although the ferrochelatase enzyme has been studied in other organisms, bacteria, yeast, mammalian cells and plants (Jones, 1968; Goldin and Little, 1969; Dailey, 1982; Martinge et al., 1994; Franco et al., 1995), there is no comprehensive study with blue-green algae. Consequently, further studies are needed to elucidate the ferrochelatase enzyme activities of blue green algae.

Acknowledgement

This work was supported by the Amasya University Scientific Research Foundation (Grant No : FMB-BAP 18-0348).

References

Al-Karadaghi, S., Hansson, M., Nikonov, S., Jönsson, B., Hederstedt, L. 1997. Crystal structure of ferrochelatase: the terminal enzyme in heme biosynthesis, Structure, 5(11): 1501-1510.

Beale, S.I. 1994. Biosynthesis of cyanobacterial tetrapyrrole pigments: hemes, chlorophylls, and phycobilins. In: Bryant, D.A. (ed.). The Molecular Biology of Cyanobacteria. Springer, Dordrecht. pp. 519-558.

Bradforf, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 72(1-2): 248-254.

Camadro, J. M., Labbe, P. 1988. Purification and properties of ferrochelatase from the yeast *Saccharomyces cerevisiae*. Evidence for a precursor form of the protein, Journal of Biological Chemistry, 263(24): 11675-11682.

Cánepa, E.T., Llambías, E.B. 1988. Purification and characterization of ferro-and cobalto-chelatases, Biochemistry and Cell Biology, 66(1): 32-39.

Dailey Jr, H.A., Lascelles, J. 1974. Ferrochelatase activity in wild-type and mutant strains of *Spirillum itersonii*: Solubilization with chaotropic reagents, Archives of Biochemistry and Biophysics, 160(2): 523-529.

Dailey, H.A. 1982. Purification and characterization of membrane-bound ferrochelatase from *Rhodopseudomonas sphaeroides*, Journal of Biological Chemistry, 257(24): 14714-14718.

Dailey, H.A. 1990. Biosynthesis of heme and chlorophylls. McGraw-Hill Companies.

Franco, R., Moura, J.J., Moura, I., Lloyd, S.G., Huynh, B.H. et al. 1995. Characterization of the iron-binding site in mammalian ferrochelatase by kinetic and Mössbauer methods, Journal of Biological Chemistry, 270(44): 26352-26357.

Goldin, B.R., Little, H.N. 1969. Metalloporphyrin chelatase from barley, Biochimica et Biophysica Acta (BBA)-Enzymology, 171(2): 321-332.

Hardison, R. 1999. The evolution of hemoglobin: studies of a very ancient protein suggest that changes in gene regulation are an important part of the evolutionary story, American Scientist, 87(2): 126-137.

Ivleva, N.B., Golden, S.S. 2007. Protein extraction, fractionation, and purification from cyanobacteria. In: Rosato, E. (ed). Circadian Rhythms. Humana Press. pp. 365-373.

Jones, O.T.G. 1968. Ferrochelatase of spinach chloroplasts, Biochemical Journal, 107(1): 113-119.

Kobayashi, K., Mochizuki, N., Yoshimura, N., Motohashi, K., Hisabori, T. et al. 2008. Functional analysis of *Arabidopsis thaliana* isoforms of the Mg-chelatase CHLI subunit, Photochemical & Photobiological Sciences, 7(10): 1188-1195.

Matringe, M., Camadro, J. M., Joyard, J., Douce, R. 1994. Localization of ferrochelatase activity within mature pea chloroplasts, Journal of Biological Chemistry, 269(21): 15010-15015.

Miyamoto, K., Kanaya, S., Morikawa, K., Inokuchi, H. 1994. Overproduction, purification, and characterization of ferrochelatase from *Escherichia coli*, The Journal of Biochemistry, 115(3): 545-551.

Porra, R., Jones, O. 1963. Studies on ferrochelatase. 1. Assay and properties of ferrochetalase from a pig-liver mitochondrial extract, Biochemical Journal, 87(1): 181-185.

Suzuki, T., Masuda, T., Singh, D.P., Tan, F.C., Tsuchiya, T. et al. 2002. Two types of ferrochelatase in photosynthetic and nonphotosynthetic tissues of cucumber their difference in phylogeny, gene expression, and localization, Journal of Biological Chemistry, 277(7): 4731-4737.

Fidan, M, Ayar, A. International Journal of Science Letters (IJSL). 2020. 2(2): 79-91.
Research Article

An *in vivo* study on *Drosophila melanogaster*, *Artemia salina*, and *Daphnia magna*: Is activated carbon used as a food additive reliable?

Mehmet Fidan[1]*, Arif Ayar[2]

[1]Institute of Science, Amasya University, Amasya/Turkey
[2]Department of Medical Services and Techniques, Sabuncuoğlu Şerefeddin Vocational School of Health Services, Amasya University, Amasya/Turkey

Abstract

Activated carbon, one of the most important adsorbents used in the industry, is a general definition used to describe carbon adsorbents, which have a large crystal and amorphous structure and wide internal pores. Activated carbon, which has started to be used in many fields in recent years, is observed to be preferred as an important additive in the food industry. This study aimed to investigate the reliability of the use of activated carbon as a food additive in different model organisms, such as *Drosophila melanogaster*, *Artemia salina*, and *Daphnia magna*. To this end, the organisms were kept alive in nutrient media containing activated carbon at different concentrations (0.1 $mgmL^{-1}$, 0.5 $mgmL^{-1}$, 1 $mgmL^{-1}$, 2.5 $mgmL^{-1}$, and 5 $mgmL^{-1}$), and changes that occurred in their percentage of survival were determined for 48 h. According to the data obtained, for all three organisms, it was found that in comparison with the control group, there was no decrease in survival percentages in any of the experimental groups in which activated carbon was used. On the contrary, there were increases depending on concentration. Especially in *A.salina*, the percentage of survival, which was 78 % in the control group, increased up to 87 % ($P < 0.05$). As a result of the study, it was concluded that activated carbon at the specified doses might be used reliably as a food additive. It was evaluated that these results should be supported by *in vivo* and *in vitro* studies to be conducted in different organisms.

Article History

Received 18.07.2020
Accepted 18.08.2020

Keywords

Activated carbon,
Artemia salina,
Daphnia magna,
Drosophila melanogaster,
Percentage of survival

1. Introduction

The carbon element present in the structure of living things exists very rarely in nature. The carbon element is also a good absorbent due to its important properties, such as pore volume, density, abrasion resistance, hardness, and grain size (Gündüzoğlu, 2008). One of the

[1]Correspondence: mfidan1980@hotmail.com

most important features of activated carbon is the wide surface area and its developed pore structure (Dermanlı, 2006; Akyıldız, 2007).

The number of studies on the use of many biomass wastes such as hazelnut husk, apricot kernel, corn cob, olive kernel, and rice stalk in the production of activated carbon is quite high. In industrial applications, mostly coal and hazelnut husk are preferred as the starting material for activated carbon production (El-Hendawy, 2005).

Activated carbon is most commonly used in adsorption and colour removal processes. The removal of colour from textile wastes and especially, the purification of wastewater are of great importance in reducing environmental problems (Köseoğlu, 2005).

There are two types of activated carbon in the food industry as an application area: 1-Medical activated carbon is used as a food additive/drug and can be consumed orally, 2-Activated carbon adsorbs unwanted compounds as a purification agent and removes them from the medium. Activated carbons, which can be consumed orally, are commercially sold as E153-coded food additive (CAS no: 7440-44-0), in the form of powder or granules, to be used for colouring food and the adsorption of toxins in the body.

In the food industry, it is benefited from the high colour adsorption feature of activated carbon to remove non-enzymatic browning reaction products in sugar, syrup, and molasses production (Ozsoy, 2010; Bernal et al., 2016). There are some studies in the wine, vinegar, and alcoholic beverage industries in which activated carbon is used to remove excess grains, unwanted colour, odour elements, sediment, turbid substances, and provide aroma isolation (Quintela et al., 2013; Lisanti et al., 2017; Tubia et al., 2018). Besides these, activated carbon is nowadays added to foods such as bread, hamburger, lemonade, and ice cream.

In the current study, the possible effects of activated carbon used in foods were investigated in three different model organisms. One of them, *Drosophila melanogaster*, is an important model organism that is frequently preferred in biological studies and known as the fruit fly. *D. melanogaster*, which was first used in experimental studies by Thomas Morgan in 1911, has many advantages in terms of use in such studies (Gui and Grant, 2008). *Artemia salina* is a species widely used in ecotoxicological studies and applications worldwide (Sanchez-Fortun et al., 1995). *A. salina*, of which the commonly known name is saltwater

shrimp, is used in many scientific fields, such as ecology, physiology, ecotoxicology, aquatic ecosystem, and genetics (Nunes et al., 2006). Another organism used in the present study is *Daphnia magna* (Water Flea) freshwater zooplanktonic crustaceans and is known as water flea among the people. Water fleas, which are rich in protein and essential fatty acids, constitute the most important food source of fish and are used as a living food source by fish farmers (Demirel, 2011). The easy and economical production of its culture due to its small structure, short life, high spawning capacity, and sensitive structure to pollution, enabled water fleas to be frequently used in aquatic toxicity tests (Demirel, 2011).

The aim of this study is to determine the toxic effect of activated carbon, which is used as a nutrient in different concentrations, on survival percentages in different model organisms such as *Drosophila melanogaster*, *Artemia salina* and *Daphnia magna* and investigate the safety of its use as a food additive.

2. Materials and Methods

2.1. Material

Activated carbon, of which effect on living things was investigated, was procured from a commercial producer. The activated carbon supplied is sold on the market in the powder form for use in foods.

The Oregon R lineage (Diptera: Drosophilidae) of *D. melanogaster* used in the experiments is a wild type (w.t.) lineage. This lineage is a homogeneous stock that has been tailored for years at the Biological Research Laboratory of Amasya University, Faculty of Science, Department of Biology. Reasons such as short life cycle (9-10 days), breeding a lot, cheap raising conditions, and easy observation of possible variations make *Drosophila* an ideal experimental organism. The eggs of *Daphnia magna*, another organism used in the study, were obtained from a commercial firm and cultured in Çorum Science and Art Center Biology Laboratory. The eggs of *Artemia salina* were obtained from a commercial company and their larvae were obtained by using the egg hatching mechanism at the Biology Laboratory of Çorum Science and Art Center.

2.2. Method

2.2.1. Preparation of experiment sets and substance application

In the study, mortality rates in 3 different model organisms living in media containing chronically activated carbon were calculated. The stock solution of the activated carbon, of which effects on different living things were investigated, was prepared in 5 different concentrations (0.1 $mgmL^{-1}$, 0.5 $mgmL^{-1}$, 1 $mgmL^{-1}$, 2.5 $mgmL^{-1}$, and 5 $mgmL^{-1}$).

In the study, the Oregon (Wild Type) race was used to obtain the 3rd stage larvae of *D. melanogaster* were procured from a commercial company operating in Turkey. Twenty-five female and twenty-five male Oregon individuals were put in bottles containing nutrients. These individuals were kept in the same medium for at least one day, and their mating was provided. The individuals were then transferred to new bottles containing nutrients and were expected to lay eggs in the medium for 8 h. Then, the individuals were transferred to other bottles. An 8-h egg collection process aimed to obtain individuals in the same larval stage. The individuals who reached the 3rd larval stage after 72 ± 4 h were separated under tap water with the help of fine porous sieves. The 3rd stage larvae collected with the help of the sieve were transferred to plastic bottles containing *Drosophila* ready-made food that was wetted by adding 9 mL of the freshly prepared activated carbon concentrations to be studied. One-two spatulas (100 larvae) of larvae were placed in each application medium. The number of flies that became mature from 100 larvae (after 84-108 h), which were placed in media containing different activated carbon concentrations, was noted. All treatment groups were fed in the culture tubes placed in an oven, which was set at 25°C and had 40- 60 % relative humidity.

In the current study, *D. magna*, another organism on which the effect of activated carbon was investigated, was reproduced in the laboratory under standard living conditions. *Daphnia magna* procured from a private company serving in Turkey was used as a trial material. As the experimental organism, firstly, individuals with eggs were selected and placed in a separate medium, and after spawning was provided, the offspring were subjected to the same feeding program (algae + yeast) for five days and used in the trial. One hundred individuals with the same size and without eggs were taken from the *D. magna* stock solution and placed in media containing activated carbon at different concentrations. The number of individuals was counted for 48 h, and immobile and dead individuals were noted. No feeding was made

during the experiments. 100 mL of the test medium containing the required activated carbon was prepared in each experimental vessel, and 10 *Daphnia magna* individuals were used for each experimental vessel. The temperature was kept at 20 ± 2°C during the experiment.

