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Research Article

EFFECTS OF CUTTING TIMES AND DOSES OF SOME AUXINS ON ROOTING OF THE HAIRY BROOM CUTTINGS [*Chamaecytisus hirsutus* (L.) LINK]

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ABSTRACT. In addition to developing ornamental plants, the world has focused on determining and promoting new genera and species that are not being produced. *Chamaecytisus hirsutus* is a deciduous shrub like this with high ornamental potential. The aim of this research was to determine the effects of different cutting excision times and different dose applications of some auxins on the rooting of *Chamaecytisus hirsutus* which is found naturally in the flora of the Mediterranean climate zone. Cuttings were taken in October, November, February, March, and April and were treated with 0.1%, 0.2%, 0.3%, 0.4% IBA or NAA solutions for 5 seconds. The results showed that the application of 0.1% (8.00), 0.2% (9.06), 0.3% (7.56), and 0.4% (8.39) NAA and 0.3% (6.89) IBA doses in April gave the best rooting rate. For the propagation of *Chamaecytisus hirsutus*, we recommend treating the cuttings with 0.1% NAA concentration to produce large amounts of planting materials economically for using less auxin levels.

Keywords: *Chamaecytisus hirsutus, cutting, IBA, NAA, propagation, timing, rooting*

INTRODUCTION

The diversity of native plant species plays a considerable role in rural and urban landscape planning [1]. Natural plants are very important and economic alternatives for landscaping, protection, and stabilization studies due to these features. When natural plants are used in landscaping and vegetation studies, they both render the studies efficient in terms of aesthetics and function and ensure a healthy eco-system by means of adapting to the natural surroundings and the surrounding flora, thereby increasing the success of the studies [2]. Increases in heat caused by global warming, which is one of the important effects in many geographies, increase the demand for drought-tolerant plants in the Mediterranean flora. Therefore, the usage of Mediterranean plants, their production, and cultivation need to be prioritized [3]. Cultivating natural genera should initially start by determining propagation methods [4]. According to Erwin [5], the initial work to be done regarding plants to be commercialized as ornamental plants are to collect data regarding the vegetative development of the plant and to work on their production.

Generative propagation techniques should be preferred in order to ensure tolerance of natural conditions and a natural adaptation to the conditions in which the material is found. However, the methods preferred for the reproduction of species produced commercially are vegetative propagation methods. Therefore, it is imperative to understand vegetative propagation as well as the generative propagation of every material to be presented to the ornamental plants producers, to encourage them to adopt these species [4]. Rooting of cuttings is the best efficient model of vegetative propagation and necessities for maintain and cultivation of wild threatened plant genetic resources [6].

Chamaecytisus hirsutus is a perennial, semi woody plant with yellow flowers (Fig. 1). It is 20-100 cm high and slightly wild; slowly and vertically grown shrub. *C. hirsutus* blooms in April to June. Naturally grows in the Aegean, Mediterranean, Marmara, and Western Black Sea Regions, ranging between 10 and 2000 m in altitude and is one out of ten *Chamaecytisus* species in the flora of Turkey [7, 8]. It is native to; Albania, Austria, Belgium, Bulgaria, Czech Republic, France, Greece, Hungary, Italy, Poland, Romania, Switzerland, Turkey and former Yugoslavia [10,11].

Chamaecytisus species which have high ornamental plant potential are also used as provender in some mediterranean countries . *C. hirsutus* can also be generatively produced, but germination rate ranges between 12.50-59.00 % [9]. These ratios are not sufficient for commercial production. For a higher rate and faster production of this species has to be produced vegetatively. It is clear that there is limited information about the propagation of *C. hirsutus* by cutting, effect of different cutting excision times, different auxins with different doses.

In this study, it is aimed to determine the effect of different cutting excision times and different dose applications of auxins on the rooting of cuttings the natural plant species *Chamaecytisus hirsutus* in line with the principles to ensure sustainable usage of natural genera in flora of the southern Europe and to convert natural resources into economic values.

MATERIALS AND METHODS

Cuttings taken from natural populations of *Chamaecytisus hirsutus* in Uludağ–Bursa as a material of this research. The map with the studied populations was given in Fig.2. The research was conducted in Atatürk Horticultural Central Research Institute' research field

Fig. 1. *Chamaecytisus hirsutus* (L.) Link

Fig. 2. *The natural population maps of* ***C. hirsutus*** *cuttings collected*

Rooting trials of cuttings excised in September, October, November, February, March, and April in the years of 2008-2009 and 2009-2010. The trials were conducted two factors (time and auxin applications**)** and 3 replicates, each replicate had 50 cuttings. Four doses (0.1%, 0.2%, 0.3% and 0.4%) of indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) were compared with control applications for rooting. The cuttings were collected from wild plants, grown in natural conditions at different ages in Bursa province. Cuttings were prepared as semi woody cuttings that were 18-20 cm long. 1-2 leaves were left on top of each cutting (Fig. 3a) and left in perlite as the rooting medium (Fig. 3c). The

temperature was adjusted to 20±1 °C during the trials. Air humidification was ensured automatic fogging system adjusted with 10 seconds of mist which was sprayed every 30 minutes.

After the cuttings were prepared, they were soaked in 0.5% Captan solution for 30 minutes, left to dry for 5 minutes, and then treated with auxin applications for 5 seconds (Fig. 3b). Auxin solution was prepared 50% alcohol and 50% distilled water. NAA and IBA applications were applied as dipping to liquid solution onto cuttings that were planted on the 10th to 15th day of each month. The rooting of cuttings was evaluated 8 weeks after they were planted, and the cuttings that had developed at least one root were considered to be rooted (Fig. 3d). The rooting trays were alternately treated with 0.25% Captan and 0.1% Benomyl every 15 days [12].

Fig. 3. *The images of* ***C. hirsutus*** *reproductions works with cuttings*
a-cuttings, b-auxin treatments, c-rooting medium and fogging system, d-rooted cuttings

The means of two years observations or measurements were used for statistical analysis. The percentage values were analyzed after transformed arcsine square root transformation. The data were analyzed using analyzis of variance (ANOVA) according to the randomized complete block design. Least Significant Differences test (LSD) was used for multiple comparison analyzes, at 95% reliability limit (α=0.05). All statistical analyzes were conducted in the JMP 7.0 (SAS Institute Inc.) package statistic programme [13,14].

RESULTS AND DISCUSSION

The variance analysis shows that the time of cutting excision and auxin applications affected the rooting of the cuttings of the *C. hirsutus* plant. The interactions between the cutting time and applications were found to be insignificant.

In this study, the best rooting rate of *C. hirsutus* cuttings rooting was obtained in April. The rooting rate in April was 15.4% on average. September, October, November, February and March months had the same effect for *C. hirsutus* cuttings rooting and they were statistically in the same group. Considering the effect of time on rooting, while the rooting percentages were low in time applications conducted in September, October, November, and February, they started to increase in March. The rooting rate reached a peak point in April (Table 1, Fig. 4). These results showed that *C. hirsutus* cuttings had higher rooting potential in spring than autumn and winter. Giatromanolaki et al. [15] and Zencirkıran and Erken [16] reported contrast findings that, autumn (November) is the best time for the rooting of *Staehelına petiolata and Platanus orientalis* cuttings and than in April which none of the cuttings rooted. The same results of our findings were reported by Alsup et al. [17] and Nhung et al. [18]. Alsup et al**.** [17] found that rooting percentage was greatest in May (1999) and April (2000), of *Acer saccharum* cuttings. Nhung et al. [18] found that rooting percentage was higher in spring and autumn than summer and winter, best time for root length is the spring season for *Dalbergia tonkinensis* cuttings. Our findings can be explained that imparted knowledge by Mascarello et al. [19], In spring, during anthesis, the plant showed the highest rooting performance when the floral buds were excised and the presence of flowers strongly inhibited the rooting process. According to Enders and Strader [20], all of these results should be considered normally as they reported that endogenous auxin content of different species may vary at different times.

Table 1. *Effect of different time on the rooting of C. hirsutus (%)*

Time	Mean Rooting	
	M ± se*	
September	4.10 ± 0.23	b**
October	3.37 ± 0.23	b
November	4.57 ± 0.24	b
February	4.33 ± 0.23	b
March	8.10 ± 0.23	b
April	15.40 ± 0.23	a

*The means (M) ± standard error of 3 replicates (SE)

**within a column followed by the same letter are not significantly different by LSD multiple range test at $p \leq 0.05$ (CV = 0.17)

According to our results 0.2%, 0.4%, 0.1%, 0.3% NAA and 0.3% IBA applications effected the rate of rooted cuttings and values are 9.06%, 8.39%, 8.00%, 7.56%, 6.89% respectively. All of NAA and 0.3% IBA doses were in the same group and had the rooting rate better than control. These applications were statistically different from others and had the best results (Table 2, Fig. 5) whereas IBA applications and doses were in the second

and third groups exception of 0.3% IBA application. These findings shows that; all doses of NAA aplications are more efficient than IBA for rooting of *C. hirsutus* cuttings, during the 8 weeks of rooting time. IBA concentrations were not statistically important than control group (Table 2).

Table 2. *Effect of different auxins applications on the rooting of C. hirsutus (%)*

Concentration of Auxin and Doses	Mean Rooting M ± se*
0.1% IBA	3.94 ± 0.13 d**
0.2% IBA	5.83 ± 0.13 bcd
0.3% IBA	6.89 ± 0.13 abc
0.4% IBA	6.67 ± 0.14 bcd
0.1% NAA	8.00 ± 0.14 ab
0.2% NAA	9.06 ± 0.13 a
0.3% NAA	7.56 ± 0.13 ab
0.4% NAA	8.39 ± 0.13 ab
Control	5.06 ± 0.14 cd

*The means (M) ± standard error of 3 replicates (SE)
**within a column followed by the same letter are not significantly different by LSD multiple range test at $p \leq 0.01$ (CV = 0.17)

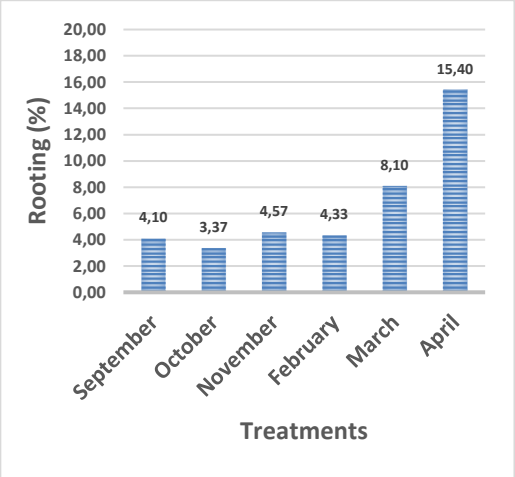

Fig. 4. *Effects of different cutting time on rooting of the C. hirsutus (%)*

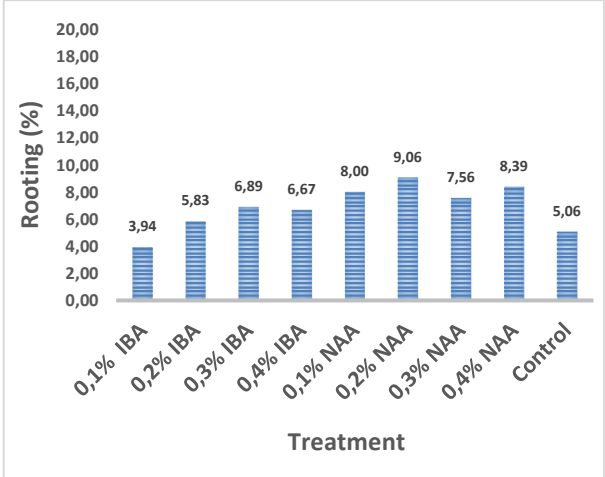

Fig. 5. *Effects of different auxin applications on rooting of the* ***C. hirsutus*** *(%) cuttings*

A similar study was carried out by Sedaghathoor et al. [21] in *Pinus mugo* cuttings and Song et al. [22] in *P. rupestris* cuttings. Sedaghathoor et al [21] conducted with the same plant growth regulators and treated same doses like our study, the best rooting was obtained from 4000 ppm NAA and 1000 ppm IBA applications. According to Song et al. [22], it was revealed that 5370 µM NAA and 4920 µM IBA applications were more effective than rooting Rooton preparations and control on rooting of *P. rupestris* cuttings.

Our observations are in compatible with announced findings of Yan et al. [23] and Yusnita et al. [24]. According to Yan et al. [23], NAA applications were found more

effective in rooting hybrid Aspen cuttings than IAA and IBA applications. In the studies of Yusnita et al. [24], they reported that NAA is more effective than IBA in rooting of *Syzygium malaccense* cuttings and that the NAA doses of 2000 and 4000 ppm are the most effective.

Hartmann et al. [12], reported that IBA induced rooting in all species it was more effective than other auxins. These declarations are in contrast to ours.

Again some results were reported by the Gehlot [25], Bayraktar et al [26], Chaudharı et al. [27], Shao et al. [28] and Nhung et al [18] were contrast to our results. In the study of rooting *Azadirachta indica* cuttings, Gehlot [25] found that IBA was more effective than NAA and IAA; 500 mg L^{-1} dose was more effective than 100, 250, 750, 1000 and 1500 mg L^{-1} doses. In the study of Bayraktar et al [26], it was determined that IBA's more effective than NAA and 5000 ppm dose was more effective than 1000 ppm upon the rooting of *Taxus baccata* cuttings. Chaudhar et al. [27] reported that 4000 ppm dose of IBA was the most effective application for rooting *Euphorbia pulcherrima* cuttings. Shao et al. [28], reported that 1500 mg L^{-1} dose of IBA was the most effective application for rooting *Zizyphus jujuba* cuttings. In the studies of Nhung et al [18], it was found that IBA was more effective than IAA and NAA in the rooting of *Dalbergia tonkinensis* cuttings.

Azamal [29] on *Grewia optiva* cuttings, Pandey et al. [30] on *Ginkgo bloba* L. cuttings, Khosh-Khui and Kaviani [31] on M*elia azedarach* cuttings and Egbe et al. [32] on *Albizia zygia* cuttings, Zencirkıran and Erken [16] on *Platanus orientalis* cuttings observed that IBA and NAA treatments were able to induce rooting and the application of IBA was found to be more effective than NAA. Different researchers reported that the application of IBA increased the rooting percentage of cuttings in some ornamental plants [17, 33, 34, 35, 36] Tiwari and Das [37] found that, IBA and NAA had a similar effect to *Embelia tsjeiam* cuttings.

While the rooting percentages increased with applications of IBA rising from 0.1% to 0.2% and 0.3%, the rooting rate decreased once again at the dose of 0.4% and statistically took place in the same group as the 0.2% dose. In the NAA dose applications, however, rooting starting at 8% with 0.1% dose and decreased once again after rising to 9.06% with 0.2% dose (Fig. 5). In the study of Azad et al. [38], although the rooting rate of *Sterculia foetida* cuttings increased with the increment of IBA doses, contrary in our study rooting rate decreased while the dose increased.

In NAA applications, however, according to statistical groups, the positive effect of NAA on the rooting of *C. hirsutus* cuttings started to decline after the 0.2% doses were applied. Holloway et al. [39] and Han et al. [40] reported that treatment with a high concentration of IBA did not improve the rooting of cuttings. Rooting rate declined with the hormone concentration increasing and high hormone concentration has side effects on the root development. Tate and Page [41] found that 3000, 4000 and 8000 ppm doses of IBA were ineffective in their study on rooting of *Santalum austrocaledonicum* cuttings. While in Nhung et al. [18]'s study revealed the effect of a single dose (1.5 g L^{-1}-IBA) clearly, but in our study any single and clear dose could be revealed.

In our experiment the values of rooting rates were found between 3.94-9.06% with NAA and IBA treatments. Chunshan et al. [42] (2007) found that rooting rate of another *Chamaecytisus* species *C. palmensis* cuttings had the average rooting rate 5-20% with ABT rooting powder treated cuttings. It is not so much higher than our findings.

CONCLUSION

The rooting percentages obtained in this study are not applied commercially in terms of levels. The cuttings were taken from natural populations and elderly plants of different ages. It was not possible to find a sufficient number of standard cuttings. In some experiments, the rooting period was delimited for 8 weeks. Lyubomirova and Iliev [43] reported that IBA had an important influence on rooting after 75 days. Sulusoglu and Cavusoglu [44] recommended, 90 days rooting time for *Prunus laurocerasus.*

Rooting ability depends on a great extent upon the age of stock plants along with physiological condition of the cuttings. This means that, the young plants must be used in the autovegetative propagation [45, 46]. It is thought that the rooting rates have remained low for these factors in this study.

According to our findings it's obtained that, cutting collecting date is an important factor in rooting, best time for excision of *C. hirsutus* cuttings is April and NAA treatments are more effective than IBA treatments. All NAA treatment doses effect rooting of the hairy broom cuttings. The rooting rates of cuttings can be increased if we would take cuttings from younger and cultivated plants.

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REFERENCES

[1] Zencirkiran, M. (2009): Determination of native woody landscape plants in Bursa and Uludag. African Journal of Biotechnology, 8 (21): 5737-5746.

[2] Topay, M., Kaya, Z. (1998): Determination of landscape values of some herbaceous plants in the Bartın-Amasra flora and possibilities of using them in urban areas. I. National Ornamental Plants Congress, 6-9 October, Yalova-Turkey, 74-82.

[3] Anonymous. (2003): Seed propagation of Mediterranean trees and shrubs. Agency Fort the Protection of the Environment and For Technical Services. ISBN 88-448-0081-0, Romap. 120.

[4] Karagüzel, O., Baktir, I., Çakmakçi, S., Ortaçeşme, V., Aydinoğlu B., Atik, M. (2002): Effects of scarification methods temperature and sowing dates on some germination characteristics of *Lupinus varius* L. II. National Ornamental Plants Congress, 22-24 October, Antalya-Turkey, pp.40-47.

[5] Erwin, J. (2007): Looking for new ornamentals: flowering studies. VI International Symposium on New Floricultural Crops, Funchal, Portugal, 11-15 June, Conference Title 813, Acta Horticulturae, pp. 61-66.

[6] Azad, M.S., Alam, M.J., Mollick, A.S., Matin, M.A., (2016): Responses of IBA on rooting, biomass production and survival of branch cuttings of *Santalum album* L., a wild threatened tropical medicinal tree species Journal of Science, Technology and Environment Informatics, 3; 195–206.

[7] Davis, P.H. (1984): Flora of Turkey and The East Aegean Island. Volume III, Edinburgh University Press, London,

[8] Malyer, H., Heper, M., Biçakçi, A.(1995): Distributions of *Chamaecytisus* (Fabaceae/ Genisteae) species in Turkey. The herb Journal of Systematic Botany, 2(1): 133-146.

[9] Erken, K. (2011): Determination of Propagating Methods and Properties Ornamental Plant of *Spartium junceum* L. *Chamaecytisus hirsutus* (L.) Link and *Genista lydia* var *lydia* Boiss Taxa. (Unpublished PhD thesis), University of Ege, Izmir, Turkey.

[10] URL 1., (2020): CABI Data Mining Invasive Species Databases. https://www.cabi.org/isc/datasheet/113179#toidentity

[11] URL 2. (2020): Plants of the world online, Kewscience, http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:485961-1#distribution-map

[12] Hartman, H. T., Kester, D. E., Davies, F.T. (1990): Plant propagation principles and practices. 5th edition, Prentice-Hall Inc., Englewood Cliffs, New Jersey, p.647.

[13] Kalayci, M. (2005): Using Jump with examples and analysis of variance models for agricultural research. Anatolia Agricultural Research Institute Publication No: 2, Eskişehir-Turkey, p. 296.

[14] Acar, M., Gizlenci, S. (2006): Using JMP for Agricultural Researchers. The Black Sea Agricultural Research Institute, Samsun-Turkey, p. 69.

[15] Giatromanolaki, A. A., Dragassaki, M., Vlahos, I., Papadimitriou, M. (2006): Vegetative propagation *in vivo* and *in vitro* of *Staehelina petiolata* (L.) Hilliard et Burtt. Propagation of Ornamental Plants, 6(4): 187-193.

[16] Zencirkiran, M., Erken, K. (2012): The Effect of Different Times Collecting Cutting and Auxin Treatments of the Rooting in *Platanus orientalis* L. (Oriental Plane Tree - Cınar), The Journal of Animal & Plant Sciences, 22(3): 764-767

[17] Alsup, C., Cole J., Claypool, L. (2003): Timing and IBA application affect rooting of *Acer saccharum* Marsh. stem tip cuttings. Propagation of Ornamental Plants, 3(1): 42-46.

[18] Nhung, N.P., Thu, P.Q.,C hi, N.M., Dell, B. (2019): Vegetative propagation of *Dalbergia tonkinensis*, a threatened, high-value tree species in South-east Asia. Southern Forests: A Journal of Forest Science (81)3, 195-200.

[19] Mascarello, C., Cucchiara G., Ruffoni, B. (2003): Effect of season and rooting agents on the rhizogenesis of *Olearıa scıllonıensıs* cuttings. Propagation of Ornamental Plants, 3(1): 47-49.

[20] Enders, T. A., Strader, L.C. (2015): Auxin activity: past, present, and future. American Journal of Botany, 102, 180–196.

[21] Sedaghathoor, S., Kayghobadi, S., Tajvar, Y. (2016): Rooting of Mugo pine (*Pinus mugo*) cuttings as affected by IBA, NAA and planting substrate. Forest Systems, Volume 25, Issue 2, eSC08. http://dx.doi.org/10.5424/fs/2016252-09087.

[22] Song, S.J., Ko, C.H., Shin, U.S., Oh, H.J., Kim, S.Y., Lee, S.Y. (2019): Successful stem cutting propagation of *Patrinia rupestris* for horticulture Rhizosphere, 9; 90-92

[23] Yan, S.P., Yang, R.H., Wang, F., Sun, L.N., Song, X.S. (2017): Effect of auxins and associated metabolic changes on cuttings of hybrid Aspen. Forests 2017, 8, 117.

[24] Yusnita, Y., Jamaludin,J., Agustiansyah, A., Hapsoro, D. (2018): A combination of IBA and NAA resulted in better rooting and shoot sprouting than single auxin on Malay apple [*Syzygium malaccense* (L.) Merr. & Perry] stem cuttings. AGRIVITA Journal of Agricultural Science, 40(1), 80–90. http://doi.org/10.17503/agrivita.v40i0.1210

[25] Gehlot, A. (2017): Impact of auxin and rooting media on root formation in semi-hard wood cuttings of *Azadirachta indica* A. Juss (Neem). Nativa, Sinop, v.5, n.4, p.283-289,

[26] Bayraktar, A., Atar, F., Yıldırım, N., Turna, I. (2018): Effects of Different Media And Hormones on Propagation By Cuttings of European Yew (*Taxus baccata* L.) Šumarski list, 9–10, CXLII; 509–516

[27] Chaudharı, B.B., Bhatt, D., Chawla, S.L., Patel, M.A., Bennurmath, P. (2018): Effect of rooting hormone and media on root induction in poinsettia (*Euphorbia pulcherrima* Willd.), Journal of Ornamental Horticulture, 21 (1&2): 7-12,

[28] Shao, F., Wang, S., Huang, W., Liu, Z. (2018): Effects of IBA on the rooting of branch cuttings of Chinese jujube (*Zizyphus jujuba* Mill.) and changes to nutrients and endogenous hormones, Journal of Forestry Research, 29(6):1557–1567

[29] Azamal, H. (2012): Changes of soluble sugars and enzymatic activities during adventitious rooting in cuttings of *Grewia optiva* as affected by age of donor plants and auxin treatments. American Journal of Plant Physiology, 7 (1): 1-16.
[30] Pandey, A., Tamta S., Giri, D. (2011): Role of auxin on adventitious root formation and subsequent growth of cutting raised plantlets of *Ginkgo biloba* L. International Journal of Biodiversity and Conservation, 3(4): 142-146.
[31] Khosh-Khui, M., Kaviani, K. (2006): Investigation on sexual and asexual propagation of Chinaberry (*Melia azedarach* L.). Journal of Plant Sciences, 1: 31-35.
[32] Egbe, E.A., Chuyong, G.B., Fonge, A.B., Tata, B.L., Tabot, P.T. (2012): The effects of different concentrations of indole-3-butyric acid (IBA) on leafy stem cuttings of four tropical timber species. Journal of Horticulture and Forestry, 4(5): 85-91.
[33] Rifaki, N., Economou, A., Scaltsoyiannes, A. (2001): Factors affecting the rooting of *Ilex aquıfolium* L. cuttings. Propagation of Ornamental Plants, 1(1): 31-35.
[34] Bielenin, M. (2003): Rooting and gas exchange of conifer cuttings treated with indole butyric acid. Journal of Fruit and Ornamental Plant Research, Vol. 11: 99-105.
[35] Mpeck, M.L.N., Tchoundjeu, Z., Asaah, E. (2003): Vegetative propagation of *Pausınystalıa johimbe* K. Schum. by leafy stem cuttings. Propagation of Ornamental Plants, 3(2): 11-18.
[36] Vakouftsis, G., Syros, T., Kostas, S., Economou, A.S., Tsoulpha, P., Scaltsoyiannes, A., Metaxas, D. (2009): Effect of IBA, time of cutting collectıon, type of cuttings and rooting substrate on vegetative propagation ın *Cupressus macrocarpa* 'Goldcrest'. Propagation of Ornamental Plants, (2): 65-70.
[37] Tiwari, R.K.S., Das, K. (2010): Effect of stem cuttings and hormonal pre-treatment on propagate on of *Embelia tsjeriam* and *Caesalpinia bonduc*, two important medicinal plant species. Journal of Medicinal Plants Research, 4 (15): 1577-1583.
[38] Azad, Md. S., Alam, Md. J., Mollick, A.S., Khan, Md. N.I. (2018): Rooting of cuttings of the wild Indian almond tree (*Sterculia foetida*) enhanced by the application of indole-3-butyric acid (IBA) under leafy and non-leafy conditions. Rhizosphere 5; 8–15
[39] Holloway, L., Krasowski, M., Smith R.F, Cameron, S.I. (2008): Enhancing the rooting of Canada yew stem cuttings wıth IBA treatments. Propagation of Ornamental Plants, 8(1): 23-27.
[40] Han, H., Zhang, S., Sun, X. (2009): A review on the molecular mechanism of plants rooting modulated by auxin. African Journal of Biotechnology. 8 (3): 348-353.
[41] Tate, H.T., Page, T. (2018): Cutting propagation of *Santalum austrocaledonicum*: the effect of genotype, cutting source, cutting size, propagation medium, IBA and irradiance. New Forests (2018) 49:551–570
[42] Chunshan, C., Guojun, C., Shaotong, M., Weiqing C., Rui, X. (2007): Breeding trial of *Chamaecytisus palmensis* in Gansu province. Scientia Silvae Sinicae; 2007-12.
[43] Lyubomırova, T., Ilıev, I. (2014): Factors Affectıng The Rootıng Of Cuttıngs Of *Syringa Vulgaris* L. Cultıvars. Muzeul Olteniei Craiova. Oltenia. Studii Şi Comunicări. Ştiinţele Naturii. Tom. 30, No. 1/2014 ISSN 1454-6914 67
[44] Sulusoglu, M., Cavusoglu, A. (2010): Vegetative propagation of Cherry laurel (*Prunus laurocerasus* L.) using semi-hardwood cuttings. African Journal of Agricultural Research 5 (23): 3196- 3202.
[45] Awang, Y., Sandrang, A.K., Mohamad, R. Selamat, A. (2011): Effect of ontogenic age on root and shoot development of *Tabebuia heterophylla* cuttings propagated in soilless culture. African Journal of Agricultural Research, 6 (24): 5422-5427.
[46] Ayan, S., Yahyaoğlu, Z., Gerçek, V., Şahin, A., Sıvacıoğlu, A. (2006): The vegetative propagation possibilities of Black Alder (*Alnus glutinosa* subsp. *barbata* (CA Mey.) Yalt.) by softwood cuttings. Pakistan Journal of Biological Sciences 9 (2): 238-242.

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Research Article

LAND DEGRADATION IN THREE LOCATIONS AND SAMPLING PERIODS IN PHYSIOGRAPHIC UNITS UNDER ARABLE CROP PRODUCTION IN SUB- HUMID TROPICS OF NIGERIA

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ABSTRACT. Understanding landscape features of cultivable agricultural lands and the influence by topographic settings and degradation has become imperative for sustainable land use planning and management. This study compared land degradation in three locations and sampling periods under arable crop production in Nsukka area. Three locations (Obukpa, Lejja and Ozi-Edem) were selected. Soil auger samples were randomly collected in triplicates fromdepth of 0-30cm in plots of 25 m^2 marked on the farmer's field. Soil core samples were collected at 0-10cm depth. Soil sampling was done at three repeated periods; April-May, July-August and October-November for 1st,2nd and 3rd sampling period, respectively. Soil samples were analyzed for physical and chemical properties and all data statistically analyzed as a 3 x 3 x 3factorial using Genstat statistical software. FAO method was employed in land degradation assessment. Results showed that most physical and chemical properties had significantly ($p < 0.05$) highest values at low slope relative to others,and also at first sampling period relative to other sampling periods. Degradation in Upper slope increased by 3.4 and 5.9 % over middle and lower slope, respectively. Obukpa had the highest level of degradation relative to other locations. Degradation was highest in 3rdsampling period and increasedby 8.4 and 14.9 %over2ndand 1stsampling period, respectively.Degree of degradation increased with sampling time. Appropriate conservation measures, inclusion of restorative crops in cropping systems, use of locally available materials and bio fertilizer are recommended to reduce effect of land degradation in arable soils.

Keywords: *slope, degradation, soil properties, land use, sub-tropic*

INTRODUCTION

Land degradation is the result of a combination of social, political and biophysical forces operating across a broad spectrum of temporal and spatial scales but essentially arises from bad management that encourages soil erosion by wind and water. Intensification rather than extensification of agriculture is widely practiced to scale up food production. This practice has escalated land degradation [13].

In the tropics and subtropics, agricultural activities have been taking place under varying dynamic context such as physiography, agro-ecology, climate and soil conditions. The success is then strongly influenced by topographic settings, degree of human interferences and underlying biographical features [11].

It was reported that extreme pressure on landscape stability is high where there is a sharp increase in population [23] and this has been causing intensive land utilization and forest clearing for cultivation even in areas that are not practical for agriculture such as in steep hill slopes or marginal lands [38]. The greatest concern to the agriculturists in developing countries is to meet the future needs of the growing population. Land has been utilized intensively for all purposes at the expense of its suitability thereby resulting in land degradation and altering the natural ecological conservational balances

in the landscape. Such imbalances pose great difficulty to soil productivity and food security [36].

In the last 50 years alone, 20% of the world's agricultural land has been irreversibly damaged due to human-induced land degradation. Thus, if the process of destruction continues at this pace, agriculture will lose 15-30% of its present productivity [18]. Land degradation is caused by poor land management practices such as slash and burn agriculture, uncontrolled livestock grazing on fragile lands, poor road construction and unplanned or poorly planned settlements in landslide-prone areas [33]. Annually colossal amounts of valuable top-soil is eroded into rivers and out to sea during heavy rains. Land degradation is a function of slope attribute, the amount of soil erosion has always been proportional to the steepness of the slope [24].

Variations in the physiography of agricultural lands have an enormous influence on soil properties and crop production. Study on the influence of different land uses and topography on soil properties in southeastern Nigeria revealed an increasing trend in soil pH, organic carbon and exchangeable bases with a decrease in slope [15]. Similar report was made for Wollo, Ethiopia [9]. Another study in northeastern Nigeriaby Ezeaku and Unagwu [13]and northeastern Ethiopia [19] also indicated that mean values of total nitrogen, organic matter and cation exchange capacity were higher in lower than upper slope land position. Report by Yimer [40]showed significant changes in soil properties on varied altitudinal ranges of Bale mountains, Ethiopia.

Other studies showed that involvement of farmers' different land use types put impact on soil fertility and productivity [19]. Their findings indicated lower soil organic carbon, total nitrogen and basic cations in cultivated lands compared to grazing or protected forest area which was attributed by continuous cultivation, absence of fallowing and erosion. Limited maintenance of soil physico-chemical health is very likely to result to poor aggregate stability, decline of soil organic matter, nutrient deficiency and unavailability to plants and stagnation of crop yields [39] and exacerbates soil degradation.

The foregoing is an indication that the soil quality is associated with biophysical setting and anthropogenic factors. Although, studies have shown effects of land use on soil properties along a toposequence [15, 6, 11, 9], there is dearth of information on degree of land degradation as influenced by location and sampling periods in physiographic units under cultivated crops. Again, soil degradation due to erosion is a major threat to sustainable agricultural production in the inland-upland continuum in Nsukka subtropical area. These necessitate the need for adequate information to intervene and solve soil degradation problem in the general area. The main objective of the study therefore is to asses land degradation as influenced by sampling period in physiographic units under cultivated crops in sub-humid tropics of Nsukka area.

MATERIALS AND METHODS

Description of Study Area

This research was carried out in three locations at Nsukka namely; Obukpa, Lejja and Ozi-Edem. These locations are in Nsukka agricultural zone of Enugu state with longitude of $6^{o}43^{I}$ - $6^{o}55^{I}$E and latitude $7^{o}18^{I}$ - $7^{o}28^{I}$N (Figure 1). The three locations were selected because they are among the major crop producing areas in Nsukka agricultural zone and are mostly affected by land degradation as was observed during the reconnaissance visits of the towns in the zone. Nsukka climate is characteristically

sub-humid tropical, with mean annual total rainfall of about 1600 mm; of which distribution is bimodal, with peaks during July and October in the first and second phases, respectively. Atmospheric temperature in Nsukka is high with mean daily minimum temperatures between 21.40and 25.00ºC, and mean daily maximum temperature ranges from 28.10 to 34.60ºC. Relative humidity ranges between 70 and 80% [16]. The soils in Nsukka of Eastern Nigeria are generally derived from the residuum of false-bedded sand-stone or upper - coal measure formation as a result of disintegration of rock (which could be alteration by physical, chemical and biological processes) [2]. During reconnaissance visits, different land uses were identified along the physiographic units (Table 1) as being practiced by local farmers.

Field Methods

Site selection

Three locations (Lejja, Obukpa and Ozi-Edem) in Nsukka local Government Area were selected and used for the study. Prior to the selection, reconnaissance visits were undertaken on five locations, out of which, three namely; Lejja, Obukpa and Ozi-Edem showed similarity in cropping systems (eg arable cropping: cassava/yam/ vegetable/ maize inter crops and cereal such as guinea corn cropping). The crops were observed to be grown along the landscape (physiographic units) positions – upper slope, middle slope and toe slope. Again, the three sites were observed to be experiencing erosion menace as shown by the presence of rill, sheet and gully erosions. The topographic map of the three selected locations is shown in Fig. 1, while Table 1 shows the coordinates and land use types obtained in the three locations.

Table 1. *Location physiographic units and land uses with their coordinates*

Location	Physiographic unit	Latitude	Longitude	Altitude (m)	Land use
Lejja	US	6.4836º N	7.2342º E	496.58	Pepper/maize/cassava
	MS	6.4837º N	7.2343º E	490.60	Pumpkin/tomatoes
	TS	6.4838º N	7.2344º E	439.91	Cocoyam/cassava/ Pepper
Obukpa	US	6.5315º N	7.2380º E	417.76	Cassava/bitter yam
	MS	6.5313º N	7.2377º E	414.32	Garden egg/ tomatoes
	TS	6.5311º N	7.2375º E	389.86	Cocoyam
Ozi-Edem	US	6.5093º N	7.2069^0 E	414.28	Pepper/maize/tomatoes
	MS	6.5094º N	7.2067^0 E	409.97	Garden egg/maize/cassava
	TS	6.5095º N	7.2065^0 E	402.94	Cassava/garden egg

NB: US= upper- slope; MS = middle- slope; TS =toe -slope. (Zea mays), Cassava (Manihot esculenta), Pumpkin (Cucurbita pepo), Cocoyam (Colocasia esculenta), bitter yam (Dioscorea bulbifera), Garden egg (Solanum melongena), Tomatoes (Solanum lycopersicum)

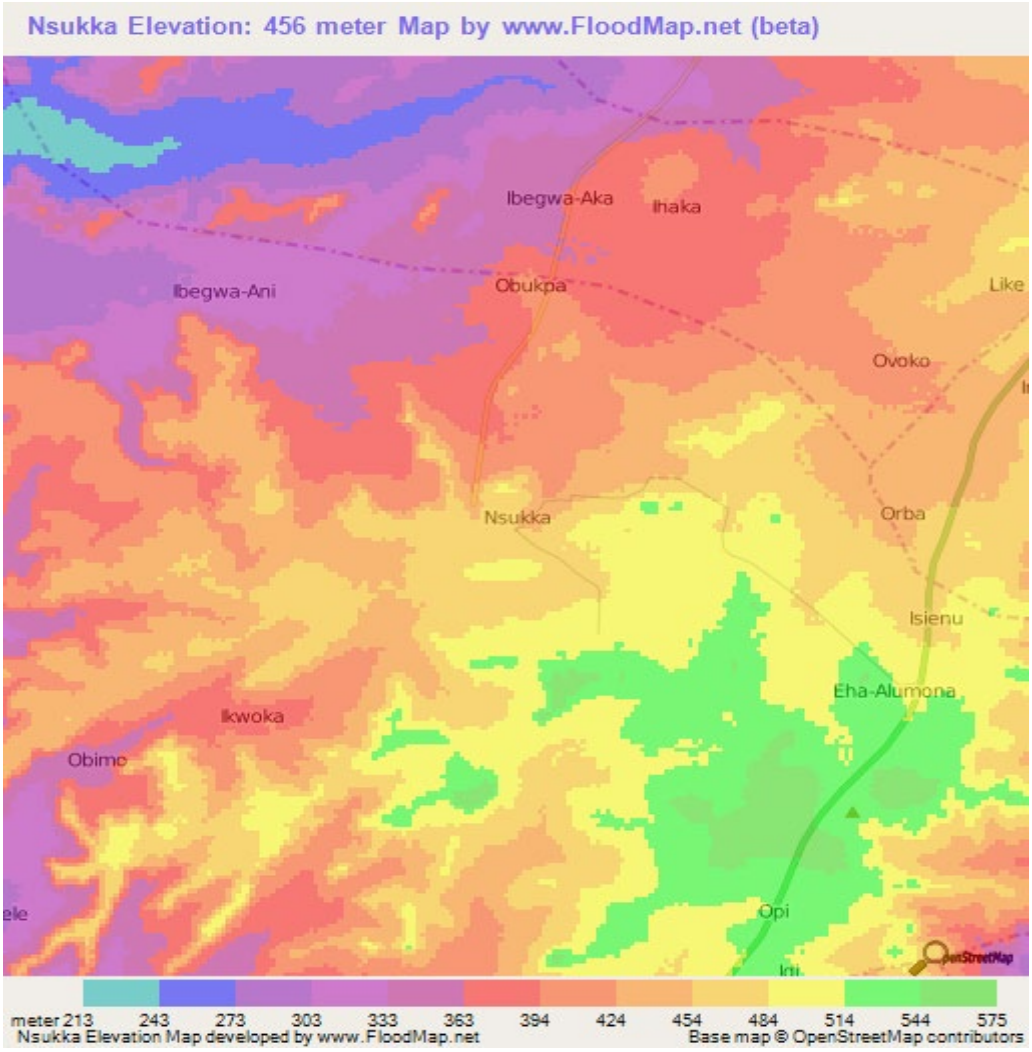

Fig.1. *Elevation, Topo and Contour map of Nsukka, Nigeria.*
The elevation map of Nsukka,Nigeria is generated using elevation data from NASA's 90m resolution SRTM data

Soil sampling

Disturbed and undisturbed soil samples were taken from the field using auger and core sampler respectively. Soil auger samples were collected in three replicates at depth of 0-30 cmat random from plots of 5 × 5 m (25m^2) marked on the farmer's field in the physiographic units (upper slope, middle slope and toe-slope). Core samples were collected at 0-10cm soil depth. The soil sampling was done at three repeated periods; April to May for the first sampling period; July to August for the second sampling period, and October to November 2015 for the third sampling period. Eighteen (18) soil samples were collected from each location giving a total of fifty-four (54) soil samples collected for the first sampling period. A total of 162 samples were collected at the end of the three sampling periods across the locations.

Laboratory Studies

After each visit, soil samples were collected and put into polyethylene bag, tied and labeled and taken to the laboratory. Thereafter, they were dried, ground and sieved

through 2 mm sieve. The samples were analyzed for physical and chemical properties like particle size distribution, bulk density, soil pH, organic carbon, total N and other macro elements.

Methods for soil physical and chemical properties determination

Particle-size distribution (PSD) was analyzed following the method by Gee and Bauder [20], bulk density by core method [3], saturated hydraulic conductivity (K_s) [27], soil chemical properties : soil pH (1:2.5 soil: water suspension) [29], soil organic carbon [30], total nitrogen [4], available phosphorus [18], exchangeable bases (Na^+, K^+, Ca^{2+},and Mg^{2+}) [7], exchangeable acidity (E.A) [37] andcation exchange capacity (CEC) [35].

Soil Degradation Assessment

The degradation status of the soils in the various locations was assessed by field observation and determined analytical indicators to compare with the standard indicators and criteria for land degradation assess*ment according to Food and Agricultural Organization [17] as shown in Table* 2. Those indicators are; soil bulk density, content of nitrogen, phosphorus, potassium and base saturation.

Aggregate degradation determination

Aggregate degradation determination is defined as the ratio of total actual class score to potential (possible) highest score. This is mathematically calculated as:

$$\text{AD (\%)} = \frac{TAC}{PHS}\ X\ 100$$

Where AD (%) = Percent aggregate degradation

TAC = Total actual class score
PHS = Potential highest score (24)

Aggregate degradation rating values have an inverse relationship with the rating of agricultural productivity potential byEzeaku [14]. The higher the aggregate degradation, the lower the suitability and agricultural productivity (Table 3)

Table 2. *Indicators and criteria for land degradation assessment*

Criteria	**Degree of Degradation**			
	1	2	3	4
Soil bulk density (g cm^{-3})	<1.5	1.5-2.5	2.5-5	>5
Content of organic matter (g kg^{-1})	>2.5	2-2.5	1.0-2	<1.0
Content of total Nitrogen element (g kg^{-1})	>0.13	0.10-0.13	0.08-0.10	<0.08
K content (cmol kg^{-1})	>0.16	0.14-0.16	0.12-0.14	<0.12
Content of Base Saturation (%)	>10%	5-10%	2.5-5%	<2.5%
Content of available phosphorus element (mg kg^{-1})	>8	7-8	6-7	<6
Aggregate Score (%)	0-25	25-50	50-75	75-100

Source: FAO [17] Key: Class1: None- slightly degraded; Class 2: Moderately degraded; Class 3: Highly Degraded'; Class 4: Very highly Degraded

Table 3. *Degree / class of degradation suitability and aggregate degradation ratings*

S/N	Degree of Degradation class	Degradation class	Suitability class	Potential agricultural productivity	Aggregate degradation (%)
1	Non to slightly degraded soil	1	S_1	75-100	0-25
2	Moderately degraded	2	S_2	50-75	25-50
3	Highly degraded	3	S_3	25-50	50-75
4	Veryhighly degraded	4	N_1	0-25	75-100

NB: S_1 : highly suitable, S_2: moderately suitable, S_3: marginally suitable, N_1: currently not suitable. Source: [17].

Statistical Analysis

All data were statistically analyzed as a 3 x 3 x 3 factorial using Genstat statistical software. Discovery Edition 4. The means were separated using Fisher's least significant differences. The factors were; location with three rates: Lejja, Obukpa and Ozi-Edem; physiographic Units: Upper- slope, Middle- slope and Toe- slope and sampling periods : first sampling period (April/May), second sampling period (July/August) and third sampling periods (October/November).

RESULTS AND DISCUSSIONS

Effect of physiographic units, sampling periods and their interactions on soil physical properties in the three study sites

The study areas are characterized by marked topographic variations in which agriculture is practiced under flat to very steep sloping topographic lands. Again, the study areas are dominated by rain feds agricultural activities. These significantly influenced most of soil physical and chemical properties. The results of soil physical properties according to their physiographic units for the three locations are presented in Table 4. It was observed that for Lejja location, there were no significant differences for silt and total sandamong the physiographic units, while all physical properties measured at Ozi-Edem showed significant ($p < 0.05$) differences among the physiographic units except for total sand (Table 4).

The soil physical properties according to the sampling period for the three locations are presented in Table 5. It was observed that there were no significant differences for total sand at the different sampling periods across the locations while at Lejja other soil physical properties such as clay, silt, bulk density, total porosity and hydraulic conductivity showed significant ($p < 0.05$) differences at the different sampling periods across the locations. At Ozi-Edem, there were no significant differences among the three sampling periods for bulk density and total porosity. All other measured physical properties such as hydraulic conductivity showed significant differences ($p < 0.05$) among the sampling periods for all the study locations.

Clay and silt contents were significantly ($p <0.05$) higher at the toe-slopefollowed by the middle-slope, whileupper-slope had the lowest value (Table 5) in all the locations. Total sand content decreased down slope while the other particle sizes increased in the same direction. This could be due to larger size of sand and its decreased transportability while silt and clay sizes are smaller and lighter hence easily moved in suspension towards the valley bottom [32].

Clay content had significantly ($p < 0.05$) higher value(Table 5) at the first sampling period (April) for all locations while the second and third sampling period values were statistically equal. Silt content was significantly ($p < 0.05$) higher at the third sampling period (6.00, 9.00) followed by the first (5.67, 8.67) and second sampling periods value (5.67) (Table 5)for both Lejja and Obukpa. At Ozi-Edem, highest value of silt (11.17) at the first sampling period was offered followed by the second (10.67) and third sampling periods (6.33). Significantly ($p < 0.05$) higher value was recorded for total sand content at second sampling period compared to the others for the three locations (Table 5).

Bulk density was significantly ($p < 0.05$) higher(Table 4) at the upper-slope regions for all the study locations while the middle and toe-slope values were not significantly different for Lejja and Obukpa and significantly ($p < 0.05$) different for Ozi-Edem. Generally, the upper-slope had higher bulk density and this could be due to its greater sand content. Bulk density normally decreases, as mineral soils become finer[8]. Bulk density for Lejja and Obukpa were significantly ($p < 0.05$) highest at the first sampling period while the second and third sampling period values were not significantly different. This could be due to the higher sand content at the first sampling period due to more intense erosion.

Total porosity was significantly ($p < 0.05$) higher at the toe-slope regions (Table 4) for all the study locations while the middle-slope and upper-slope values were not significantly different. Increase in bulk density could be due to more compaction of finer particles that reduced porosity probablydue to machinery traction and heavy raindrops [28].

Upper, middle and toe-slopepositions along the landscape had significantly ($p < 0.05$) higher value for saturated hydraulic conductivity (K_s) at Lejja(Table 4).Saturated hydraulic conductivity decreases with increasing bulk density as a response of the smaller volume of coarse pores (Dee, et al., 2008) and this could explain the higher $K_s$in the toe-slope of Obukpa. Saturated hydraulic conductivity values significantly decreased in the order: 1st sampling period> 2nd sampling period > 3rd sampling period for all the study locations.This is to say that Lejja had: 67.37>63.18>27.33cm hr^{-1},while Obukpa recorded: 42.72> 25.09>41.08 cm hr^{-1} and Ozi-Edem:39.03>33.34>16.33 cm hr^{-1}, respectively (Table 5).

Table 6 also shows the interactive effect of physiographic units and sampling period on the soil physical properties for the three locations. It was observed that most of the soil physical properties had highest value at an interaction between the upper-slope and first sampling period except for bulk density which had highest value (1.71 g cm^{-3}) (Table 6) at the interaction between the toe-slope and third sampling period.

Table 4. *Main effect of physiographic units on soil physical properties in the three sites*

Location	Unit	Clay	Silt	T. Sand	BD	TP	K_{Sat}
		→	g kg^{-1}	←	g cm^{-3}	%	Cm h^{-1}
Lejja	US	8.33	5.00	86.34	1.61	39.24	53.89
	MS	8.66	5.00	85.34	1.55	41.72	55.68
	TS	10.00	6.33	85.00	1.53	42.11	47.30
	LSD(0.05%)	0.49	NS	NS	0.04	1.62	4.42
Obukpa	US	12.00	5.33	82.67	1.63	38.53	36.08
	MS	12.16	7.00	80.84	1.53	42.68	31.58
	TS	12.33	10.83	76.84	1.49	43.03	41.22
	LSD(0.05%)	NS	0.52	NS	0.04	1.94	2.22
Ozi – Edem	US	20.16	8.17	71.67	1.24	50.00	35.61
	MS	20.67	8.82	70.51	1.22	53.89	26.05
	TS	21.00	11.33	67.67	1.17	55.89	27.08
	LSD(0.05%)	0.51	0.52	NS	0.06	6.74	1.86

NB: BD = bulk density TP = total porosity K_S = hydraulic conductivity, T.sand = total Sand, US = upper-slope, MS = middle-slope, TS = toe-slope, NS= not significant

Table 5. *Main effect of sampling period on soil physical properties in the three*

Location	sampling period	Clay	Silt	T.Sand	BD	TP	K_S
		→	(g kg^{-1})	←	g cm^{-3}	%	Cm $^{h-1}$
Lejja	1st	9.00	5.67	85.33	1.62	42.28	67.37
	2nd	8.66	5.67	85.67	1.54	41.72	62.18
	3rd	8.33	6.00	85.67	1.53	39.08	27.33
	LSD(0.05%)	0.49	0.17	NS	0.04	1.62	4.42
Obukpa	1st	12.67	8.67	78.66	1.58	42.11	42.72
	2nd	11.33	5.67	83.00	1.53	40.55	25.09
	3rd	12.33	9.00	78.67	1.54	41.60	41.08
	LSD(0.05%)	0.19	0.52	NS	0.04	1.94	2.22
Ozi – Edem	1st	21.00	11.17	67.83	1.23	55.18	39.03
	2nd	20.67	10.67	68.66	1.21	50.83	33.34
	3rd	20.33	6.33	73.33	1.19	53.75	16.33
	LSD(0.05%)	0.51	0.52	NS	NS	NS	1.86

NB: BD = bulk density TP = total porosity K_S = hydraulic conductivity, T.sand = total Sand, NS= not significant

Table 6. *Interactive effects of physiographic units and sampling period on soil physical properties in the three study sites*

Location	Unit	sampling period	Clay	Silt	g kg^{-1}	CS	FS	BD g/cm^{-3}	TP %	Ks Cm h^{-1}
	US	1st	9.00	4.00		46.00	45.50	1.58	40.25	69.75
Lejja	US	2nd	8.00	5.00		45.00	42.50	1.57	40.88	67.51
	US	3rd	8.00	6.00		42.00	43.00	1.49	44.03	24.41
	MS	1st	9.00	4.00		43.50	41.00	1.44	45.45	73.27
	MS	2nd	8.00	5.00		43.50	41.50	1.51	43.02	63.48
	MS	3rd	8.00	6.00		42.00	40.50	1.65	37.86	30.30
	TS	1st	12.00	6.00		43.50	44.00	1.56	41.13	59.07
	TS	2nd	10.00	6.00		42.50	42.00	1.56	41.26	55.56
	TS	3rd	9.00	7.00		40.50	41.50	**1.71**	35.35	27.27
		LSD(0.05)	0.86	0.86		0.86	0.86	0.08	2.80	7.65
	US	1st	12.00	5.00		49.00	46.50	1.68	36.60	41.91
	US	2nd	9.00	5.00		37.00	49.00	1.59	40.13	28.45
	US	3rd	11.00	6.00		36.00	49.00	1.62	38.87	37.88
Obukpa	MS	1st	13.00	8.00		38.00	45.50	1.50	43.90	39.20
	MS	2nd	12.00	6.00		35.00	47.00	1.54	41.76	17.68
	MS	3rd	12.00	9.00		28.00	47.00	1.54	42.39	37.88
	TS	1st	13.00	13.00		34.00	29.50	1.43	45.82	47.06
	TS	2nd	13.00	7.00		28.00	46.00	1.60	39.75	29.12
	TS	3rd	13.00	12.00		30.00	45.50	1.43	43.53	47.47
		LSD(0.05)	0.93	0.19		0.91	0.89	0.08	3.35	3.85
	US	1st	20.00	10.00		32.00	44.00	1.11	58.00	50.43
	US	2nd	11.00	9.00		32.00	51.00	1.18	55.50	41.24
Ozi– Edem	US	3rd	20.00.	5.00		29.50	45.00	1.21	54.20	15.15
	MS	1st	21.00	10.00		31.50	39.00	1.19	55.10	30.01
	MS	2nd	21.00	10.00		27.00	44.50	1.24	53.2	27.45
	MS	3rd	20.00	6.00		24.50	45.50	1.24	53.3	20.71
	TS	1st	21.00	13.00		27.00	37.00	1.26	52.50	36.78
	TS	2nd	21.00	11.00		22.00	39.00	1.22	43.80	31.33
	TS	3rd	21.00	8.00		26.00	42.50	1.24	53.70	13.13
		LSD(0.05)	0.89	0.19		0.91	0.93	0.09	11.67	3.215

NB: BD = bulk density TP = total porosity Ks = hydraulic conductivity FS = fine sand CS = coarse sand. UP = upper-slope, MS = middle-slope and TS = toe-slope

Effect of physiographic units, sampling period and their interactions on soil chemical properties in the three study sites

The soil chemical properties according to their physiographic units and sampling periods for the three locations are presented in Table 7. It was observed that for Lejja, Obukpa and Ozi-Edem, there were no significant differences for EA, OM, K^+ and Na^+ among the physiographic units. However, soil pH, total nitrogen, Ca^{2+}, Mg^{2+}, CEC, base saturation and available phosphorus were significantly different ($p < 0.05$).

The soil chemical properties according to the sampling period for the three locations are presented in Table 8. It was observed that there were no significant differences for soil pH, K^+, and Na^{2+} at the different sampling periods. At Lejja and Ozi-Edem, there were no significant differences among the three sampling periods for CEC, organic matter and total nitrogen.

Soil pH had significantly ($p <0.05$) higher value at toe-slope regions for all the study locations. Exchangeable acidity was significantly ($p <0.05$) higher at the middle-slope followed by the toe-slope with the middle-slope having the lowest value for Ozi-Edem. Exchangeable acidity was only found significant at Obukpa among the various sampling periods where the third and second sampling periods had the lowest value. The increase of soil pH down the slope may be attributed to possible loss of basic cations down the slope which finally accumulate at the lowland [32,26].

Organic matter was only significantly different ($p <0.05$)at Lejja among the various sampling periods with the third sampling period being significantly higher than the second sampling period which was statistically equal with the first sampling period. This should be expected as the lowlands are enriched of the plant nutrients by flood waters. This is in agreement with earlier reports [1, 25,12].

Significantly ($p < 0.05$) higher value was recorded for total nitrogen at toe-slope compared to the middle and upper-slope regions at Lejja location. This could be associated to the process of washing soil nutrientelements downslope by runoff waters. This accords earlier reports [1, 25, 12]. Ozi Edem and Obukpa recorded highestvalue of nitrogen at the upper-slope compared to the middle and toe-slope regions, and could be due to minimal water erosion at the upper slope.

Exchangeable Ca^{2+} and Mg^{2+} were the only cations that were significantly different both among the physiographic units and the sampling periods for all the study locations. It was observed that for both Ca^{2+} and Mg^{2+}, toe-slope regions in Ozi Edem and Lejja had significantly ($p < 0.05$) higher values when compared to the other physiographic units. At Obukpa, upper-slope had highest value for Mg^{2+}. The higher Ca^{2+} and Mg^{2+} contents at thetoe-slope could be as a result of runoff carrying the cations down the slope. This is synonymous with the results of Pillai and Natarajan [34].

Among the various sampling periods, Ca^{2+} had significantly ($p < 0.05$) highest value at the third sampling period for Ozi-Edem while Mg^{2+} had the highest value at the second and first sampling periods for Lejja and Obukpa, respectively. The value of Ca^{2+}, Mg^{2+} and Na^+ decreases irregularly along the physiographic units through mid-slope and toe-slope which could be as a result of runoff. Higher value of Ca^{2+}, Mg^{2+} and Na^+ at TS could beattributed to elements eroded from upper-slope during run off and deposited on flood plains by flood water [31].

Generally, toe-slope soils were having significantly ($p< 0.05$) higher base saturation than upper-slope indicating high degree of leaching in upper slopes. These findings are in concurrence with previous results [34]. Among the sampling periods, CEC was significantly highest (16.27 cmol kg^{-1}) at the third sampling period for Ozi-Edem,while

base saturation was highest in the following order: second (96.97 %)> first (96.15 %) > third (93.82 %) sampling periods for Lejja, Obukpa and Ozi-Edem, respectively (Table 8). This variation could be due to slight differences in the land use at various locations. Obukpa location had highest value of % base saturation may be due to higher vegetation cover of the land surface.

Available phosphorus at Lejja was significantly ($p < 0.05$) higher at the middle-slope followed by the toe-slope and upper-slope. High available phosphorus at the middle-slope could be due to higher biological activities and accumulation of organic matter in the middle-slope [21]. At Obukpa, available phosphorus concentration followed the order upper-slope > middle-slope > toe-slope while at Ozi-Edem the reverse occurred. Increase and decrease of phosphorus along the physiographic units could be attributed to run off and leaching.

Among the sampling periods, the second sampling period of available phosphorus was significantly ($p < 0.05$) higherthan the first sampling period but was not significantly different to the third sampling period for Obukpa. While the third sampling period was significantly higher than the first sampling period this was found statistically equal to the second sampling period for Ozi-Edem. High phosphorus availability could be attributed to higher organic matter content in the soils.

The interactive effect of physiographic units and sampling period on the soil chemical properties for the three locations is presented in Table 9. Results show that most properties such as base saturation (96.81%) had highest value at an interaction between the toe-slope and first sampling period.

Table 7. *Main effect of physiographic units on soil chemical properties in the three study sites*

Location	Unit	Soil pH H_20	KCL	EA cmol kg^{-1}	OM	TN	Exchangeable Bases (cmolkg$^-$) Ca^{2+}	Mg^{2+}	K^+	NA^{2+}	CEC	B.S	AP
					($g\ kg^{-1}$)				cmol kg^{-1}			%	mg kg^{-1}
Lejja	US	5.1	4.35	1.20	6.9	0.6	12.05	1.14	0.05	0.03	14.15	94.70	7.01
	MS	5.27	4.54	1.67	6.6	0.9	14.05	1.30	0.07	0.02	16.18	97.91	**26.27**
	TS	**5.50**	**4.79**	1.45	7.3	1.7	**12.68**	1.60	0.07	0.03	15.47	93.10	11.04
	LSD(0.05%)	0.17	0.16	NS	NS	0.1	0.34	0.26	NS	NS	0.35	0.27	0.27
Obukpa	US	4.70	3.88	1.47	9.4	1.0	12.05	1.80	0.06	0.03	14.57	96.14	20.08
	MS	4.87	3.97	1.84	9.6	0.8	12.80	1.57	0.06	0.03	15.10	95.41	12.11
	TS	**5.15**	**4.42**	1.91	8.3	0.7	12.95	1.52	0.06	0.03	15.57	94.87	9.81
	LSD(0.05%)	0.17	0.16	NS	NS	0.1	0.28	0.31	NS	NS	0.36	0.31	0.27
Ozi–Edem	US	4.49	3.80	1.40	23.2	1.2	12.60	1.97	0.08	0.02	15.70	93.66	6.41
	MS	4.52	3.89	**1.87**	22.6	1.1	12.70	1.73	0.08	0.02	15.10	90.48	4.53
	TS	**4.64**	**3.95**	1.77	22.6	0.9	**13.80**	1.64	0.08	0.02	15.57	95.98	6.86
	LSD (0.05%)	NS	NS	0.35	NS	0.1	0.40	0.28	NS	NS	0.45	0.25	0.26

Table 8. *Main effect of sampling period on soil chemical properties in the three study sites*

Location	Sampling period	Soil pH H_20	KCL	EA cmol kg^{-1}	OM	TN	Exchangeable Bases (cmolkg$^-$) Ca^{2+}	Mg^{2+}	K^+	NA^{2+}	CEC	B.S	AP
					$g\ kg^{-1}$				cmolkg^{-1}			%	mg kg^{-1}
Lejja	1st	5.42	4.57	1.60	7.5	0.7	12.92	1.33	0.06	0.03	15.23	95.08	14.77
	2nd	5.25	4.58	1.40	7.3	0.7	12.82	**1.50**	0.06	0.03	15.00	**96.97**	14.77
	3rd	5.20	4.52	1.33	**5.9**	**1.8**	13.05	1.20	0.06	0.02	15.30	94.55	14.78
	LSD(0.05%)	0.17	NS	**NS**	0.6	0.1	NS	0.26	NS	NS	NS	0.27	NS
Obukpa	1st	5.05	4.15	1.63	9.7	1.0	12.95	**1.87**	0.06	0.03	15.67	96.15	10.93
	2nd	4.72	4.07	2.03	8.0	0.5	12.10	1.50	0.06	0.02	14.40	94.75	11.31
	3rd	4.95	4.05	1.50	9.5	1.0	12.83	1.52	0.06	0.03	15.17	95.51	10.88
	LSD(0.05%	0.17	NS	0.19	NS	**NS**	0.28	0.31	NS	NS	0.36	0.31	0.27
Ozi–Edem	1st	4.57	3.88	1.90	23.0	1.0	12.90	1.87	0.08	0.02	**16.27**	93.36	4.58
	2nd	4.48	3.87	1.80	22.4	0.9	12.87	1.83	0.08	0.02	15.93	92.94	4.51
	3rd	4.58	3.88	1.87	23.0	**1.2**	**13.33**	1.63	0.08	0.01	16.08	93.82	8.70
	LSD (0.05%)	NS	NS	NS	**NS**	0.1	0.40	NS	NS	NS	**NS**	0.25	0.26

Table 9. *Interactive effect of physiographic unit and sampling period on soil chemical properties in the three study sites*

Location	Unit	Sampling period	Soil pH		EA	OM	TN	Exchangeable Bases (cmol kg^{-})						AP
			H_20	KCL	Cmol kg^{-1}	(g kg^{-1})		Ca^{2+}	Mg^{2+}	K^{+} Cmol kg^{-1}	NA^{2+}	CEC	B.S %	mg kg^{-1}
	US	1st	5.03	4.10	1.30	7.4	0.6	12.20	1.10	0.05	0..03	14.10	94.81	7.96
	US	2nd	4.95	4.20	1.00	7.2	2.0	12.30	1.10	0.05	0.03	14.00	96.20	7.93
	US	3rd	5.30	4.75	1.20	6.0	0.6	12.10	1.20	0.08	0.02	14.35	93.10	5.13
Lejja	MS	1st	5.50	4.65	1.90	7.4	0.8	14.30	1.60	0.06	0.03	16.2	98.68	23.28
	MS	2nd	5.25	4.60	1.80	7.2	2.7	14.10	1.20	0.07	0.03	15.90	96.72	23.31
	MS	3rd	5.05	4.35	1.10	5.1	1.0	14.20	1.10	0.08	0.02	15.65	98.32	32.21
	TS	1st	5.70	5.25	1.60	7.2	3.1	12.70	1.30	0.07	0.04	15.40	98.68	13.06
	TS	2nd	5.55	4.95	1.11	7.5	0.6	12.50	2.45	0.07	0.03	15.10	97.98	13.06
	TS	3rd	5.25	4.45	1.30	6.6	3.9	13.10	1.30	0.07	0.02	15.40	94.16	7.00
		LSD(0.05)	0.29	0.28	0.54	2.8	0.2	0.58	0.45	0.00	0.00	0.61	0.47	0.46
	US	1st	4.85	3.90	1.80	10.2	1.2	12.40	2.50	0.06	0.03	15.30	91.88	9.38
	US	2nd	5.00	4.15	1.60	1.8	0.5	11.80	1.40	0.06	0.02	13.90	95.49	14.92
	US	3rd	4.75	3.85	1.70	9.9	1.2	12.20	1.50	0.06	0.03	14.50	95.04	4.39
Obukpa	MS	1st	4.80	3.85	1.60	10.0	0.9	13.40	1.50	0.05	0.04	15.60	96.06	13.58
	MS	2nd	4.60	4.05	1.30	8.7	0.6	11.70	1.90	0.05	0.03	14.60	93.57	9.23
	MS	3rd	4.70	3.75	2.60	9.9	0.9	13.30	1.30	0.06	0.03	15.20	96.61	4.71
	TS	1st	5.50	4.70	1.10	8.9	0.9	13.05	2.05	0.06	0.04	16.10	94.52	9.84
	TS	2nd	4.55	4.00	2.66	7.2	0.4	12.80	1.20	0.06	0.02	14.80	95.19	9.80
	TS	3rd	5.40	4.55	1.80	8.7	0.9	13.00	1.90	0.06	0.03	15.80	94.89	2.24
		LSD(0.05)	0.29	0.28	2.00	3.2	0.1	0.49	0.54	0.00	0.01	0.63	0.53	0.46
	US	1st	4.65	3.95	2.00	22.6	01.0	12.10	2.10	0.08	0.02	15.20	94.08	4.30
	US	2nd	4.50	3.90	1.60	22.2	1.0	12.10	2.20	0.09	0.02	15.20	94.75	4.20
Ozi– Edem	US	3rd	4.75	4.00	1.80	24.8	1.5	13.70	1.60	0.08	0.02	16.70	92.15	10.73
	MS	1st	4.6	3.90	1.60	23.1	1.2	12.80	1.80	0.08	0.02	16.50	89.20	4.71
	MS	2nd	4.50	3.90	2.00	22.2	1.1	12.90	1.70	0.09	0.02	16.50	89.22	4.67
	MS	3rd	4.45	3.85	2.00	22.4	1.1	12.40	1.70	0.08	0.01	15.24	93.04	4.20
	TS	1st	4.45	3.80	1.80	23.2	1.4	13.80	1.70	0.08	0.02	16.10	96.81	4.72
	TS	2nd	4.45	3.80	2.00	22.8	1.2	13.60	1.60	0.08	0.02	16.10	94.85	4.67
	TS	3rd	4.55	3.80	0.61	21.9	1.0	14.00	1.60	0.08	0.02	16.30	96.28	11.19
		LSD(0.05)	0.13	0.32	0.61	2.8	0.1	0.69	0.49	0.00	0.01	0.78	0.44	0.45

NB: OM = organic carbon, TN = total nitrogen, CEC = cation exchange capacity, AP = available phosphorus, BS = base saturation, EA = exchangeable acidity, US=upper-slope, MS middle-slope and TS= toe-slope

Degree of degradation class scores of the soil properties at the three locations based on sampling periods

This revealed that soil physical and chemical properties degraded differently under the different physiographic units. The soil properties degradation score ranges from 1, 2 through 3 to 4, representing non-slightly degraded, moderately degraded through highly degraded to very highly degraded, respectively. Soil properties with the highest-class score of degradation class 3 (highly degraded) and class 4 (very highly degraded) are total nitrogen, exchangeable K^+, base saturation and available phosphorus. The implication is that most arable crops will not perform optimally since they need these basic elements more in the soil.

Results in Table 11 show the overall aggregate degradation class score. The aggregate degradation scores varied from 37.5 to 66.7% representing moderate (S_2) to marginally suitable (S_3) land within the upper slope to toe slope continuum across the three locations.