The eggs of *A. salina*, which was the other organism used in the present study, were procured from a commercial company operating in Turkey. The eggs obtained were first immersed in demineralized water at + 4°C and kept waiting for 1 h. During this period, cysts that sank to the bottom of the water and remained on the surface were separated. The cysts obtained were placed in the medium that had been previously prepared by dissolving 25 g sea salt in 1 L of water. Nauplii larvae hatched from the eggs, which were exposed to powerful aeration for 24 h, were drained and used in the experiments. The obtained *Artemia* individuals were kept alive in media containing different concentrations of activated carbon, as 100 individuals in each. It was checked for 48 h, and immobile and dead individuals were counted and noted. All experiments were repeated three times.

2.2.2. Statistical analysis

The statistical analysis of the data obtained from the experiments investigating the effects of the examined substances on the percentage of survival was performed using the SPSS (Statistical Package for the Social Sciences) 15.0 program.

3. Results

Activated carbon, which was applied in different doses to determine larval toxicity, did not reduce the survival rates of *D. melanogaster* in stage 3 larvae (Table 1). As observed in Table 1, the survival rate, which was 94 % in the negative control group, increased to 95 % in the highest treatment group of activated carbon (5 $mgmL^{-1}$) depending on the dose increase. This increase was found to be statistically insignificant ($P<0.05$). As a result of this study, it was detected that activated carbon did not show any toxic effects in *D. melanogaster* larvae at any concentration and did not increase larval mortality (Figure 1).

Table 1. Survival and mortality rates of *Drosophila melanogaster* larvae chronically fed with different concentrations of activated carbon after 84-108 h

Treatment Groups	Concentration ($mgmL^{-1}$)	Number of Larvae	Mortality Rate ± SE	Survival Percentage (%)
Control (Distilled Water)		100	5.66 ± 0.33^{a}	94^{a}
Activated Carbon ($mgmL^{-1}$)	0.1	100	6.66 ± 0.66^{a}	94^{a}
	0.5	100	4.66 ± 0.66^{b}	96^{b}
	1	100	5.0 ± 0.15^{a}	95^{a}
	2.5	100	6.33 ± 0.33^{a}	94^{a}
	5.0	100	5.0 ± 0.15^{a}	95^{a}

SE: Standard Error; Statistical evaluations of the difference between the groups were made within the group. Values shown with different letters in the same column are significant at the level of $p<0.05$.

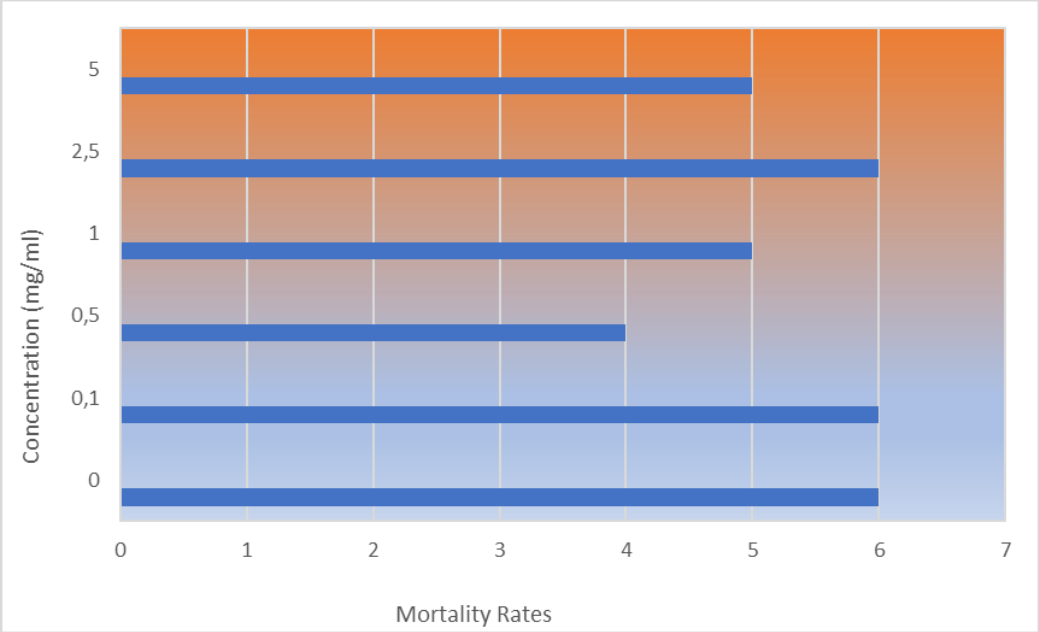

Figure 1. Comparison of the mortality rates of *Drosophila melanogaster* larvae raised in media containing different concentrations of activated carbon

Afterwards, in the study, 100 individuals were used for each concentration to determine the effects of activated carbon on *D. magna* (Table 2). Activated carbon, which was applied in different doses in order to determine toxicity in *D.magna* adults, did not reduce survival rates in *D. magna* individuals (Table 2 and Figure 2). As observed in Table 2, the survival rate, which was 84 % in the negative control group, increased until 92 % in the highest treatment group of activated carbon (5 $mgmL^{-1}$) depending on the dose increase. This increase was found to be statistically significant ($P<0.05$).

Table 2. Survival and mortality rates of *Daphnia magna* fed with different concentrations of activated carbon after 48 h

Treatment Groups	Concentration ($mgmL^{-1}$)	Number of Adult Individuals	Mortality Rates ± SE	Survival Percentage (%)
Control (Distilled Water)		100	16.0 ± 1.15^{a}	84^{a}
Activated Carbon ($mgmL^{-1}$)	0.1	100	13.66 ± 0.33^{b}	86^{a}
	0.5	100	14.0 ± 1.15^{b}	86^{a}
	1.0	100	12.66 ± 0.33^{c}	87^{a}
	2.5	100	10.33 ± 1.15^{d}	90^{b}
	5.0	100	8.33 ± 0.33^{e}	92^{c}

SE: Standard Error; Statistical evaluations of the difference between the groups were made within the group. Values shown with different letters in the same column are significant at the level of $p<0.05$.

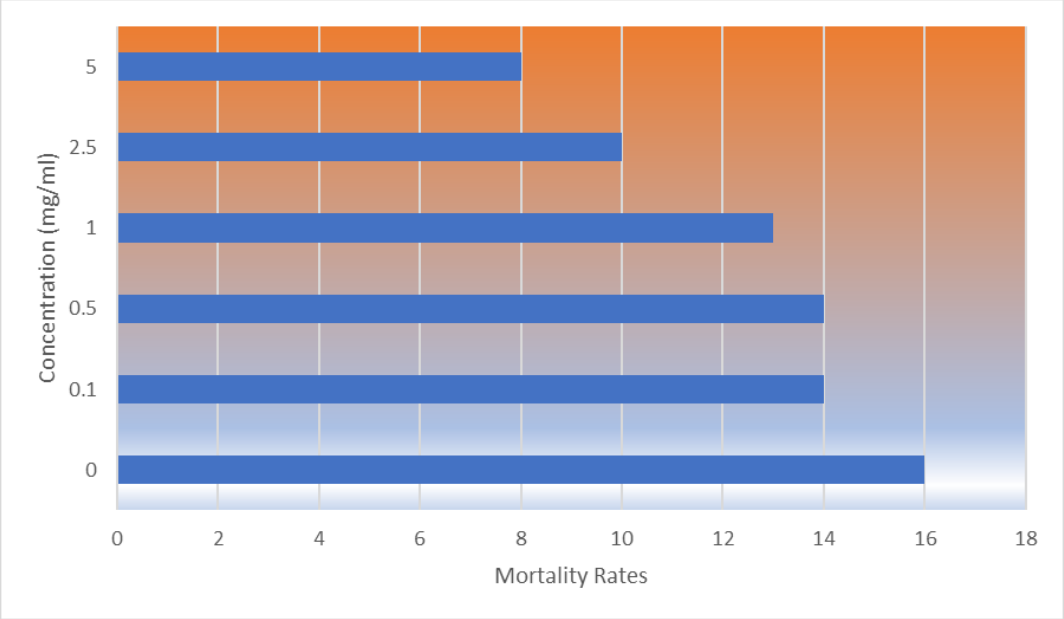

Figure 2. Comparison of the survival percentages of *Daphnia magna* raised in media containing different concentrations of activated carbon

The data obtained as a result of the feeding of *A. salina*, which is commonly used in ecotoxicological studies and applications, with activated carbon are presented in Table 3. In *A. salina*, 100 larvae were used for each concentration. Activated carbon, which was applied at different doses to determine toxicity in *A. salina* nauplii larvae, did not reduce survival rates in *A. salina* individuals (Table 3 and Figure 3). As observed in Table 3, the survival rate, which was 78 % in the negative control group, increased up to 87 % in the highest treatment group of activated carbon (5 $mgmL^{-1}$) depending on the dose increase. This increase was found to be statistically significant ($P<0.05$).

Table 3. Survival and mortality rates of *Artemia salina* (nauplii) fed with different concentrations of activated carbon after 48 h

Treatment Groups	Concentration ($mgmL^{-1}$)	Number of Larvae	Mortality Rates ± SE	Survival percentage (%)
Control (Distilled Water)		100	22.0 ± 0.57^{a}	78^{a}
Activated Carbon ($mgmL^{-1}$)	0.1	100	22.0 ± 0.33^{a}	78^{a}
	0.5	100	22.0 ± 0.66^{a}	78^{a}
	1.0	100	18.0 ± 0.33^{b}	82^{b}
	2.5	100	16.33 ± 0.88^{b}	84^{b}
	5.0	100	13.66 ± 0.66^{c}	87^{c}

SE: Standard Error; Statistical evaluations of the difference between the groups were made within the group. Values shown with different letters in the same column are significant at the level of $p<0.05$.

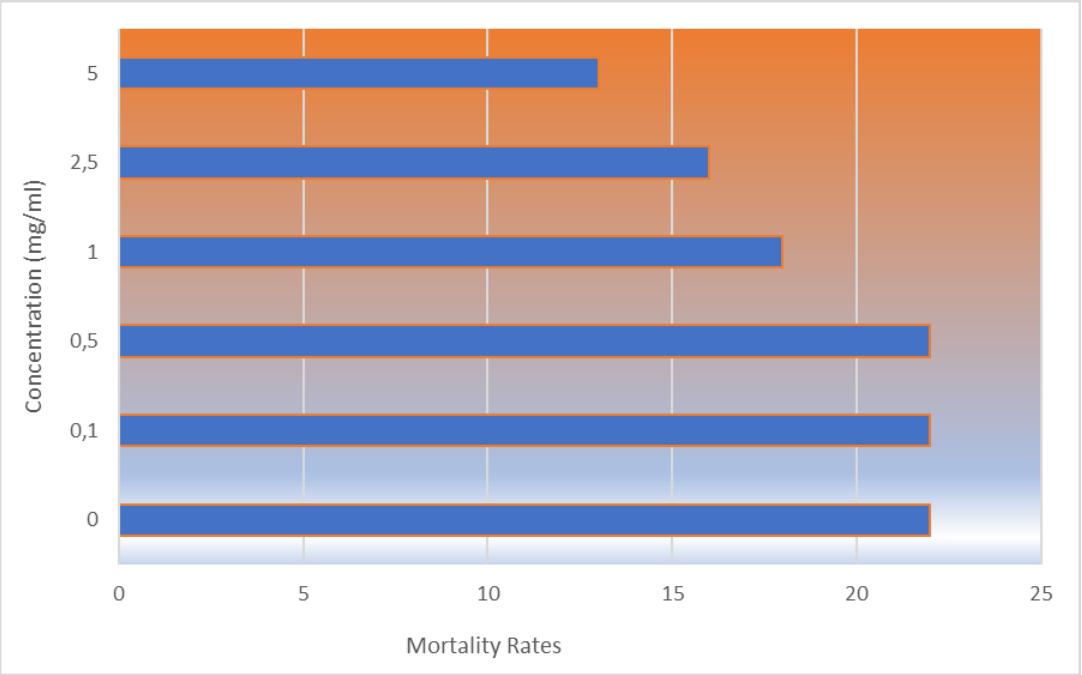

Figure 3. Comparison of the survival percentages of *Artemia salina* nauplii larvae raised in media containing different concentrations of activated carbon

4. Discussion

In the study, the effect of activated carbon on mortality rates in 3 separate organisms was investigated. When firstly, the data on *Drosophila melanogaster*, which was the organism on which the effect of activated carbon was investigated, were examined, the survival percentage was 94% in the control group, while the same or higher data were obtained in the treatment

groups (Table 1). When the data on *Daphnia magna*, which is a freshwater organism that is frequently used in toxicity studies, were examined, while the survival percentage in the control group was 84 % after 48 h, an increase occurred in survival percentages depending on the increase in concentration in the experimental groups. The highest rate was observed to be 92 % in *Daphnia* fed in the medium containing 5 $mgmL^{-1}$activated carbon (Table 2). When the effect of activated carbon on *Artemia salina*, a brine organism, was examined, the survival percentage in the control group was 78 %, while the survival percentage at 5 $mgmL^{-1}$ concentration increased to 87 % (Table 3).

When the obtained results were examined, no negative effect of activated carbon was found in 3 different organisms. It is thought that the results are significant in terms of eliminating concerns about the use of activated carbon in foods. The use of activated carbon in main consumption materials such as bread, hamburger, and lemonade, which are important nutrients, is a subject that interests all people. Therefore, it is thought that this study should be supported by other studies that will be conducted.

New application areas of activated carbon in the food industry are listed as follows: retaining volatile fatty acids in anaerobic digestion processes, increasing methane gas production performance with ammonia nitrogen rate control, purifying food additives, CO_2 gas control in modified atmospheric applications, new generation antimicrobial agent, removal of volatile organic compounds, recovery of aroma components, activated carbon electrochemical sensors (EDCL electrode), and efficient separation processes with electromagnetic activated carbons.

In the production of renewable energy (biofuels) from food waste, the overloading of the reactor with volatile fatty acids in anaerobic digestion processes and increased ammonia nitrogen, which is toxic to microorganisms with its high protein content, reduce the process efficiency. Therefore, activated carbon applications are used to remove these components from the reactor environment (Capson-Tojo et al., 2018).

Activated carbon is also used in the purification of additives such as monosodium glutamate (MSG), glutamic acid, and hydrolyzed vegetable proteins (HVPs), which are commonly used in the food industry (Wang et al., 2017; Kobayashi et al., 2018). Hydrocarbons, cyclic structures, and aromatics, released in food production stages such as

fermentation, cooking, evaporating, condensation, heating, and drying processes, are volatile organic compounds that generally produce bad smell-taste in foods and show toxic, mutagenic, and carcinogenic effects. Furthermore, during the cleaning and disinfection steps, chlorine compounds and other polluting gasses such as H_2S, NOX must be removed from the medium. Activated carbons are more economical and reusable adsorbents for removing volatile organic compounds from the medium (Olgun et al., 2017).

In a study, magnetic carbons that were produced with $FeCl_3$, $FeCl_2$, and urea-catalyzed hydrothermal reaction, in which carbohydrates (fructose, glucose, and sucrose) of expired beverages were used as carbon sources, were activated with KOH. Then, their removal with water-soluble methylene blue dye with a 404.73 mgg^{-1} adsorption capacity was achieved (Liu et al., 2019). In another study, in order to increase the absorption of triazine herbicides from model food samples such as milk and rice, the separation of triazine herbicides depending on the hydrophobic interaction between triazine herbicides coated with magnetic modified activated carbon on a nonionic silicon surface and the adsorbent was achieved at a rate of 81 % in food samples (Mohd et al., 2019).

The effects of various food additives on the model organisms used in the study have been tested in different studies. Some of these studies are as follows: It was determined that various preservatives (sorbic acid, potassium sorbate, benzoic acid, sodium benzoate, potassium acetate, sodium metabisulfite, potassium metabisulfite, sodium tetraborate, sodium sulfite, and boric acid) had different effects and that among these substances, potassium acetate had the least effect and sorbic acid had the highest effect. Benzaldehyde, which is used to give flavour to foods, was revealed to be mutagenic and genotoxic (Güneş, 2016).

Activated carbon causing a colour change in the consumed foods leads to the emergence of an unusual appearance such as black toothpaste, black hamburger. In a study conducted with different food dyes, these food dyes were determined to cause a mutagenic effect. The genotoxic effect of tartrazine, one of the food dyes, was investigated by Niraj et al. (1989) with somatic mutation and recombination test in *Drosophila*. As a result of 0.06 % and 0.03 % tartrazine application, the researchers detected that tartrazine had both mutagenic and recombinogenic effects (Niraj et al., 1989).

Among food dyes (erythrosine, indigo carmine, patent blue, amaranth, and carminic acid), the use of more than 25 mg of patent blue, more than 20 mg of carminic acid, more than 6 mg of erythrosine, more than 2 mg of indigo carmine, and more than 10 mg of amaranth were determined to show a lethal effect on the insect, and benzaldehyde, which is used to give flavour to foods, was determined to be mutagenic and genotoxic (Güneş, 2016).

In another study conducted with *Daphnia magna*, the effect of monosodium glutamate (MSG), which is a highly controversial compound and found in the American diet, was investigated. This compound, which is generally used as a supplement for flavour, was reported as being considered safe (U.S. Food and Drug Administration, 2012).

In another study conducted with *Daphnia magna*, the effect of food dyes was evaluated. In the study conducted by Abe et al. in 2017, Basic Red 51 (BR51) dye was observed to be extremely toxic. An increase was detected in the respiratory levels of *D.magna* in the short term, and negative effects were detected in its reproduction in the long term. *Artemia salina*, another organism used in the study, is an organism commonly used in toxicity and ecotoxicity tests.

Activated carbon, which has started to be also used in food products in recent years, attracts attention due to the change it causes in the colour of food products. Its presence also in frequently consumed foods, such as bread, hamburgers, and lemonade, has raised doubts in consumers about its benefits and harms. Determining the genotoxic potential of preservatives used in foods is of great importance in terms of food safety, human health, and quality of life. Even if the preservatives commonly used in foods are used in amounts that do not harm the health, it should be taken into consideration that these substances may accumulate in the body over time and thus threaten human health directly or indirectly. It is necessary to show the utmost care and sensitivity in the use of food additives, to increase the level of awareness of manufacturers and consumers in terms of food safety and to strengthen the control mechanisms related to food health. In this respect, the current study is thought to be valuable in terms of eliminating doubts. Not observing any negative effects in *Drosophila melanogaster*, *Artemia salina*, and *Daphnia magna*, which are important model organisms, creates a hope about the subject that these products containing activated carbon will be consumed safely.

References

Abe, F.R., Mendonça, J.N., Moraes, L.A.B., Gravato, C., Soares, A.M.V.M. et al. 2017. Toxicological and behavioral responses as a tool to assess the effects of natural and synthetic dyes on zebrafish early life, Chemosphere, 178: 282-290.

Akyıldız, H. 2007. Activated carbon production from the olive kernel with H_3PO_4 activation. MSc thesis. Yildiz Technical University, p. 115, Istanbul, Turkey.

Bernal, M., Ruiz M.O., Geanta, R.M., Benito, J.M., Escudero, I. 2016. Colour removal from beet molasses by ultrafiltration with activated charcoal, Chemical Engineering Journal, 283: 313-332.

Capson-Tojo, G., Moscoviz, R., Ruiz, D., Santa-Catalina, G., Trably, E. et al. 2018. Addition of granular activated carbon and trace elements to favor volatile fatty acid consumption during anaerobic digestion of food waste, Bioresource Technology, 260: 157-168.

Demirel, T. 2011. Investigation of the acute toxic effect of zinc on *Daphnia magna* (straus, 1820) (Crustacea: Cladocera). MSc thesis. Ankara University, p. 54, Ankara, Turkey.

Dermanlı, Y. 2006. Production of activated carbon from food fabrication waste and investigation of bleaching performance in soybean oil. MSc Thesis. Istanbul Technical University, p. 62, Istanbul, Turkey.

El-Hendawy, A.A. 2005. Surface and adsorptive properties of carbons prepared from biomass, Applied Surface Science, 252: 287-295.

Gündüzoğlu, G. 2008. Preparation and characterization of activated carbon from sugar beet bagasse. MSc Thesis. Eskişehir Osmangazi University, p. 150, Eskişehir, Turkey.

Güneş, E. 2016. *Drosophila* in food and nutrition studies, Kahramanmaraş Sütçü İmam University Journal of Agriculture and Nature, 19(3): 236-243.

Gui, Y., Grant, A. 2008. Joint effects of density dependence and toxicant exposure on *Drosophila melanogaster* populations, Ecotoxicology and Environmental Safety, 70(2): 236-243.

Kobayashi, K., Tanaka, M., Tanabe, S., Yatsukawa, Y., Tanaka, M. 2018. Distinguishing acid in foodstuffs and monosodium glutamate used as seasoning by stable carbon and nitrogen isotope ratios, Heliyon, 4: 1-17.

Köseoğlu, E. 2005. The characterization of activated carbons prepared from agriculture side product with chemical activation and the investigation of cation adsorption from aqueous solution. MSc thesis. Inönü University, p. 102, Malatya, Turkey.

Lisanti, M.T., Gambuti, A., Genovese, A., Piombino, P., Moio, L. 2017. Treatment by fining agents of red wine affected by phenolic offodour, European Food Research and Technology, 243(3): 501-510.

Liu, Y., Huo, Z., Song, Z., Zhang, C., Ren, D. et al. 2019. Preparing a magnetic activated carbon with expired beverage as carbon source and KOH as activator, Journal of the Taiwan Institute of Chemical Engineers, 96: 575-587.

Mohd, N.I., Gopal, K., Raoov, M., Mohamad, S., Yahaya, N. et al. 2019. Evaluation of a magnetic activated charcoal modified with nonionic silicone surfactant as a new magnetic solid phase extraction sorbent with triazine herbicides as model compounds in selected milk and rice samples, Talanta, 196: 217- 225.

Niraj K.T., Kalyani K.P., Nabi M.J. 1989. Genotoxcicity of tartrazine studied in two somatic assays of *Drosophila melanogaster*, Mutation Research, 224: 479-483.

Nunes, B. S., Carvalho, F. D., Guilhermino, L. M., Van Stappen, G. 2006. Use of the genus *Artemia* in ecotoxicity testing, Environmental Pollution, 144(2): 453-462.

Olgun, B., Erdem, A., Asiltürk, M., Doğan, G. Evaluation of volatile organic compound removal in ambient air with nanoparticle-attached activated carbon. VII. National Air Pollution and Control Symposium, 1-3 November 2017, Antalya, Turkey, p. 830-841.

Ozmak, M. 2010. Activated carbon production from biomass waste. PhD thesis. Ankara University, p. 203, Ankara, Turkey.

Ozsoy, H.D., van Leeuwen, J.H. 2010. Removal of color from fruit candy waste by activated carbon adsorption, Journal of Food Engineering, 101(1): 106-112.

Patrick, J.W. 1995. Porosity in carbons, Edward Arnold, London.

Quintela, S., Villaran, M.C., Armentia, I.L., Elejalde, E. 2013. Ochratoxin A removal in wine: A review, Food Control, 30: 439-445.

Sanchez-Fortun, S., Sanz-Barrera, F., Barahona-Gomariz, M.V. 1995. Acute toxicities of selected insecticides to the aquatic arthropod *Artemia salina*. Bulletin of Environmental Contamination and Toxicology, 54(1): 76-82.

Tubia, I., Prasad, K., Perez-Lorenzo, E., Abadin, C., Zumarraga, M. et al. 2018. Beverage spoilage yeast detection methods and control technologies: A review of *Brettanomyces*, International Journal of Food Microbiology, 283: 65-76.

U.S. Food & Drug Administration. 2012. Food Additives & Ingredients - Questions and Answers on Monosodium glutamate (MSG).

Wang, F., Liang, J., Wang, W. Fu, D., Xiao, W. 2017. A new and efficient method for purification of poly-γ- glutamic acid from high viscosity fermentation broth, Tropical Journal of Pharmaceutical Research, 16(6): 1267-1275.