Using the % aggregate score (AS) to group the soils, it was observed that the soils were highly and moderately degraded across the three sampling periods along the physiographic units. Obukpa up-slope in the 3rd sampling period has the highest aggregate score (66.7) while Ozi-Edem up-slope and toe-slope (3rd sampling period) has the least aggregate score (37.5). The 1st sampling period has the highest level of degradation followed by the 2nd sampling period and least at the 3rd sampling period.

Table 10. *Degree of degradation class scores of the three study areas based on sampling period and physiographic units across the location*

April/May									
Location	**Lejja**			**Obukpa**			**Ozi – Edem**		
Physiographic Unit	**US**	**MS**	**TS**	**US**	**MS**	**TS**	**US**	**MS**	**TS**
Bulk density	2	1	2	2	2	1	1	1	1
Organic matter	1	1	1	1	1	1	1	1	1
Total Nitrogen	4	3	1	4	3	3	4	4	4
Exchangeable K^+	4	4	4	4	4	4	1	1	1
Base saturation	1	1	1	1	1	1	1	1	1
Available phosphorus	2	1	1	1	1	1	4	4	4
% aggregate Score	**58.3**	**45.8**	**41.7**	**54.2**	**50,0**	**45.8**	**50.0**	**50.0**	**50.0**
July/August									
Bulk density	2	2	2	2	2	2	1	1	1
Organic matter	1	1	1	2	1	1	1	1	1
Total Nitrogen	4	1	1	4	4	4	1	1	1
Exchangeable K^+	4	4	4	4	4	4	4	4	4
Base saturation	1	1	1	1	1\	1	1	1	1
Available phosphorus	2’	1	1	1	1	1	4	4	4
% aggregate Score	**58.3**	**45.8**	**41.7**	**59.3**	**54.2**	**54.2**	**50.0**	**50.0**	**50,0**
October/November									
Bulk density	1	2	2	2	2	1	1	1	1
Organic matter (%)	1	1	1	1	1	1	1	1	1
Total Nitrogen	1	1	1	4	3	3	1	1	1
Exchangeable K^{+4}	4	4	4	4	4	4	4	4	4
Base saturation	1	1	1	1	1	1	1	1	1
Available phosphorus	4	1	3	4	4	4	1	4	1
% Aggregate Score	**50.0**	**41.7**	**50.0**	66.7	**62.5**	**58.3**	**37.5**	**50.0**	**37.5**

NB: Aggregate scores:-1. Non - slightly degraded soil (0- 25 %). 2. Moderately degraded soil (25-50%). 3. Highly degraded soil (50-75%). Very highly degraded soil (75-100 %). US= upper slope, MS= middle slope and TS=toe-slope

Table 11. *Percentage aggregate score of the degradation class of the physiographic units in the three locations*

Location	Physiographic Unit	% Aggregate Score	Degree of Degradation Class
	US	58.3	Highly Degraded
Lejja	MS	45.8	Moderately Degraded
	TS	41.7	Moderately Degraded
	US	54.2	Moderately Degraded
Obukpa	MS	50.0	Highly Degraded
	TS	45.8	Moderately Degraded
	US	50.0	Highly Degraded
Ozi – Edem	MS	50.0	Highly Degraded
	TS	50.0	Highly Degraded
	US	58.3	Highly Degraded
Lejja			
	MS	45.8	Moderately Degraded
	TS	41.7	Moderately Degraded
	US	58.3	Highly Degraded
Obukpa	MS	54.2	Highly Degraded
	TS	54.2	Highly Degraded
	US	50.0	Moderately Degraded
Ozi – Edem	MS	50.0	Highly Degraded
	TS	50.0	Moderately Degraded
	US	50.0	Highly Degraded
Lejja	MS	41.7	Moderately Degraded
	TS	50.0	Highly Degraded
Obukpa	US	66.7	Highly Degraded
	MS	62.5	Highly Degraded
	TS	58.3	Highly Degraded
Ozi - Edem	US	37.5	Moderately Degraded
	MS	50.0	Highly Degraded
	TS	37.5	Moderately Degraded

NB: Aggregate scores:-1. Non - slightly degraded soil (0- 25 %). 2. Moderately degraded soil (25-50 %). 3. Highly degraded soil (50-75%). Very highly degraded soil (75-100 %). US= upper slope, MS= middle slope and TS=toe-slope

Effect of location, physiographic unit and sampling period on the degree of degradation

The degree of degradation across locations, physiographic units and sampling periods are shown in figure 2-4. Figure 2 shows degree of degradation across physiographic units. The up-slope had the highest level of degradation followed by the middle-slope and least at the toe-slope. The up-slope had the highest level of degradation with a percent increase of 3.4 and 5.9 over the middle slope and toe-slope which had the lowest level of degradation. The mean % AS of the middle-slope was 2.6% higher than the toe-slope.

Upper slope being more degraded when compared to toe-slope, an implication that up- slope to be more prone to erosion. It has been shown that steeper land is more

erosion prone [5] and a more level landscape reduces the chance of extreme soil erosion [22].

Percent aggregate score in relation to the degree of degradation across locations is shown in Fig 3. Obukpa had the highest level of degradation with a percent increase of 0.62 and 10.8 over Lejja and Ozi-Edem, respectively. However, the mean % AS of Lejja was 10.2% higher than Ozi-Edem with the lowest % AS. The overall degradation score obtained in the upper-slope at Obukpa was higher (56.1%) than Lejja (48.0%) and Ozi -Edem (47.2%), representing a difference of 13.2% and 24.6% respectively. Also the aggregate degradation score for Obukpa is higher than Ozi -Edem by 13.1%. Given these, it can be said that Ozi -Edem upper-slope is less prone to degradation when compared to Obukpa and Lejja that have less vegetation covers.

In terms of sampling periods, figure 4 shows the recorded mean % aggregate score of 463.5%, 454.2% and 445.8% for third, second and first sampling periods, respectively. The third sampling period had the highest level of degradation with a percent increase of 7.56 and 13.7 over the second and first sampling periods, respectively. The mean % AS of the second sampling period was 50.% higher than the first sampling period. This shows the increment of degradation status of the soils: Oct/Nov > July/Aug > April/May. This phenomenon may be associated to soil erosion and leaching as well as other anthropogenic influences like crop harvesting and animal grazing.

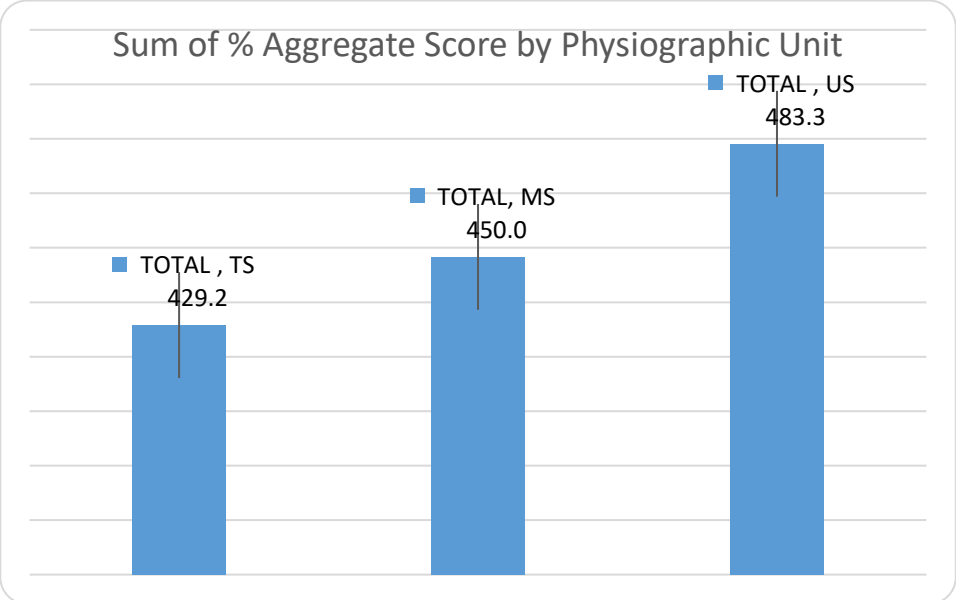

Fig. 2. *Percent aggregate score in relation to degree of degradation across the physiographic units in the study locations*

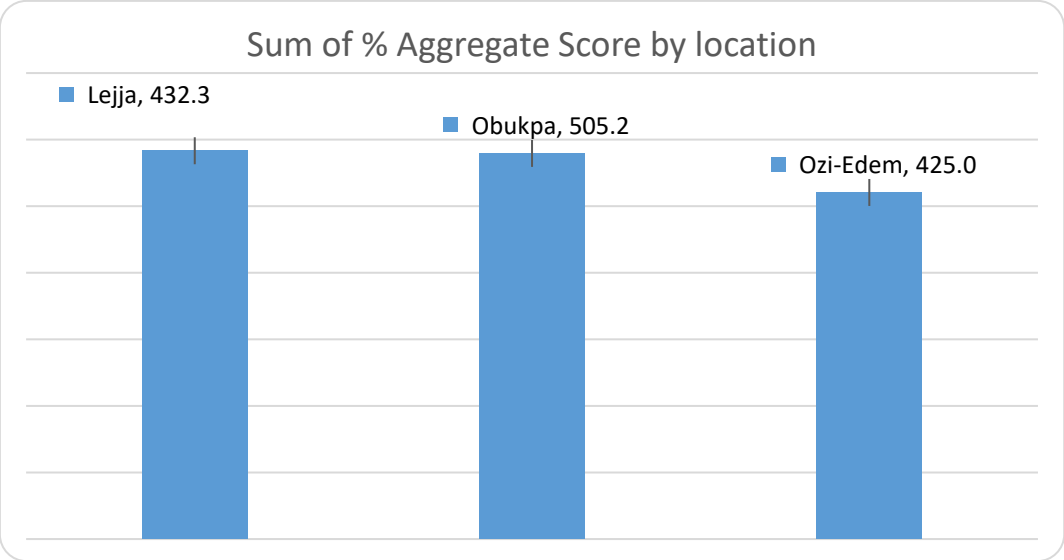

Fig. 3. *Percent aggregate score in relation to the degree of degradation across the three study locations*

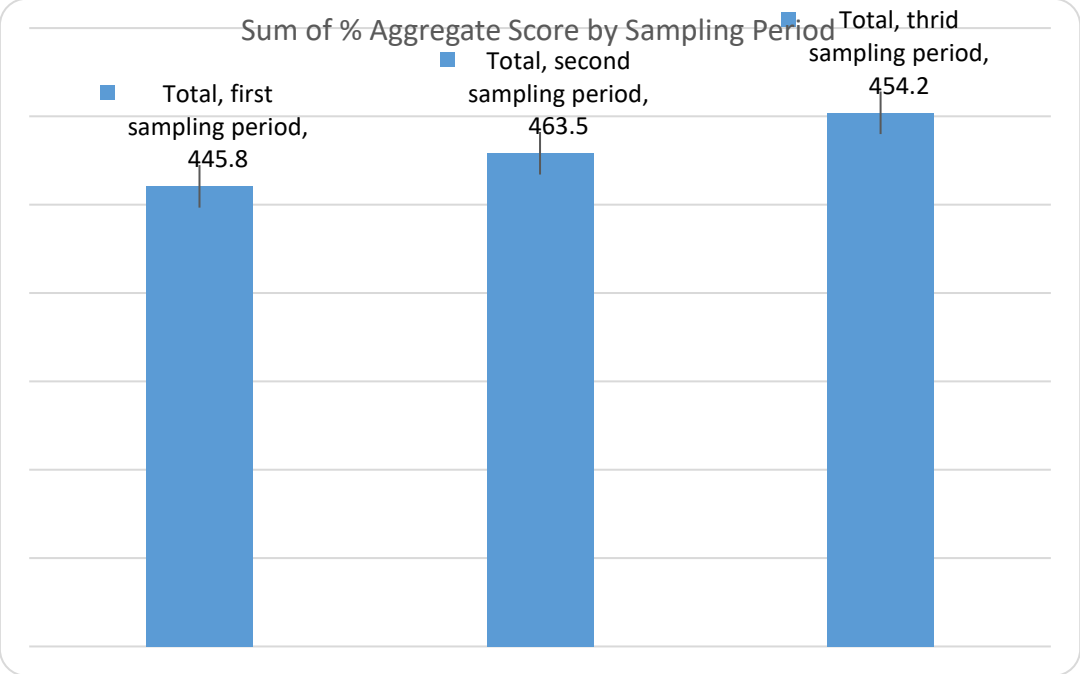

Fig. 4. *Degree of degradation across the sampling periods in the three study areas*

CONCLUSION

The results revealed that the study areas were moderately and highly degraded. The physical and chemical properties varied within the various physiographic units with the lower slope having higher nutrient contents due to minimal degradation relative to middle and upper slopes; suggesting that toe slope land could be better utilized for crop production. In terms of location, Obukpa had the highest level of soil degradation followed by Lejja and Ozi-Edem having the lowest degradation and were not

significantly different. Highest soil degradation was recorded during October/November periods relative to July/August and April/May periods.

Farmers need monitoring tools such as local level monitoring approach to help them assess the status of their soils since most farming soils in the study locations are vulnerable to erosion and by the time that degradation becomes visible and irreversible, it might be too late or very expensive to reverse it.There is potential for possible increase in crop yield if appropriate soil management practices are applied in the study locations. Therefore, integrating the following activities such as appropriate soil conservation measures, inclusion of restorative crops in cropping systems, use of locally available materials, and use of bio fertilizers are recommended to combat soil degradation problems and exploring the potential crop productivity. This study under pines the need for adequate information to intervene and solve soil degradation problems in the sub-humid tropics of Nigeria.

REFERENCES

[1] Ahn, P. M. (1970): West African soils. Oxford University Press, Oxford.
[2] Asadu, C.L.A. (1990): A Comparative characterization of two foot-slope soils in Nsukka area of Eastern Nigeria. Soil Science, 150: 527-534.
[3] Blake, G.R. and Hartge, K.H (1986): Bulk density, pp363-376. In A. Klute (ed.) Methods of Soil Analysis. American society of Agronomy/ Soil Science Society of America Monograph. No 9 Maidson Wisconain.
[4] Bremmer, J. M. and Mulverney, G.S. (1982): Nitrogen Total In; page et al (eds). Methods of Soil Analysis, part ASA No Madison Wisousm pp 595-624.
[5] Briones, R. M. (2009): Land Degradation and Rehabilitation in the Philippines final report submitted to: The World Bank Prepared by: Roehlano M., Consultant Senior Research Fellow Philippine Institute for Development Studies February. The Philippines Country Environmental Analysis.
[6] Chaberlin J. and Emily S. (2011): Ethiopian agric: adynamic geographic perspective, Development Strategy Support and Governance Division, Int. food Policy Res. Inst, Ethiopia Strategy Support Program Ethiopia. Google Scholars.
[7] Chapman, H. D. (1982): Total exchangeable bases. In C. A. Black (ed). Method of soil analysis Part II. Am. Soc . Agron., 9: 902 – 904
[8] Cresswell H.P and Hamilton (2002): Particles size analysis. In: Soil Physical Measurement and Interpretation for land evaluation. Eds. N.J. Mckenzie, H.P Cresswell and K.J. Coughlan) CSIRO Publishing: Collingwood, Victoria pp 224-239.
[9] Damene S. (2012): Effectiveness of wateras soil conservation measures for land restoration in the Wello area, northern Ethiopia high lands, In: Ecology and Dev. Series No. 29, Doctoral Dissertation, Univ. of Bonn, Germany. Grogle Scholar.
[10] Dee D., Dorne, J., Fazekas, O. B. and R. Horn (2008): Effect of bulk density on hydraulic properties of homogenized and structured soils. J. Soil Sc. Plant Nutr., 8 (1):1-13.
[11] Diwediga B, Wala K, Folega F, Dourma M, WoeganY.A., Akpagara K and G.B. Le (2015): Biophysical and anthropogenic determinants of landscape pattern and degradation of plant communities in Mo Hilly Basin (Togo).Ecol. Eng, 85 :132.
[12] Esu, I. E. (1983): Characterization and classification of dark clay soil of Biu plain. Nig. J. Soil Sci., 4: 80-91.
[13] Ezeaku P.I. and B.O. Unagwu (2014): Evaluating soil fertility and degradation rates as influenced by land use change, management and catenary sequence in north southeastern Nigeria. Chapter 6, pp 103-126. In : Bharti P.Kr and P.I. Ezeaku (eds) Water Resources

and Agriculture. Published by Discovery Publishing House PVT, Ltd. New Delhi-110 002, India. P. 214.

[14] Ezeaku, P. I. (2011): Methodologies for agricultural land use planning: sustainable soil management and productivity. Published by Great AP Express Publishers Ltd, Nsukka, Nigeria. Pp 248.

[15] Ezeaku, P.I. and F.U. Eze (2014): Effect of landuse in relation to slope position on soil property variations in a semi-humid Nsukka area, southeastern Nigeria. J. Agric. Res., 52 (3):369-381.

[16] Ezeokpube, N.D and C.J. Obiora (2013): Quantity of indiscriminately disposed sachet wastes generated in Nsukka Urban, Enugu State, Nigeria. Journal of Engineering and Applied Sciences, 2(1) :1-7.

[17] FAO (1979): Report on the Agro ecological zones project. Vol. 12. Results from South – West Asia. World soils resources rept. 48/2, FAO. Rome.

[18] Olsen S.R, and Sommers L.E (1982): Phosphorus. p. 403-434. In: Page *et al* (eds) Methods of Soil Analysis. Part 2. Agron 9. ASA, SSSA. Madison, W.I

[19] Gatreselassie Y; Anemut F. and S. Adclisu (2015): The effect of land use types, management practices and slope classes on selected physico-chemical properties in Zikre watershed, North-Western Ethiopia Springer open J. Env. Sys Res., 4: 1-7.

[20] Gee, G.W. and Bauder, J.W. (1986): Particle size analysis. P. 383-411. In: Klute, A (ed). Methods of Soil Analysis. Part 2, 2nd ed. Agron. Monog. 9. ASA and SSSA, Madison.

[21] Gidena T.R. (2016): Verification and Demonstration of Soil Test Based Phosphorus Fertilizer Recommendation Rate on yield of Teff (Eraghostis Tef (ZUCC) trotter) in vertisols of Northern Ethiapia. Journal of Natural Sciences Research. ISSN 2224-3186 (paper) ISSN 2225-0921. Vol. 6, No 1.

[22] Gilroy, A.M. (2006): Detecting Change in Soil Erosion in 8 Digit Hydrologic Units in Iowa: Correction between level of soil erosion and active conservation practices from 1992-1997. Resource Analysis, 8: 1-10.

[23] Haile W. and S. Boko (2011): Response of Irish potato (Solanum terberosum) to the application of K at acidic soils of chencha, southern Ethiopia. Int. J. Agric. Biol, 13:598 Google Scholar.

[24] Igwe CA. (1994): The applicability of SLEMSA and USLE erosion models on soils of southeastern Nigeria. PhD Thesis, University of Nigeria, Nsukka; 1994.

[25] Jones, M J. and Wild, A. (1975): Soils of the West African Savanna. Technical communication No. 55. Commonwealth Bureau of Soils. Harpenden. Pp 9-50.

[26] Khan, M.A. and Kamalakar, J. (2012): Physical and chemical properties of soils of newly established Agro-biodiversity Park of Acharya NG Ranga Agricultural University, Hyderabad, Andhra Pradesh. International Journal of Farm Sciences 2(2) :102-116

[27] Klute A. and C. Dirkson (1986): Hydraulic conductivity and diffusivity. Laboratory methods. In: Klute, A.(Ed), Methods of Soil Science Society of America, Madison, WI, 687-732.

[28] Leelavathi, GP., Naidu, M.V.S., Ramavatharam, N. and S.G. Karuna (2009): Studies on genesis, classification and evaluation of soils for sustainable land use planning in Yerpedu mandal of Chittoor district, Andhra Pradesh. J. Indian Soc. Soil Sci. 57:109-120

[29] Mclean, E.O. (1982): Soil pH and lime requirement. In: A.L. Page et.al. (eds.),Methods of soil analyses (No. 9, part 2), 199-224. American Society of Agronomy; Soil Science Society of American Inc. Madison, Wisconsin, U.S.A.

[30] Nelson D.W. and Sommers. I.E. (1982): Total Carbon, organic carbon and organic matter, in page, et al ed, method of soil analysis, part 2, 2nd 291 ed Agronomy Monograph No 9, ASA and SSSA, Madison, WI,PP 539-579.

[31] Ogbodo, E.N.(2012): Soil dynamic and vegetation diversity of an ecology, subjected to seasonal bush burning in Enugu State, Southeastern Nigeria. Nig. J, Soil Sci., 22 (2):298-307

[32] Onweremadu, E.U. and Mbah, C.N. (2009): Changes in epipedal development in soils of a gravelly hilly terrain. Nature and Science, 7(2): 40 -46
[33] Paix, M.J., Lanhai, L., Ahmed S. and Varenyam A. (2011): Soil Degradation and altered flood Risk as a consequence of Deforestation. https://doi.org/10./002/idr.1147
[34] Pillai, M.Y. and Natarajan, A. (2004): Characterization and classification of dominant soils of parts of Garakahalli watershed using remote sensing technique. The Mysore J. Agric. Sci., 38: 193-200.
[35] Rhodes, J.O. (1982): CEC in A.L. page R.H. Miller and D.R Keeney (eds) methods of soil analysis, part 2, Chemical and microbial properties. Madison Wisconsin: pp149-157.
[36] Senjobi, B. A. (2007): Parametric and conventional approaches for potential evaluation in three ecological zones of Southern Nigeria. Moor Journal of Agricultural Research, 2 (2): 91-102.
[37] Tel, D. and Rao F. (1982):Automated and semi-auto-mated methods for soil and plant analysis, IITA manual series No. 17:15-25.
[38] Thiemann S, Schutt B. and G. Forch (2015): Assessment of erosion and soil erosion processes-a case study for the northern Ethiopia highland. Vol 3, In: Tropic of Integrated Watershed Management-Proceedings. Pp 173-185 Google scholar.
[39] Yengoh G.F. (2012): Determinants of yield differences in small scale food crop farming systems in Cameroun. Agric. Food Security. 1:19 Google scholars.
[40] Yimer F. and A. Abdulkadir (2011): The effect of crop land fallowing on soil nutrient restoration in Bale mountains, Ethiopia. J. Sci. Dev., 1:43-51 Google scholar.

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Research Article

PHYTOCHEMICAL ANALYSIS AND ANTI-BACTERIAL SCREENING OF *Ajuga iva* L. EXTRACT FROM ALGERIA

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ABSTRACT. The objectives assigned to the present study are the phytochemical screening of several secondary metabolites and the evaluation of the antibacterial activity of the methanolic extracts of the different organs of *Ajuga iva* L. Phytochemical screening revealed the presence of substances with high therapeutic values (flavonoids, tannins, polyphenols, coumarin, etc.). The antibacterial activity of the extracts is carried out by the diffusion method on agar medium vis-à-vis twenty-three bacterial strains chosen according to the traditional use of this species in Algeria: *Bacillus cereus, Escherichia coli* (ATCC22), *Escherichia coli* (BLSE), *Escherichia coli* (ciproR), *Escherichia coli* (mcr1), *Klebsiella pneumoniae* (C+), *Klebsiella pneumoniae* (C-), *Klebsiella pneumoniae* Nassey Marseille, *Serratia sp, Salmonella sp, Pseudomonas aeruginosa* (ATCC53), *Pseudomonas sp, Pseudomonas aeruginosa* (VIM21), *Pseudomonas aeruginosa* (VIM22), *Acinetobacter* (NDM1), *Acinetobacter* (OXA23), *Staphylococcus aureus, Staphylococcus aureus* (23), *Staphylococcus aureus* (13), *Enterobacter cloacae* (FOSR1), *Enterobacter cloacae* (FOSR2), *Enterococcus faecalis* (Vanc R), *Enterococcus faecalis* (ATCC12). The study shows a remarkable antibacterial activity against all the microbial strains tested with the exception of *Enterobacter cloacae* (FOSR1).

Keywords: *Ajuga iva L., Guelma, phytochemical screening, methanolic extract, antibacterial effect*

INTRODUCTION

Medicinal plants represent a rich source of antimicrobial agents. A wide variety of natural products are used in the treatment of common infection in traditional medicine in developing countries [1]. The routin use of antibiotic has led to the development of one or more antibiotics resistant infectious bacteria. This issue has resulted on the failure of the treatments to numerous microbial causing infectious diseases. Previous investigations pointed out to a number of medicinal plant extracts by constituting a group of potent natural antimicrobial agents [2].

Ajuga iva is a small (5-10 cm) wooly, aromatic perennial herb with well documented anti-ulcerous, hypoglycemic and anti- inflammatory activities. Topically, it has been used for wound healing and breast hardness [3]. It was mentioned as "*Chendgoura"* in traditional Algerian medicine.

Ethnopharmacological surveys have revealed that some 20 species of *Ajuga* plants are used in traditional medicine mostly in Africa, Asia and China. In North Africa, *Ajuga* plants are used to treat diabetes and hypertension [4, 5]. Other reported activities of *Ajuga* plants include antibacterial, antifungal, anti-inflammatory, antimalarial/antiplasmodial, antimycobacterial, antioxidant, antipyretic, larvae and insect antifeedant and insect growth inhibitor activity [6].

The aim of this study was to evaluate anti-bacterial effects of methanolic extract from leaf, stem and root of *Ajuga iva* L. against twenty-three bacteria. Furthermore,

phytochemical analysis was also carried out for justifying the possible reason of antibacterial efficacy.

MATERIALS AND METHODS

Plant Material

The species *Ajuga iva* L. was harvested in the region of Guelma (north-eastern Algeria) in February 2019.

The identification of the plant was done with the key to determining the flora of Quezel and Santa [7]. Specimens were kept at the Laboratory of Cryptogamy and Medical Botany, Department of Pharmacy, Faculty of Medicine Annaba-Algeria.

Preparation of Methanolic Extracts

Dry parts (stem, leaf and root) of *Ajuga iva* L. have been ground and stored in glass bottles, hermetically sealed at low temperatures. 10 g of the vegetable powder was macerated in 100 ml of methanol with stirring for 24 hours at a temperature of 25 ± 2° C. The extract obtained was filtered and evaporated to dryness under reduced pressure at 50° C on a rotavapor. The dry residue is taken up in 3 ml of methanol and stored at -18 ° C until it is used [8].

The yield of the methanolic extracts was calculated by the following formula: R (%) = (M/M_0) ×100. With: R (%): yield expressed in%; M: mass in grams of the resulting dry extract; M_0: mass in grams of the plant material to be treated.

Phytochemical Screening

Phytochemical tests of the powders of the stem, leaf and root of *Ajuga iva* L. obtained are made from precipitation reactions or characteristic staining in order to highlight chemical groups that may be present in this species. The tests are carried out according to the protocols described by Diallo and al., and Senhadji et al. [9, 10].

The results are classified according to the appearance in:
Frankly positive reaction: +++;
Positive reaction: ++;
Moderately positive reaction: +;
Shady reaction: ± ;
Negative reaction: -.

Antibacterial test

The test of the sensitivity of the bacteria is carried out by the diffusion method in agar medium (the disk method). It is a method similar to that of the antibiogram which consists in determining the sensitivity of a bacterial strain vis-à-vis one or more substances [8].

The antibacterial activity of the methanolic extracts of the stem, leaf and root of *Ajuga iva* L. is evaluated vis-à-vis twenty-three bacterial strains chosen according to the traditional use of this species in Algeria: *Bacillus cereus, Escherichia coli* (ATCC22), *Escherichia coli* (BLSE), *Escherichia coli* (ciproR), *Escherichia coli* (mcr1), *Klebsiella pneumoniae* (C+), *Klebsiella pneumoniae* (C-), *Klebsiella pneumoniae* Nassey Marseille, *Serratia sp, Salmonella sp, Pseudomonas aeruginosa* (ATCC53),

Pseudomonas sp, Pseudomonas aeruginosa (VIM21), *Pseudomonas aeruginosa* (VIM22), *Acinetobacter* (NDM1), *Acinetobacter* (OXA23), *Staphylococcus aureus, Staphylococcus aureus* (23), *Staphylococcus aureus* (13), *Enterobacter cloacae* (FOSR1), *Enterobacter cloacae* (FOSR2), *Enterococcus faecalis* (Vanc R), *Enterococcus faecalis* (ATCC12). These strains were kindly provided by the Microbiology Laboratory Manager at Annaba Medical School, Algeria.

Preparation of The Inoculum

By taking twenty-three tubes that each contains 5 ml of sterile physiological saline. Using a platinum loop, some well isolated colonies are scraped from each of the bacteria, each of which will be discharged into a tube.

For the preparation of the different concentrations of extracts, 2, 5 mg of each freeze-dried extract (methanolic extract of leaf and stem), are introduced into a labeled tube, in which we added 1 ml of dimethylsulfoxide (DMSO), solvent without any antibacterial effect. The tubes are vortexed until complete dissolution of the extract, and the dilutions are prepared to obtain X/4, X/8 and X/16 concentrations from the stock solution.

Seeding should be done within 15 minutes after the preparation of the inoculum. In 69 sterile Petri® dishes, 20 ml of agar are poured. After solidification of the medium, the latter is inoculated with 1 ml of bacteria to be studied. Then, it is spread on the surface using a glass rake.

Sterile 5 mm diameter disks prepared in Whatman® n°1 papers are impregnated with a sterile metal forceps in each concentration and placed on the surface of the solidified medium (Mueller-Hinton). The dishes were incubated for half an hour at room temperature, then for 24 to 48 hours in an oven at 37 ° C.

The reading is carried out by measuring the diameter of the inhibition zone (Ø), which translates into a translucent halo around each disc; the presence or absence of a halo would explain the sensitivity or the resistance of the germs vis-a-vis extracts tested; according to a symbolic notation scale from - to +++ [11].

Table 1. *Sensitivity of microbial strains according to zones of inhibition*

Sensitivity	Inhibition zone
Not sensitive or resistant (-)	Diameter <10 mm
Sensitive (+)	Diameter between 10 to 16 mm
Very sensitive (++)	Diameter between 16 to 25 mm
Extremely sensitive (+++)	Diameter > 25 mm

RESULTS AND DISCUSSION

Phytochemical Screening

The phytochemical screening tests shown in Table 2 show the following results:

-The presence of mucilage, polyphenols, coumarins, tannins, flavonoids, reducing compounds, saponosides**,** flavonoids, carotenoids, iridoids in the plant studied;

-The absence of anthocyanins, alkaloids, triterpenes and starch in the all parts of the plant.

Table 2. *Phytochemical Screening Tests*

		Leaf	Stem	Root
Mucilage		+++	+++	+++
Polyphenols		+++	+++	-
Anthocyanins		-	-	-
Coumarins		+++	+++	++
Tannins	Condensed tannins	+++	+++	+++
	Hydrolyzable tannins	+++	+++	+++
Flavonoids	Flavonoids	+++	+++	+++
	Flavonols	Flavonols	Flavanones	Flavones
Reducing compounds		+++	++	++
Alkaloids		-	-	-
Saponosides		++ (foam index less than 100)	+++ (foam index= 157)	-
Anthracene derivatives	Free derivatives	-	-	-
	O-glycoside	++	++	-
	C-glycoside	+++	+++	+++
Cardiotonic glycosides		-	+	+
Sterols, triterpenes, carotenoids, iridoids	Triterpenes	-	-	-
	Sterols	+++	++	-
	Carotenoids	+++	++	+
	Iridoids	+++	+++	+++
	Starch	-	-	-

Extraction Yield

The yield, appearance and color of the methanolic extracts of the stem, leaf and root of *Ajuga iva* L. are shown in Table 3.

These results show that the yield of the methanolic extract of the leaf is the highest, with a percentage of 6.33%, and it is pasty dark green.

Table 3. *Extraction yield of Ajuga iva L., by the methanol mixture*

	Extraction Solvent	Color of the extract	Aspect	Yield in%
Stem	Absolute methanol 99%	Green	Pasty	1.33%
Leaf	Absolute methanol 99%	Dark green	Pasty	6.33%
Root	Absolute methanol 99%	Dark yellow	Pasty	1%

Reading Antibiograms

Results showed that the leaf, stem and root of *Ajuga iva* L. possessed antibacterial activity against all the microbial strains tested with the exception of *Enterobacter cloacae* (FOSR1). However, the most antibacterial effect has been observed against *Escherichia coli* (ATCC22) and *Klebsiella pneumoniae* Nassey Marseille (diameter of

zone of inhibition= 19,2mm “leaf/root” and 15,3mm “leaf”, respectively). The zone of inhibition by each organ extract in the nutrient agar media is represented in Table 4.

Table 4. *Inhibition Diameter (mm) of methanolic extracts of Ajuga iva* L.