Türkmen, BN, Tükmen, E. International Journal of Science Letters (IJSL). 2020. 2(2): 92-97.
Research Article

Coatomic refinable modules

Burcu Nişancı Türkmen[1], Ergül Türkmen[1*]

[1]Department of Mathematics, Faculty of Arts and Science, Amasya University, Amasya/Turkey

Abstract

In this article, we define the concept of (strongly) coatomic refinable modules as a proper generalization of (strongly) refinable modules. It is shown that: (1) every direct summand of a coatomic refinable module is coatomic refinable; (2) over a left max ring a module M is (strongly) coatomic refinable if and only if it is (strongly) refinable; (3) if a coatomic refinable module M is $\pi-$projective, then it is strongly coatomic refinable; (4) if coatomic direct summands lift modulo every coatomic submodule of M, then M is coatomic refinable.

Article History

Received 28.07.2020
Accepted 21.08.2020

Keywords

Refinable module,
Coatomic module,
Coatomic refinable module

1. Introduction

In this paper, all rings are associative with identity and all modules are left modules. Let R be such a ring and let M be an R-module. The notation $N\subset M$ ($N\subseteq M$) means that N is a (proper) submodule of M. A submodule U is called *small* (in M), denoted by $U<<M$, if $M = U + K$ for every proper submodule K of M (Wisbauer, 1991). Following (Wisbauer, 1991), a module M is called *hollow* if every proper submodule of U is small in M. M is called *local* if it is hollow and finitely generated. By $Rad(M)$, namely *radical*, we will denote the sum of all small submodules of M. It is well known that $Rad(M)$ is the intersection of all maximal submodules of M. It is clear that if a module M is hollow, then $Rad(M)<<M$ or $M = Rad(M)$.

Let M be a module. M is called *coatomic* if every proper submodule of M is contained in a maximal submodule of M (Zöschinger, 1974). Every finitely generated module is coatomic, and coatomic modules have small radical. It can be seen that if a coatomic submodule N of a module M is contained in $Rad(M)$, then N is a small submodule of M. Also, over a Dedekind domain every small submodule of a module is coatomic. The class of coatomic modules is closed under factor modules and extensions. In general, a submodule of a coatomic module

[1]Correspondence: ergulturkmen@hotmail.com

need not be coatomic. Over a commutative noetherian ring, every submodule of a coatomic module is coatomic. A ring R is called *a left max ring* if every nonzero left R-module have a maximal submodule. Left perfect rings (over which every module has a projective cover) are left max rings. It is known that a ring R is a left max ring if and only if every nonzero left R-module is coatomic.

For any ring R, an R-module M is called *supplemented* if every submodule N of M has a *supplement*, that is a submodule K minimal with respect to $N + K = M$. K is a supplement of N in M if and only if $N + K = M$ and $N \cap K << K$ (Wisbauer, 1991). Every direct summand of a module M is a supplement submodule of M, and supplemented modules are a proper generalization of Artinian modules.

Mohamed and Muller (1990) call a module M $\oplus$-*supplemented* if every submodule N of M has a supplement that is a direct summand of M (Mohamed and Müller, 1990). Every $\oplus$-supplemented module is supplemented, but a supplemented module need not be $\oplus$-supplemented in general (see Mohamed and Müller, 1990, Lemma A.4 (2)). It is shown in Mohamed and Müller (1990), 3, Proposition A.7 and Proposition A.8] that if R is a Dedekind domain, every supplemented R-module is $\oplus$-supplemented. Hollow modules are $\oplus$-supplemented. Characterizations and the structure of supplemented and $\oplus$-supplemented modules are extensively studied by many authors.

A module M is *lifting* if every submodule N of M contains a direct summand L of M such that $M = L \oplus K$ and $N \cap K << K$ (see Clark et al., 2006). Every projective module over a left Artinian ring is lifting, and lifting modules are $\oplus$-supplemented (see Wisbauer, 1991, 41.15).

In Wisbauer (1996), the class of (strongly) refinable modules is introduced as a proper generalization of lifting modules. An R-module M is called *refinable* if, for any submodules $U, V \subseteq M$ with $M = U + V$, there exists a direct summand U of M with $A \subseteq U$ and $M = A + V$. Every finitely generated regular module is refinable. A module M is said to be *strongly refinable* if, in the given situation, there exist submodules $A \subseteq U$, $B \subseteq V$ of M with $M = A \oplus B$. Hollow modules and semisimple modules are strongly refinable.

In this paper, we define the concept of coatomic (strongly) refinable modules. The class of

(strongly) coatomic refinable modules properly contains the class of refinable module. We prove that every direct summand of a coatomic refinable module is coatomic refinable. We show that if a coatomic refinable module M is π-projective, then it is strongly coatomic refinable. We also prove that if coatomic direct summands lift modulo every coatomic submodule of M, then M is coatomic refinable.

2. Coatomic Refinable Modules

In this section, we give basic properties of (strongly) coatomic refinable modules. In particular, we show that every direct summand of a coatomic refinable module is coatomic refinable.

Definition 2.1. Let M be a module. M is called *coatomic refinable* if, for any coatomic submodule N of M and any submodule K of M with $M = N + K$, there exists a direct summand N of M with $N' \subseteq N$ and $M = N' + K$, and M is called *strongly coatomic refinable* if, for any coatomic submodule N of M and any submodule K of M with $M = N + K$, there exist submodules N' and K' of M with $N' \subseteq N$, $K' \subseteq K$, $M = N' + K$ and $M = N' \oplus K'$.

Clearly, (strongly) refinable modules are (strongly) coatomic refinable. The following example shows that a strongly coatomic refinable module need not be refinable. The following well-known fact is given for completeness.

Lemma 2.2. Let R be a Dedekind domain and M be an R-module. Assume that a submodule N of M is contained in Rad(M). Then, N is a small submodule of M if and only if N is coatomic.

Example 2.3. Consider the $\mathbb{Z}$-module $M = {}_{\mathbb{Z}}\mathbb{Q}$. Therefore, we have that $M = Rad(M)$. If N is a coatomic submodule of M, it follows from Lemma 2.2 that N is a small submodule of M. So we can write $M = N + M = 0 \oplus M$ and $0 \subset N$. It means that M is a strongly coatomic refinable module. On the other hand, M is not refinable.

Proposition 2.4. Let R be a commutative noetherian ring and M be a (strongly) coatomic refinable R-module. If M is coatomic, then it is (strongly) refinable.

Proof. Let M be a coatomic R-module. Since R is commutative noetherian, every submodule of M is coatomic. By the assumption, M is (strongly) refinable.

Proposition 2.5. Let R be a left max ring and M be an R-module. Then, M is (strongly) coatomic refinable if and only if it is (strongly) refinable.

Proof. ($\Rightarrow$) Let M be a (strongly) coatomic refinable module. Since R is a left max ring, it follows that every submodule of M is coatomic. Hence M is (strongly) refinable.

($\Leftarrow$) It is clear.

Recall from [4, 41.13] that M is called *π-projective* if for every submodule N, K of M and identity homomorphism $I_M : M \longrightarrow M$ with $M = N + K$ there exists $y \in End(M)$ with $I_M(y) \subseteq N$ and $Im(I_M - y) \subseteq K$.

Proposition 2.6. Let M be a coatomic refinable module and π-projective. Then M is strongly coatomic refinable.

Proof. Let N be any coatomic submodule of M. Suppose that there exists a submodule K of M such that $M = N + K$. By the hypothesis, there exist submodules N, L of M such that $N' \subseteq N$, $M = N' \oplus L$ and $M = N' + K$. By Clark et al. (2006, 4.14), there exists a submodule K' of M such that $K' \subseteq K$ and $M = N \oplus K$. Thus M is strongly coatomic refinable.

Now we prove that the property coatomic refinable is inherited by direct summands.

Theorem 2.7. Let M be a module. If M is a coatomic refinable, then every direct summand of M is coatomic refinable.

Proof. Let N be any direct summand of M. Then there exists a submodule K of M such that $M = N \oplus K$. Let U be any coatomic submodule of N. Suppose that $N = U + V$ for some submodule V of N. Then $M = U + (V + K)$. Since U is coatomic submodule of M and M is a coatomic refinable module, then there exist submodules U, T of M such that $U' \subseteq U$, $M = U' \oplus T$ and $M = U' + (V + K)$.

By the modularity, we have $N = N \cap [U' + (V + K)] = U' + [V + (N \cap K)] = U' + V$. Thus U is a coatomic refinable module.

Recall from Wisbauer (1991) that A submodule N of M is called *fully invariant* if $f(N)$ is contained in N for every $f \in End(M)$. A module M is called *a duo module* provided every submodule of M is fully invariant (Ozcan et al., 2006).

Theorem 2.8. *Let $\{M_i\}_{i\in I}$ be a family of coatomic refinable modules and $M = \bigoplus_{i\in I} M_i$. If M is a duo module, then M is coatomic refinable.*

Proof. Let U be any coatomic submodule of M. Suppose that there exists a submodule V of M such that $M = U + V$. By the hypothesis, $U = \bigoplus_{i\in I} (U \cap M_i)$. Since, for every $i \in I$, $U \cap M_i$ is homomorphic image of U, $U \cap M_i$ is a coatomic submodule of U. So, for every $i \in I$, $U \cap M_i$ is a coatomic submodule of M_i. Now we can write

$$M_i = M_i \cap (U + V)$$
$$= M_i \cap [\bigoplus_{i\in I} (U \cap M_i) + V]$$
$$= (U \cap M_i) + M_i \cap [\bigoplus_{i\neq j\in I} (U \cap M_j) + V]$$

for every $i \in I$. Since M_i is coatomic refinable for every $i \in I$, we obtain that

$M_i = U_i + [\bigoplus_{i\neq j\in I} (U \cap M_j) + V$ and $M_i = U_i \oplus V_i$

Where U_i and V_i are submodules of M_i with $U_i \subseteq U \cap M_i$. So, $\bigoplus_{i\in I} U_i \subseteq U$ and $M = (\bigoplus_{i\in I} U_i) + (\bigoplus_{i\in I} V_i)$ Note that

$$M = \bigoplus_{i\in I} M_i$$
$$= \bigoplus_{i\in I} \{U_i + [[\bigoplus_{i\neq j\in I} (U \cap Mj) + V]\}$$
$$= \bigoplus_{i\in I} U_i + \bigoplus_{i\in I} [\bigoplus_{i\neq j\in I} (U \cap Mj) + V]$$
$$= \bigoplus_{i\in I} U_i + \bigoplus_{i\in I} (V \cap M_i)$$

It follows from the hypothesis that $V = \bigoplus_{i\in I} (V \cap M_i)$. Therefore $M = \bigoplus_{i\in I} U_i + V$. Thus M is coatomic refinable.

Recall from Clark et al. (2006, 11.26) that a module M is called *direct summands lift modulo a submodule N of M* if, under the canonical projection $p : M \longrightarrow M/N$, every direct summand of M/N is an image of a direct summand of M. Similarly, a module M is called

(finite) decompositions lift modulo N if, whenever *M/N* is expressed as a (finite) direct sum of submodules M_i/N, then $M = \bigoplus_{i\in I} K_i$, where $p(K_i) = K_i/N$ for each $i \in I$.

Proposition 2.9. Let M be a module. If coatomic direct summands lift modulo every coatomic submodule of M, then M is coatomic refinable.

Proof. Let *N* be any coatomic submodule of *M*. Suppose that a submodule *K* of *M* with $M = N + K$ and the canonical projection $p : M \longrightarrow M/K$. Since *N* is coatomic submodule of *M*, then N+K/K is coatomic direct summand of *M/K*. By the hypothesis, *N* is a coatomic direct summand of *M*. So *M* is coatomic refinable.

Proposition 2.10. Let M be a module. If coatomic decompositions lift modulo every coatomic submodule of M, then M is strongly coatomic refinable.

Proof. Let N be any coatomic submodule of M. Suppose that a submodule K of M with M=N+K. Say L=N∩K. Then M/L=N/L ⊕ K/L. Suppose the canonical projection g:M⟶M/L. Since g(N)=N/L and g(K)=K/L, we have M=N⊕K by the hypothesis. Therefore M is strongly coatomic refinable.

References

Clark, J., Lomp, C., Vanaja, N. Wisbauer, R. 2006. Lifting modules supplements and projectivity in module theory. Frontiers in Mathematics, Birkhauser Basel.

Mohamed, S.H., Müller, B.J. 1990. Continuous and discrete modules, London Mathematical Society Lecture Note Series 147. Cambridge University Press, New York.

Ozcan, A.C, Harmancı, A. Smith, P.F. 2006. Duo modules, Glasgow Mathematical Journal, 48: 533-545.

Wisbauer, R. 1991. Foundations of modules and rings. Gordon and Breach.

Wisbauer, R. 1996. Modules and algebras: bimodule structure and group actions on algebras, Pitman Monographs *and Surveys* in Pure and Applied Mathematics 81. Longman.

Zöschinger, H. 1974. Komplementierte moduln uber Dedekindringen, Journal of Algebra, 29: 42-56.

Kocaman, B, Toy, S, Marakli, S. International Journal of Science Letters (IJSL). 2020. 2(2): 98-113.

Review

Application of different molecular markers in biotechnology

Berna Kocaman[1], Sevim Toy[2], Sevgi Marakli[1,3,4*]

[1]Department of Biotechnology, Institution of Science, Amasya University, Amasya/Turkey
[2]Department of Biology, Institution of Science, Amasya University, Amasya/Turkey
[3]Department of Medical Services and Techniques, Sabuncuoglu Serefeddin Health Services Vocational School, Amasya University/Turkey
[4]Amasya University, Central Research Laboratory, Amasya/Turkey

Abstract

Several molecular markers have been developed to explore genetic diversity, resistance against biotic/abiotic stress, disease, biomarker and evolutionary relationships in different genomes. These markers could be classified as sequence, hybridisation, PCR and retrotransposon based techniques. In this study, procedure and applications of AFLP (Amplified Fragment Length Polymorphism), ITS (Internal Transcribed Spacer), IRAP (Inter Retrotransposon Amplified Polymorphism), SSR (Simple Sequence Repeats), VNTR (Variable Number Tandem Repeats), SNP (single nucleotide polymorphism), CAPS (Cleaved Amplified Polymorphic Sequences), SCoT (Start Codon Targeted Polymorphism), SSCP (Single Strand Conformational Polymorphism) markers in plant, animal and human genomes were discussed.

Article History

Received 15.07.2020
Accepted 24.08.2020

Keywords

AFLP,
CAPS,
IRAP,
ITS,
SCoT,
SNP,
SSCP
SSR,
VNTR

1. Introduction

Markers are properties that could be used to distinguish intra- and/or inter-populations. When compare to morphological markers, molecular markers are more useful, more robust and independent of environmental conditions. Moreover, the strength of these markers has been increased with advances in next-generation sequencing technologies (Grover and Sharma, 2016; Nadeem et al., 2018). Many studies are using these markers in different genomes. However, plant genomes are mainly investigated to analyse genetic diversity by using these markers. Here, we present a detailed procedure and application of various molecular marker techniques in animal and human in addition to plant genomes.

[1]Correspondence: sevgi.marakli@amasya.edu.tr

2. AFLP (Amplified Fragment Length Polymorphism)

AFLP is one of the PCR-based molecular marker technologies using DNA fragments after digestion with restriction enzymes and compares fingerprints to analyse differences in DNA sequences (Figure 1). The advantage of this method is as follows: no prior information about target genome, high reproducibility and sensitivity. AFLP technique has been used for genetic diversity within and between species, genetic map and evolutionary relationships. Moreover, transcriptomic variations and epigenetic studies such as DNA methylation are also analysed by using this technique (Paun and Schönswetter, 2012).

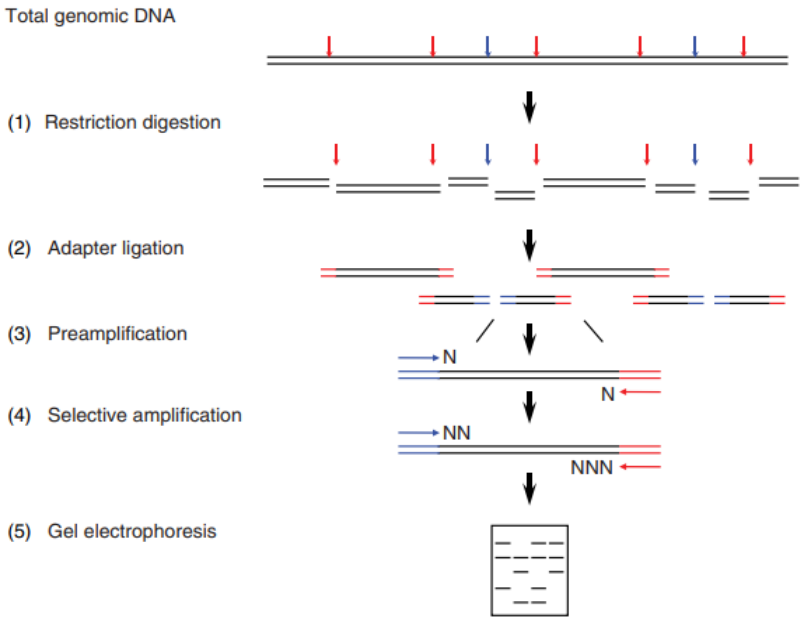

Figure 1. Procedure of AFLP molecular marker. 1. Genomic DNA is digested with combination of *Eco*RI (blue) and *Mse*I (red) restriction enzymes. 2. *Eco*RI- (blue) and *Mse*I (red)- specific adapters are ligated to the fragment ends. 3. In pre-amplification step; primers-specific to adapter sequences are used to amplification of fragments. 4. In selective PCR amplification step, selective nucleotides are added to *Eco*RI and *Mse*I primers. 5. Amplification products are analysed by using denaturing polyacrylamide gels (Vuylsteke et al., 2007)

There are numerous reports to identify polymorphisms among plants via AFLP molecular marker. One of them was carried out by Moya-Hernández et al. (2018). They performed comparative analyses related to genetic diversity of *C. ficifolia* found in some regions of Mexico, reporting a total of 195 bands with 24.6% polymorphism. Similar to this study,

Ovesna et al. (2018) used AFLP markers to investigate diversity in a different plant, *S. sonchifolius*. They found higher polymorphism ratio (97.3%) among plants and reported that this ratio much higher than previous studies.

In some studies, molecular marker combinations have been used to better understand efficiency of markers to investigate polymorphism and comparison the results. Cao et al. (2019) performed one of these studies by using Simple Sequence Repeat (SSR) and (AFLP) markers. They investigated genetic diversity of Pyrus pyrifolia var. Nakai. As a result of SSR, AFLP and SSR+AFLP analyses, they observed rich genetic diversity in Nakai varieties. Similarly, Hadipour et al. (2020) also analysed AFLP and ISSR primer combinations in 67 P.bracteatum genotypes. Among populations, 52% for ISSR and 48% for AFLP polymorphism ratios were detected.

3. ITS (Internal Transcribed Spacer)

Ribosomal RNA gene and spacer regions have provided phylogenetic knowledge in prokaryotes and eukaryotes. Ribosomal DNA consists of coding regions (18S, 5.8S and 28S) together with two ITS regions and one NTS (non-transcribed spacer) (Wei et al., 2006) (Figure 2). ITS is a degraded region during maturation in ribosomal transcript.

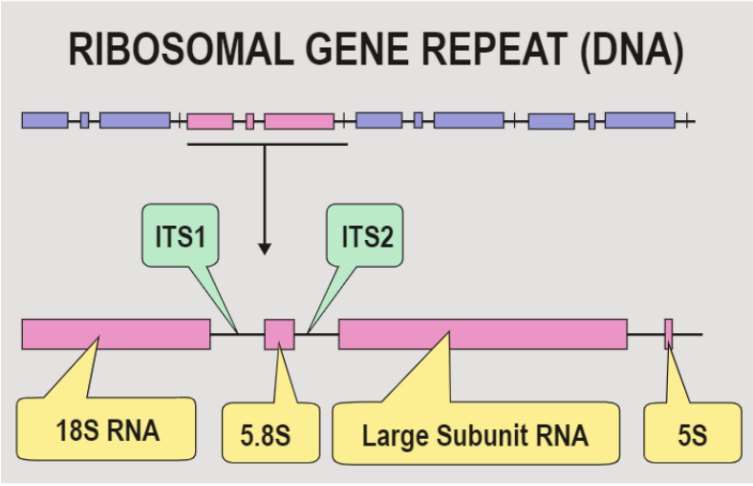

Figure 2. ITS regions together with ribosomal subunits (Choudhary et al., 2015)

ITS due to more variation properties than ribosomal sequences have been commonly used for molecular markers to figure out phylogenetic analysis and/or identification of bacteria, plant and animal strains and/or species (Lee et al., 2017; Rocha et al., 2019; Ali et al., 2019;

Fazeli-Nasab et al., 2020). Pourahmad et al. (2019) performed sequence analysis of the mycobacterial 16S-23S ITS region to determine aquatic mycobacteria species, reporting effectiveness of this marker. In another study, Yu et al. (2020) evaluated the species specificity of ITS. For this purpose, they improved a determination method based on 500 ITS sequences. They concluded that the accuracy ratio of this procedure was 99.3% and 100% for species level and genus level, respectively in Streptococcus. A similar study was performed in a fungal community. Deng et al. (2020) investigated the specificity of ITS sequences by using *in silico* and even experimental analyses. They suggested that primer selection could affect the finding of amplicon-based metabarcoding studies due to different taxonomic levels.

4. IRAP (Inter Retrotransposon Amplified Polymorphism)

IRAP is a retrotransposon-based molecular marker technique, using primers face outwards from LTR (Long Terminal Repeats) regions. Single primer or different primers depending on LTR sequences are used for amplifying genomic DNA regions between retrotransposons (Kalendar and Schulman, 2006) (Figure 3). In this technique, high polymorphism levels could detect without DNA digestion, ligations or probe hybridisation.

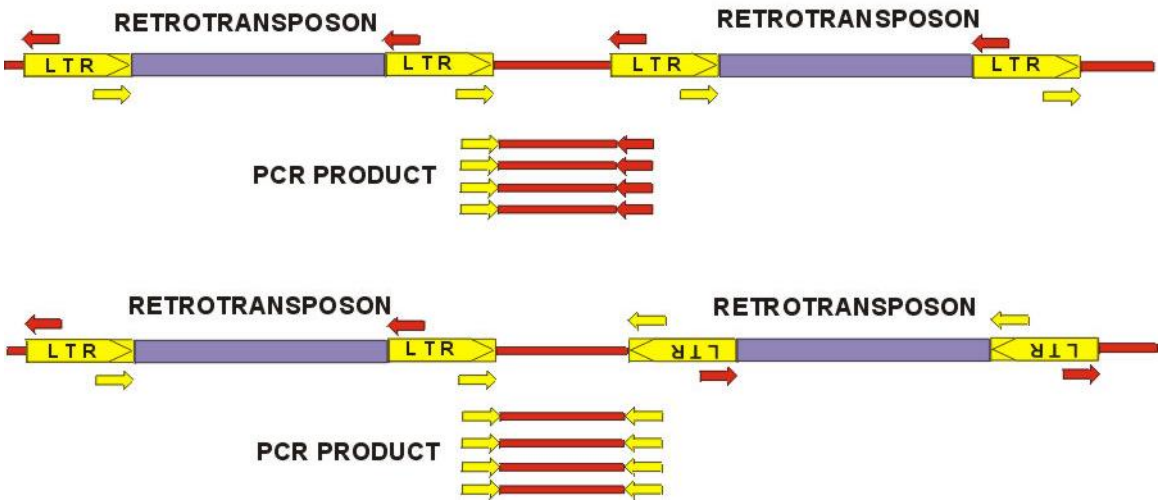

Figure 3. Procedure of IRAP molecular marker
(http://www.biocenter.helsinki.fi/bi/genomedynamics/markers.html)

There are many retrotransposons based molecular markers but IRAP method has been used for several studies to investigate genetic diversity, especially plant species (Noormohammadi et al, 2018; Lancíková and Žiarovská, 2020). Furthermore, there are also different studies

using combination of two retrotransposon markers: IRAP and REMAP (Retrotransposon-Microsatellite Amplified Polymorphisms). One of them was carried out by Holasou et al. (2019) to evaluate genetic diversity in wheat (*Triticum aestivum* L.). They reported that both methods produced highly polymorphic bands in samples. Shingote et al. (2019) also identified that IRAP system was superior to ISSR in terms of marker in terms of index, resolving power and polymorphic loci per assay.