	Dilutions of leaf extract				**Dilutions of stem extract**				**Dilutions of root extract**			
	x/4 (47.5 mg/ml)	x/8 (23.75 mg/ml)	x/16 (11.87 mg/ml)	x/32 (5.93 mg/ml)	x/4 (10 mg/ml)	x/8 (5 mg/ml)	x/16 (2.5 mg/ml)	x/32 (1.25 mg/ml)	x/4 (7.5 mg/ml)	x/8 (3.75mg/ml)	x/16 (1.87mg/ml)	x/32 (0.93mg/ml)
***Escherichia coli* (ATCC22)**	10.8 mm	19.2 mm	16.7 mm	9.5 mm	6.2 mm	8.5 mm	11.7 mm	7.6 mm	19.2 mm	10.3 mm	8.1 mm	9.5 mm
***Escherichia coli* (BLSE)**	8.1 mm	9.2 mm	9.7 mm	11.2 mm	8.4 mm	10.5 mm	10.1 mm	9.1 mm	6.5 mm	10.1 mm	6.2 mm	6.2 mm
***Escherichia coli* (ciproR)**	8.5 mm	9.2 mm	9.5 mm	10.5 mm	6.7 mm	9.2 mm	8.1 mm	8.5 mm	7.7 mm	8.1 mm	8.5 mm	7.4 mm
***Escherichia coli* (mcr1)**	11.1 mm	11.3 mm	11.1 mm	11.1 mm	10.6 mm	11.1 mm	11.1 mm	11.3 mm	12.1 mm	11.2 mm	11.4 mm	10.5 mm
***Klebsiella pneumoniae* (C+)**	8.7 mm	9.1 mm	10.5 mm	10.8 mm	6.2 mm	9.2 mm	8.5 mm	9.3 mm	11.4 mm	11.4 mm	10.1 mm	11.6 mm
***Klebsiella pneumoniae* (C-)**	10.1 mm	10.5 mm	7.5 mm	6.4 mm	6.2 mm	9.7 mm	8.5 mm	8.5 mm	11.8 mm	9.5 mm	10.9 mm	10.9 mm
***Klebsiella pneumoniae* Nassey Marseille**	14.5 mm	15.3 mm	15.3 mm	9.5 mm	7.2 mm	11.5 mm	11.7 mm	11.7 mm	11.1 mm	12.9 mm	8.3 mm	10.1 mm
Serratia sp	8.5 mm	9.2 mm	10.1 mm	8.5 mm	6.4 mm	8.2 mm	8.4 mm	6.2 mm	9.6 mm	9.5 mm	7.2 mm	7.2 mm
Salmonella sp	9.5 mm	6.2 mm	6.2 mm	9.1 mm	6.2 mm	6.2 mm	10.1 mm	9.3 mm	6.8 mm	6.8 mm	7.1 mm	6.4 mm
***Enterobacter cloacae* (FOSR1)**	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm	6.2 mm
***Enterobacter cloacae* (FOSR2)**	9.1 mm	6.6 mm	6.6 mm	6.8 mm	6.8 mm	6.3 mm	6.3 mm	6.2 mm	8.6 mm	9.8 mm	6.5 mm	6.2 mm
Bacillus cereus	7.1 mm	9.1 mm	10.7 mm	6.2 mm	6.2 mm	8.1 mm	8.1 mm	9.1 mm	10.1 mm	10.2 mm	10.7 mm	12.6 mm
***Pseudomonas aeruginosa* (ATCC53)**	9.2 mm	9.2 mm	9.2 mm	6.3 mm	6.3 mm	9.1 mm	6.3 mm	6.3 mm	16.1 mm	15.6 mm	8.1 mm	7.6 mm
Pseudomonas sp	9.1 mm	8.8 mm	10.3 mm	10.3 mm	7.6 mm	9.1 mm	12.5 mm	12.8 mm	10.2 mm	8.4 mm	9.1 mm	9.1 mm
***Pseudomonas aeruginosa* (VIM21)**	9.8 mm	10.2 mm	10.4 mm	9.3 mm	10.4 mm	8.1 mm	8.1 mm	10.5 mm	10.4 mm	10.9 mm	10.9 mm	10.9 mm
Pseudomonas aeruginosa	7.7 mm	7.7 mm	10.1 mm	10.1 mm	6.4 mm	9.1 mm	9.2 mm	8.5 mm	6.3 mm	8.2 mm	7.6 mm	7.6 mm

(VIM22)												
***Acinetobacter* (NDM1)**	6.3 mm	8.7 mm	9.5 mm	6.3 mm	6.3 mm	6.8 mm	6.2 mm	6.4 mm	7.1 mm	7.1 mm	6.2 mm	9.1 mm
***Acinetobacter* (OXA23)**	8.5 mm	8.4 mm	6.3 mm	6.3 mm	6.2 mm	8.2 mm	9.3 mm	8.8 mm	6.3 mm	6.3 mm	8.1 mm	10.1 mm
Staphylococcus aureus	6.7 mm	6.8 mm	6.7 mm	8.5 mm	6.3 mm	9.1 mm	9.7 mm	6.7 mm	10.1 mm	6.2 mm	7m m	7m m
***Staphylococcus aureus* (23)**	9.4 mm	10.5 mm	10.2 mm	10.1 mm	8.1 mm	10.1 mm	10.1 mm	10.1 mm	7.4 mm	8.1 mm	9.1 mm	9.1 mm
***Staphylococcus aureus* (13)**	7.1 mm	8.5 mm	9.2 mm	7.3 mm	7.1 mm	7.2 mm	8.4 mm	9.7 mm	11 mm	8.5 mm	10.5 mm	11.1 mm
***Enterococcus faecalis* (Vanc R)**	9.8 mm	10.1 mm	10.2 mm	10.1 mm	8.1 mm	8.1 mm	9.4 mm	9.8 mm	9.8 mm	10.5 mm	10.5 mm	10.5 mm
***Enterococcus faecalis* (ATCC12)**	6.4 mm	7.5 mm	7.5 mm	7.5 mm	6.7 mm	9.1 mm	7.5 mm	8.5 mm	13.5 mm	13.2 mm	10.1 mm	12.4 mm

The variation in yield and biological activity could be attributed to difference in the type and amount phytochemicals concentrated during growth of the plant. The variation in phytochemicals is an attribute of differences in soil, age, seasons, climate and type of vegetation among the ecological zones [12], phytochemical production in plants varies with the geographical location. Plant developmental stage influences secondary metabolism; defense compounds are generally more concentrated and diverse when plants are young and more "apparent" to herbivores, but they are known to decrease with age as structural defenses are developed [13,14].

The phytoconstituents alkaloids, glycosides, flavonoids and saponins are antibiotic principles of plants, these antibiotic principles are actually the defensive mechanism of the plants against different pathogens [1].

The *Ajuga iva* L. methanolic extracts showed varied antimicrobial activity against the bacteria used in this study (Table 4). Among the tested microorganisms, *Escherichia coli* (ATCC22) was the most susceptible microorganism against leaf and root extracts (19,2mm) followed by *Klebsiella pneumoniae* Nassey Marseille against leaf extract (15,3mm).

The high antibacterial efficacy in the methanolic extract possibly is due to the presence of tannins, flavonoids and terpenoids. These medically bioactive ingredients practice antimicrobial activity through various mechanisms. Flavonoids, which have been found to be effective antimicrobial substances against a wide array of microorganisms in vitro, are known to be synthesized in response to microbial infection by plants. They have been found to exhibit antimicrobial activity through various mechanisms like inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and energy metabolism [15]. They have also the ability to complex with extracellular and soluble proteins and to complex with bacterial walls [16]. The saponins seems to involve membranolytic properties [17], they have the capacity to rise leakage of metabolites from the cell [18]. Tannins cause cell wall synthesis inhibition by forming irreversible complexes with prolene rich protein [19]. Terpenoids cause dissolution of the cell wall of microorganism by undermining the membranous tissue.

There is also some evidence that minor components have a critical part in antibacterial activity, possibly by producing a synergistic effect between other components [1].

The absence of inhibition zone does not necessarily mean that compounds are inactive. For example, non-polar compounds may not diffuse into the culture medium [1].

In previous studies, antibacterial effect of extract from *Ajuga iva* L. were investigated: *Neo*-Clerodane diterpenoids (lupulins A, B and D) isolated from *A. lupulima* exhibited antibacterial activity against *Pseudomonas aeruginose* and *Escherichia coli* [20]. According to the report of Chaari et al. [21], phytoecdysteroids from *A. pseudovia* and also 8-o-actetylharpagide from *Ajuga iva* can possess antibacterial activity. While Mamadalieva et al. [22] showed that the isolated ecdysteroids from *Ajuga turkestanica* were weak antimicrobial compounds. Nevertheless, the chloroform extract of *Ajuga turkestanica* showed antimicrobial activity against multi resistant strains such as *Staphylococcus aureus* MRSA ATCC 1000/93 and *Streptococcus pyogenes* ATCC 12344 [20,22]. Additionally, two isolated compounds 14, 15-dihydroajugapitin and 8-o-acetylharpagide from the aerial parts of *Ajuga bracteosa* showed antibacterial activity against *Escherichia coli* with zone of inhibitions of 25.0 ± 1.4 mm and 22.6 ± 0.9 mm respectively [22].

CONCLUSION

The preliminary phytochemical screening indicates that the plant consist different types of phytoconstituents such as flavonoids, tannins, polyphenols, coumarin, etc., possess significant bioactive properties. The study of antibacterial efficacy against the twenty-three bacterial strains revealed that the methanolic extracts of the leaf, stem and root of the *Ajuga iva* L. from Guelma, Algeria have ability to kill or inhibit the growth of bacteria which experimentally support the use of this plant in traditional medicine by traditional healers.

Extensive research should be carried out on phytochemicals of this plant for the development of cost effective drugs for future.

REFERENCES

[1] Tirumalasetty, J., Basavaraju, A.P. (2014): Antimicrobial activity of methanolic extracts of *Azadirachta indica*, *Rosmarinus officinalis* and *Lagenaria siceraria* leaves on some important pathogenic organisms. J. Chem. Pharm. Res 6(4):766-770.

[2] Jaafreh, M., Khleifat, K.M., Qaralleh, H., Al-limoun, M.O. (2019): Antibacterial and Antioxidant Activities of *Centeurea damascena* Methanolic Extract . Journal of basic and applied Research in Biomedicine 5(1): 55-63.

[3] Sadati Lamardi, S.N., Tahroodi, S., Khanavi, M., Hosseini Doust, R. (2017) : Antibacterial Activity of Aerial Part Extracts and Fractions of *Ajuga chamaecistus* ssp. *tomentella.* Trad Integr Med 2(2): 61-66.

[4] Eddouks, M., Ouahidi, M.L., Farid, O., Moufid, A., Khalidi, A. Lemhadri, A. (2007): The use of medicinal plants in the treatment of diabetes in Morocco. Phytotherapie 5: 194-203.

[5] Tahraoui, A., El-Hilaly, J., Israili, Z.H., Lyoussi, B. (2007): Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). J. Ethnopharmacol 110: 105-117.

[6] Israili, Z.H., Lyoussi, B. (2009): Ethnopharmacology of the plants of the genus *Ajuga*. Pak. J. Pharm. Sci 22(4): 425-462.
[7] Quezel, P., Santa, S. (1963): Nouvelle flore de l'Algérie et des régions désertiques méridionales. Ed Centre national de la recherche scientifique, 1170 p.
[8] Falleh, H., Ksouri, R., Chaieb, K., Karray-Bouraoui, N., Trabelsi, N., Boulaaba, M., Abdelly, C. (2008): Phenolic composition of *Cynara cardunculus* L. organs, and their biological activities. Comptes Rendus de Biologie331: 372-379.
[9] Diallo, A. (2005): Étude de la phytochimie et des activités biologiquesde *Syzygium guineense* Willd. (Myrtaceae). Thèse de pharmacie, Bamako 98 p.
[10] Senhadji, O., Faid, M., Elyachioui, M., et al. (2005): Étude de l'activité antifongique de divers extraits de cannelle. J Mycol Med 15: 220–9.
[11] Ounaissia, K., Bennadja, S., Aliane, L., Djahoudi, A. (2020): Phytochemical screening and anti-bacterial activity of methanolic extracts of the aerial parts of *Atriplex halimus* L., from Biskra (Algeria). International Journal of Agricultural and Natural Sciences 13(1): 26-33, 2020.
[12] Daniel, B., Innocent, E., Mbwambo, Z.H., Musharaf, S.G. (2011): Comparison of mosquito larvicidal activity of *Annona squamosal* leaves growing in different eco-zones in Tanzania. International Journal of Pharmacology and Bio Sciences 2(4): 566-572.
[13] Feeny, P. (1976): Plant apparency and chemical defense. In: Wallace, J. W., Mansell, R.L. (Eds.), Biochemical Interactions between Plants and Insects. Recent Advances in Phytochemistry, vol. 10: Plenum, NY, pp.1–40.
[14] Shannon, E.B., John, T.A., Bernard, R.B. (2002): Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). Biochemical Systematics and Ecology 30: 837–854.
[15] Cushnie, T.P., Lamb, A.J. (2005): Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 26: 343–56.
[16] Cowan, M. M. (1999): Plant products as antimicrobial agents. Clinical microbiology reviews, 12(4), 564-582.
[17] Firas, A., Hassan, F. (2008): Antibacterial and antifungal activities of different parts of *Tribulus terrestris* L. growing in Iraq. J Zhejiang Univ Sci B 9(2):154-59.
[18] Zablotowicz, R.M., Hoagland, R. E., & Wagner, S. C. (1996): Effect of saponins on the growth and activity of rhizosphere bacteria. In Saponins Used in Food and Agriculture. Springer, Boston, MA, pp. 83-95.
[19] Mamtha, B., Kavitha, K., Srinivasan, K. K., & Shivananda, P. G. (2004): An in vitro study of the effect of Centella asiatica [Indian pennywort] on enteric pathogens. *Indian Journal of Pharmacology 36*(1), 41.
[20] Chen, H., Tan, R.X., Liu, Z.L., Zhang, Y., Yang, L. (1996): Antibacterial neoclerodane diterpenoids from *Ajuga lupulina*. J Nat Prod 59:668-670.
[21] Chaari, A., Jannet, H.B., Mighri, Z. (2000): Isolation and structural determination of a novel antibacterial phytoecdysteroid and of two cinnamic acid derivatives based on *Ajuga pseudoiva* Leaves. J Soc Chim 4:789-799.
[22] Mamadalieva, N.Z., El-Read, M.Z., Ovidi, E., Ashou, M.L., Hamoud, R., Sagdullaev, S.S. (2013): Antiproliferative, antimicrobial and antioxidant activities of the chemical constituents of *Ajuga turkestanica*. Phytopharmacology 4:1-18.

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Research Article

MARKER ASSISTED BACKCROSS BREEDING FOR FUSARIUM WILT (*Fusarium Oxysporum Schlecht. F. Sp. Melongenae*) IN EGGPLANT

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ABSTRACT. Eggplants are produced in both greenhouses and open fields. Plant diseases and pests, and disease causes significant yield, thus economic losses loss. Fusarium wilt (*Fusarium oxysporum Schlecht.f. sp. melongenae*, FOM) is a major soil-borne pathogen, causing vascular wilt disease in eggplant. A molecular marker tightly linked to single dominant gene (FOM) was developed for use in marker assisted selection (MAS). The aim of the study is to develop eggplant lines resistant against Fusarium wilt using a marker assisted backcross breeding approach. Donor parents were carrying the *Fusarium wilt* resistance gene that six commercial hybrids claimed to have fusarium wilt resistance. The eggplant breeding materials (F1 to F8) was first screened with molecular markers linked to the FOM gene. Then, the 533 young seedlings were root-dip inoculated with FOM isolate. The seedlings identified to be resistant using the markers all survived in the inoculation. Although, the six hybrids that did not possess the marker locus for resistance against FOM were all resistant according to classical test. BC1F1, BC2F1 and BC3F1 population were developed from sensitive female and resistance male(commercial hybrids) crossing and all populations tested as classical and molecular. From resistance BC3F1 progenies 25 DH plants were obtained from each combination and resistance status of these plants was determined through initially molecular then classical testings. Results indicate that the marker was reliable to develop eggplant lines resistant against fusarium wilt, and there may can be another source of resistance that is independent from the known resistance gene originating from LS2436.

Keywords: *Disease, Hybrid Eggplant, Marker Assisted Selection, Resistance*

INTRODUCTION

Eggplant (*Solanum melongena* L.) is the third most economically important *Solanaceous* crop after potato and tomato [1]. Eggplant is one of the most cultivated fruit plants worldwide with an 1.858.253 ha harvested area and worldwide annual production is, production is more than 50 million tons in the world [2].

Eggplant cultivated lands are mostly located production area are mainly within subtropical zones and productions are performed either in for both greenhouses or and open fields. , worldwide production area and total yield is condensed in Asia, Africa, Mediterranean Basin and South America are the major eggplant producer continents [3].

In European countries, eggplant is an outlandish vegetable but in Asia and the Mediterranean, it is an important and valuable source of nutrient ingredient, thus it is called as the “king of vegetables” [4].

Eggplant offers the possibility of improvement through heterosis breeding and continues to be a choice of breeders for exploitation of heterosis due to the hardy nature of the plant, comparatively large size of flowers, and large numbers of seeds produced by a single act of pollination. Increased productivity can be achieved in the shortest time can be achieved through heterosis breeding [5]. Highly varied consumer preferences have acceptance directed researchers to demands development of high-yielding F1 hybrids. Exploitation of hybrid vigor has become a potential tool for improvement in eggplant [6], [7]; [8]. The estimation of heterosis for yield and its component characters would be useful in determining the best hybrid combination. Knowledge on genetics of resistance helps in determining the most appropriate breeding method.

Eggplant is susceptible to various diseases especially *fusarium*, *verticillum* and *bacterial wilt* [9]. Soil-borne diseases (e.g. bacterial and fungal wilts, nematodes) and insects are the most serious diseases causing of great losses reduces in yield and quality of eggplants both in greenhouses and in open fields cultivations. Fungal wilts caused by *Verticillium dahliae* (Vd) Kleb. and *Fusarium oxysporum f. sp. melongenae* (FOM) are two of the main diseases in eggplant.

Fusarium wilt, is one of the most devastating and widespread outspreaded diseases of eggplant. Matsuo and Ishigami [10] was published the first study for *Fusarium Wilt* and then, fundamental researches have been conducted printed with the aim of identify resistant eggplant allies.

Fungus penetrates into the roots and proliferates in the vascular tissue. Wilting progresses from lower to upper leaves, followed by collapse of the plant. When the stem and roots are cut diagonally, reddish-brown streaks are visible in the vascular tissues. [11]. Pathogen can live in the soil for many years [12].

Fungicides cannot control Fusarium wilt effectively, other solutions, such as soil fumigation or grafting might work well but they constitute are either additional cost items and may exert threats on hazardous to the environment and human healthy [13]. For economic and safety reasons, resistant crop breeding is a most efficient way to avoid from this disease [14].

A source of resistance against Fusarium wilt was resistance source has been identified in *S. aethiopicum* Gilo Group and *S. aethiopicum* Aculeatum Group both are relatives of which are *Solanum melongena*'s relatives [15]. LS1934, LS174, and LS2436, have resistance and these eggplants were been defined as to be completely source of resistance source [16]; [17]. *Rf1* is a single dominant identifed as a resistance locus with Cleaved Amplified Polymorphic Sequences (CAPS) tightly linked to the gene of interest[18]. SCAR markers linked to a *Fusarium* Resistance locus in eggplant line, LS2436 with bulked segregant analyses were published by Mutlu in [15].

Conventional breeding and molecular marker analysis can be used to increase disease resistance and, improve yield traits of and use cultivated eggplant. A molecular marker tightly linked to single dominant gene (FOM) was developed for use in marker assisted selection (MAS).

The primary objective of the present study aim of the study is to develop eggplant lines resistant against Fusarium wilt with the use of using a marker- assisted backcross breeding approach. This makes available economic damage to producers can be prevented. And breeding with molecular marker assisted will make time consuming for breeders.

MATERIALS AND METHODS

Plant Materials

120 eggplant lines, 4 commercial cultivars, pGM1F1, pGM2F1 and pGM3F1 progenies were used as plant materials.

Molecular Marker Screening

Molecular marker assisted selections were performed in accordance with Mutlu et al. (2010). Parent (F1, BC1F1, BC2F1, BC3F1 and DH plants) DNA was extracted from young leaves using a modified CTAB extraction protocol (Doyle and Doyle 1990). PCR reactions were performed in 15µL volumes in Akdeniz University, Agricultural Biotechnology Laboratory (MJ RESEARCH PTC-225 Peltier Thermal Cycler). All PCR products were separated on a 1.5% agarose gel (Thermo scientific Gel Tank), visualized with ethidium bromide staining under ultraviolet light, and photographed with Minilumi, DNR Bio-Imaging Systems. Classical testing for *Fusarium Wilt* Resistance Using *Fusarium Oxysporum f.sp.* Melongeae Isolate The *Fusarium oxysporum f.sp.* Melongeae isolate was supplied from BATEM institute, Antalya, Turkey. The *Fusarium oxysporum* melongenae isolate was grown on the potato dextrose agar (PDA) at 24°C in dark for 10 days. Liquid medium was prepared from this culture. Liquid cultures were shaken at 50 rpm in a rotary shaker for 8 days at 24 - 25°C. The suspensions were filtered through cheesecloth. The spores were re-suspended and spore density was adjusted to 1 × 106 conidia/ml. Seedling roots were washed with clean tap water and freed from the soil. The 1/3 of roots were first trimmed with a sterile scissor to create scar tissue to promote infection. Wounded roots were submerged into the beaker containing 106 concentration of FOM isolate for 5 minutes (Herman and Perl-Treves, 2007; Karimi et al. 2010). For control groups, 12 seedlings from each of parents were submerged either into distilled water or into FOM isolate. The seedlings were planted into small pots and maintained in the nursery. Seedlings were planted into 48-well trays containing sterile torf. After inoculation, seedlings were kept at 27°C/18°C under 12-h photoperiod. Five weeks after inoculation, disease symptoms were recorded as 1 (resistant) no symptoms of disease and 0 (susceptible) dead plant.

The best proper stage for anther culture in eggplant is the off-centered position of microspors at the end of mono-nucleus stage. It corresponds to growth stage with 2 mm long and light yellow-yellow petal. Flower buds were disinfected properly, then buds were opened over sterile filter papers with a pens and bistouries, anthers were separated from the filaments and sown into growth media as to dorsal sections touched the media(MS 01. Mg/kinetin and 0.1 mg mg/l 2,4 D +MS + 30 gr sucrose [19]).

The anthers placed into MS were subjected to pre-treatment in an incubator at +35ºC under dark conditions for 8 days, then petri dishes were kept in a climate chamber at 3600 lux light intensity, 25±1°C temperature under 16:8 light/dark photoperiod for 8 days. Proper adaptation producers were followed while taking cultures to outdoor conditions.

For plodiy analysis, very small leaf samples in a petri dish was supplemented with 1.5 ml cystatin solution. About 0.3 ml of resultant mixture was placed into plastic Eppendorf tube and placed into flow cytometry device. Based on device peak ranges, chromosome pattern (haploid or diploid) was determined. The 0.5% colchicine-absorbed cottons were placed over the buds and kept for 2 hours for stratification of haploid plants.

RESULTS AND DISCUSSION

Initially, 120 eggplant lines and 4 commercial cultivars which declared as resistant were tested with molecular markers [15]. While 120 pure eggplant lines in gene pool were found to be susceptible, 4 commercial hybrid cultivars were found to be resistant. These 4 hybrid cultivars found to be resistant in marker analysis were then subjected to classical testing and status of resistance was verified. Later on, following the general combination tests, 4 pure lines with high heterosis ability, but without FOM resistance were selected from the gene pool. These 4 lines were hybridized for BC program with resistant sources of which FOM resistance was proved through molecular markers and classical testing and F1 hybrids were obtained. In hybridizations performed with 4 pure lines as mother and 4 commercial cultivars as father, 100 plants from each hybrid combination were subjected to molecular tests and heterozygote resistant (about 50% resistant) plants were selected and transplanted into a greenhouse. Observations were made since the initial flowering and following the first fruit set, selected 10 plants were backcrossed to main plant to get pGM1F1(Table 1, Figure 1). Following the backcrossing, observations were made until the end of harvest season and seeds of the backcrossed hybrid combination with the best performance throughout the entire season were harvested. In the next season, seeds of hybrid pGM1F1 hybrid combinations were sown into viols and subjected to marker tests. Resistant plants were transplanted into greenhouse. Again, observations were made since the initial flowering and following the first fruit set, selected 10 plants were backcrossed to main plant to get pGM2F1. Following the backcrossing, observations were made until the end of harvest season and seeds of the backcrossed hybrid combination with the best performance throughout the entire season were harvested. Then in the next season, hybrid pGM2F1 hybrid combinations were sown into viols and subjected to marker tests. Resistant plants were transplanted into greenhouse. Again, observations were made since the initial flowering and following the first fruit set, selected 10 plants were backcrossed to main plant to get pGM3F1. Following the backcrossing, observations were made until the end of harvest season and seeds of the backcrossed hybrid combination with the best performance throughout the entire season were harvested. In subsequent season, seed of hybrid pGM3F1 hybrid combinations were sown into viols and subjected to marker tests. The 20 pGM3F1 plants from 4 hybrid combinations (total 80 plants) identified as resistant with molecular tests were transplanted into greenhouse. Buds were taken from these plants and anther culture was performed. Following the anther culture, 25 DH plants were obtained from each combination and resistance status of these plants was determined through initially molecular then classical testings.

Table 1. *Clasical and molecular testing results of parents and pGM1F1 population for Fusarium oxysporum Schlecht. f sp. Melongenae resistance*

	Inoculated Plants No	Dead Plants No	Healthy Plants No	SCAR Marker resistance band
Resistant (Parent) 1	12	0	12	12
Resistant (Parent) 2	3	0	3	3
Resistant (Parent) 3	3	0	3	3
Susceptible (control) 1	8	8	0	0
Susceptible (Parent) 2	3	3	0	0
Susceptible (Parent) 3	3	3	0	0
Susceptible (Parent) 4	3	3	0	0
Susceptible (Parent) 5	3	3	0	0
Susceptible (Parent) 6	3	3	0	0
PBC 1A-1	24	11	13	12
PBC 1A-2	24	6	18	18
PBC 1A-4	24	19	5	5
PBC 2A-1	24	16	8	8
PBC 2A-2	24	12	12	12
PBC 2A-4	24	6	18	17
PBC 3A-1	24	20	4	4
PBC 3A-2	24	10	14	14
PBC 3A-4	24	12	12	12
PBC 4A-1	24	9	15	15
PBC 4A-2	24	15	9	8
PBC 4A-4	24	15	9	9
PBC 5A -1	24	11	13	12
PBC 5A -2	24	10	14	13
PBC 5A -4	24	15	9	9
PBC 6A-1	24	12	12	12
PBC 6A-2	24	4	20	18
PBC 6A-4	24	7	17	17
Total	**432**	**210**	**222**	**215**

More than 500 seedlings belonging to various genetic background were inoculated and 380 plants derived from 11 different genetic materials were identified as resistant. One month after root dip inoculation, resistant and susceptible plants were able to be identified.

Same genetic materials were tested with molecular markers reported by Mutlu at all. [15] with the use of FOM markers, 3 genotypes were identified as resistant. These genotypes were able to survive with the root-dip inoculation. Six commercial hybrids

that did not possess the marker locus for resistance against FOM were all identified as resistant according to the classical testing.

Present findings indicated that the marker was reliable to develop eggplant lines resistant against fusarium wilt and there may be another source of resistance independent from the known resistance gene originating from LS2436.

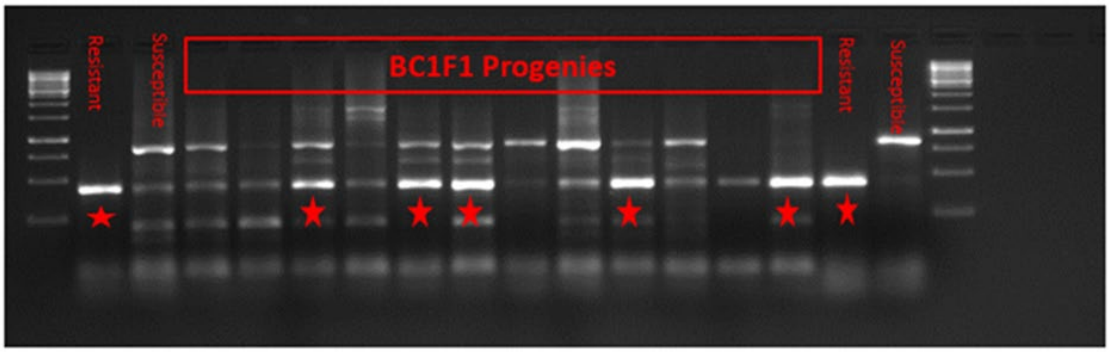

Figure 1. *Gel image for molecular testing using BC1F1 population (L=1kb ladder; Resistant bands have been marked with red color and R=Resistant, S=Susceptible)*

X^2 test results for classical and molecular testing as dominant one gene for Fusarium oxysporum Schlecht. f sp. Melongenae resistance using BC1F1 population were given in Table 2. 432 BC1F1 progenies were used for classical testing and X^2 test results showed to 1:1 mendelian ratio (p 0,99), and 88 BC1F1 progenies were used for molecular testing [15] and also X^2 test results showed to 1:1 mendelian ratio (p 0.96).

Table 2. *X^2 test results for classical and molecular testing as dominant one gene for Fusarium oxysporum Schlecht. f sp. Melongenae resistance using BC1F1 population*

Population	Resistant plants (no.)	Susceptible plants (no.)	Expected ratio	$\chi 2$	Probability (P)
BC1F1	222	210	1:1	0.82	0.99

Population	Resistant plants (no.)	Susceptible plants (no.)	Expected ratio	$\chi 2$	Probability (P)
BC1F1	40	44	1:1	0.76	0.96

In breeding studies, rapid scanning of disease-resistant lines using molecular markers saves time, space and provide reliability in selection of the material obtained with the desired genotypes and selection of hundreds of plants in a single day [20]. Goth and Webb [21] reported that there were no eggplant varieties that were resistant to *Verticillium wilt* solitude, while certain eggplant varieties were tolerant to Fusarium wilt. Genetic resistance studies on FOM demonstrated that resistance was controlled by a single dominant gene. However, studies that aimed to transfer the resistance in wild forms to the culture plants were not quite successful. Mochizuki et al. [22] reported that the LS174 line was resistant to Fusarium, and found that the resistance in LS174 was managed by a single dominant gene. In a study by Monma et al. [23], it was reported that the LS 1934 and LS 2436 genotypes of the *S. melongena* species were resistant to

FOM. Boyaci and Abak [24] investigated the inheritance of resistance between the resistant genotypes LS 1934 and LS 2436 of the *S. melongena* species and the susceptible genotype NSFB-99 and found that the resistance was monogenic dominant for both resistant genotypes. Genomic mapping was conducted on the F2 and BC1 populations obtained by cross-breeding the FOM-resistant genotype LS 2436 and sensitive NSFB-99 and the H-12 primary marker with a distance of 2.6 cM to the resistance gene was identified. However, in a RADP marker rehabilitation study, it was demonstrated that the inheritance of resistance could not be determined as homozygous or heterozygous, and the reproducibility was difficult, so it was reported that further studies were needed. Mutlu et al. [15] developed SRAP, SRAP-RGA, RAPD and SCAR markers to determine the resistance to FOM pathogen. It was determined as a result of 2.316 primer combinations that three markers were the closest to gene with 2.6 cM in the study. It was determined that the codominant SRAP marker Me8/Em5 and the dominant SRAP-RGA marker Em12/GLPL2 were linked to the resistance gene with 1.2 cM, while the RAPD marker H12 was linked with 2.6 cM to both alleles. In the study, it was determined that the SRAP marker F2 and backward hybrid 3 generations were linked to the resistance gene, and it was transformed to two dominant SCAR markers. It was reported that two SCAR markers designed in that study might be useful in MAS-selective breeding studies to determine resistance to Fusarium wilt in eggplants. The preference of the closest primer used in breeding studies is important for the reliability of the results. In the present study, eggplant genotypes were tested with the SCAR426 primer, which was found to be linked to the resistance gene at 1.2 cM. The use of SCAR markers is quite reliable, accurate and easily detectable on agarose gel due to its close proximity to the gene in homozygous or heterozygous determinations of inheritance in F2 and F3 populations in breeding studies when compared to the other SRAP RAPD and RGA [19-27]. The findings of the present study once more demonstrated that by selecting adequate genotypes with MAS selection in breeding studies would save money, time and contribute to the rapid commercialization of the lines in cross-breeding programs. Mutlu et al. [15] stated that disease resistance should be verified with classical tests that would be conducted in regular intervals on breeding material that were identified as resistant with markers in breeding programs, although these types of markers, such as SCAR426 and SCAR347, are very close to the gene and their recombination rate is quite low. As a result of molecular work conducted in the present study, verification of the resistance of 4 eggplant genotypes to FOM pathogen using SCAR426 primer was conducted with classical testing. As a result of the classical testing conducted with this purpose, evaluations of the plant and the root demonstrated that there were no FOM symptoms [25]. Eggplant (*Solanum melongena* L.) has the potential for improvement through heterosis breeding, which can be further utilized for development of desirable recombinants. A Line × Tester mating design was used to determine heterosis over better parent, combining ability, and gene action for 11 characters in eggplant. Crosses showing high specific combining ability (sca) and yield involved parents showing high general combining ability (gca) for fruit weight, fruit diameter, or fruit length. Two hybrids, ‘VNR-218’ × ‘BCB-11’ and ‘Arka Nidhi’ × ‘KS-331’ were selected on the basis of their per se (mean) performance: heterosis and the sca effects. These hybrids could be used commercially due to high yield and low percentage disease index (PDI) values for bacterial wilt disease. The preponderance of nonadditive gene action was evident for control of all characters studied. Parental lines and hybrids were categorized according to disease reaction. Average large vessel area of

stems and roots of the hybrids was negatively correlated with PDI. Larger vessel area in the vascular bundle needs to be considered in the selection of hybrids for resistance to bacterial wilt disease [26]. The introgression of its disease resistance gene into cultivated eggplants would allow for breeding disease resistant eggplants. In this study, interspecific hybridization and subsequent backcrossing between PI388846 and cultivated eggplants were performed. The results showed that *Verticillium wilt* resistance was successfully introduced into the cultivated eggplants, and the agronomic traits of the interspecific hybrid progeny were improved by continuous backcrossing with the cultivated eggplants. In addition, a gene specific marker for the Ve homolog in PI388846 was developed to detect *Verticillium wilt* resistance in the backcross population. The results represent a positive beginning for the genetic enhancement of cultivated eggplants for *Verticillium* wilt resistance (Liu et al., 2015). Sousa et al., (1998) carried out a study to obtain estimates of heterosis in crosses between seven eggplant cultivars (Embu = E; Santa Genebra = SG; Viserba = V; Aubergine de Barbentane = AB; Florida Market 10 = FM; Black Beauty = BB, and Melitino = M) and two breeding lines (B-14-07 = B1 and B-31-06 = B2). The F1 hybrids used were: E x FM; E x BB; E x M; E x B1; E x B2; SG x FM; SG x BB; SG x M; SG x B1; SG x B2; V x FM; V x B1; V x B2; AB x FM; AB x M; AB x B1; AB x B2 and M x FM. Cultivars, lines and hybrids were evaluated at the ESAL experimental field in Lavras, MG, from February to October 1992. A randomized complete block design with three replications was used. Significant heterosis relative to the parental means was detected for all traits studied. Their values ranged from +41.23% to +113.31% for total fruit yield, from -11.45% to +26.17% for average fruit weight, and from +27.98% to +141.81% for early production. Heterosis relative to the superior parent ranged from +13.89% to +92.51% for total fruit yield. Hybrid pairs: SG x FM and AB x B1, V x FM and AB x FM, E x M and AB x B1 were the most heterotic relative to the parental mean for total fruit production, mean fruit weight and early production, respectively. The hybrids displaying highest heterosis relative to the superior parent for total yield were AB x B1 and SG x FM.