In addition to REMAP and ISSR, SCoT (start codon-targeted) markers together with IRAP has also been used to analyse relationships among samples. Guan et al. (2020) investigate genetic diversity of 268 *Diospyros* accessions from different regions in China, determining 90 and 97 polymorphic alleles from nine SCoT and nine IRAP markers. Shehata et al. (2020) performed different study in both yeast (*Saccharomyces cerevisiae* L.) and barley (*Hordeum vulgare* L.) by using IRAP and SCoT. Obtaining findings indicated that different band patterns observed between control and salt treatments, and even the high levels of salinity could cause new retrotranspositions.

5. SSR (Simple Sequence Repeats)

Genomes consist of repetitive elements classified as interspersed repeats and tandem repeats depending on distribution SSR (microsatellites) with units of 1 to 6–10 bp are subclass of microsatellites in tandem repeats (Dumbovic et al., 2017) (Figure 4). There are two main approaches to develop SRR primers: i. Analysing known SSR primers already improved for related species and ii. production of genomic library and improving SSRs b using NGS technologies (Csencsics et al., 2010). In addition to genomic analyses, de novo transcriptome sequencing (RNA-Seq) is also reliable approach for SSR development in different species, (Taheri et al., 2018).

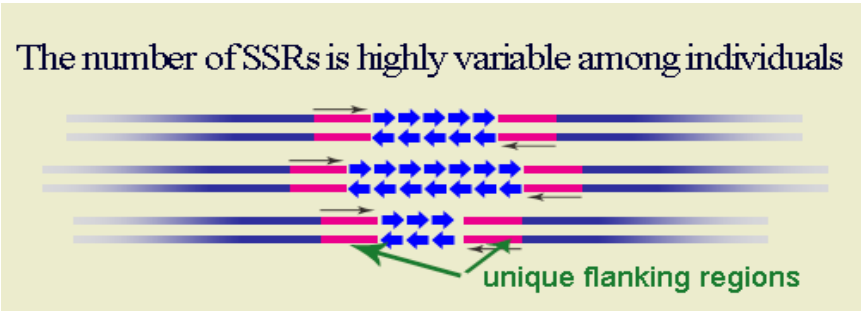

Figure 4. SSR markers (https://www.ncbi.nlm.nih.gov/probe/docs/techsts)

SSR markers have also been widely used in genetic diversity studies especially in plants such as rice (Jasim Aljumaili et al., 2018), walnut (Bernard et al., 2018), torch ginger (Ismail et al., 2019), maize (Adu et al., 2019), Norway spruce (Bínová et al., 2020) and chickpea (Asadi et al., 2020). Moreover, development of EST-SSR markers have been also studied in Indian mulberry (Thumilan et al., 2016), Lycium barbarum (Chen et al., 2017), Bletilla striata (Xu et al., 2018), Lilium (Biswas et al., 2018), Chinese Hawthorn (Ma et al., 2019) and opium poppy (Vašek et al., 2020). In addition to plants, these markers are also used for animal (Li et al., 2020; Silva Junior et al., 2020) and human (Pai et al., 2016).

6. VNTR (Variable Number Tandem Repeats)

VNTR also known as minisatellites are a member of repetitive DNA sequences dispersed in genome. They organised as tandem repeat units of a 10–60 base motif, flanked by conserved DNA restriction sites (Figure 5). These sequences show variations in length (number of repeats) among individuals (Singh et al., 2008).

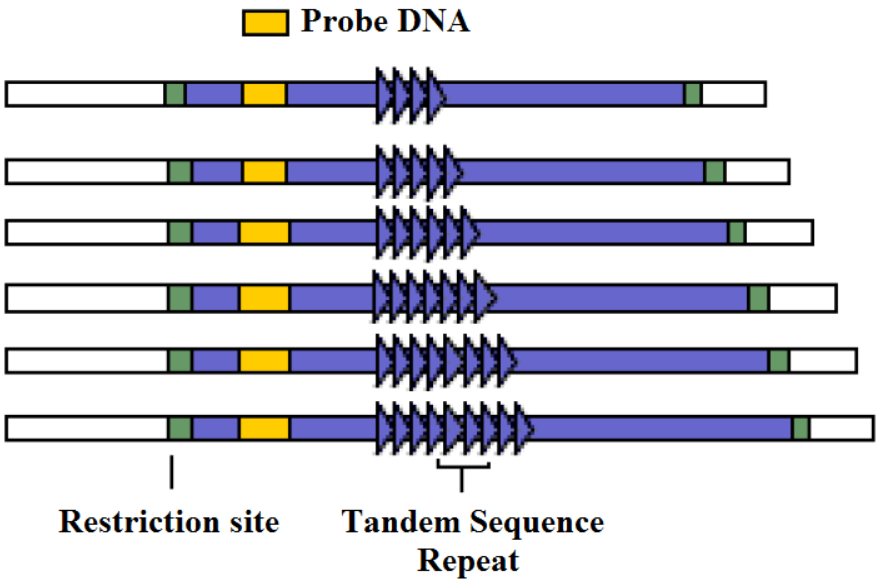

Figure 5. SSR markers (https://www.ncbi.nlm.nih.gov/probe/docs/techsts)

VNTRs are an important source of RFLP (Restriction Fragment Length Polymorphism) markers in linkage analysis (mapping) of genomes. Similar to other molecular markers, VNTR method has been also utilised for genetic diversity and understand evolutionary relationships between species (Apablaza et al., 2015; Hu et al., 2015; Ghielmetti et al., 2017). The versatility of this marker allows it to be easily used in different organisms. Mathema et al.

(2019) evaluated six microsatellite markers were developed and tested in 37 *P. malariae* isolates, concluding sufficient heterozygosity among samples. Furthermore, Sneideris et al. (2019) reported high genetic diversity as a result of VNTR tests on F. *graminearum* isolates. Najar-Peerayeh et al. (2019) studied with other basteria species, *A. baumannii*, determining high level of polymorphism similar to Sneideris et al. (2019).

7. SNP (Single Nucleotide Polymorphism)

Single nucleotide polymorphisms (SNP) are found almost all living things. If more than 1% of a population not carry a same nucleotide at a specific DNA position, then this variation can be named as a SNP. SNPs can occur once every 1000 bases or so. Not only a SNP is a nucleotide difference in DNA but also causes genetic polymorphisms among individuals and even populations (Perkel, 2008) (Figure 6).

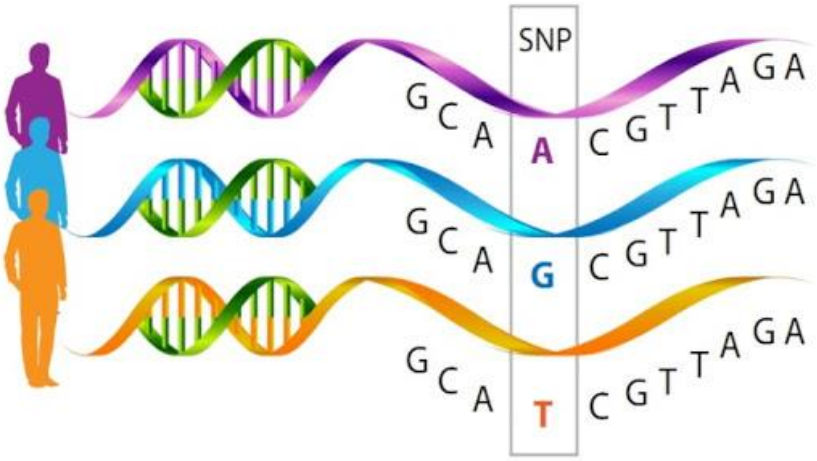

Figure 6. SNPs are DNA differences found in a specific location (https://genetics.thetech.org/ask-a-geneticist)

SNPs are used to identify gene or genes responsible for traits (Li et al., 2020; Ayala-Usma et al., 2020). In human, animal and plant genomes, there are many studies in terms of SNPs analyses. Among them, Kuhn et al. (2019) investigated biallelic SNP markers in mango germplasm. Zafar et al. (2020) studied with the relationships between oleic acid and SNPs in *Brassica napus*. Moreover, Amanullah et al. (2020) developed SNPs based cleaved amplified polymorphism sequence (CAPS) markers in melon. In a different analysis, Zhao et al. (2019) studied with meat from yak and cattle, identifying higher polymorphism in the cattle population and monomorphism in the yak population. Fatai et al. (2020) performed *in silico*

analyses to identify functional and structural effects of inhibin A gene which is a growth factor and relationships between this gene and SNPs. In addition to correlating trait and SNPs, some SNPs can also be associated with certain diseases (Liu et al., 2020; Shibeshi et al., 2020).

8. CAPS (Cleaved Amplified Polymorphic Sequences)

CAPS are DNA fragments amplified by PCR using specific primers, then digestion of these amplicons with a restriction enzyme. Length polymorphisms as a result of variation in restriction sites are determined by gel electrophoresis (Figure 7). Therefore, this technique is also referred to PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) (Singh et al., 2008).

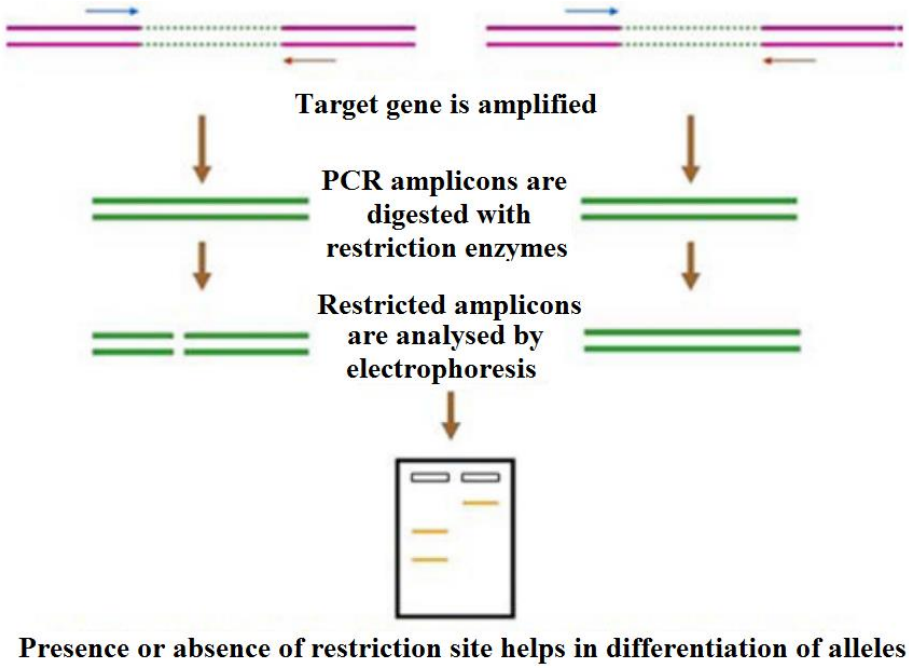

Figure 7. Procedures of CAPS technique (Agarwal et al., 2008)

Polymorphisms analyses by using CAPS markers can be used for analysing structural properties (Babu et al., 2017), biotic/abiotic stress (Hubhachen et al., 2020) in addition to genetic diversity and molecular breeding (Kawahara et al., 2020). In one study, Gunaydin and Kafkas (2017) characterised strawberry varieties by using SSR and CAPS markers. They suggested that SSR was considered to be better than CAPS in terms of polymorphism rates.

Amanullah et al. (2020) combined SNP-CAPS markers to investigate in *Cucumis melo* L, identifying 7 QTLs for melon ovary traits.

9. SCoT (Start Codon Targeted Polymorphism)

SCoT is depended on short conserved region in genes surrounding the ATG translation start (or initiation) codon. Therefore, primers are designed according to the short conserved region flanking the ATG start codon (Figure 8). Amplicons are evaluated by standard gel electrophoresis with agarose gels (Collard and Mackill, 2009).