Colak Ates et al. [25] conducted a study to determine FOM resistance, 77 eggplant genotypes were screened with the SCAR426 marker, which was identified to be the closest to the gene at 1.2 cM, and it was determined that the four eggplant genotypes, namely P11, P29, P49 and P52 were heterozygous resistant against FOM. FOM-resistant lines were verified by classical testing. As a result of classical testing conducted to determine PVY and RN resistance of eggplant genotypes, it was determined that all lines were PVY-sensitive and P29 and P52 genotypes were resistant to FOM and RN. Disease-resistant genotypes determined in the present study would contribute to the development of the new F1 hybrid eggplant cultivar.

CONCLUSION

MAS should be continued up to BC3F2 generation and allelism test should be initiated to understand whether a new and unique gene exists for resistance against FOM. DH is being used at BC1-BC3 generations to develop FOM resistant eggplant lines and to transfer the resistance gene into parental lines of the hybrid.

REFERENCES

[1] Rotino, G. L., Sihachakr, D., Rizza, F., Valè, G., Tacconi, M. G., Alberti, P., ... & Acciarri, N. (2005): Current status in production and utilization of dihaploids from somatic hybrids between eggplant (Solanum melongena L.) and its wild relatives. Acta physiologiae plantarum, 27(4), 723-733.

[2] FAO, 2019. www.faostat.org

[3] Mutlu, N., Boyacı, F.H., Göçmen, M., Abak, K. (2008): Development of SRAP, SRAP-RGA, RAPD and SCAR markers linked with a Fusarium wilt resistance gene in eggplant. Theoretical and Applied Genetics, 117, 1303-1312.

[4] Sękara, A., Cebula, S., & Kunicki, E. (2007): Cultivated eggplants–origin, breeding objectives and genetic resources, a review. Folia Horticulturae, 19(1), 97-114.

[5] Kakizaki, Y. (1931). Hybrid vigor in egg-plants and its practical utilization. Genetics, 16(1), 1.

[6] Bavage, M.S., Madalageri, M.B. and Mulge, R. (2005): Hybrid performance of round fruited brinjal (Solanum melongena L.). Karnataka J. Hort., 1: 95–97.

[7] Dharwad, N., Patil, S.A. and Salimath, P.M. (2011): Study on genetic diversity and its relation to heterosis in brinjal (Solanum melongena L.). Karnataka J. Agr. Sci., 24(2): 110–113.

[8] Prabhu, M., Natarajan, S. and Pugalendhi, L. (2005): Studies on heterosis and mean performance in brinjal (Solanum melongena L.). Veg. Sci., 32(1): 86–87.

[9] Kalloo, G. (1993): Eggplant: Solanum melongena L. In Genetic improvement of vegetable crops (pp. 587-604). Pergamon.

[10] Matsuo T, Ishigami K (1958): On the wilt of Solanum melongena L. and its casual fungus Fusarium oxysporum f. melongenae n.f. Phytopathol Soc Jpn 23:189–192 (in Japanese with English summary)

[11] Altınok, H.H. (2005): First Report of Fusarium wilt of eggplant caused by Fusarium oxysporum f. sp. melongenae in Turkey. Plant Pathology, 54(4), 277

[12] Altınok, H.H. (2006): Doğu Akdeniz Bölgesi'nde Patlıcanda Fusarium Solgunluğu Hastalığı (Fusarium oxysporum Schlecht. f. sp. melongenae Matuo and Ishigami)'nın Yaygınlığı, Etmenin Moleküler Karakterizasyonu ve Bitkide Hastalığa Karşı Dayanıklılığın Uyarılması. Tez (Doktora)-Çukurova Üniversitesi, Fen Bilimleri Enstitüsü, Bitki Koruma Anabilim Dalı, Adana.

[13] King, S. R., Davis, A. R., Zhang, X., & Crosby, K. (2010): Genetics, breeding and selection of rootstocks for Solanaceae and Cucurbitaceae. Scientia horticulturae, 127(2), 106-111.

[14] Kaur, P., Duhan, J. S., & Thakur, R. (2018): Comparative pot studies of chitosan and chitosan-metal nanocomposites as nano-agrochemicals against fusarium wilt of chickpea (Cicer arietinum L.). Biocatalysis and Agricultural Biotechnology, 14, 466-471.

[15] Mutlu, N., Boyacı, F. H., Göçmen, M., & Abak, K. (2008): Development of SRAP, SRAP-RGA, RAPD and SCAR markers linked with a Fusarium wilt resistance gene in eggplant. Theoretical and Applied Genetics, 117(8), 1303.

[16] Monma S, Sato T, Matsunaga H (1996): Evaluation of resistance to bacterial, Fusarium and Verticillium wilt in eggplant and eggplantrelated species collected in Ghana. Capsicum Eggplant Nwsl 15:71–72

[17] Sakata, Y., Monma, S., Narikawa, T., & Komochi, S. (1996): Evaluation of resistance to bacterial wilt and Verticillium wilt in eggplants (Solanum melongena L.) collected in Malaysia. Journal of the Japanese Society for Horticultural Science, 65(1), 81-88.

[18] Toppino, L., Valè, G., & Rotino, G. L. (2008): Inheritance of Fusarium wilt resistance introgressed from Solanum aethiopicum Gilo and Aculeatum groups into cultivated eggplant (S. melongena) and development of associated PCR-based markers. Molecular Breeding, 22(2), 237-250.

[19] Matsubara S, Yamamoto M, Man-HyunJo MurakamiK, Man HJ (1998): Embryoid and callus formation from microspores by anther culture from July to November in pepper (Capsicum annuum L.). Sci Rep Faculty Agric Okayama Univ 87:117–122

[20] Barone, A., Ercolano, M.R., Langella, R., Monti, L., Frusciante, L. (2005): Molecular marker-asisted selection for pyramiding resistance genes in tomato. Adv. Hort. Sci. 19, 147-152.

[21] Goth, R.W., Webb, R.E. (1981): Sources and genetics of host resistance in vegetable crops. in: "Fungal Wilt Diseases of Plant", ed: Mace M.E., Bell, A.A., and Beckman, C. H.Academic Press, London, 377-409 pp.,

[22] Mochizuki, H., Sakata, Y., Yamakawa, K., Nishio, T., Komochi, S., Nariakawa, T., Monma, S. (1997): Eggplant parental line 1 and Eggplant Breeding Line Resistant to Fusarium wilt. Bulletin of the National Research Institute of Vegetables, Ornamental Plants and Tea. Series A: Vegetables and Ornamental Plants. (12), 85-90.

[23] Monma, S., Sato, I., Matsunaga, H. (1996): Evaluation of resistance to bacterial, Fusarium and Verticillium wilt in eggplant and eggplant releated species collected in Ghana. Capsicum and Eggplant Newsletter. 15, 71-72.

[24] Boyaci, H. F., Abak K., (2008): Patlicanlarda Fusarium Solgunluğuna Dayaniklilik Kaynaklari ve Dayanikliliğin Kalitimi. Çukurova Üniversitesi, Fen Bilimleri Enstitüsü, Doktora Tezi.

[25] Colak-Ates, A., Fidan, H., Ozarslandan, A., & Ata, A. (2018): Determınatıon of The Resıstance of Certain Eggplant Lines Against Fusarıum Wilt, Potato Y Potyvirus and Root-Knot Nematode Usıng Molecular and Classıc Methods. Fresenıus Envıronmental Bulletın, 27(11), 7446-7453.

[26] Chattopadhyay, A., Seth, T., Dutta, S., Ghosh, P. P., Chattopadhyay, S. B., Majumder, D., & Hazra, P. (2012): Breeding eggplant for higher productivity and bacterial wilt tolerance. International journal of vegetable science, 18(4), 376-392.

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Research Article

DETERMINATION OF FATTY ACID COMPOSITION AND ANTIOXIDANT ACTIVITY OF FIG SEED OIL

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ABSTRACT. *Ficus carica* is a tree that grows in the Eastern Mediterranean and Southwest Asia. It is among the first cultivated plants by humans. *Ficus carica*, called figs, has a very large family and more than 700 known species. Different parts of the plant like its fruit, latex, leaves, and seeds have a very important medical value. However, studies on fig seed oils are very limited. In this study, the fatty acid composition of fig seed oils was determined by gas chromatography/mass spectrophotometry (GC/MS). Total antioxidant activity analysis was made through DPPH (2,2-diphenyl-1-picrylhydrazyl) method and antioxidant activity value was determined as trolox equivalent (TE). While the total oil yield was determined as 14.08%, the antioxidant capacity was determined to be 140.19 mg TE/100 g. According to GC/MS analysis, it was determined that the highest fatty acid was α-Linolenic acid (26.31%), followed by linoleic acid (24.27%) and oleic acid (19.65%). With these results and limited literature information, it is clear that figs can be evaluated outside of common consumption.

Keywords: *Ficus carica, fatty acid, seed oil, antioxidant capacity, DPPH, fig*

INTRODUCTION

The genus *Ficus* is one of the largest medicinal plants, with more than 750 woody plants, trees, and shrubs that can grow in subtropical and tropical areas of the World [1]. Ficus is an essential source of genes owing to its nutritional value and high economic and is a substantial part of biodiversity in the rain forest ecosystem [2, 3].

Ficus carica L., also called as fig, is one of the important members of the genus *Ficus*. Fig is one of the first plants that are native to Southwest Asia and the Eastern Mediterranean and cultivated by humans [4]. Because figs are practical to use and belief objects, they have special importance for tropical regions. Besides, these plants attract the attention of many researchers with their biological activities. In traditional medical systems such as Ayurveda, Unani, Homoeopathy, and Siddha, the therapeutic benefits of *F. carica* L. have been emphasized. It is known that these traditional medicine systems are used in the treatment of many diseases such as cardiovascular, hypertensive, ulcerative, infectious, and cancer diseases [5, 6].

F. carica L. continues to be cultivated due to its edible fruits. According to TUIK (Turkish statistical institute) data, it is known that the production in our country was 306.499 tons in 2018. Moreover, according to FAO (Food and Agriculture Organization) data of 2017 [7], the world fig production in Turkey is in first place with 305 689 tonnes of world production. This is followed by Egypt (177.135 tons), Morocco (137.934 tons) and Algeria (128.684 tons), respectively. More detailed data can be found in Fig 1.

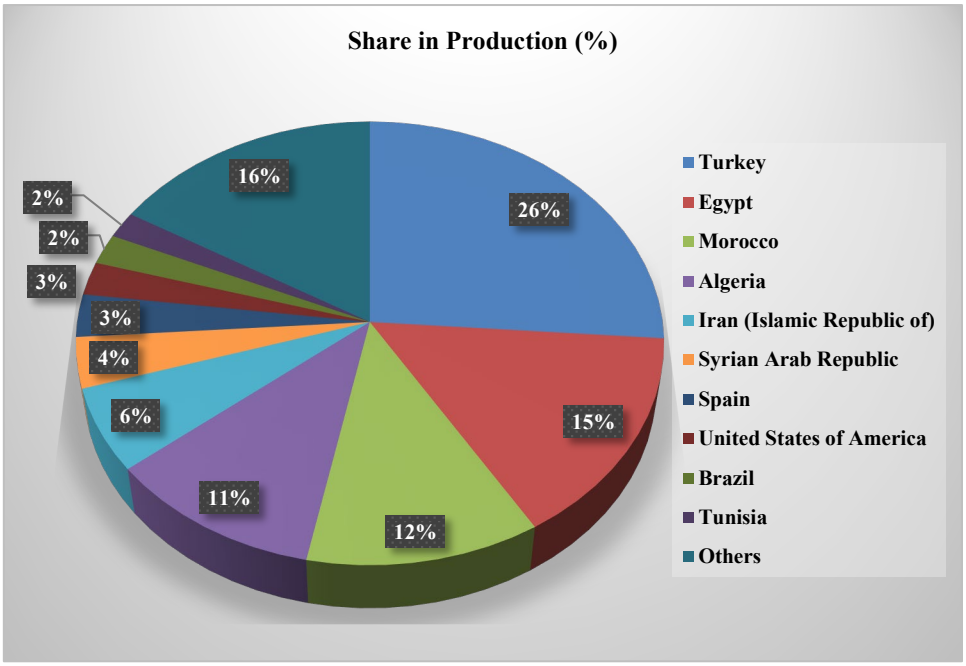

Fig. 1. *Distribution of fig production in the world by country (2017) [7]*

In the world, fig consumption is in the form of dry and table fresh consumption. Both dried and fresh figs have high levels of fiber and polyphenols [8, 9]. Besides, it has been reported by many researchers that dried fruits are an important source of sugar, vitamins, minerals, protein, organic acid, carbohydrates, and phenolic compounds [10, 11, 12, 13]. Due to these properties, fig has a substantial place among foods and a very large consumption area. It also has very important antioxidant activities for human health in terms of many compounds it contains.

Antioxidants are defined as compounds that take place under the effect of atmospheric oxygen or reactive oxygen species, which can delay or inhibit oxidation processes. They are employed to stabilize the polymeric products of cosmetics, pharmaceuticals, petrochemicals, and food items [14].

One of the parts of figs that makes them important both in terms of nutritional values and health is seeds. These seeds can be large, medium and small, and the number of seeds per fruit (30 to 1600) varies. There are many edible seeds in one fig and the seeds are hollow unless pollinated. On the other hand, pollinated seeds give the characteristic nutty taste of fig. Fig seeds attract researchers' interest due to the rich chemical compounds they contain [15, 16].

In this study, it is aimed to determine the composition of fig core fatty acids by gas chromatography/mass spectrophotometry (GC/MS). Moreover, it is predicted that determining antioxidant capacity will contribute to the literature.

MATERIALS AND METHODS

Material

Fig seeds used in this study were obtained from local companies and kept at room temperature until analysis.

Method

Fatty Acid Analysis

The oil extraction of the fig seeds we preserved was made with n-hexane. Analysis of fatty acids was done according to the method of IUPAC IID19 [17]. Gas chromatography (GC; Perkin Elmer, Shelton, USA) was used to determine the fatty acid composition. Flame ionization detector (FID) and column (30 m × 0.25 mm ID, 0.25-μm film thickness) were used for chromatographic separation. The oven temperature was raised to 120°C (2 min) and 220°C with 5°C / minute and kept for 10 minutes; Injector and detector temperatures were set at 280°C and 260°C, respectively. The results are calculated in % with their average deviations.

Total Antioxidant Activity Analysis

Total antioxidant activity analysis in fig seeds was done using DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Readings were made using a spectrophotometer at a wavelength of 515nm. The antioxidant capacity of fig seed oil was calculated as trolox equivalent.

Statistical Analysis

The fatty acid analysis was performed in three replications according to random plot trial design. The data obtained were analyzed by employing the SAS-JMP statistical program (SAS Institute Inc., Cary, NC) and variance analysis was performed. The differences between them are compared with the LSD multiple comparison test.

RESULTS AND DISCUSSION

In this study, analysis of total oil amount, fatty acid composition, and total antioxidant activity in *F. carica* L. seeds were performed. According to the obtained results, the total amount of fat was determined as 14.08%. In the study, the fatty acid composition was determined by Gas Chromatography (GC) and presented in detail in Table 1.

Accordingly, it was determined that the highest fatty acid was α-Linolenic acid (26.31%) followed by linoleic acid (24.27%) and oleic acid (19.65%). All three together constitute 70% of the total core oil. a-Linolenic acid is an important herbal Omega-3 source. Omega-3 is a very important fatty acid in terms of health and reduces risk factors for many diseases. Omega-3 fatty acids are defined as anti-inflammatory and structural essential dietary oils for retinal photoreceptors and nerve tissue [18]. It has been determined in the analysis that fig seed oil is a vital α-Linolenic acid source and it has been observed that there are very limited studies in this context. It has been reported that the amount of α-linolenic acid in other plants is 55% for flaxseed, 10% for canola oil and 10% for walnut [19, 20].

Table 1. *Fatty acid composition of F. carica L. Seed (Figures in parentheses are angular transformation values of percentage of response)*

	Fatty Acid	Percentage (%)
1	Palmitic Acid (C16: 0)	15.10^{d} (22.86)
2	Palmitoleic Acid (C16: 1)	0.07^{k} (1.51)
3	Stearic Acid (C18: 0)	5.89^{e} (14.04)
4	Oleic Acid (C18: 1n9c)	19.65^{c} (26.31)
5	Eleaidic Acid (C18: 1n9t)	0.04^{l} (1.14)
6	Linoleic Acid (C18: 2n6c)	24.27^{b} (29.51)
7	a-Linolenic Acid (C18: 3n3)	26.31^{a} (30.85)
8	γ-Linolenic Acid (C18: 3n6)	0.11^{j} (1.90)
9	Arachidic Acid (C20: 0)	0.37^{g} (3.48)
10	Eicosenoic Acid (C20: 1n9c)	0.29^{h} (3.08)
11	Behenic Acid (C22: 0)	0.17^{i} (2,36)
12	Caproic Acid (C6: 0)	0.13^{j} (2.06)
13	Lignoceric Acid (C24: 0)	0.08^{k} (1.62)
14	Caprylic Acid (C8: 0)	0.56^{f} (4.29)
15	Capric Acid (C10: 0)	0.04^{l} (1.14)
16	Myristic Acid (C14: 0)	0.12^{j} (1.98)
17	Undecanoic Acid (C17: 0)	0.08^{k} (1.62)
18	Heptadecanoic Acid (C17: 0)	0.16^{i} (2.29)
19	Pentadecanoic Acid (C15: 0)	0.04^{l} (1.14)

*LSD: 0,17*** , P<0.05*, P<0.01**, P<0.001****

In a study on dried figs, Jeong and Lachance found that the most dominant fatty acid in milled fruits was linolenic acid (53.1%), followed by linolenic acid (21.1%), palmic acid (13.8%) and oleic acid (9.8%) have determined that [10]. Similarly, Yarosh and Umarov [21] obtained high linolenic acid (trienic acid) amount from ground fig seeds.

Linoleic acid, one of the other essential fatty acids, is an omega-6 fatty acid. It is one of the fatty acids very important in determining oil quality. The percentage amounts of linoleic (omega-6), oleic (omega-9), and linolenic (omega-3) acids among fatty acids in oil quality are very important. It has been reported that oleic acid is effective in having the desired frying properties [22] and linoleic acid decreases the level of cholesterol in the blood [23, 24]. Since oleic acid provides long stability, it has an area of use in the chemical industry, especially in the food and cosmetics sector [25].

As with many seeds, the total oil yield is very important in fig seeds. Kim et al. [20] reported the amount of oil in 2 different local fig varieties as 0.31-0.27%. It is a very low value compared to the fig seed used in this study. It is thought that this difference in the amount of oil is due to the locality, harvest time, and the variety of figs studied.

In a different study, Hssaini et al. [26] obtained the highest oil content from local varieties 'C7A14' (29.65 ± 1.21%) and 'C11A21' (28.96 ± 0.62%). The lowest oil

content was observed in 'Borjassoute Noir' (21.54 ± 1.71%) and 'White Adriatic' (24.71 ± 2.14%) varieties.

Berry [27] determined that the oil content in fresh and boiled durian (*Durio zibethinus* Murr) Seeds was 1.8%. He found that about 82% of all seed oil consists of unsaturated fatty acids.

Taoufik et al. [28] investigated *Opuntia ficus indica* cactus seed oils of different origins. They found the oil yields of a total of 17 cactus seeds between 5.4% and 9.9%. They emphasized that the main fatty acids of cactus oil are palmitic acid (11.6–12.4g / 100g), oleic acid (18.2–22.3g / 100g), and linoleic acid (60.2–64.6g / 100g).

In a review covering the nutritional values of 10 different fruit seed oils, it was reported that the oil content in the seeds was between 1.8% and 49.0%, and the protein content was 6% to 40.0%. As in our determination, it has been emphasized in this review that the varying oil content may be due to differences in plant varieties and geographical differences [29].

According to the total antioxidant activity analysis, the antioxidant capacity of fig seed oils was observed to be 140.19 mg TE / 100 g. With the antioxidant capacity value obtained, it has been proven that it is precious in this respect as well. However, this value is not stable and may change with many factors. A different study conducted proves this; while researchers working on light and dark fig fruits found the highest antioxidant (DPPH) value in the dark-colored Bouankik (45.25%) variety, they determined the lowest amount in Taghanimt (28.33%) [30]. Accordingly, it is seen that colors, types, and methods have different effects on antioxidant amounts. In different studies; It has been emphasized that fruits, for instance, blueberries, blackberries, and strawberries have high levels of antioxidant capacity due to the high levels of polyphenol and anthocyanin they contain [31, 32]. Turkey to performing work on Smyrna figs Halvorsen et al. [33] the total antioxidant amount 0.73 mmol / 100g been found not. Accordingly, it has been determined that fig seed has less total antioxidant capacity than pomegranate, grape, and plum, but higher than papaya, mango, apple, apricot, and banana.

Hssaini et al. [34] compared the morphological and biochemical characteristics of 11 local fig varieties grown in the Moroccan climate. According to DPPH free radical scavenging capacity, the highest antioxidant value is in 'Nabout' [88.1 ± 3.37 mmol TE / g dw (dry weight)] and 'Breval Blanca' (83.16 ± 6.93 mmol TE / g dw) varieties, while the lowest values are in 'El Quoti Lbied' and They have been observed in "Snowden" (14.28 ± 1.42 and 14.76 ± 1.61 mmol TE / g dw, respectively). The highest antioxidant activity was obtained with DPPH test.

Having researched the antioxidant activity of different fruits, Reddy et al. [35] observed that fresh fruits ranged from 32 to 891mg TE / 100g as a result of DPPH radical scavenging, while the highest antioxidant activity was seen in guava (Psidium guajava) (891) and the lowest in watermelon (Citrullus lanatus) (32). In dried fruits, the highest activity was found in walnut (*Juglans regia*) (1541 mg TE / 100 g) and the lowest activity in piyal (*Pistacia vera*) seeds (271 mg TE / 100 g). These results are quite high compared to our study.

CONCLUSION

In this study, fig seed oil yield was determined as 14.08%. As a result of the analysis of the fatty acid composition, it was determined that the highest fatty acids were α-

Linolenic acid, linoleic acid and oleic acid, respectively. It is expected that this study will be very useful due to the limited number of studies on fig seed fatty acids. It is very important in terms of completing the deficiency in the literature and providing data on how fig seed fatty acids can be evaluated in the future. It is suggested that a separate window should be opened for the seeds of fig fruits, which are mostly evaluated for human consumption and health. It should not be forgotten that it can be an alternative source of linolenic acid to other animal and vegetable oils.

REFERENCES

[1] Joseph, B., & Raj, S. J. (2011): Pharmacognostic and phytochemical properties of *Ficus carica* Linn–An overview. International journal of pharmtech research 3(1): 8-12.

[2] Rønsted, N., Salvo, G., & Savolainen, V. (2007): Biogeographical and phylogenetic origins of African fig species (Ficus section Galoglychia). Molecular phylogenetics and Evolution 43(1): 190-201.

[3] Mawa, S., Husain, K., & Jantan, I. (2013): *Ficus carica* L.(Moraceae): phytochemistry, traditional uses and biological activities. Evidence-Based Complementary and Alternative Medicine 2013: 8

[4] Dueñas, M., Pérez-Alonso, J. J., Santos-Buelga, C., & Escribano-Bailón, T. (2008): Anthocyanin composition in fig (*Ficus carica* L.). Journal of Food Composition and Analysis 21(2): 107-115.

[5] Badgujar, S. B., Patel, V. V., Bandivdekar, A. H., & Mahajan, R. T. (2014): Traditional uses, phytochemistry and pharmacology of *Ficus carica*: A review. Pharmaceutical biology 52(11): 1487-1503.

[6] Prasad, P. V., Subhaktha, P. K., Narayana, A., & Rao, M. M. (2006): Medico-historical study of "aśvattha" (sacred fig tree). Bulletin of the Indian Institute of History of Medicine (Hyderabad) 36(1): 1-20.

[7] FAO, (2020): Food and Agriculture Organization of The United Nations, http:faostat3.fao.org/download/Q/QC/E, (Date of access:06.07.2020)

[8] Vinson, J. A. (1999): The functional food properties of figs. Cereal foods world 44(2): 82-87.

[9] Vinson, J. A., Zubik, L., Bose, P., Samman, N., Proch, J. (2005): Dried fruits: excellent in vitro and in vivo antioxidants. Journal of the American College of Nutrition 24(1): 44-50.

[10] Jeong, W. S., Lachance, P. A. (2001): Phytosterols and fatty acids in fig (*Ficus carica* var. Mission) fruit and tree components. Journal of food science 66(2): 278-281.

[11] Veberic, R., Jakopic, J., Stampar, F. (2008): Internal fruit quality of figs (*Ficus carica* L.) in the Northern Mediterranean Region. Italian Journal of Food Science 20(2): 255-262.

[12] Mawa, S., Husain, K., Jantan, I. (2013): *Ficus carica* L. (Moraceae): phytochemistry, traditional uses and biological activities. Evidence-Based Complementary and Alternative Medicine 2013.

[13] Oliveira, A. P., Valentão, P., Pereira, J. A., Silva, B. M., Tavares, F., Andrade, P. B. (2009): *Ficus carica* L.: Metabolic and biological screening. Food and Chemical Toxicology 47(11): 2841-2846.

[14] Pisoschi, A. M., Negulescu, G. P. (2011): Methods for total antioxidant activity determination: a review. Biochem Anal Biochem 1(1): 106.

[15] Badgujar, S. B., Patel, V. V., Bandivdekar, A. H., Mahajan, R. T. (2014): Traditional uses, phytochemistry and pharmacology of *Ficus carica*: A review. Pharmaceutical biology 52(11): 1487-1503.

[16] Joseph B., Raj S.J. (2011): Pharmacognostic and phytochemical properties of *Ficus carica* Linn –An overview, Int.J. PharmTech Res 3(1): 8-12

[17] IUPAC - International Union of Pure and Applied Chemistry. (1987): IUPAC standard method 2.507. In: Standart methods for the analysis of oils, fats and derivatives. 7th ed.

IUPAC IID19. The International Union of Pure and Applied Chemistry, Blackwell, Oxford.

[18] Richer, S., Ulanski, L., Popenko, N. A., Pratt, S. G., Hitchmoth, D., Chous, P., ... & Sardi, B. (2016): Age-related Macular Degeneration Beyond the Age-related Eye Disease Study II. Advances in Ophthalmology and Optometry 1(1): 335-369.

[19] İşleroğlu, H., Yıldırım, Z., Yıldırım, M. (2005): Fonksiyonel bir gıda olarak keten tohumu. Gaziosmanpaşa Üniversitesi Ziraat Fakültesi Dergisi 22(2): 23-30.

[20] Kim, S. S., Lee, C. H., Oh, S. L., Chung, D. H. (1992): Chemical components in the two cultivars of Korean figs (*Ficus carical* L.). Applied Biological Chemistry 35(1): 51-54.

[21] Yarosh E. A., Umarov A.U. (1971): A study of the oil of the seeds of *Ficus carica*. Chem Nat Compd Engl Transl 71(1): 99.

[22] Yeilaghi, H., Arzani A., Ghaderian M., Fotovat R., Feizi M., Pourdad S.S. (2012): Effect of salinity on seed oil content and fatty acid composition of safflower (*Carthamus tinctorius* L.) genotypes. Food Chemistry J 130: 618–625.

[23] Mohsennia, O., Jalilian, J. (2012): Response of safflower seed quality characteristics to different soil fertility systems and irrigation disruption. International Research Journal of Applied and Basic Sciences 3(5): 968-976

[24] Wilson, T. A., Nicolosi, R. J., Saati, A., Kotyla, T., Kritchevsky, D. (2006): Conjugated linoleic acid isomers reduce blood cholesterol levels but not aortic cholesterol accumulation in hypercholesterolemic hamsters. Lipids J 41: 41–48.

[25] Duru, S., Konuşkan, D. B. (2014): Bitkisel Yağlarda Oleik Asit Miktarının Arttırılması ve Yağ Kalitesi Üzerine Etkileri. GIDA 39(6).

[26] Hssaini, L., Hanine, H., Charafi, J., Razouk, R., Elantari, A., Ennahli, S., ... & Ouaabou, R. (2020): First report on fatty acids composition, total phenolics and antioxidant activity in seeds oil of four fig cultivars (*Ficus carica* L.) grown in Morocco. OCL 27: 8.

[27] Berry, S. K. (1980): Cyclopropene fatty acids in some Malaysian edible seeds and nuts: I. Durian (*Durio zibethinus* Murr.). Lipids, 15(6): 452-455.

[28] Taoufik, F., Zine, S., El Hadek, M., Idrissi Hassani, L. M., Gharby, S., Harhar, H., & Matthäus, B. (2015): Oil content and main constituents of cactus seed oils *Opuntia Ficus Indica* of different origin in Morocco. Mediterranean Journal of Nutrition and Metabolism 8(2): 85-92.

[29] Raihana, A. N., Marikkar, J. M. N., Amin, I., Shuhaimi, M. (2015): A review on food values of selected tropical fruits' seeds. International Journal of Food Properties 18(11): 2380-2392.

[30] Bey, M. B., Louaileche, H. (2015): A comparative study of phytochemical profile and *in vitro* antioxidant activities of dark and light dried fig (*Ficus carica* L.) varieties. The J. Phyto 4(1): 41-48.

[31] Sellappan, S., Akoh, C. C., Krewer, G. (2002): Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. Journal of agricultural and food chemistry 50(8): 2432-2438.

[32] Rababah, T. M., Ereifej, K. I., Howard, L. (2005): Effect of ascorbic acid and dehydration on concentrations of total phenolics, antioxidant capacity, anthocyanins, and color in fruits. Journal of agricultural and food chemistry 53(11): 4444-4447.

[33] Halvorsen, B. L., Holte, K., Myhrstad, M. C., Barikmo, I., Hvattum, E., Remberg, S. F., ... & Moskaug, Ø. (2002): A systematic screening of total antioxidants in dietary plants. The Journal of nutrition 132(3): 461-471.

[34] Hssaini, L., Charafi, J., Razouk, R., Hernández, F., Fauconnier, M. L., Ennahli, S., Hanine, H. (2020): Assessment of Morphological Traits and Fruit Metabolites in Eleven Fig Varieties (*Ficus carica* L.). International Journal of Fruit Science 1-21.

[35] Reddy, C. V. K., Sreeramulu, D., Raghunath, M. (2010): Antioxidant activity of fresh and dry fruits commonly consumed in India. Food research international 43(1): 285-288.

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Research Article

BIOCONTROL OF BACTERIAL DISEASES WITH BENEFICIAL BACTERIA IN LETTUCE

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ABSTRACT. In this study it was aimed to investigate the preventive effect of commercial *Bacillus subtilis* strain QST 713, endophytic bacteria isolated from healthy lettuce leaves, and the effect of application time of these treatments against *Pseudomonas cichorii* and *Pseudomonas viridiflava* infections on lettuce. *Bacillus subtilis* strain QST 713 was applied as recommended by the manufacturer. Thirty-two endophytic bacteria were isolated and the antagonistic effect of them was investigated by the disc diffusion method *in vitro*. The best promising strains were selected according to the antagonistic effect against the plant pathogenic bacteria *in vitro* and identified as *Pseudomonas gessardii* and *Bacillus mojavensis* by MALDI-TOF MS. *In vivo* tests were conducted on healthy lettuce plantlets. Statistical analysis revealed that commercial *Bacillus subtilis* strain QST 713 was an effective treatment against both pathogens at almost all application times. *Pseudomonas gessardii* and *Bacillus mojavensis* strains prevented *Pseudomonas viridiflava* infection at 0 and 24h prior application and decrease the infection at all application times. *Bacillus mojavensis* strain was found the most effective treatment at 24h prior application against *Pseudomonas cichorii* infection statistically.

Keywords: *Endophytic bacteria, Pseudomonas cichorii, Pseudomonas viridiflava, Bacillus subtilis QST 713*

INTRODUCTION

Lettuce (*Lactuca sativa*) is a one-year plant and leaves of the plant that are generally used as vegetables. Like all vegetables, lettuce is threatened by many plant pathogens. *Pseudomonas cichorii* (*Pc*) and *Pseudomonas viridiflava* (*Pv*) are important multi-host plant pathogenic bacteria of lettuce. *Pc* causes shiny dark brown, firm, necrotic spots that occur on the inner crop leaves as varnish spot and midrib rot of lettuce [1]. *Pv* is a non-fluorescence member of *P. syringae* group. Necrotic areas on the leaf, stem necrosis, and stem and root rot are the common symptoms of the pathogen on different host plants [2]. Both bacteria were reported on lettuce in Turkey previously [3, 4]. The yield loss can be economically important especially in wet seasons. There is not a successful or remarkable application to prevent or to cure the infection of both pathogens on lettuce.