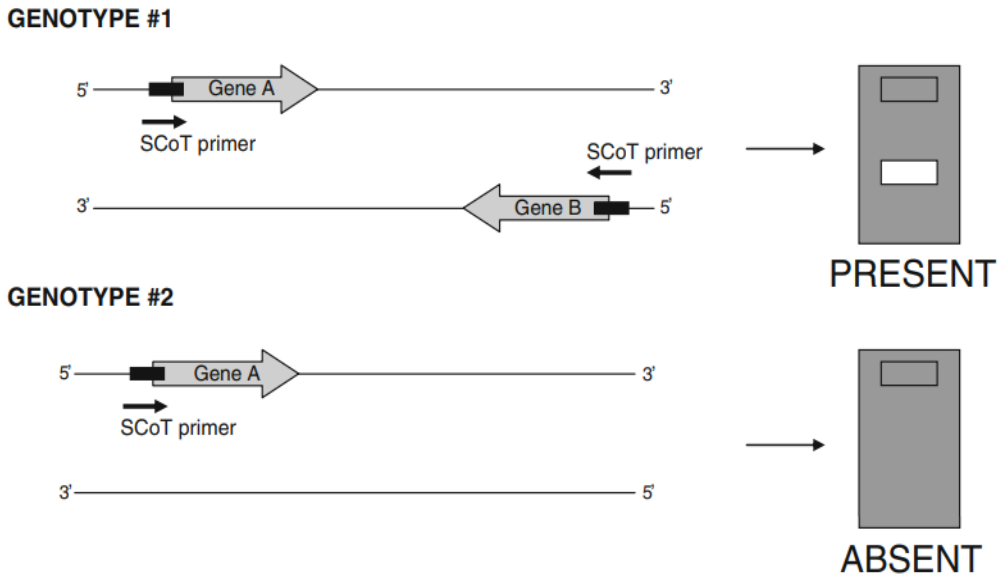

Figure 8. Principle of SCoT analysis (Collard and Mackill, 2009)

Similar to other molecular markers, SCoT could be successfully practised for genetic diversity (Vivodík et al., 2019; Vanijajiva et al., 2020; Xiao et al., 2020). In some studies, SCoT markers were combined with other molecular markers. Etminan et al. (2016) investigated SCoT and ISSR markers together to analyse durum wheat genotypes. In another study, Shekhawat et al. (2018) used SCoT and CBDP (CAAT-box derived polymorphism) markers for *Prosopis cineraria*. Similarly, Gholamian et al. (2019) also studied with *T. urartu* by using SCoT and CBDP markers. El-Fiki and Adly combined RAPD and SCoT markers to characterise potato cultivars.

10. SSCP (Single Strand Conformational Polymorphism)

SSCP is based on the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel. This conformational difference depends on the length of strand, sequence, location and number of base pairs (Gasser et al., 2006) (Figure 9).

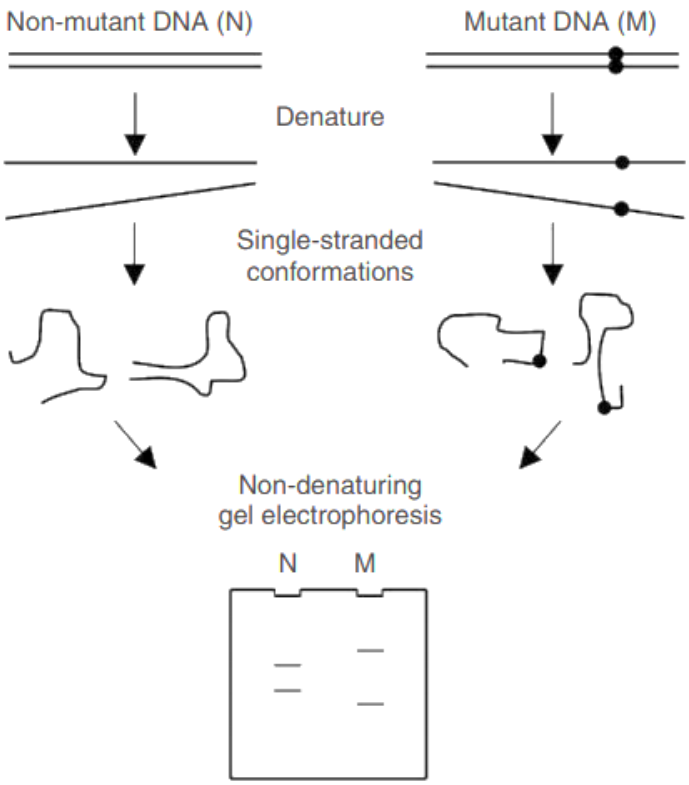

Figure 9. The principle of SSCP analysis. Dot indicates point mutation cause single-strand conformations (Gasser et al., 2006)

Youssef and Shalaby (2016) studied with *Citrus Tristeza Virus* (CTV), an important virus for citrus, to characterise populations of CTV and id determine haplotypes in populations by using SSCP marker. Zheng et al. (2016) also screened CRISRP-Cas9-mediated targeted mutagenesis in rice via SSCP application. They determined small indels and multiple mutants. In another study, Tchouomene-Labou et al. (2020) used SSCP technique to explore genetic differentiation, gene flow, demographic history and phylogenetic relationship in mitochondrial genes of *Glossina palpalis palpalis* populations.

11. Conclusion

Each marker system has advantages and disadvantages. Therefore, many studies have been performed by using combination of different markers to eliminate disadvantages. Moreover, the continuous development of molecular markers with advancements in sequencing technologies has provided a powerful tool in biotechnological research. It is important to know procedures and applications of molecular markers to improve existing markers and production of new markers.

References

Ali, F.S., Ismail, M., Mamoon, A. 2019. Comparative molecular identification of genus Dicentrarchus using mitochondrial genes and internal transcribed spacer region, Egyptian Journal of Aquatic Biology and Fisheries, 23(3): 371-384.

Amanullah, S., Saroj, A., Osae, B. A., Liu, S., Liu, H. et al. 2020. Detection of putative QTL regions associated with ovary traits in melon using SNP-CAPS markers, Scientia Horticulturae, 270: 109445.

Adu, G.B., Awuku, F.J., Amegbor, I.K., Haruna, A., Manigben, K.A. et al. 2019. Genetic characterization and population structure of maize populations using SSR markers, Annals of Agricultural Sciences, 64(1): 47-54.

Agarwal, M., Shirvastava, N., Padh, H. 2008. Advances in molecular marker techniques and their applications in plant sciences, Plant Cell Reports, 27: 617-631.

Apablaza, P., Brevik, Ø. J., Mjøs, S., Valdebenito, S., Ilardi, P. et al. 2015. Variable Number of Tandem Repeats (VNTR) analysis of *Flavobacterium psychrophilum* from salmonids in Chile and Norway, BMC Veterinary Research, 11(1): 150.

Asadi, A., Ebrahimi, A., Rashidi-Monfared, S., Basiri, M., Akbari-Afjani, J. 2020. Comprehensive functional analysis and mapping of SSR markers in the chickpea genome (*Cicer arietinum* L.), Computational Biology and Chemistry, 84: 107169.

Ayala-Usma, D.A., Danies, G., Myers, K., Bond, M.O., Romero-Navarro, J.A. et al. 2020. Genome-wide association study identifies single nucleotide polymorphism markers associated with mycelial growth (at 15, 20, and 25^{o}C), mefenoxam resistance, and mating type in *Phytophthora infestans*, Phytopathology, 110(4): 822-833.

Babu, B.K., Mathur, R.K., Kumar, P.N., Ramajayam, D., Ravichandran, G. et al. 2017. Development, identification and validation of CAPS marker for SHELL trait which governs dura, pisifera and tenera fruit forms in oil palm (*Elaeis guineensis* Jacq.), PLoS ONE, 12(2): e0171933.

Bernard, A., Barreneche, T., Lheureux, F., Dirlewanger, E. 2018. Analysis of genetic diversity and structure in a worldwide walnut (*Juglans regia* L.) germplasm using SSR markers, PLoS ONE, 13(11): e0208021.

Bínová, Z., Korecký, J., Dvořák, J., Bílý, J., Zádrapová, D. et al. 2020. Genetic structure of norway spruce ecotypes studied by SSR markers, Forests, 11(1): 110.

Biswas, M.K., Nath, U.K., Howlader, J., Bagchi, M., Natarajan, S. et al. 2018. Exploration and exploitation of novel SSR markers for candidate transcription factor genes in *Lilium* species, Genes, 9: 97.

Cao, J., Zhou, Z., Tu, J., Cheng, S., Yao, J. et al. 2019. Genetic diversity and population structure analysis of sand pear (*Pyrus pyrifolia*) 'Nakai 'varieties using SSR and AFLP markers, Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 47(3): 970-979.

Chen, C.L., Xu, M.L., Wang, C.P., Qiao, G.X., Wang, W.W. et al. 2017. Characterization of the *Lycium barbarum* fruit transcriptome and development of EST-SSR markers, PLoS ONE, 12: e0187738.

Choudhary, K., Verma, A.K., Swaroop, S., Agrawal, N. 2015. A review on the molecular characterization of digenean parasites using molecular markers with special reference to ITS region, Helminthologia, 52(3): 167-187.

Collard, B.C., Mackill, D.J. 2009. Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants, Plant Molecular Biology Reporter, 27(1): 86-93.

Csencsics, D., Brodbeck, S., Holderegger, R. 2010. Cost-Effective, species-specific microsatellite development for the endangered dwarf bulrush (*Typha minima*) using next-generation sequencing technology, Journal of Heredity, 101: 789-793.

Dumbovic, G., Forcales, S.V., Perucho, M. 2017. Emerging roles of macrosatellite repeats in genome organization and disease development, Epigenetics, 12(7): 515-526.

El-Fiki, A., Adly, M. 2019. Genetic variation and molecular characterization in some potato cultivars using random amplified polymorphic DNA and start codon targeted markers, Egyptian Journal of Biotechnology, 59.

Etminan, A., Pour-Aboughadareh, A., Mohammadi, R., Ahmadi-Rad, A., Noori, A. et al. 2016. Applicability of start codon targeted (SCoT) and inter-simple sequence repeat (ISSR) markers for genetic diversity analysis in durum wheat genotypes, Biotechnology & Biotechnological Equipment, 30(6): 1075-1081.

Fatai, R.B., Akinyemi, M.O., Osaiyuwu, O.H. 2020. In silico analysis of single nucleotide polymorphism in inha gene of sheep and goats, Biotechnology Journal International, 12-21.

Fazeli-Nasab, B., Sayyed, R.Z., Farsi, M., Ansari, S., El-Enshasy, H.A. 2020. Genetic assessment of the internal transcribed spacer region (ITS1. 2) in *Mangifera indica* L. landraces, Physiology and Molecular Biology of Plants, 26(1): 107-117.

Gasser, R.B., Hu, M., Chilton, N.B., Campbell, B.E., Jex, A.J. et al. 2006. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation, Nature Protocols, 1(6): 3121.

Günaydın, S., Kafkas, S. Characterization of strawberry cultivars by SSR and CAPS Markers. VIII International Strawberry Symposium, 13-17 August 2016, p. 171-178.

Ghielmetti, G., Scherrer, S., Friedel, U., Frei, D., Suter, D. et al. 2017. Epidemiological tracing of bovine tuberculosis in Switzerland, multilocus variable number of tandem repeat analysis of *Mycobacterium bovis* and *Mycobacterium caprae*, PLoS ONE, 12(2): e0172474.

Gholamian, F., Etminan, A., Changizi, M., Khaghani, S., Gomarian, M. 2019. Assessment of genetic diversity in *Triticum urartu* Thumanjan ex Gandilyan accessions using start codon targeted polymorphism (SCoT) and CAAT-box derived polymorphism (CBDP) markers, Biotechnology & Biotechnological Equipment, 33(1): 1653-1662.

Grover, A., Sharma, P.C. 2016. Development and use of molecular markers: past and present, Critical Reviews in Biotechnology, 36(2): 290-302.

Guan, C., Chachar, S., Zhang, P., Hu, C., Wang, R. et al. 2020. Inter-and intra-specific genetic diversity in *Diospyros* using SCoT and IRAP markers, Horticultural Plant Journal, 6(2): 71-80.

Hadipour, M., Kazemitabar, S.K., Yaghini, H., Dayani, S. 2020. Genetic diversity and species differentiation of medicinal plant Persian Poppy (*Papaver bracteatum* L.) using AFLP and ISSR markers, Ecological Genetics and Genomics, 100058.

Holasou, H.A., Rahmati, F., Rahmani, F., Imani, M., Talebzadeh, Z. 2019. Elucidate genetic diversity and population structure of bread wheat (*Triticum aestivum* L.) cultivars using IRAP and REMAP markers, Journal of Crop Science and Biotechnology, 22(2): 139-151.

Hu, Y., Li, B.Q., da Jin, Z., He, L.H., Tao, X.X. et al. 2015. Identification of Variable-Number Tandem-Repeat (VNTR) sequences in *Acinetobacter pittii* and development of an optimized multiple-locus VNTR analysis typing scheme, Biomedical and Environmental Sciences, 28(12): 855-63.