Treatment with endophytic bacteria (EB), which is derived from plants but does not have any pathogenicity to the plant it is hosted in, is a new approach to combat plant pathogens in recent years. Endophytic bacteria can provide nutrients, promotes growth with the synthesis of plant growth regulators [5], protect plants from environmental stress factors with the synthesis of osmoprotectants and exopolysaccharides [6], supports the plant defense mechanism inducing systemic resistance (ISR) and inhibits some plant pathogens with antimicrobial metabolites [7]. As obtained from different parts of the plants, there are also known commercial formulations. *Bacillus subtilis* strain QST 713

(*Bs*) is one of the registered commercial *Bs* formulations used widely around the world as a bio-formulated pesticide.

The purposes of this study were to observe the preventive effect of commercial *Bs* strain QST 713 and two different EB which were isolated from healthy lettuce leaves in this study and the effect of application time of these beneficial bacteria against two important bacterial pathogens of lettuce, *Pc* and *Pv*.

MATERIAL AND METHODS

Isolation and identification of endophytic bacteria

Healthy lettuce leaves were surface disinfected in 1% NaOCl for 3 min and then rinsed at least three times with sterile distilled water (sdw). The disinfected leaves were dried on sterile filter papers and 3 cm diameters of the leaves were taken. The leaf pieces were ground in sterile extraction bags (Bioreba) which consist of 5ml 0.9% NaCl. The suspension was diluted to 10^4 and 50µl was streaked onto King's B medium [8] by a sterile loop. The Petri dishes were incubated at 28 °C in an incubator and after 48 h colonies were observed under binocular. Fluorescence single colonies were selected and re-streaked onto King's medium B for bacterial purification. The opaque, fuzzy white and irregular edges colonies were selected and re-streaked onto nutrient agar (NA). The selected bacteria were performed on *in vitro* to observe the antagonistic effect against pathogenic *Pc* and *Pv.*

Identification of Inhibitory Effect of Endophytic Bacteria in vitro

Pc. and *Pv.* strains were cultured on nutrient broth (NB) for 48h in a rotary shaker at 160 rpm at 28 °C. The bacterial suspension was transferred to microcentrifuge tubes as 2 ml and was centrifuged at 5 000 rpm for 5 minutes. The pellet was resuspended with 1ml 1X PBS (0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24g/L KH_2PO_4, H_2O, pH 7.4). The bacterial concentration was adjusted on 10^9 CFU/mL with a spectrophotometer at OD: 600 and 100 µl of bacterial suspension was streaked onto NA surface. After 30 min of streaking the pathogenic bacteria, sterile blank disks (Oxoid) were soaked into EB suspensions at 10^9 CFU/ml concentration individually and evenly spaced on the Petri dish surface. The Petri dishes were incubated at 28 °C for 48h. The inhibitory effect was evaluated by transparent zone occurrence and the diameter of the zone around the EB-soaked disks. The diameter of the transparent inhibition zone around the EB colony and the diameter of the EB colony were measured and the antagonistic index value was calculated by proportioning these values to each other [9]. Each endophytic bacterium was tested three times.

EB that had an inhibitory effect *in vitro* screening on *Pc* and *Pv* were identified with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics GmbH, Bremen, Germany) and were analyzed as described by Pavlovic et al [10]. For identification, the bacteria were grown as mentioned below and 18h culture was used as instructed.

Molecular identification by 16S rRNA sequence analysis

Genomic DNA of the selected EB strains which had an inhibitory effect *in vitro* screening on *Pc* and *Pv* was isolated by a EURX GeneMATRIX DNA purification kit

according to the manufacturer's instructions. The concentration of the extracted DNA was measured with a nanodrop (Nano2000, Thermo Fisher), and 20 ng of genomic DNA was used as a template for polymerase chain reaction (PCR). The 16S rRNA gene was amplified for the identification of the strains. The universal 16S rRNA primer pair 63f/1387r [11] was used for PCR. PCR was performed with GoTaq flexi master mix (Promega, Madison, WI), 0.2 µM each primer, and 10 µl of dH_2O, with a final volume of 25µL. The PCRs were conducted with the following steps: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec; and a final extension at 72 °C for 10 min. Aliquots of 5 mL from the PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized under ultraviolet (UV) light after staining the gel with ethidium bromide. 16S rRNA PCR products of selected strains were sequenced from both directions with the primer pair 63f/1396r. DNA sequences were trimmed manually based on the pick quality and aligned, and a consensus sequence was obtained by using MEGA version X. The obtained sequences were blasted and deposited in The National Center for Biotechnology Information (NCBI).

Application of Bs strain QST713 and endophytic bacteria to lettuce

Preventive effect of *Bs* strain QST 713 and selected EB were tested on Romain type lettuce (*Lactuca sativa* var. Romaine) variety Sangio. *Bs* strain QST 713 were mixed in water at the highest labeled rates recommended by the manufacturers. The experiment was conducted in a completely randomized block design and three replicates were used for each treatment. Healthy, 5 weeks old lettuce plantlets were used in the experiment. Selected EB were grown from pure cultures in NB in a rotary shaker at 160 rpm at 28 °C for 24h. The bacterial suspension was prepared as mentioned below. All suspensions were sprayed to the lettuce plantlets by a hand spray and the foliar parts of each plantlet were covered with suspension. The experiment was repeated twice with the same number of repetitions.

Pathogenic *Pc* (G5) and *Pv* (P 5.1) strains were used as inocula which were previously isolated from lettuce. The pathogenicity of the selected strains was recorded as moderate on lettuce from the previous studies (Canik Orel, unpublished). The pathogenic *Pc* and *Pv* inocula were also prepared as mentioned below and were sprayed onto plantlets as 10^6 CFU/ml at 0, 24, 48, 72, and 96 h after foliar application. After the pathogen inoculation, inoculated plantlets were covered with a thin, transparent polyethylene layer, to keep moisture in, which were removed after 24 h. Plantlets were kept in a controlled climate room at 26°C, 50% humidity, and 14h light/10h dark conditions. The results were evaluated 14 days after inoculation (dai). Nine plantlets were used for each application. Sdw only sprayed plantlets were used as healthy control and pathogen only sprayed plantlets were used as infected control. Disease severity was assessed according to a qualitative rating scale as described by Thirthamallappa Lohithaswa [12] with some modifications on a 0-to-4 scale, in which 0: Symptomless, 1: <10% of the leaf area, 2: 10-25 % of the leaf area, 3: 26-49 % of the leaf area, 4: 50-100 % of the leaf area.

Statistical analysis

Obtained results were analyzed statistically by variance analysis (ANOVA) and Tukey's t-test at $P \leq 0.05$ by using the Minitab Statistic Software Version 18.0 (Minitab Inc.).

RESULTS AND DISCUSSION

Isolation and identification of endophytic bacteria

Totally, 32 endophytic bacteria were obtained after isolation from the healthy lettuce leaves (Table 1). Eleven of the isolated bacteria were fluorescence pseudomonads. MALDI-TOF analysis result showed that the most common strains were *Serratia liquifaciens* and *Pseudomonas gessardii* (Table 1).

Table 1. *MALDI-TOF MS analysis of endophytic bacteria obtained from healthy lettuce leaves and the antagonistic index value of the strains against pathogenic Pv and Pc*

MALDI TOF MS identification result	**Strain no**	**Antagonistic index value**	
		P.cichorii	***P. viridiflava***
Serratia liquefaciens	Lt 1	1.2	2.2
	Lt 4	2.2	4
	Lt 5	2.3	6.2
	Lt 6	1.1	-
	Lt 8	-	-
	Lt 9	-	-
	Lt 10	1.1	2
	Lt 12	1.4	4.6
Serratia proteamaculans	Lt 2	1.3	-
	Lt 3	1.4	1.6
	Lt 7	2.3	8
	Lt 11	1.2	-
Pseudomonas gessardii	Lt 13	4.6	3.9
	Lt 14	2.2	1.4
	Lt 15	2.2	1.8
	Lt 16	3.1	2.8
	Lt 17	2.8	2.1
	Lt 18	3.1	2.0
	Lt 19	2.9	1.5
	Lt 20	2.4	2.6
Pseudomonas putida	Lt 22	3.1	2.8
Pseudomonas jessenii	Lt 21	1.1	-
Pseudomonas caricapapayae	Lt 23	-	-
Citrobacter braakii	Lt 28	-	-
Rheinheimera soli	Lt 30	-	-
Bacillus mojavensis	Lt 24	4.0	3.4
Not reliable identification	Lt 25	1.1	-
	Lt 26	1.1	-
	Lt 27	1.4	-
	Lt 29	-	-
	Lt 31	-	-
	Lt 32	-	-

Identification of inhibitory effect of endophytic bacteria in vitro

In vitro inhibitory effect of the strains on pathogenic *Pc* and *Pv* strains were evaluated according to the antagonistic index value which was calculated by proportioning the

diameter of the transparent inhibition zone around the EB colony to the diameter of the EB colony (Table 1). The most inhibitory effects were obtained from fluorescence *Pseudomonas* strain Lt 13 and *Bacillus* strain Lt 24 and were identified by MALDI-TOF MS analysis as *Pseudomonas gessardii* (*Pg*) and *Bacillus mojavensis* (*Bv*), respectively (Figure 1a, b). *Pg* strain LT 13 and *Bm* strain Lt 24 were selected for *in vivo* tests and were applied as the antagonistic EB to confirm the inhibitory effect on lettuce plantlets against pathogenic *Pc* and *Pv* strains.

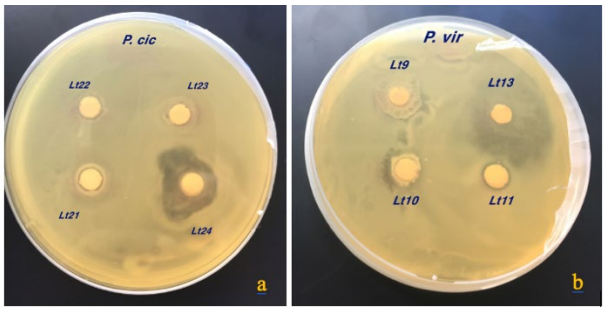

Fig. 1. *The inhibitory zone of different endophytic bacteria obtained from healthy lettuce leaves against Pc (a) and Pv (b) on NA after 24h incubation at 28℃.*

Molecular identification by 16S rRNA sequence analysis

The universal 16S rRNA primer pair 63f/1396r was used for PCR analysis and sequencing of the strains. When the obtained sequences were blasted, strain Lt 13 was found as *P. gessardii* with 99,79% and Lt 24 was found as *B. mojavensis* with 99,39% similarity to the respective reference strains. The sequences were submitted to GenBank under the accession number MT_856830 for Lt 13 and MT_856906 for Lt 24.

Application of Bs strain QST713 and endophytic bacteria to lettuce

The first symptoms on inoculated lettuce plantlets were observed at the 5th dai for both pathogenic bacteria. The effect of treatments and application time were assessed at 14th dai. The statistical analysis showed that there were differences between application time and treatments on *Pc* and *Pv* infections on lettuce (Table 2, Table 3). When the results were evaluated for *Pv* infection (Table 3), *Bs* strain QST 713 was found effective at 0, 24, 48, and 72 h prior applications. All-time applications of Lt 13 showed a preventive effect against *Pv* statistically ($p<0.05$). *Bm* strain Lt 24 was also found effective statistically at all application times ($p<0.05$). The most effective application times of the treatments were 0h and 24h prior applications of Lt13 and Lt 24, and 24h prior application of *Bs* strain QST 713 (Fig. 2).

Fig. 2. *Effect of different treatments on different times against Pv infection on lettuce, a: Lt13 0h, b: Lt 13 24h, c: Lt 24 0h, d: Lt 24 24h, e: Bs QST 713 24h, f: Bs QST 713 96h, g: Disease control, h: Healthy control*

Table 2. Inhibitory effect of Bs strain QST 713, Pg and Bm applications against Pv infection on lettuce plantlets at different times

	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Bs QST 713	0,7±0,0bCD	0,3±0,5bB	0,8±0,0bB	1,2±0,6bB	2,7±0,6aA
Lt 13	0,4±0,6abD	0,0±0,0bB	0,9±0,0aB	0,7±0,6abC	1,0±0,0abB
Lt 24	0,3±0,6aD	0,3±0,5aB	1,0±0,0aB	1,3±0,6aB	0,7±0,6aB
Pv 5.1	3,1±0,0aA	3,1±0,3aA	3,1±0,0aA	3,3±0,6aA	3,0±0,0aA
dH_2O	0.0±0.0aD	0.0±0.0aB	0.0±0.0aC	0.0±0.0aD	0.0±0.0aC

[1] Lowercase letters refer to treatment vs time, [2] Capital letters refer to disease vs time. There is no statistical difference between the same letters following the same column (Tukey's t-test, $P<0.05$)

When the results were evaluated for *Pc* infection (Table 3), *Bs* strain QST 713 was effective at all application times ($p<0.05$). The most effective treatment was found the 24h prior application of *Pg* strain Lt 13 against *Pc* infection. After 24h application, the effect of the strain Lt13 decreased. When *Bm* strain Lt 24 assessed, while it was not effective at the 0h application, it was found effective in the 24 and 48 h prior applications and ineffective in the 72 and 96h to prevent *Pc* infection (Figure 3).

Fig. 3. *Effect of different treatments on different times against Pc infection on lettuce, a: Lt13 24h, b: Lt 24 24h, c: Lt 24 48h, d: Bs QST 713 96h, e: Disease control, f: Healthy control*

Table 3. Inhibitory effect of Bs strain QST 713, Pg, and Bm applications on Pc infection against lettuce plantlets at different times

	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Bs QST 713	0,7±0,5a[1]B[2]	1,0±0,0aB	0,6±0,5aCD	1,3±0,6aB	1,3±0,6aC
Lt 13	0,8±0,4bcB	0,3±0,5cC	2,6±0,5aB	1,8±0,0abB	2,4±0,6aB
Lt 24	2,7±0,5aA	1,0±0,0bB	1,1±0,3bC	2,7±0,6aA	2,9±0,0aAB
***Pc* G5**	3,1±0,3aA	3,0±0,0aA	3,2±0,4aA	3,0±0,0aA	3,0±0,0aA
dH2O	0.0±0.0aC	0.0±0.0aD	0.0±0.0aD	0.0±0.0aC	0.0±0.0aD

[1] Lowercase letters refer to treatment vs time, [2] Capital letters refer to disease vs time. There is no statistical difference between the same letters following the same column (Tukey' t-test, $P<0.05$)

When all treatments and application time data were evaluated together statistically, it can be said that the difference was significant between different treatments and application time (Tukey' t-test, $P<0.05$) to prevent *Pc* and *Pv* infections on lettuce.

Bacterial plant diseases are difficult to be managed with certain pesticides such as fungicides or herbicides in agriculture. Starting with clean propagation material is the most important issue for plant propagation. Since there are plenty of inoculum sources in the environment such as soil, infected plant debris, weeds, vectors, etc. preventive applications are necessary for all crop systems. There are not many studies on bacterial disease management on lettuce crop systems. In this study, the preventive effect of commercial *Bs* strain QST 713 and two different endophytic bacteria against *Pc* and *Pv*, and the effect of application time on the lettuce were investigated.

Glick [13] indicated the widely used of EB in agriculture despite the limited understanding of endophytic bacteria-plant interactions. In this study, commercial *Bacillus* strain *Bs* QST 713 was found as the most effective treatment against both *Pc* and *Pv* infection on lettuce based on the treatments. Similarly, *Bs* strain QST 713 and basic copper sulfate mixture were previously used as an effective treatment on lettuce bacterial leaf spot disease caused by *Xanthomonas campestris* pv. *vitians* [14]. *Bacillus subtilis* strain QST 713 is used widely around the world as bio-fungicide [15]. Our results showed that the preventive effect of *Bs* strain QST 713 strain was not only on fungal diseases but also on bacterial plant diseases.

MALDI-TOF is one of the popular instruments used in biological sciences, due to its rapid and precise identification of genus and species of an extensive range of Gram-negative and -positive bacteria [16]. 16S ribosomal RNA sequences have been used extensively in the classification and identification of *Bacteria* and Archaea [17, 18, 19]. The results of MALDI TOF and 16S rRNA sequence results of the promising EB strains, Lt 13 and Lt 24, were found to be compatible with each other.

Pseudomonads have many members of EB which show an antagonistic effect on different plant pathogens. Especially, fluorescent pseudomonads are known as the antagonistic agents through direct antagonistic effect and/or by inducing plant defense system against many diseases [20]. They have important traits in bacterial fitness such as the ability to adhere to soil particles and the rhizosphere, motility, antibiotic synthesis, and hydrolytic enzyme production. Fluorescent pseudomonads were also reported to grow faster than many organisms and to be more competitive in different environments [21]. Except for *P. caripapayae* strain, all fluorescence strains had different levels of the antagonistic effect against both *Pc* and *Pv* strains *in vitro* tests in this study. *In vivo* studies showed that *P. gessardii* strain Lt 13 was more effective on *Pv* infection than *Pc* infection. This result can be explained that *Pg* strain Lt13 may act more competible on *Pv* from the

beginning to the fifth day of application on lettuce than *Pc*. Although both of the plant pathogens belong to the genus *Pseudomonas*, the durability of the pathogenic traits at the same host can show differences. Pseudomonads divided 19 certain groups according to multilocus sequence analysis that *P. gessardii* belong to group II which was named *P. gessardii* group while *Pv* and *Pc* belong to the group XIX was named *P. syringae* group [22]. *Pv* and *Pc* showed difference within the *P. syringe* group genotypically. This difference might be the reason for the difference in tolerance to the same antagonist at the same host.

Bacillus spp. have also important endophytic members which invade plants by entering through stomata on the leaf surface, triggers ISR that protects non-infected plant parts and accelerate closure of the stomata in response to pathogen attack [23, 24, 25]. In this study, *Bm* strain Lt 24 was found effective on 24 and 48h prior applications. As well as the competitive effect of the bacterial strain, another aspect is, some foliar pathogens invade plants by entering through stomata on the leaf surface. This endophyte-induced priming for enhanced stomatal closure represents yet another structural barrier that can delay disease progression in plants. *B. subtilis* FB17-ISR was previously reported to accelerate closure of the stomata in response to pathogen attack [25]. In this case, it can be said that *Bm* strain Lt 24 can play a competitive role with *Pc* at the plant surface at the first 48h after application or it may cause an effect of stoma closure to prevent the infection as reported previously.

Endophytic bacteria can be applied to the plants by seed treatments, dipping the roots to the bacterial suspension or by foliar applications. Vasudevan et al. [26] also reported that some *Bacillus* spp. could decrease rice leaf blight by seed coating, root applications, and foliar spraying. In this study, all the treatments were applied as the foliar application by spraying to the leaf surface and were found effective to prevent pathogenic *Pc* and *Pv* infections on lettuce. The results of this study showed that foliar applications may provide advantageous on leaf pathogens, such as *Pc* and *Pv* because of the direct action on the pathogen on the leaf surface during or after the leaf inoculation.

CONCLUSION

It is difficult to manage bacterial diseases when the host plant was infected. As a part of plant disease management, application time is as important as the selection of the best treatment for control of the diseases. This study revealed the preventive effect of *Bs* QST 713 strain, and two bacterial endophytes isolated from healthy lettuce plants and the effect of the application time against *Pc* and *Pv* on lettuce. Further studies should be conducted to reveal the effect of these effective components in a mixture of different active ingredients and the effect of the strains on the field conditions.

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REFERENCES

Hikichi, Y., Saito, A., Suzuki, K. (1996): Infection sites of *Pseudomonas cichorii* into head leaf of lettuce. Annual Phytopathological Society of Japan 62, 125–129.

[1] Bartoli, C., Berge, O., Monteil, C.L., Guilbaud, C., Balestra, G.M. *et al.* (2014): The *Pseudomonas viridiflava* phylogroups in the *P. syringae* species complex are characterized by genetic variability and phenotypic plasticity of pathogenicity-related traits. Environmental Microbiology 16, 2301-2315.

[2] Mirik, M., Aysan, Y., Sahin, F. (2011): Characterization of *Pseudomonas cichorii* isolated from different hosts in Turkey. International Journal of Agriculture and Biology 13, 203–209.

[3] Aksoy, H.M., Ozturk, M., Kilic, N. (2018): First report on *Pseudomonas viridiflava* causing bacterial leaf spot of curly lettuce in Turkey. Journal of Plant Pathology 100, 121.

[4] Beneduzi, A., Ambrosini, A., Passaglia, L.M. (2012): Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genetics and Molecular Biology 35,1044-1051.

[5] Berg, G., Grube, M., Schloter, M., Smalla, K. (2014): Unraveling the plant microbiome: Looking back and future perspectives. Frontiers in Microbiology 5, 148.

[6] Gond, S.K., Bergen, M.S., Torres, M.S. (2015): Endophytic bacillus spp. Produce antifungal lipopeptides and induce host defense gene expression in maize. Microbiological Researches 172, 79–87.

[7] King, E.O., Ward, M.K., Raney, D.E. (1954): Two simple media for the demonstration of pyocyanine and fluorescin. Journal of Laboratory and Clinical Medicine 44, 301–307.

[8] El-Sayed, W.S., Akhkha, A., El-Naggar, M.Y., Elbadry, M. (2014): *In vitro* antagonistic activity, plant growth promoting traits and phylogenetic affiliation of rhizobacteria associated with wild plants grown in arid soil. Frontiers in Microbiology 5, 651.

[9] Pavlovic, M.; Konrad, R.; Iwobi, A.N., Sing, A.; Busch, U.; Huber, I. (2012): A dual approach employing MALDI-TOF MS and real-time PCR for fast species identification within the *Enterobacter cloacae* complex. FEMS Microbiology Letters 328, 46–53.

[10] Marchesi, J.S., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. et al. (1998): Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S-rRNA. Applied and Environmental Microbiology 64, 795-799.

[11] Thirthamallappa Lohithaswa, H.C. (2000): Genetics of resistance to early blight (*Alternaria solani* Sorauer) in tomato (*Lycopersicon esculentum* L.). Euphytica 113, 187–193.

[12] Glick, B.R. (2012): Plant growth-promoting bacteria: mechanisms and applications. Scientifica, Article number: 963401:1- 963401:15.

[13] Bull, C.T., Koike, S.T. (2005): Evaluating the efficacy of commercial products for management of bacterial leaf spot on lettuce. Plant Health Progress 6, doi:10.1094/PHP-2005-1121-01-RS.

[14] Serrano, L., Manker, D., Brandi, F., Cali, T. (2013): The use of *Bacillus subtilis* QST 713 and Bacillus pumilus QST 2808 as protectant fungicides in conventional application programs for black leaf streak control. Acta Horticulture 986, 149-155.

[15] Hou, T-Y, Chiang-Ni, Y., Teng, S-H. (2019). Current status of MALDI-TOF mass spectrometry in clinical microbiology. Journal of Food Drug Analysis 27, 404-414.

[16] Kim, M., Chun, J. (2014). 16SrRNA Gene-Based Identification of Bacteria and Archeae Using the EZTaxon Server. In: *Methods in Microbiology*; Elsevier

[17] Raina, V., Nayak, T., Ray, L., Kumari, K., Suar, M. (2019): Approach for Designation and Description of Novel Microbial Species. In: Microbial Diversity in the Genomic Era, Elsevier, India.

[18] Islam, N., Ali, S., Choi, S-J., Park, Y-I., Baek, K-H. (2020): Salicilic acid producing endophytic bacteria increase nicotine accumulation and resistance against wildfire disease in tobacco plants. Microorganisms 8, 31.

[19] Cartieaux, F., Thibaud, M.C., Zimmerli, L., Lessard, P., Sarrobert, C., David, P. et al. (2003): Transcriptome analysis of Arabidopsis colonized by a plant-growth promoting rhizobacterium reveals a general effect on disease resistance. Plant Journal 36, 177–188.

[20] Panpatte, D.G., Jhala, Y.K., Shelat, H.N., Vyas, R.V. D.P. Singh et al. (eds) (2016): Microbial Inoculants in Sustainable Agricultural Productivity 1, Springer, New Delhi, India.
[21] Gomila, M., Peña, A., Mulet, M., Lalucat, J., García-Valdés, E. (2015): Phylogenomics and systematics in *Pseudomonas*. Frontiers in Microbioogy 6, 214.
[22] Kloepper, J.W.; Ryu, C.M., Zhang, S. (2004): Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94, 1259–1266.
[23] Shoresh, M., Harman, G.E., Mastouri, F. (2010): Induced systemic resistance and plant responses to fungal biocontrol agents. Annual Review of Phytopatholgy 48, 21–43.
[24] Kumar, A.S., Lakshmanan, V., Caplan, J.L., Powell, D., Czymmek, K.J., et al. (2012): Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomata. Plant Journal 72, 694–706.
[25] Vasudevan, P., Kavitha, S., Priyadarisini, V.B., Babujee, L., Gnanamanickam, S.S. (2002). Biological control of rice diseases. In: Biological Control of Crop Diseases, Marcel Decker, New York USA.

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Review Article

IN VITRO CULTURE OF PRIMULA: A REVIEW

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ABSTRACT. Primula is the largest genus belongs to Primulaceae family. The majority of the genus is comprising short-lived perennial herbaceous plants. Members of the genus have attractive flowers and are cultivated as bedding plants and flowering potted plants. Additionally, some of the species among the genus are economically important for ornamental plant industry. In commercial production systems, hybrids are especially propagated by seed, while primroses can also be propagated by dividing clumps. However, some problems such as irregular or late germination and low germination rate of the seeds in many primula species prevent mass propagation. Therefore, tissue culture techniques are valuable tools in breeding program, enlargement of genetic pool, gene transformation, as well as in conservation plant genetic materials in primula. In this review, common plant tissue culture techniques used in *Primula* species were briefly presented.

Keywords: *primula, primrose, regeneration, tissue culture*

INTRODUCTION

Primula is the largest genus with approximately 500 species in the Primulaceae family. The first time the genus was described by Carl Linne in 1753 who named the genus as *Primula* derived from the word first in Latin, referring to the early blooming in spring [1]. Primula species are mostly originated from Northern Hemisphere and are naturally grown in Europe, South America, Asia, temperate zones of the Northern Africa [2]. Most of the native plants require humid and cool climate and grow in the forest belt, plain meadows, Alpine lawns, and meadow tundras [3]. The majority of the genus *Primula* is that it comprises short-lived perennial herbaceous plants with leaves arranged in basal rosettes. Leaves are revolute or involute when they are young, often with a white or yellow farinose coating. Leaf margins vary by species as entire, dentate or serrate [4]. Inflorescence of the species in the genus are single-flowered, or arranged in umbels, several superimposed whorls, heads, or spikes. The color of the flowers in genus varies by species as white, yellow, pink, red, purple, violet, or blue, but the mouth of the tube in a contrasting color [5]. *Primula* species are the most popular example of dimorphic floral development known as heterostyly in order to understand evolutionary, developmental biology, population genetics and recently molecular genetics of the system. There are two types flower morphology, named ‘pin’ and ‘thrum’, in dimorphic species. The major differences in heteromorph flowers are that the male and female organs are placed reciprocal position in flower tube which prevent self-pollination. Pin flower type has a long style and stigma can be seen easily at the mouth of the flower, whereas thrum flowers has a short style and anthers are located at the mouth of the flower [6] (Fig. 1).

Members of the genus have attractive flowers and are cultivated as bedding plants and flowering potted plants [1]. Some of the species among the genus are economically important for ornamental plant industry and they are classified as English primrose or

acaulis primula (*Primula vulgaris* synonym *P. acaulis*) (2n=22), polyanthus, polyantha primrose or hybrid primrose (P.×polyantha), fairy primrose or baby primrose (*P. malacoides*) (2n= 18, 36, 72), German primrose or poison primrose (*P. obconica*) (2n= 24, 48), cowslip (*P. veris*) (2n= 22) and Chinese primrose (*P. sinensis*) (2n= 24, 36, 48) [7].

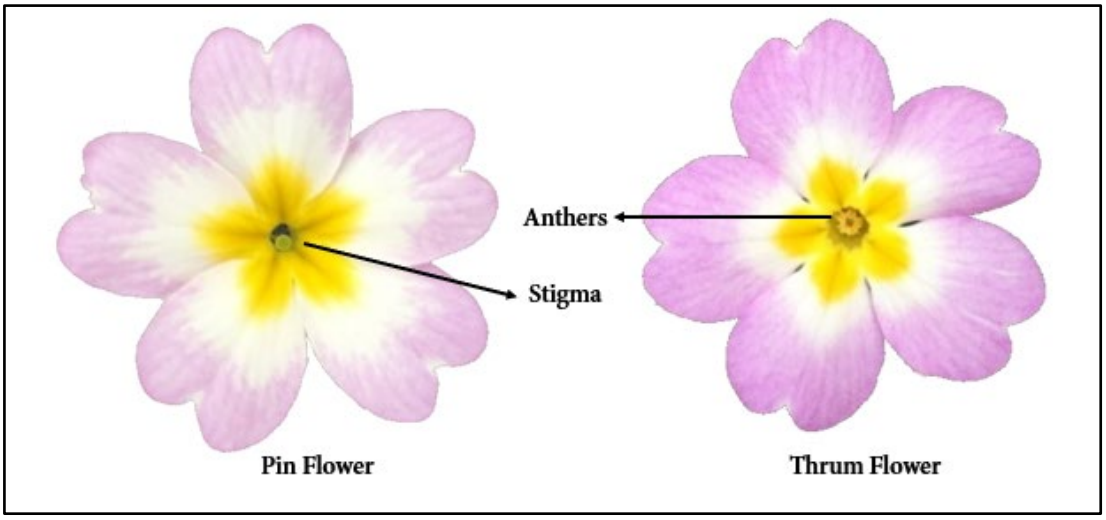

Fig. 1. *Pin and Thrum flower types in Primula vulgaris*

Primula or primrose breeding started in the early 1900s and many primula hybrids have been improved in different countries mainly through intraspecific crossings and partly through interspecific hybridization. In commercial production systems, hybrids are especially propagated by seed, while primroses can also be propagated by dividing clumps (Fig. 2). However, some problems such as irregular or late germination and low germination rate of the seeds in many primula species prevent mass propagation [8, 9, 10].

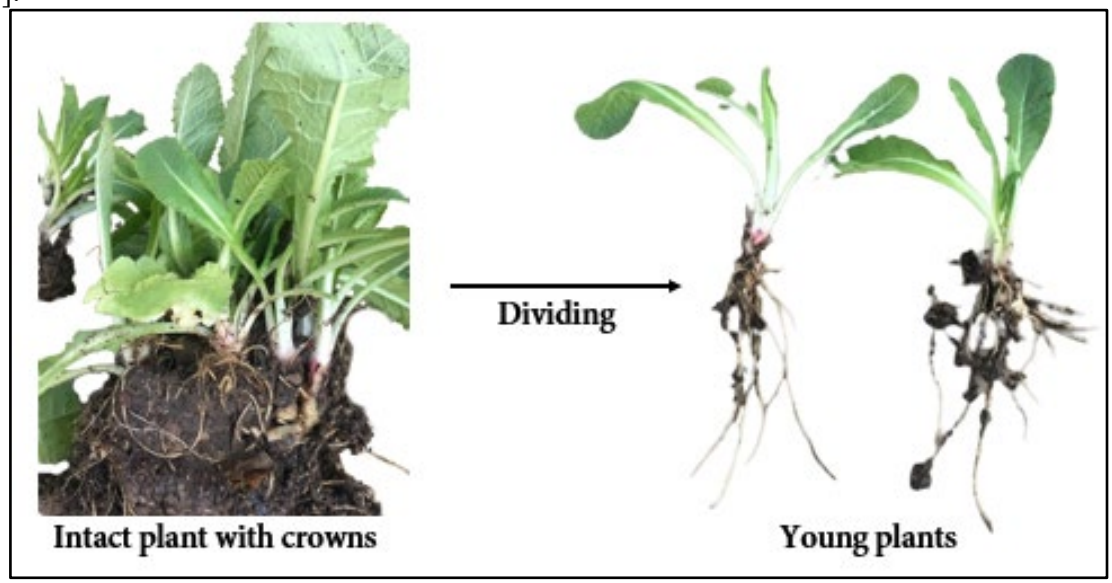

Fig. 2. *Propagation of primroses by dividing clumps.*

In addition, heteromorphic floral development and sporophytic incompatibility due to the S allels are observed and these obstacles in reproductive biology of the plant cause self-incompatibility. Therefore, tissue culture techniques are valuable tools in breeding

program, enlargement of genetic pool, gene transformation, as well as in conservation plant genetic materials in primula.

SOME OF THE IN VITRO TECHNIQUES USED IN PRIMULA

Mass propagation of Primula

Primula is the rich genus with approximately 500 species and member of the genus naturally grown in many countries, however some of them are endangered or rare due to habitat loses or other anthropologic factors. Additionally, some of the species such as *Primula veris* [11], *P. denticulata* [12] and *P. macrophylla* [13] have valuable medicinal metabolites and tissue culture techniques are often used to propagate plant materials to obtain stable high-quality metabolites [14]. The optimization an *in vitro* propagation method is also beneficial for propagation important floral characteristics such as doubled flowers for plants which do not produce seed and parental lines of commercial cultivars [15].

In vitro culture studies of *Primula* species started in late 1970s and it is focused on mass propagation due to irregular or delayed seed germination of *P. obconica* which is economically important pot plant worldwide. At present, several of the *Primula* species are successfully propagated by *in vitro* techniques, but the studies are still limited. The determination explant types, culture medium and plant growth regulators are essential prior to the *in vitro* culture. Coumans et al. [10] reported that inflorescence tip explants of *P. obconica* gave best vegetative proliferation when cultured on MS medium supplemented with 1 mg/L benzyladenine (BA) and 1 mg/L napthaleneacetic acid (NAA). Further subculture of explants allowed proliferation of vegetative buds and fivefold propagation ratio per month. Shimada et al. [16] reported that Ohashi and Mii [17], successfully obtained plantlets regenerated from leaf explants cultured on MS medium supplemented with 1 mg/L IAA (Indole-3-acetic acid) and 5 mg/L Zeatin in *P. obconica* and *P. malacoides*. Yamamoto et al. [18] cultured explants prepared from distal and proximal half of the young leaves of *P. sieboldii* on MS medium supplemented with different concentrations of BA (6-Benzylaminopurine) and NAA (1-Naphthaleneacetic acid). They determined best propagation results for shoot formation in MS medium supplemented with 1 mg/L BA and 0.1 mg/L NAA. Additionally, shoot regeneration was not obtained from the medium without NAA and distal part of the leaves of *in vitro* grown plants gave better results than explants taken from *in vivo* donor plants. Morozowska et al. [9] used shoot tip explants of *P. vulgaris* for micropropagation and MS supplemented with 4.44 µM BA and 1.13 µM 2,4-D (2,4-dichlorophenoxyacetic acid) was found to be optimal regeneration medium.

Mass propagation studies of *Primula* species conducted by many researchers and they were successful on inducing adventitious shoots from different explants cultured on medium supplemented with various concentrations of auxin and cytokinine. In our lab, wild *P. vulgaris* plants cultured ex situ and seeds were harvested after flowering period. Surface sterilized seeds were cultured on MS medium supplemented with 1.0-1.5 mg/L BA, 3% sucrose and a few seeds were germinated and turn into plantlets. Then each clump was dived and plantlets were sub-cultured on same medium. Further subculture was performed approximately every two months. We observed 4-8 fold propagation ratio in each subculture and some of the plantlets were rooted during *in vitro* culture (Fig 3a).

Additionally, some of the plants were died, when the subculture period extended (data not published) (Fig 3b).

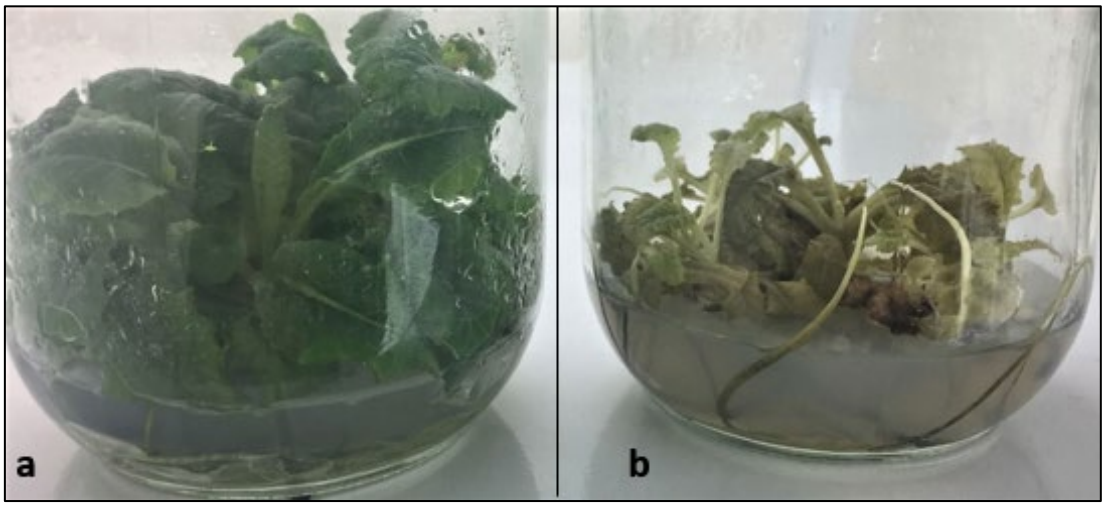

Fig. 3. *In vitro culture of wild P. vulgaris genotypes (a: rooted plantlets during culture, b: dead plants in extended subculture period)*

The regeneration capacity of *Primula* species varies depending on culture conditions, explant types, subculture interval, concentrations of plant growth regulator. As a result, lower concentrations of cytokinine or combination with auxine in tissue culture media gave successful results for mass propagation. On the other hand, concentrations of surface sterilization agents should be lower, when the young tissues such as young leaves and shoot tips will be used as explants. Moreover, endangered or rare plants can be propagated *ex situ* before tissue culture studies in order to prevent losing plant material or seeds can be chosen as an explant resource.

Organogenesis and somatic embryogenesis

One of the important key points in tissue culture studies is planning which explants will be cultured and which developmental stages will be followed. The explants cultured on medium follows two regeneration pathways, organogenesis and somatic embryogenesis. In the somatic embriyogenesis pathway, bipolar structures regenerate from a single cell and they have no vascular connection [19]. Unlike somatic embryogenesis, unipolar structures occur in the organogenesis pathway and vascular connection is formed between the formed structures and the donor plant. Structurally, the main difference of both pathways from each other is the formation of bipolar or unipolar structures as a result of the development phase [20].

According to previous studies of direct and indirect organogenesis, leaf explants commonly were used to induce regeneration in *Primula*. The regeneration was also obtained from root and pedicle explants in limited number of the studies. This is probably due to higher regeneration capacity of leaf explants or to overcome taking explants depended vegetation period of the donor plants. Additionally, leaves can be superior taken from *in vitro* grown seedling are preferred to overcome contamination problem and seasonal limitations.

The explant cultured on tissue culture media follow two organogenic pathways in organogenesis. In indirect organogenesis, a callus phase develops from explant cultured on medium, while shoots or roots are regenerated in direct organogenesis. The callus

phase can be obtained from explants cultured on media containing higher auxin than cytokinine plant growth regulators. Different concentrations of 2,4-D and TDZ are major growth regulators to induce callus in *Primula* studies in order to obtain adventitious shoots through indirect organogenesis technique (Table 1). Hayta et al. [21] reported that TDZ and auxin concentrations were crucial for promoting dedifferentiation and regeneration in *P. vulgaris*. They observed that the media supplemented with 4.0 mgL^{-1} 2,4-D+2.0 mgL^{-1} TDZ, and 2.0 mgL^{-1} TDZ+0.5 mgL^{-1} NAA and 3.0 mgL^{-1} TDZ+ 0.3 mgL^{-1} NAA had the highest callus induction rates (100%). Additionally, they indicated that TDZ alone was detrimental to the plant material and failed to initiate callus. However, Shimada et al. [16] reported that using media supplemented with 1.0 mg/L TDZ alone induced somatic embryogenesis from leaf explants of *P. cuneifolia* var. *hakusanensis*. Takihira et al. [22] successfully obtained adventitious shoots from explants cultured on LS + 0.2 TDZ mg/L +2.0 mg/L NAA in *P. x pubescens* cv. 'Borders mixed', while Hamidoghli et al. [23] reported that adventitious shoots regenerated from explants cultured on MS + 2.0 mg/L TDZ + 1.0 mg/L NAA (Table 1). This differences in regeneration response occurred in explants cultured on TDZ containing media could be reasoned chemical behavior of TDZ which auxin and cytokinin like affects due to its ability to modulate endogenous growth regulators [21]. Additionally, Schween and Schwenkel [24] observed genotypic effects on regeneration of six cultivars of *Primula*. The callus regeneration rates ranged from 25% to 96% and shoots regeneration rates were 0.0-11.6% depending on genotypes, the duration of plant growth regulator treatment.

In vitro androgenesis and gynogenesis

Haploid and double haploid plants are one of the key factors in plant breeding program. It is superior to conventional techniques due to facilitate obtaining complete homozygosity in short time. The gynogenesis comprises culture of unfertilized ovary, ovule, or flower bud, while anther and microspore cultures are used in androgenesis. In both pathways, not only haploid plants can be regenerated from explants but also the plant with same chromosome level as donor plant can be regenerated on culture media due to undesired regeneration from somatic tissues.

In a number of studies androgenesis and gynogenesis conducted on *Primula* species. Ovule or ovary culture studies mainly conducted for embryo rescue processes in interspecific hybridization of *Primula* species, while anther culture preferred to induce haploidisation. Bajaj [26] reported that plantlets regenerated from anther culture of *P. obconica* frozen in liquid nitrogen aiming possibility of long-term preservation of haploid germplasm. The young anthers at the uninucleate pollen stage frozen in liquid nitrogen according to cryopreservation protocol of the author, then some of the explants cultured on MS medium supplemented with 0.5 mg/L IAA + 0.5 mg/L 2,4-D and 2.0 mg/L zeatin. Plantlets obtained directly from pollen-embryos, and indirectly through the differentiation of callus. Additionally, Jia et al. [27] demonstrated that lengths of 4.0–5.0 mm flower buds were at the appropriate microspore developmental stage and the highest callus induction was obtained from explants cultured on MS + 1.0 mg/L BAP + 0.5 mg/L 2,4-D. The highest induction and proliferation of indefinite buds (55.2%) was produced on MS + 0.2 mg/L BAP + 0.01 mg/L NAA. As a result of their results, totally 516 plantlets were obtained and 2% of the plantlets were haploid according to cytological analysis and flow cytometry.

Table 1. *Studies on somatic embryogenesis and organogenesis in Primula spp.*

Species	Path	Exp.	Media	Resp.	References
P. cuneifolia	SE	L.	LS + 1.0 mg/L TDZ or 5.0-10.0 mg/L Zeatin	Emb.	[16]
Primula spp.	IDO	P.	Callus induction media: ½ MS + 4.0 mg/L 2,4-D + 2.0 mg/L TDZ, shoots regenerated on hormone free media	ASh.	[25]
P. vulgaris	IDO	P.	Callus induction media: ½ MS 4.0 mg/L 2,4-D + 2.0 mg/L TDZ, shoots regenerated on hormone free media	ASh.	[24]
P. x pubescens	DO	L.	LS + 0.2 TDZ mg/L 2.0 mg/L NAA	ASh.	[22]
P. heterochroma	DO	L.	MS + 2.0 mg/L TDZ + 1.0 mg/L NAA	ASh.	[23]
P. veris	IDO	R.	Callus induction media: MS + 0.1 mg/L BA + 5.0 mg/L PIC, Shoot regeneration: 0.5 mg/L TDZ + 0.1 mg/L NAA	ASh.	[14]
P. vulgaris	IDO	L.	Callus induction media: B5 macro salts + ½ MS micro salts (basal media) + 4.0 mg/L 2,4-D + 2.0 mg/L TDZ, shoots regenerated on basal medium +3.0 mg/L TDZ + 0.3 mg/L NAA	ASh.	[21]

ASh.: Adventitious shoot, DO: Direct organogenesis, Emb.: Embryo, Exp.: Explant, IDO: Indirect organogenesis, L.: Leaf, P.: Pedicle, Resp. Response, R.: Root, SE: Somatic embryo.

CONCLUSIONS

The genus *Primula* is the largest genus in Primulaceae family and it has valuable species for ornamental plant industry. The member of the genus has various reproductive barriers such as heteromorph flower or self-incompatibility. Additionally, some of the species are under threat due to habitat loses or other anthropological affects. Modern biotechnological methods including plant tissue culture have been taken part in breeding strategies of plant. Several biotechnological methods could be applied to plant to have better ones in the process of plant breeding. Plant tissue culture techniques serve essential

tools for conservation of genetic diversity or development new cultivar through breeding [28, 29]. In different *Primula* species, *in vitro* culture techniques were successfully performed. It has been seen that *Primula* species can be easily propagated using appropriate explants, plant growth regulators and tissue culture media. However, these factors should be optimized individually for each species to obtain high efficiency in *in vitro* culture studies.

REFERENCES

[1] Enache, M., Băbeanu, N. (2008): Micropropagation Of Garden Plants (2): Rare Primulas Micropropagarea Plantelor De Grădină (2): Primule Rare. Scientific Bulletin Biotechnology, U. Ş. A. M. V. Bucharest, Serie F, Vol. XIII, 5-9.

[2] Smith, W.W., Forrest, G. (1929): The sections of the genus *Primula*. Journal of the Royal Horticultural Society of London 54, 1–50.

[3] Kovtonyuk, N. K., Goncharov, A. A. (2009): Phylogenetic relationships in the genus *Primula* L. (Primulaceae) inferred from the ITS region sequences of nuclear rDNA. Russian Journal of Genetics 45(6), 663-670.

[4] Anderberg, A. (2004): Primulaceae, In: Kubitzki, K. (Eds) The Families and Genera of Vascular Plants. Flowering Plants, Dicotyledons: Celastrales, Oxalidales, Rosales, Cornales, Ericales. Springer-Verlag Berlin Heidelberg. New York, 313-319.

[5] Trift, I., Källersjö, M., Anderberg, A. A. (2002): The monophyly of *Primula* (Primulaceae) evaluated by analysis of sequences from the chloroplast gene rbcL. Systematic Botany 27(2), 396-407.

[6] Li, J., Webster, M. A., Smith, M. C., Gilmartin, P. M. (2011): Floral heteromorphy in *Primula vulgaris*: progress towards isolation and characterization of the S locus. Annals of botany 108(4), 715-726.

[7] Karlsson, M. G. (2001): Primula culture and production. Horttechnology 11(4), 627-635.

[8] Coumans, M., Coumans-Gilles, M. F., Delhez, J., Gaspar, T. (1979): Mass propagation of *Primula obconica*. Acta Horticulturae 91, 287-294.

[9] Morozowska, M., Cieszkowski, A., Polskiego, W. (2004): In vitro clonal propagation of *Primula veris* L. and preliminary phytochemical analysis. Acta Biologica Cracoviensia Series Botanica 46: 169-175.

[10] Sharaf, A. R. N., Hamidoghli, Y., Zakizadeh, H. (2011): In vitro seed germination and micropropagation of primrose (*Primula heterochroma* Stapf.) an endemic endangered Iranian species via shoot tip explants. Horticulture Environment and Biotechnology 52(3), 298-302.

[11] Başbülbül, G., Özmen, A., Biyik, H. H., Şen, Ö. (2008): Antimitotic and antibacterial effects of the *Primula veris* L. flower extracts. Caryologia 61(1), 88-91.

[12] Aslam, K., Nawchoo, I. A., Ganai, B. A. (2015): In vitro antioxidant, antibacterial activity and phytochemical studies of *Primula denticulata* an important medicinal plant of Kashmir Himalaya. International Journal of Pharmacological Research *5*(3), 49-56.

[13] Najmus-Saqib, Q., Alam, F., Ahmad, M. (2009): Antimicrobial and cytotoxicity activities of the medicinal plant Primula macrophylla. Journal of Enzyme Inhibition and Medicinal Chemistry 24(3), 697-701.

[14] Morozowska, M., Wesołowska, M., Głowicka-Wołoszyn, R., Kosińska, A. (2013): Effect of plant growth regulators and explant types on induction and growth of callus of *Primula veris* L. Herba Polonica 59(3), 17-25.

[15] Kanda, M. (1996): Somaclonal Variation in Primula. In Somaclonal Variation in Crop Improvement II. Springer Berlin, Heidelberg. pp. 305-314.

[16] Shimada, T., Matsushita, T., Otani, M. (1997): Plant regeneration from leaf explants of *Primula cuneifolia* var. *hakusanensis*,"Hakusan-kozakura". Plant Biotechnology 14(1), 47-50.

[17] Ohashi, H., Mii, M. (1988): In Higuchi, S (Eds) In Vitro Culture of Horticultural Plants. Shibata Hario Garasu Ltd.Tokyo. p. 236- 237
[18] Yamamoto, T., Magaya, Y., Maruyama, Y. (1999): Mass propagation of *Primula sieboldii* E. Morr. through leaf segment culture. Bulletin of the Faculty of Horticulture Minamikyushu University. Series A. Natural Science (Japan).
[19] Haccius, B. (1978): Question of unicellar origin of non-zygotic embryos in callus cultures. Phytomorphology 28:74-81.
[20] Brown, D. C. W., Thorpe, T. A. (1986): Plant Regeneration by Organogenesis. In: Vasil IK (Ed) Cell Culture and Somatic Cell Genetic of Plants Vol. 3: Plant Regeneration and Genetic Variability. Academic Press Inc. Florida, USA, pp. 49-65.
[21] Hayta, S., Smedley, M. A., Li, J., Harwood, W. A., Gilmartin, P. M. (2016): Plant regeneration from leaf-derived callus cultures of primrose (*Primula vulgaris*). HortScience 51(5), 558-562.
[22] Takihira, M., Otani, M., Tsuchiya, S., Shimada, T. (2007). Plant regeneration from leaf explants of auricula cultivars (*Primula× pubescens* Jacq.). Plant Biotechnology *24*(4), 425-427.
[23] Hamidoghli, Y., Noroozi Sharaf, A. R., Zakizadeh, H. (2011): Organogenesis from seedling derived leaf explants of primrose (*Primula heterochroma* Stapf.) as a scarce Iranian Plant. Australian Journal of Crop Science 5(4), 391.
[24] Schween, G., Schwenkel, H. G. (2003): Effect of genotype on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in the greenhouse of *Primula* ssp. Plant Cell, Tissue and Organ Culture *72*(1), 53-61.
[25] Schween, G., Schwenkel, H. G. (2002): In vitro regeneration in *Primula* ssp. via organogenesis. Plant Cell Reports 20(11), 1006-1010.
[26] Bajaj, Y. P. S. (1981): Regeneration of plants from ultra-low frozen anthers of *Primula obconica*. Scientia Horticulturae 14(1), 93-95.
[27] Jia, Y., Zhang, Q. X., Pan, H. T., Wang, S. Q., Liu, Q. L., Sun, L. X. (2014): Callus induction and haploid plant regeneration from baby primrose (*Primula forbesii* Franch.) anther culture. Scientia Horticulturae *176*, 273-281.
[28] Dönmez, D., Şimşek, Ö., Kaçar, Y. A. (2016): Genetic engineering techniques in fruit science. International Journal of Environmental and Agriculture Research, 2(12), 115-128.
[29] Simsek, O., Kacar, Y. A. (2010). Discovery of mutations with TILLING and ECOTILLING in plant genomes. Scientific Research and Essays, 5(24), 3799-3802.

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Review Article

AN ANTHOLOGY OF CYTOGENETIC END POINTS LIKE MICRONUCLEUS TEST, COMET ASSAY AND CHROMOSOMAL ABERRATION ASSAY IN PISCES

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ABSTRACT. Growing awareness for chemicals with potential hazards has stimulated significant interest to use fishes as indicators for mutagens, carcinogens and teratogens in aquatic ecosystem as they take higher place in the food chain forming significant source of food and nutrition for human affecting health directly. They are generally considered as best animal model for studies with advantages of monitoring genotoxicity owing to their ability to metabolize xenobiotics and accumulate pollutants. This has led todevelop several biological tests to detect and identify such effectsthrough various cytogenetic endpoints which are very sensitive genetic assays to detect environmental mutagens at sub-toxic levels. Micronuclei tests (MN), comet assay and chromosomal aberrations test (CA) in fishes were reported as useful biomarker of in vivo techniques from time to time with potential for in situ monitoring of water quality. Various techniques of cytogenetic end point assay using fish in vitro and in vivo and their applications to environmental monitoring and eco genotoxicology has gained considerable significance from time to time. This article aims to present an anthology of such studies in piscine models to realize their significance in environmental mutagenesis. It justifies the use of piscine models in such studies like any other experimental animal model.

Keywords: *Fishes, Genetic Disorder, Hazards, Mutagens, Pollutants*

INTRODUCTION

Since last few decades, human civilization has been exposed to a very huge number of chemicals with potential hazards to genomeeither knowingly or inadvertently, while accomplishing all regular day to day activities. The industrial development and rapid urbanization has led to development of polluted zones discharging potentially toxic compounds in the environment. Quite a large number of chemical pollutants have proven record of potential hazards to affected organisms. Thousands of natural or synthetic materials in their ionic, simpler or complex molecular forms are used for different purposes in pharmaceuticals, drugs, beverages, cosmetics, perfumes, confectionaries, tobacco industries, food and food industries, food colorants, dyes, paints, enamels, cement, asbestos etc. and many more. These are added regularly to the environment in a pursuit to improve life style and living standard. In addition, various organic and inorganic effluents from different mills or industries, primary or secondary metabolites, heavy metals like Pb, Al, Cr, Ni, As, Sb, Zn, Sn, Hg and agrochemicals like fertilizers, pesticides, insecticides, herbicides increase the bulk content of this list of hazardous materials. Many of them can induce death of exposed organisms orcause physiological alterations or genetic disorders.

Mutagenicity induced by heavy metals even in sub-lethal concentrations can cause development of tumor [1] or undesirable alterations in genetic materials [2, 3]. Some of these chemicals in low concentrations may not cause acute detectable effects but in the

long run may reduce the life span [4] of the organisms exposed to them. Higher level of Pb and Hg can cause wide range of toxicity like muscular and neurological degeneration, destructive growth inhibition, mortality, reproductive problems and paralysis [5]. Various metallic ions act as genotoxins at particular concentrations due to their ability to bind to thiol groups and induce instability in the spindle formation in the cells [6]. Some heavy metals, such as copper, iron, nickel and chromium are importantmetals due to their essential functions in living systems, whereas cadmium and Pb are non-essential and are toxic even in trace amounts [7]. Certain agrochemicals inhibit cell division, induce chromosomal abnormalities and damage the genome [8]. This article aims to present an anthology of such studies in various fishes with particular reference to cytogenetic end points realizing their significance in environmental genotoxicity.

Why Piscine models?

Aquatic environment covers more than two-thirds of the earth inhabited by more than 28,000 fish species [9]. Growing awareness for the aquatic pollutant generated potential hazards has stimulated significant interest in their use as indicators for monitoring of environmental mutagens, carcinogens and teratogens [10]. They have been used in numerous biochemical and toxicological studies linked to development, carcinogenicity and teratogenicity both in vitro and in vivo [11, 12, 13] due to their easiness to handle and maintain ad libitum by relatively low cost methods [14]. Aquatic environment remains the ultimate recipient of an increasing number of agrochemicals where many of themare able to interact with DNA leading to gene mutation or genetic syndromes [15, 16] in aquatic organisms, particularly fishes. For different assay/analytical activities at genomic/cellular level, various species of fishes have been proved as suitable piscine model. Rodriguez-Cea et al. [17] noted that some fish species are more sensitive to genotoxic pollutants than other species such as eel (Anguilla anguilla) or minnow (Phoxinus phoxinus). As per Braunbeck et al. [18] and Osman et al. [19], they are considered to be efficient and cost effective as best toxicity indicators (Ruperelia et al. [20] and Barse et al. [21]) for evaluation of potentially teratogenic and carcinogenic substances [22]. At the top of the aquatic food chain they may directly affect human health by making them significant for their bio monitoring [23, 24]. Any change in the natural conditions of aquatic medium causes several physiological adjustments in them [25]. Fishes can take up both essential metals and nonessential metals which accumulate in their tissues [26]. As per Lavanya et al. [27], contaminants get accumulated in major aquatic organisms and Adeogun and Chukwuka [28] reported that, they are the final sink for many chemicals with long term effect on reproduction and gene pool of organisms. Rivero-Wendt et al. [29] proved lack of genotoxicity of 17α-Methyltestosterone (MT) in Oreochromis niloticus and Astyanax bimaculatus by cytogenetic studies. Some of their advantages as suitable model for monitoring aquatic genotoxicity owe to their ability to metabolize xenobiotics and accumulate pollutants [30, 31, 32]. Akpoilih [33] examined the use of ecogenotoxicology in environmental monitoring, the role of fish in genotoxicity testing of pollutants, genetic basis in genotoxicological assessment, current methods of ecogenotoxicological hazard assessment using fish in vitro and in vivo and their applications to environmental monitoring as well as recent advances in the field of fish eco genotoxicology and highlighted limitations and recommendations for further research on the use of eco genotoxicology.

Cytogenetic end points

Krishnaja and Rege [34] showed Boleophthalmus dussumieri as a satisfactory cytogenetic model in vivo for mutagenic studies. Manna [35] informed that, cytogenetic analysis constitutes important short term assay system for evaluation of genotoxic potentials of environmental agents which were applicable earlier to non piscine models formetaphase analysis [35], micronucleus test [36, 37], sister chromatid exchange analysis [38] and spermatocyte chromosome analysis of in vivo models. According to Manna [39], fishes are employed to assess the cytogenetic effects of environmental and manmade mutagens [40, 41, 42] in the aquatic ecosystems supported by Braunbeck et al. [18], Mitchell and Kennedy [43] and Akiyama et al. [44]. Gopal Krishna [45] reported on detection of damage due to genotoxicants, radiation, and apoptosiscausing depletion of fish resources could be addressed through cytogenetic end points. Chromosomal aberrations test (CA) and micronuclei tests (MN) in fishes were reported as useful biomarker of in vivo techniques [46, 47]. Alink et al. [48] reported that, Eastern mudminnow (Umbra pygmaea L.) exposed for 11 days to Rhine water had a significantly higher number of SCE and an increased comet tail length compared with control fish exposed to groundwaterand concluded that genotoxins are still present in the river Rhine, but the genotoxic potential has markedly decreased as compared. Genotoxic studies using cytogenetic analysis in fishes have been demonstrated by a number of workers [2, 46, 49, 50, 51, 52, 53]. Insecticides/pesticides lead to DNA damage in form of micronucleus formation, chromosome aberrations and mitotic aberrations [54, 55, 56]. Mahboob et al. [57] reported cytogenetic effect of heavy metal in Clarias gariepinus using the micronucleus test, chromosomal aberrations and sister chromatid exchange suggesting that $HgCl_2$ caused genotoxic effects in fish. For agrochemicals, Cypermethrin induced genotoxicity was studied by various cytogenetic end points in different organisms like Simoniello et al. [58] in Prochilodus lineatus evaluating DNA damage using alkaline comet assay, Ansari et al. [59] in Channa punctatus by MN studies, Rana [60] in Channa punctatus by chromosomes analysis; Rakesh [61] in Labeo rohita inducing chromosomal aberrations. Similarly, genotoxicity of Malathion was reported by various workers in fishes like Kumar et al. [62] in Channa punctatus by micronucleus and comet assay; Parveen and Shaadab [63] in the same species revealing clastogeneicity of chromosomes. Thus many chemicals were tested from time to time in different piscine models revealing the importance of cytogenetic end point analysis in genotoxicity studies.

a) Micronucleus test (MN)

The MN test, developed by Schmidt [64] is an in vivo and in vitro short time screening method widely used to detect genotoxic effects. These are cytoplasmic mass of chromatin with appearance of small nuclei arising from chromosomes lagging behind in anaphase. Using them, scientists evaluated potential clastogenicity of inhaled substances like cigarette smoke, methyl isocyanate, ozone and many other chemicals by analyzing bone marrow cells and blood lymphocytes of Chinese hamsters, rats and mice [65, 66, 67] and MN were first described in cytoplasm of erythrocytes as “fragment of nuclear material” by Howell or “intra-globularies corpuscles” in terminology of Jolly in late 18th century and early 1900 known to haematologists as Howell-Jolly bodies [68]. According to Heddle et al. [69], clastogenic and aneugenic agents are known to affect the spindle apparatus and can be differentiated on the basis of the relatively induced micronucleus sizes. Heddle and Salmone [70] described it as one of the simplest,

reliable, least expensive, sensitive and rapid screening system for both clastogenic and aneugenic effects [46, 71, 72] The micronucleus test in circulating erythrocytes of fish has been widely employed for both in situ exposure to environmental waters [73, 74, 75] and laboratory treatments in vivo [49, 76, 77, 78] in particular reference to Cyprinus carpio [79, 80, 81]. Their count has served as an index of chromosome break and mitotic spindle dysfunction [82]. It is widely employed to assess the biological impacts of aquatic pollutants [83, 84, 85] since it is associated with chromosome aberrations [86]. MN is the most widely used assay due to its proven correctness for fish [46]. Their presence in cells reflects structural and/or numerical chromosomal aberrations arising during mitosis [69, 87, 88] and its frequency is extensively used as a biomarker of genomic stability [88]. The mean frequencies of MN in piscivorous species have been shown to be almost five fold higher in the detrivorous and/or omnivorous species [89] and also reported significantly higher mean frequencies of MN in Prochilodus nigricans (detritivorous), Mylossoma duriventris (omnivorous) and Hoplias malabaricus (piscivorous) from the Madeira River compared to the frequencies observed in the same species in the Solimoes River. The MN has been employed successfully in various fish species to detect mutagenic changes caused by aquatic pollutants [90]. As per Fenech et al. [91]) and Udroiu [92] it is one of the most popular tests of environmental genotoxicity serving as an index of cytogenetic damage. According to Kirsch Volders et al. [93], the assay is a multi endpoint test of genotoxic responses to clastogens. The assay is sensitive for evaluating genotoxicity of compounds in fish [94] commonly used for the estimation of biological impacts of water pollutants in fish [95]. Their appearance in the cytoplasm is considered as biomarker of DNA damage [96]. Micronuclei are very small fragments of chromatin material developed from broken section of chromosome or from the chromosomes that could not be incorporated into daughter nuclei [97]. Erythrocytes of fish present a high frequency of MN and NAs after exposure to different heavy metals [46, 98, 99]. Their presence in cell reflects structural and/or numerical chromosomal aberrations [100]. The assay is an easy and ideal monitoring system to assess genotoxicity of water [101] allowing quick result for bio monitoring [84] of aquatic pollutants [102]. MN ensures continuous and effective evaluation of metallic pollution in aquatic environments [103]. MN is applied to evaluate genotoxicity of chemicals in fishes and their biological monitoring [104, 105].