Hubhachen, Z., Jiang, H., Schlipalius, D., Park, Y., Guedes, R.N. et al. 2020. A CAPS marker for determination of strong phosphine resistance in *Tribolium castaneum* from Brazil, Journal of Pest Science, 93(1): 127-134.

Imwong, M., Mathema, V. B., Nakeesathit, S., Pagornrat, W., Smithuis, F. et al. 2019. Polymorphic markers for identification of parasite population in *Plasmodium malariae*, Malaria Journal, 19: 48.

Ismail, N.A., Rafii, M.Y., Mahmud, T.M.M., Hanafi, M.M., Miah, G. 2019. Genetic diversity of torch ginger (*Etlingera elatior*) germplasm revealed by ISSR and SSR markers, BioMed Research International, doi: 10.1155/2019/5904804.

Jasim Aljumaili, S., Rafii, M. Y., Latif, M. A., Sakimin, S. Z., Arolu, I. W. et al. 2018. Genetic diversity of aromatic rice germplasm revealed by SSR markers, BioMed Research International, doi: 10.1155/2018/7658032.

Kalendar, R., Schulman, A. 2006. IRAP and REMAP for retrotransposon-based genotyping fingerprinting, Nature Protocols, 1(5): 2478-2484.

Kawahara, Y., Endo, T., Omura, M., Teramoto, Y., Itoh, T. et al. 2020. Mikan Genome Database (MiGD): integrated database of genome annotation, genomic diversity, and CAPS marker information for mandarin molecular breeding, Breeding Science, doi: 10.1270/jsbbs.19097.

Kuhn, D.N., Dillon, N., Bally, I., Groh, A., Rahaman, J. et al. 2019. Estimation of genetic diversity and relatedness in a mango germplasm collection using SNP markers and a simplified visual analysis method, Scientia Horticulturae, 252: 156-168.

Lancíková, V., Žiarovská, J. 2020. Inter-retrotransposon amplified polymorphism markers revealed long terminal repeat retrotransposon insertion polymorphism in flax cultivated on the experimental fields around Chernobyl, Journal of Environmental Science and Health, Part A Toxic/Hazardous Substances and Environmental Engineering, 55(8): 957-963.

Lee, S.Y., Mohamed, R., Faridah-Hanum, I., Lamasudin, D.U. 2017. Utilization of the internal transcribed spacer (ITS) DNA sequence to trace the geographical sources of *Aquilaria malaccensis* Lam. Populations, Plant Genetic Resources, 16(2): 103-111.

Li, J., Hou, L., Sun, Y., Xing, J., Jiang, Y. et al. 2020. Single nucleotide polymorphism rs737028527 (G> A) affect miR-1b-3p and effects on chicken egg-laying traits, Animal Reproduction Science, 106476.

Li, S., Deng, Y., Wang, Z., Zhang, Z., Kong, X. et al. 2020. Exploring the accuracy of amplicon-based internal transcribed spacer markers for a fungal community, Molecular Ecology Resources, 20(1): 170-184.

Li, X., Tang, Y., Zhang, R., Tian, F., Zhao, K. 2020. Characterization and development of SSR markers of schizothoracine fish (Cypriniformes: Cyprinidae) based on SLAF-seq Technique, Journal of Applied Ichthyology, doi: 10.1111/jai.14032.

Liu, Z., Yin, X., Mai, H., Li, G., Lin, Z. et al. 2020. SCD rs41290540 single-nucleotide polymorphism modifies miR-498 binding and is associated with a decreased risk of coronary artery disease, Molecular Genetics & Genomic Medicine, 8(3): e1136.

Ma, S.L.Y., Dong, W.S., Lyu, T., Lyu, Y.M. 2019. An RNA sequencing transcriptome analysis and development of EST-SSR markers in Chinese hawthorn through Illumina sequencing, Forests, 10: 82.

Moya-Hernández, A., Bosquez-Molina, E., Serrato-Díaz, A., Blancas-Flores, G., Alarcón-Aguilar, F.J. 2018. Analysis of genetic diversity of *Cucurbita ficifolia* Bouché from different regions of Mexico, using AFLP markers and study of its hypoglycemic effect in mice, South African Journal of Botany, 116: 110-115.

Nadeem, M. A., Nawaz, M. A., Shahid, M. Q., Doğan, Y., Comertpay, G. et al. 2018. DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing, Biotechnology & Biotechnological Equipment, 32(2): 261-285.

Najar-Peerayeh, S., Karmostaji, A. 2019. Evaluation of multilocus Variable-Number Tandem-Repeat (MLVA-8 Orsay) for Typing of carbapenem-resistant *Acinetobacter baumannii* isolated from patients in Tehran, Iran, Archives of Clinical Infectious Diseases, 14(1): e64402s

Noormohammadi, Z., Ibrahim-Khalili, N., Sheidai, M., Alishah, O. 2018. Genetic fingerprinting of diploid and tetraploid cotton cultivars by retrotransposon-based markers, The Nucleus, 61(2): 137-143.

Ovesná, J., Russo, D., Frescura, D., Cusimamani, E.F., Svobodova, E. et al. 2018. Assessment of genetic diversity of *Smallanthus sonchifolius* (Poepp. & Endl.) h. Robinson landraces by using AFLP markers, Genetika, 50(3): 803-816.

Pai, T.W., Chen, C. M. 2016. SSRs as genetic markers in the human genome and their observable relationship to hereditary diseases, Biomarkers in Medicine, 10(6): 563-566.

Paun, O., Schönswetter, P. 2012. Amplified fragment length polymorphism: an invaluable fingerprinting technique for genomic, transcriptomic, and epigenetic studies. In: Sucher, N.J., Hennell, J.R., Carles, M.C. (Eds.). Plant DNA Fingerprinting and Barcoding. pp. 75-87, Humana Press.

Perkel, J. 2008. SNP genotyping: six technologies that keyed a revolution, Nature Methods, 5(5): 447-453.

Pourahmad, F., Adams, A., Thompson, K.D., Richards, R.H. 2019. Identification of aquatic mycobacteria based on sequence analysis of the 16S-23S rRNA internal transcribed spacer region, Journal of Medical Microbiology, 68(2): 221-229.

Rocha, G.M., Martinelli, A.B., Utz, L.R.P., Fernandes, L.F.L. 2019. Using internal transcribed spacers for Calanoida (Crustacea, Copepoda) species identification, Marine Biodiversity, 49(3): 1565-1571.

Shehata, M.M., Fahmy, E.M., Mohamed Badawy, F., Mostafa Kamal Sayed, L. 2020. Measuring of the alteration of retrotransposition in the response of salinity stress using IRAP and SCoT markers, Arab Universities Journal of Agricultural Sciences, doi: s10.21608/ajs.2019.18888.1107

Shekhawat, J.K., Rai, M.K., Shekhawat, N.S., Kataria, V. 2018. Exploring genetic variability in *Prosopis cineraria* using two gene targeted CAAT box-derived polymorphism (CBDP) and start codon targeted (SCoT) polymorphism markers, Molecular Biology Reports, 45(6): 2359-2367.

Shibeshi, S., Adetimirin, V.O., Kumar, L., Abebe, M., Gedil, M. 2020. Verification of single nucleotide polymorphism (SNP) markers associated with maize (*Zea mays*. L) streak virus resistance in early generation maize lines, International Journal of Genetics and Molecular Biology, 12(1): 36-45.

Shingote, P.R., Mithra, S.A., Sharma, P., Devanna, N.B., Arora, K. et al. 2019. LTR retrotransposons and highly informative ISSRs in combination are potential markers for genetic fidelity testing of tissue culture-raised plants in sugarcane, Molecular Breeding, 39(2): 25.

Silva Junior, M.S.F.S.D., Borges, A.A.D.S., Santos, S.R.N., Moura, V.G.D., Silvana, G.R.D. et al. 2020. SSR and ISSR markers in assessing genetic diversity in *Gallus gallus domesticus*: a quantitative analysis of scientific production, Ciência Rural, 50(7): e20190401.

Singh, R.K., Mishra, G.P., Thakur, A.K., Singh, S.B. 2008. Molecular markers in plants. In: Singh, R.K., Singh, R., Ye, G.Y., Selvi, A., Rao, G.P. (Eds.). Molecular Plant Breeding: Principle, Method And Application, Studium Press LLC, Houston, pp. 35-78.

Sneideris, D., Ivanauskas, A., Suproniene, S., Kadziene, G., Sakalauskas, S. 2019. Genetic diversity of *Fusarium graminearum* isolated from weeds, European Journal of Plant Pathology, 153(2): 639-643.

Taheri, S., Lee Abdullah, T., Yusop, M., Hanafi, M., Sahebi, M. et al. 2018. Mining and development of novel SSR markers using Next Generation Sequencing (NGS) Data in Plants, Molecules, 23(2): 399.

Tchouomene-Labou, J., Melachio-Tanekou, T.T., Simo, G., Kaba, D., Ravel, S. et al. 2020. Single-strand conformation polymorphism (SSCP) of mitochondrial genes helps to estimate genetic differentiation, demographic parameters and phylogeny of *Glossina palpalis palpalis* populations from West and Central Africa, Infection, Genetics and Evolution, 104303.

Thumilan, B.M., Sajeevan, R.S., Madhuri, B.J.T., Nataraja, K.N., Sreeman, S.M. 2016. Development and characterization of genic SSR markers from Indian mulberry transcriptome and their transferability to related species of Moraceae, PLoS ONE, 11: e0162909.

Vanijajiva, O. 2020. Start codon targeted (SCoT) polymorphism reveals genetic diversity of Manilkara in Thailand, Biodiversitas Journal of Biological Diversity, 21: 666-673.

Vašek, J., Čílová, D., Melounová, M., Svoboda, P., Vejl, P. et al. 2020. New EST-SSR markers for individual genotyping of opium poppy cultivars (*Papaver somniferum* L.), Plants, 9(1): 10.

Vivodík, M., Balážová, Ž., Gálová, Z., Petrovičová, L. 2019. Start codon targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated castor (*Ricinus communis* L.) genotypes, Genetika, 51(1): 137-146.

Vuylsteke, M., Peleman, J.D., Van Eijk, M.J. 2007. AFLP technology for DNA fingerprinting, Nature Protocols, 2(6): 1387.

Wei, N.W.V., Wallace, C.C., Dai, C.F., Pillay, K.M., Chen, C.A. 2006. Analyses of the ribosomal Internal Transcribed Spacers (ITS) and 5.8 S gene indicate that extremely high rDNA heterogeneity is a unique feature in the scleractinian coral genus *Acropora* (Scleractinia; Acroporidae), Zoological Studies, 45(3): 404.

Xiao, P., Liu, H., Wang, D., Tang, W., Yang, H. et al. 2020. Assessment of genetic diversity in *Camellia oleifera* Abel. accessions using inter-simple sequence repeat (ISSR) and start codon targeted (SCoT) polymorphic markers, Genetic Resources and Crop Evolution, 67: 1115-1124.

Xu, D., Chen, H., Aci, M., Pan, Y., Shangguan, Y. et al. 2018. *De novo* assembly, characterization and development of EST-SSRs from *Bletilla striata* transcriptomes profiled throughout the whole growing period, PLoS ONE, 13: e0205954.

Youssef, S.A., Shalaby, A.A. 2016. Single-Strand Conformation Polymorphism (SSCP) and nucleotide sequence analysis of *Citrus tristeza virus* in Egypt, International Journal of Advanced Research in Biological Science, 3(5): 84-92.

Yu, J., Zhou, T., Zhu, B., Wei, Y., Li, X. et al. 2020. Species-specific identification of *Streptococcus* based on DNA marker in 16S-23S rDNA internal transcribed spacer, Current Microbiology, 77, 1569-1579.

Zafar, S., Tang, M.Q., Wang, Y.K., Sarwar, R., Liu, S.Y. et al. 2020. Candidate genes-association study to identify loci related to oleic acid in *Brassica napus* using SNP markers and their heterologous expression in yeast, Plant Physiology and Biochemistry, 146: 294-302.

Zhao, J., Xu, Z., Chen, A., You, X., Zhao, Y. et al. 2019. Identification of meat from yak and cattle using SNP markers with integrated allele-specific polymerase chain reaction–capillary electrophoresis method, Meat Science, 148: 120-126.

Zheng, X., Yang, S., Zhang, D., Zhong, Z., Tang, X. et al. 2016. Effective screen of CRISPR/Cas9-induced mutants in rice by single-strand conformation polymorphism, Plant Cell Reports, 35(7): 1545-1554.