Fig. 1. *Micronuclei induced infishes due to genotoxicity of chemicals*

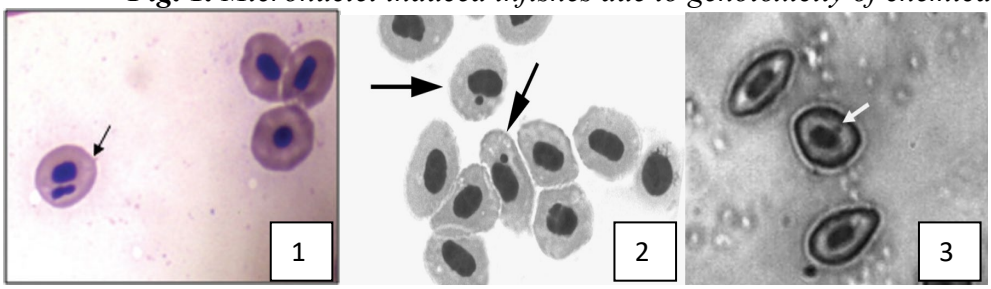

Plate 1: MN of Cirrhinus mrigala after exposure to Chlorpyrifos [106], **Plate 2:** MN of Heteropneustes fossilis induced by synthetic Sindoor [107], **Plate 3:** MN of C. macropomum treated with Methyl Mercury [108]

Table 1. *Micronuclei induced in different fishes*

Sl No	Source	Year	Species	Conclusion
1	Bahari et al. [109]	1994	Clarias gariepinus	Concentration and time dependent increase in frequency
2	Sandra et al. [110]	1996	Barbus plebejus	MN for in situ mutagens in freshwaters
3	Svobodova et al. [111]	1997	Cyprinus carpio	Malachite green induces MN
4	Campana et al. [112]	1999	Cheridon interruptus interruptus	Genotoxicity of pyrethroid lambda cyhalothrin
5	De Lemos et al. [113]	2001	Pimephales promelas	Significant induction of micronucleated erythrocytes exposed to chromium (VI)
6	Gustavino et al. [81]	2001	Cyprinus carpio	Dose dependent increase in MN due to X- rays
7	Ale et al. [114]	2004	Oreochromis niloticus	Evaluated genotoxic effect of $(NO_3)_2Pb$
8	Ferraro et al. [115]	2004	Hoplias malabaricus	Evaluated mutagenic potential of tributyltin (TBT) and inorganic lead(PbII)
9	Farah et al. [116]	2006	Channa punctatus	Possible anti mutagenic potential of ethanolic extract of neem leaves
10	Jiraungkoorskul et al. [117]	2007	O. niloticus, Poronotus triacanthus and Puntius altus.	Induction of MN and other nuclear abnormalities
11	Ali et al. [118]	2008	Channa punctatus	Increasing effect on MN frequency with concentration of Chlorpyrifos
12	Galindo and Moreira [119]	2009	Bathygobius soporator	Verified the efficiency of MN and NAs
13	Rocha et al.[108]	2009	Colossoma macropomum	MN and other NAs due to Methyl Mercury
14	Candioti et al. [120]	2010	Cnesterodon decemmaculatus	Genotoxicity of Aficida® by inducing MN
15	Ahmed et al. [121]	2011	Oreochromis mossambicus	Concentration dependent increase due to As
16	Ansari et al. [59]	2011	Channa punctatus	Cytogenetic effects of Cypermethrin using CA and MN
17	Ghisi et al. [122]	2011	Rhamdia quelen	MN and NAs due to Fipronil
18	Guner et al. [123]	2011	G. affinis	Significantly increased frequency of NAdue to Cu and Cd
19	Ansoar Rodriguez et al. [124]	2015	Oreochromis niloticus	Effect of Imidacloprid on genetic material using MN test and comet assay
20	Dar et al. [31]	2015	Carassius carassius (Cyprinidae)	Genotoxicity of Endosulfan by MN

MN: Micronuclei, CA; Chromosomal Aberration; NA: Nuclear Abnormalities, As: arsenic, Pb: Lead; Cu: Copper; Cd: Cadmium

b) Chromosomal aberration test (CA)

Chromosomal aberrations in fishes exposed to polluted aquatic environment were reported by several authors [125, 126, 127, 128, 129]. Carrasco et al. [130] reported formation of morphological nuclear abnormalities (NAs) in fish erythrocytes which includes lobbed (LB), blebbed (BL) and notched (NT) nuclei and bi nucleated (BN)

cells. According to Matter et al. [131], chromosomal aberration results from abnormalities in DNAduplication during S-phase. As per Das and John [132], genotoxic potential of methyl parathion and phosphamidon could be studied through induction of sister chromatid exchanges (SCE) and chromosome aberrations in gill tissues of Etroplus suratensis. Anitha et al. [133] showed the importance of aberration instudying the genotoxic effect of heat shock at different temperatures on gold fish Carassius auratus. Ouseph et al. [134] reported impact of physicochemical characteristics of river Cooum in Madras on the karyology of a native fish species Mystus vittatus where the species from the polluted river Cooum shows polyploidy, endo reduplication and condensed nature of chromosomal morphology causing irreparable damage to the genetic material of the fish as they are indicators of aquatic pollution. As per Mahrous and Abdou [135], water pollutants caused significant changesin chromosomal structures and centromeric attenuation in Oreochromis niloticus and Clarias lazera. Lopez-Poleza [136]) evaluated genotoxic effects of methyl mercury (CH_3Hg^+) in Hoplias malabaricus, using CA, MN and Comet assay. Cestari et al. [137] reported effects of clastogenic or mutagenic agents in neotropical fish Hoplias malabaricus using the comet (SCGE) assay and by testing for chromosomal aberrations showing that exposure leading significantly to increase frequency of chromosomal aberrations and the frequency of tailed cell nuclei indicating DNA damage. Chandra and Khuda-Bukhsh [138] studied the genotoxic effects of cadmium chloride ($CdCl_2$) and azadirachtin (Aza) singly and conjointly in a fish, Oreochromis mossambicus, with endpoints such as chromosome aberrations, abnormal red cell nuclei, abnormal sperm morphology and protein content. The bi-nucleation is an indicator of abnormal cell division due to blocking of cytokinesis resulting in genetic imbalance in the cells, may be involved in carcinogenesis [3]. Gadhia et al. [139] reported mitotic chromosomes from the gills of Boleophthalmus dussumieri for induction of CA after in vivo treatments with Bleomycin, Mitomycin-C and Doxorubicin revealing dose and time dependent increase in CA observing chromatid breaks, acentric fragments, dicentric and ring configurations. An increase in chromatid breaks andchromosomal exchange due to fluoride was reported by Chaurasia and Kumari [140]. Palikova et al. [141] reported genotoxicity of semi purified compound of microcystins and crude extract of cyanobacteria using detection of chromosomal aberrations in early life stages of weather fish revealing chromatid (gaps) and chromosomal aberrations (rings, dicentrics), percentage of which increased with the increased concentration of microcystins and the higher doses of crude cyanobacterial extract. Mohamed et al. [142] explored the capability of copper sulfate ($CuSO_4$) and lead acetate $(CHCOO)_3$ Pb in inducing chromosomal aberrations in aquatic organisms choosing Oreochromis niloticus and found that, effect of both chemicals on fish chromosomes and mitotic indices in gill cells displayed lower mitotic activity and positively induced macro-DNA damage represented by different types of aberrations e.g., chromatid deletions, chromatid breaks, gaps, fragments, stickness, translocations, ring chromosomes and centromeric attenuation. An increase in chromatid break and chromosomal exchange has been reportedby Rita and Milton [143] in Orechromis mosambicus on exposure to chromium. Kaur et al. [144] employed chromosomal aberration to study genotoxicity caused by dyeing industry effluent on a freshwater fish Cirrhinus mrigala and found chromosomal aberrations like chromosomal fragmentations (Cf), ring chromosomes (Rc), terminal chromatid deletions (Tcd), minutes (M), centromeric gaps (Cg), stickiness (Stk), clumping (C), pycnosis (Py), stretching (Stch) and pulverization (P). Yadav et al. [145] reported significant

frequencies of chromosomal aberration in a time dependent response in Cirrhinus mrigala exposed to Butachlor, showing stickiness and clumping of chromosomes demonstrating its genotoxic potential suggesting that, it interferes with cellular activities in fishes at genetic level inducing chromosomal aberrations. Promsid et al. [146], investigated chromosomal aberrations of snake head fish in a leachate-affected reservoir containing lead and mercury in water sediment observing four types of chromosomal breakages: single chromatid gap, isochromatid gap, single chromatid breaks and isochromatid breaks. Rana [60] revealed genotoxic potential of cypermethrin in Channa punctatus indicating the possibility of using fish chromosomes as indicators of genotoxic factors. Tengjaroenkul et al. [147] investigated chromosomal aberration in Rasbora tornieridue to arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb) contamination in water near gold mine area with higher chromosomal aberrations showing six types of chromosomal aberrations including centric fragmentation (CF), centric gap, single chromatid gap, fragmentation, deletion and polyploidy. Abd Ali et al. [148] described chromosomal aberration effects of electro fishing on Poecilia latipinna, located in Shat Al-Arab river in Al-garmma city (south of Iraq) showing decrease of mitotic index and significant increase in the most frequent aberration per 150 metaphase was chromosome break, fragment, range chromosome and the sticky chromosome mean were higher in comparison to non exposed organisms. Rakesh [61] carried cytogenetic study of Labeo rohita to check effects of lethal concentration (0.06 ppm) and acute lethal concentration (0.1 ppm) of Cypermethrin inducing chromosomal aberrations like acentric fragments, rings, double minutes and chromosome break, endo-reduplication, premature separation of chromosome and pulverizarion.

Fig. 2. *Chromosomal aberration in fishes induced by genotoxic chemicals*

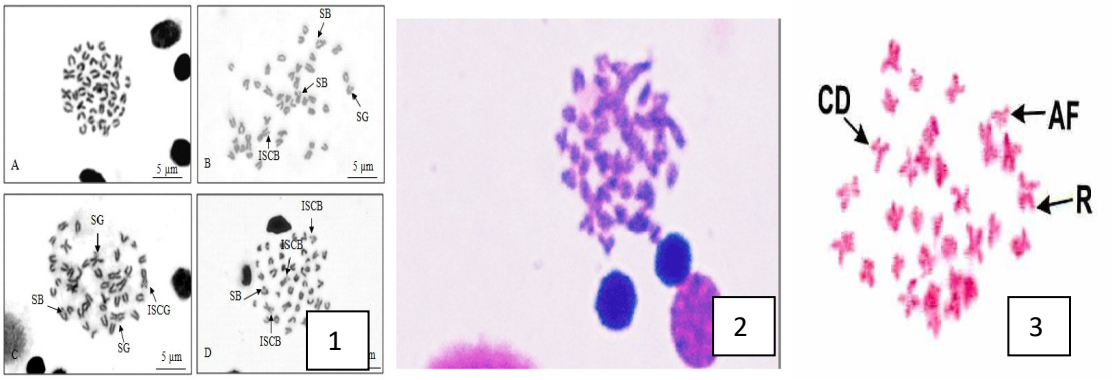

Plate-1: Chromosomal aberrations in C. striata like single chromatid gap (SG), isochromatid gap (ISCG), single chromatid breaks (SB) and isochromatid breaks (ISCB) affected by leachate of Pb and Hg [146]
Plate-2: A photomicrogragh shows a mitotic metaphase stage of O. niloticus with chromosomal fusion after treatment with Pb [142]
Plate-3: Metaphase spread with chromatid deletions (CD), acentric fragments (AF) and ring chromosomes (R) after treatment with Mitomycin-C in C. batrachus [149]

c) Single-cell gel electrophoresis (COMET) assay

The Comet assay was introduced by Ostling and Johanson [150] under neutral lysis and electrophoresis (pH 9.5) conditions as a result of studies undertaken to develop methodology of DNA electrophoresis in micro gel improved by Singh et al. [151] who launched the alkaline single cell gel electrophoresis (SCGE) based on principle of presence of single strand breaks of DNA fragments moving from the nucleoid core

towards the anode resulting in 'Comet' formation [152]. In this assay, cells are mixed with agarose and layered on microscope slides for lysis and electrophoresis; stained with fluorescent dyes i.e. DAPI or ethidium bromide for microscopic visualization of "Comets". DNA containing breaks unwinds and migrates away from the "head" (the nucleus), forming a "tail". Quantification of the amount of DNA in tails and in heads of provides an estimate of frequency of strand breaks. The molecular events that occur during processing of the cells and DNA to generate comets involve the DNA in chromatin arranged in "matrix attachment sites" and "loops" which are tightly supercoiled in undamaged dividing cells. One single-strand break is sufficient to release the superhelix tension in a loop, which is then free and can extend out from the nucleus. When the amount of damage is such that several loops have been affected, they form a "halo" that can be seen around the more intensely stained nucleus as stated by Mullenders et al [153]. Padrangi et al. [154] and Mitchelmore and Chipman [155] reported comet assay in red breast sunfish (Lepomis auritus), hard head cat fish (Anus felis), bullhead (Ameirurus nebulosus) and carp (C. carpio). DNA strand breakage could be detected by alkaline single cell gel electrophoresis (Comet assay) been applied in aquatic vertebrate and invertebrate [155, 156, 157, 158] also in several fishes showing sensitivity to genotoxic effects [155, 159, 160]. Lee and Steinert [161] informed that, exposure to genotoxins can damage the DNA of living cells and if these DNA lesions are not repaired, they can commence a cascade of biological consequences at the cellular, individual, community and finally at the population level. This has been employed since mid 1980s to study effects of environmental pollutants and occupational hazards, safety of therapeutic compounds, toxicology and to assess DNA repair capacity in human, animal and plant populations [162, 163] and to detect genetic damage in the form of DNA strand break in aquatic environments [158]. According to Ali et al. [118], Vanzella et al. [164] and Frenzilli et al. [165], comet assay has been successfully applied in many fish species exposed to different genotoxic agents, allowing the evaluation of DNA alterations with advantages like size and ploidy independency and mitotic activity is not a prerequisite as in metabolic rate and index in fish fluctuate considerably with temperature. de Campos Ventura et al. [166] showed that the assay in fishes is efficient to detect genotoxicity. In different modifications, the assay reflects variety of DNA damage in fish [167, 168, 169] and other aquatic animals [170, 171].

Comet assay has proved to be a useful tool for measuring the relationship between DNA damage and exposure of aquatic organisms to genotoxic pollutants [172] and considered more sensitive than cytogenetic techniques. According to Russo et al. [173] and Bucker et al. [174], MN is less sensitive than comet assay demonstrating genomic lesions that can be repaired reducing the number of stable lesions in DNA. Several international research groups have recommended protocols and criteria for comet assay, to establish high standards for valid, reproducible and accurate data [175, 176] increasingly used in testing of industrial chemicals, biocides, agrochemical, food additives and pharmaceuticals [176]. It is advantageous as per [118] due to its sensitivity for detecting low levels of DNA damage (0.1 DNA break/10^9 Daltons). According to Muid et al. [177], it is a suitable and rapid test for DNA damaging potential in environmental and biomonitoring studies. Nagarani et al. [178] reported utility of the assay for in vivo laboratory studies using fish.7

Table 2. *Comet Assay reported in different fishes to assess genotoxicity of chemicals*

Sl No	Source	Year	Species	Conclusion
1	Buschini et al. [179]	2004	Cyprinus carpio	Genotoxic damage due to water disinfected with sodium hypochlorite and chloride dioxide
2	Bucker et al. [174]	2006	Eingenmannia virescens	Benzene induced no significant results by MN but comet assay suggested genotoxicity in dose-dependent response
3	Vanzella et al. [164]	2007	Prochilodus lineatus	Genotoxicity of the soluble fraction of diesel (SFD) using the comet and MN
4	Wirzinger et al. [180]	2007	Gasterosteus aculeatus L.	Genotoxic potential of surface waters in Germany my MN and comet assay
5	Christofoletti et al.[181]	2008	Oreochromis niloticus	Methodological comparison of application of comet assay
6	Simoniello et al. [58]	2009	Prochilodus lineatus	Evaluated DNA damage using alkaline comet assay after in vivo exposure to Cypermethrin
7	Mitkovska et al. [182]	2017	Cyprinus carpio	Comet assay for in vitro exposure to heavy metals like Ni and Pb

MN: Micronuclei; SFD: Solid fraction of diesel; Ni: Nickel; Pb: Lead

Plate 1 **Plate 2** **Plate 3**

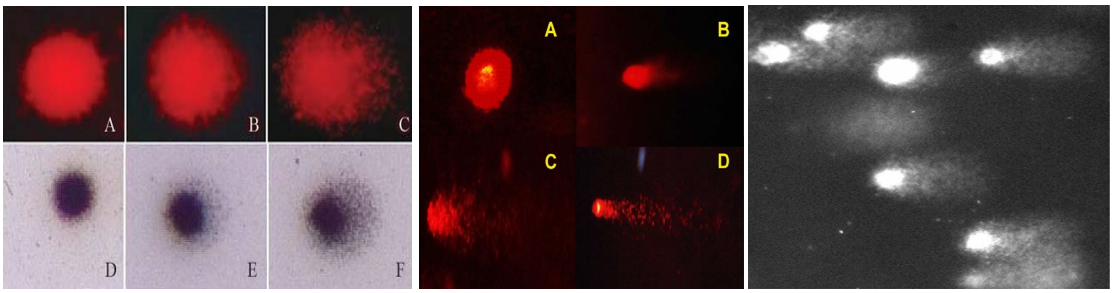

Fig. 3. *Comet test in different fish cells to reveal genotoxicity of pollutants*
Plate 1: In erythrocytes of O. niloticus using pH 12.1 stained with ethidium bromide and silver [181]
Plate 2: DNA damage in Catla catla exposed to chromium for different days [183]
Plate 3: Comets after single cell gel electrophoresis of gill cell DNA from Eastern mud minnows exposed to Rhine water [48]

Cytogenetic end point evaluations in fishes of India

Running parallel with technical advancements to assess the genotoxic potentials of various pollutants, Indian scientists were also well aware about the facts and employed fishes involving such techniques from time to time as evidenced from such vast array of references. Moorthy and Moorthy [184] analysed SCE, MN and CA in rodents exposed to mosquito coil smoke. Tripathy [185] reported that, CAs are quite significant in cytogenetic end point evaluations, including gap, chromatid break, fragments where gaps are achromatic lesion including unstained part of a chromatid appearing like an interruption; breaks are distinct dislocations of chromatid continuity and acentric fragments placed anywhere in the field of traceable origin or untraceable origin or some

available fine dots probably originated from terminal deletions. As per Bajpayee et al. [186], the comet assay is sensitive, rapid, and reliable method of quantitatively measuring DNA damage. According to Talapatra and Banerjee [187], detection of MN and NAs in fish helps us to assess the status of water quality. Sarangi [188] recommended use of MN in fish as sensitive indicator of aquatic pollution. Selection of peripheral blood erythrocytes of fish as target cell to investigate genotoxic damage is based on important role of blood in movement of toxic substances [189]. However, the anthology of references is still larger.

Table 3. *Cytogenetic assays in different fishes of India*

Sl No	Source	Year	Species	Conclusion
1	Manna et al. [190]	1985	Oreochromis mossambicus	MN induced by Aldrin, $CdCl_2$ and D-glucose amine hydrochloride as well as X-rays in fishes
2	Al Sabti [191]	1986 b	Cyprinus carpio, tench, Tinca tinca and grass carp	MN induced by aflatoxin B1, arochlor 1254, benzidene, benzo(a) pyrene and 20- methylchlo anthrene
3	Manna and Biswas [192]	1986	Labeo rohita, Catla catla, C.mrigala and O. mossambicus	MN in the blood smear of kidney and gill cells to assess the clastogenic potential of the bacterium Pseudomonas aeruginosa
4	Manna and Sadhukhan [193]	1986	Oreochromis mossambicus	MN in gill and kidney cells
5	Manna [194]	1989	Oreochromis mossambicus	Genotoxic potentiality through cytogenetic assays like somatic and germinal CA, mito-depression, MN, sperm head abnormality, dominant lethal test
6	Tripathy [195]	1993	Chela atapar, Mystus vittatus	Significant increase in incidence of MN in fishes exposed to paper mill effluent
7	Rishi and Sunita [51]	1995	Channa punctatus	Dichlorvos caused chromatid gaps, centromeric gaps, attenuation, chromatid breaks, extra fragments and stubbed arm
8	Ansy and Jahageerdar [2]	1999	C. punctata	Exposer to Pb induced CA
9	Sahoo and Bhunya [196]	2002	Heteropneustes fossilis	Carbaryl (Sevin ®) possess genotoxic potential
10	Farah et al. [197]	2003	Channa punctatus	Time dependent increase in the MN due to PCP and 2, 4–D Chlorpyrifos toxicity
11	Velmurugan et al. [198]	2006	Mystus gulio	Genotoxicity of pyrethroid pesticide lambda cyhalothrin by chromosomal aberration
12	Yadav and Trivedi [199]	2006	Channa punctata	Genotoxic potential of chromium [Cr (VI)] on aquatic biosystem causing chromatid breaks, chromosome breaks, chromatid deletions etc
13	Sharma et al. [200]	2007	Mystus vittatus	Single-cell DNA strand breaks induced by Endosulfan

14	Malla and Ganesh [201]	2009	H. fossilis	Increased incidence of CA including fragments and acrocentric associations
15	Tripathi et al. [149]	2009	Clarias batrachus	Fluoride is able to induce genotoxic effects in catfish
16	Yadav and Trivedi [202]	2009	Channa punctata	Chromosomal aberrations induced by heavy metals revealing chromatid and chromosome breaks, ring and di-centric chromosomes
17	Kumar et al. [62]	2010	Channa punctatus	Assessed genotoxic potential of Malathion using MN and comet assay
18	Nwani et al. [203]	2010	Channa punctatus	MN induction on exposure to Carbosulphan reporting concentration and duration dependency
19	Saxena and Chaudhuri [204]	2010	Channa punctatus	Exposure to Fenvalerate caused chromatid separation, chromatid break, deletion, fragments, gaps and ring type chromosomes
20	Yadav et al. [205]	2010	Cirrhinus mrigala	Significant frequencies of MN as a time dependent response to Butachlor observing broken egg (BE) and multiple micronuclei
21	Mohanty et al. [206]	2011	Labeo rohita	The phorate an organophosphate pesticide induces genotoxicity in fingerlings
22	Nwani et al. [207]	2011	C. punctatus	Exposer to Atrazine caused increase in DNA damage
23	Parveen and Shadab [63]	2011	C. punctatus	Clastogenicity of Malathion
24	Tahir et al. [107]	2011	H. fossilis	Genotoxicity of synthetic Sindoor
25	Kushwaha et al [208]	2012	C. punctatus, Mystus vittatus	Genotoxic potential of polluted water of river Gomti using MN and comet assay
26	Patowary et al. [209]	2012	C. punctatus	Significantly higher MN frequency due to arsenic exposure
27	Parveen and Shadab [210]	2012	C. punctatus	Genotoxic effect of heavy metal through MN, CA and SCE
28	Pavan et al. [211]	2012	C. punctatus	Significantly higher DNA damage in both lymphocyte and gill cells and micronuclei
29	Arunachalam et al. [183]	2013	Catla catla	Acute toxicity of chromium in fingerlings by MN and comet assay
30	Gadhave et al. [212]	2014	Labeo rohita	λ-cyhalothrin was genotoxic by MN assay
31	Ismail et al. [213]	2014	Labeo rohita	Chlorpyrifos is a genotoxic and neurotoxic insecticide causing DNA damage
32	Marques et al [214]	2014	Anguilla anguilla	Roundup® herbicide evaluated for genotoxicity
33	Chaudhari and Saxena [215]	2015	C. punctatus	Bifenthrin caused genotoxicity by using chromosomal aberration test

34	Nagpure et al. [216]	2015	Labeo rohita	Mutagenic and genotoxic effects of potassium dichromate by MN test and comet assay
35	Srivastava and Singh [217]	2015	Clarias batrachus	Genotoxic effects of Propiconazole by evaluating MN
36	Tripathi et al. [218]	2015	Labeo rohita	Genotoxic and mutagenic effects by formation of micronuclei, binucleated and multinucleated cells, pyknotic nucleus etc due to various chlorinated and phosphorylated insecticides/ pesticides and fertilizers
37	Bhatnagar et al. [106]	2016	Cirrhinus mrigala	Analyzed the incidence of NAs using MN assay due to acute toxicity of Chlorpyrifos
38	Rajan and Anandan [219]	2017	Clarias batrachus	Use of food colour containing Allura red and orange red inducing genotoxicity
39	Hussain et al. [220]	2018	Labeo rohita	Significant DNA fragmentation in river Chenab population
40	Tasneem and Yasmeen [221]	2018	Cyprinus carpio	Genotoxicity of sub lethal concentration of Karanjin

CONCLUSION

This present compilation of literary citations named as an anthology of cytogenetic end points like micronucleus test, comet assay and chromosomal aberration assay in pisces represents only a part of the huge references existing till to date in the same line and reflects the amicability and utility of different cytogenetic end point techniques as described above with particular reference to piscine models to test the genotoxic potential of various chemicals. Micronucleus test, comet assay and chromosomal abnormalities are truly significant assays in fishes to detect possible hazardous mutagens, carcinogens or teratogens in aquatic environment and the results are reproducible, replicable and reliable in fishes as well as in non piscine model organisms as per the need of the experiments. Their development, application and adaptation in different parts of the world as well as in Indian context are praiseworthy due to collective efforts of various individuals, research agencies or organizations with particular reference to fishes from time to time. These are significant to realize the genotoxic potential of various chemical pollutants in different fish models.

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REFERENCES

[1] Folmar, L. C. (1993): Effects of chemical contaminants on blood chemistry of teleostean fish: a bibliography and synopsis of selected effects. Environmental Toxicology and Chemistry, 12 (2): 337-375.

[2] Ansy, M. N. P., Jahageerdar, S. (1999): Effect of heavy metal on the karyotype of Channa punctatus. Indian Journal of Fisheries, 46: 167-72.

[3] Tolga, C. N., Garanko, N., Victor, V. A. (2005): Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. Food Chemical Toxicology, 43(4): 569-74.

[4] Nehls, S., Segner, H. (2001): Detection of DNA damage in two cell lines from rainbow trout RTG-W1 using the comet assay. Environmental Toxicology, 16 (4):321–329.

[5] Tekale, N. S. (2003): Idol immersion: a critical analysis of environmental impacts on urban lakes and remedial measures. In: Souvenir on national Conference on Urban lakes-Environmental status, Economics and Management Options, Hyderabad, India, pp: 61-63.

[6] Patra, M., Bhowmik, N., Bandopadhyay, B., Sharma, A. (2004): Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. Environmental and Experimental Botany, 52 (3): 199-223.

[7] Fernandes, C., Fontainhas-Fernandes, A., Cabral, D., Salgado, M. (2008): Heavy metals in water, sediment and tissues of Liza saliens from Esmoriz-Paramos lagoon. Portugal Environment Monitoring Assesment, 136: 267-275.

[8] Silva, J., Heuser, V., Andrade, V. (2003): Biomonitoramento Ambiental. Genetica toxicologica, Alcance, Porto Alegre, 167-170.

[9] Nelson, J. S. (2006): Fishes of the World. 4th ed: John Wiley and Sons.

[10] Krishnaja, A. P., Rege, M. S. (1982): Induction of chromosomal aberrations in fish Boleophthalmus dussumieri after exposure in vivo to Mitomycin-C and heavy metals mercury, selenium and chromium. Mutation Research/Genetic Toxicology, 102(1):71-82.

[11] Nakatsuru, Y., Nemoto, N., Nakagawa, K., Masahito, P., Ishikawa, T. (1987): O-6 metylguanine DNA metyl transferase activity in liver from various fish species. Carcinogenesis, 8(8): 1123-1127.

[12] de Flora, S., Bagnasco, M., Zanacchi, P. (1991): Genotoxic, carcinogenic and teratogenic hazards in the marine environment with special reference to the Mediterranean Sea. Mutation Research, 258 (3): 285-320.

[13] Bailey, G., Hendricks, J., Dashwood, R. (1992): Anticarcinogenesis in fish. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 267(2): 243-250.

[14] Hayashi, M., Ueda, T., Uyeno, K., Wada, K., Kinae, N., Saotme, K., Tanaka, N., Takai, A., Sasaki, Y. F., Asano, N. et al. (1998): Development of genotoxicity assay systems that use aquatic organisms. Mutation Research, 399:125-133.

[15] Maccubbin, A. E., Ersing, N., Frank, M. E. (1991): Mutagenicity of sediments from the Detroit River. Journal of Great Lakes Research, 17(3):314-21.

[16] Kurelec, B. (1993): The genotoxic disease syndrome. Marine Environmental Research, 35(4):341-348.

[17] Rodriguez-Cea, A., Ayllon, F., Garcia-Vasquez, E. (2003): Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. Ecotoxicology and Environmental Safety, 56(3):442–448.

[18] Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N. (2005): Towards an alternative for the acute fish LC (50) test in chemical assessment: the fish embryo toxicity test goes multi-species- an update. Altex, 22(2):87-102.

[19] Osman, A. G. M., Wuertz, S., Mekkawy, I. A., Exner, H. J., Kirschbaum, F. (2007): Lead induced malformations in embryos of the African catfish Clarias gariepinus (Burchell, 1822). Environmental Toxicology, 22(4): 375–389.

[20] Ruperelia, S. G, Verma, Y., Hargan, M. C. (2001): Toxicity testing of effluents from pesticide industries using fish model. Ecology and Environment, 7:137-140.
[21] Barse, A. V, Chakrabarti, T., Ghosh, T. K., Pal, A. K., Jadhao, S. B. (2007): Endocrine disruption and metabolic changes following exposure of Cyprinus carpio to diethyl phthalate. Pesticides Biochemistry and Physiology, 88 (1):36-42.
[22] Matsumoto, S. T., Mantovani, M. S., Malagutti, M. I. A., Dias, A. L., Fonseca, I. C., Marin-Morales, M. A. (2006): Genotoxicity and mutagenicity of water contaminated with tannery effluents as evaluated by the micronucleus test and comet assay using the fish Oreochromis niloticusand chromosome aberrations in onion root-tips. Genetics and Molecular Biology,29(1):148–158.
[23] Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G. (2008): Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal Chim Acta, 606 (2): 135-150.
[24] Foster, W. J., Chatelet, A. D. E., Rogerson, M. (2012): Testing benthic foraminiferal distributions as acontemporary quantitative approach tobiomonitoring estuarine heavy metal pollution. Marine Pollution Bulletin, 64:1039-1048.
[25] Garg, S., Gupta, R. K., Jain, K. L. (2009): Sub lethal effects of heavy metals on biochemical composition and their recovery in Indian major carps. Journal of Hazardous Materials, 163 (2-3): 1369-1384.
[26] Yılmaz, A. B., Sangun, M. K., Yaglıoglu, D., Turan, C. (2010): Metals (major, essential to non-essential) composition of the different tissues of three demersal fish species from Iskenderun Bay, Turkey. Food Chemistry, 123: 410-415.
[27] Lavanya, S., Ramesh, M., Kavitha, C., Malarvizhi, A. (2011): Hematological, biochemical and iono regulatory responses of Indian major carp Catla catla during chronic sub lethal exposure to inorganic arsenic. Chemosphere, 82: 977-985.
[28] Adeogun, A. O., Chukwuka, A. V. (2012): Toxicity of industrial waste water acting singly or in joint ratios on Clarias gariepinus. American Journal of Environmental Science, 8 (4): 366-375.
[29] Rivero-Wendt, C. L. G., Miranda-Vilela, A. L., Ferreira, M. F. N., Amorim, F. S., da Silva, V. A. G., Louvandini, H., Grisolia, C. K. (2013): Lack of genotoxicity in Astyanax bimaculatus and Oreochromis niloticus of 17 α-methyl testosterone used in fish hatcheries to produce male monosex populations. Genetics and Molecular Research, 12 (4): 5013-5022.
[30] Grisolia, C. K., Cordeiro, C. M. T. (2000): Variability in micronucleus induction with different mutagens applied to several species of fish. Genetics and Molecular Biology, 23 (1): 235–239.
[31] Dar, S. A., Yousuf, A. R., Balkhi, M. H., Ganai, F. A., Bhat, F. A. (2015): Assessment of endosulfan induced genotoxicity and mutagenicity manifested by oxidative stress pathways in freshwater cyprinid fish crucian carp (Carassius carassius L.). Chemosphere, 120: 273-283.
[32] Dar, S. A., Yousuf, A. R., Balkhi, M. H. (2016): An introduction about genotoxicology methods as tools for monitoring aquatic ecosystem: Present Status and Future Perspectives. Fisheries and Aquaculture Journal, 7 (1): 1-11.
[33] Akpoilih, B. U. (2012): Fish ecogenotoxicology: An emerging science, an emerging tool for environmental monitoring and risk assessment. Bulletin Environment, Pharmacology and Life Science, 2(1): 53- 64.
[34] Krishnaja, A. P, Rege, M. S. (1980): Some observation on the chromosome of certain teleosts using a simple method. Indian Journal of Experimental Biology, 18, 268-270.
[35] Manna, G. K. (1986): Mouse bone marrow as a mean of testing cytogenetic agents. Nucleus, 29:141-168.
[36] Das, R. K., Roy, B. B. (1988): A simplified method for micronucleus preparation from hepatic cells. Stain Technology, 63: 71-74.

[37] Roy, B., Das, R. K. (1990): Evaluation of genotoxicity of clofazimine, an antileprosy drug in mice in vivo II, Micronucleus test in bone marrow and hepatocytes. Mutation Research, 241: 169-173.
[38] Dash, B. C., Roy, B., Das, R. K. (1990): Evaluation of genotoxicity of Clofazimine, an antileprosy drug in mice in vivo III sister chromatid exchange analysis in bone marrow cells. In Vivo, 41.
[39] Manna, G. K. (1984): Progress in fish cytogenetics. The Nucleus, 27 (3): 203-231.
[40] Longwell, A. C., Hughes, J. B. (1980): Cytologic, cytogenetic and developmental state of Atlantic mackerel eggs from sea surface waters of the New York Bight and prospects for biological effect monitoring with ichthyoplankton. Rapp. P-V Reun. Cons. Int. Explor. Mer., 179: 275-276.
[41] Kligerman, A. D. (1982): Fishes as biological detectors of the effects of genotoxic agents. In: Mutagenecity, New Horizons in Genetic Toxicology, J. H. Heddle (ed.), New York, Academic Press.
[42] Manna, G. K. (1983): Cytogenetic studies on fishes and amphibia. In: Genetical researches in India. XV Int. Cong. Genet., Delhi, ICAR publication.
[43] Mitchell, S., Kennedy, S. (1992): Tissue concentrations of organochlorine compound in common seals from the coasts of Northern Ireland. Science of Total Environment, 115: 235-240.
[44] Akiyama, M., Oshima, H., Nakamura, M. (2001): Genotoxicity of mercury used in chromosome aberration tests. Toxicology in Vitro, 15 (4-5): 463-467.
[45] Gopal Krishna (2000): Genotoxicity and environmental monitoring. In: Training Manual of Summer School on Recent Advancement in Marine Biotechnology, 7-27 August, 2000. Organized by Fish Genetics and Biotechnology Division, Central Institute of Fisheries Education, ICAR, India: pp. 41-43.
[46] Al-Sabti, K., Metcalfe, C. D. (1995): Fish micronuclei for assessing genotoxicity in water. Mutation Research, 343 (2-3): 121-135.
[47] Kushwaha, B., Nagpure, N. S., Srivastava, S. K., Ravindra, K., Verma, M. S. (2003): Variation of micronuclei in peripheral blood cells of Channa punctatus. Indian Journal of Animal Science, 73 (10): 1192-1193.
[48] Alink, G. M., Quik, J. T. K., Penders, E. J. M., Spenkelink, A., Rotteveel, S. G. P., Maas, J. L., Hoogenboezem, W. (2007): Genotoxic effects in the Eastern mudminnow (Umbra pygmaea L.) after exposure to Rhine water, as assessed by use of the SCE and Comet assays: A comparison between 1978 and 2005. Mutation Research, 631 (2): 93–100. doi: 10.1016/j.mrgentox.2007.03.011.
[49] Das, R. K., Nanda, N. K. (1986): Induction of micronuclei in peripheral erythrocytes of fish Heteropneustes fossilis by mitomycin-C and paper mill effluent. Mutation Research, 175: 67-71.
[50] Al Sabti, K., Franko, M., Andrijanie, B., Kenz, S., Stegnar, P. (1994): Chromium induced micronuclei in fish. Journal of Applied Toxicology, 14 (5): 333-6.
[51] Rishi, K. K., Sunita, G. (1995): Chromosome aberration test for the insecticide, dichlorvos of fish chromosomes. Mutation Research, 344 (1-2): 1-4.
[52] Clarice, T. D. L., Patricia, M. R., Nara, R. T., Bernardoo, E. (2001): Evaluation of basal micronucleus frequency and hexavalent chromium effects in fish erythrocytes. Environmental Toxicology and Chemistry, 20(6): 1320-1324.
[53] Maples, N. L., Bain, L. J. (2004): Trivalent chromium alters gene expression in the mummichog (Fundulus heteroclitus). Environmental Toxicology and Chemistry, 23 (3): 626-631.
[54] Sankar, P., Telang, A. G., Manimaran, A. (2010): Curcumin protects against cypermethrin-induced genotoxicity in rats. Environmental Toxicology and Pharmacology, 30 (3): 289-291.

[55] Hussain, R., Mahmood, F., Khan, M. Z., Khan, A., Muhammad, F. (2011): Pathological and genotoxic effects of atrazine in male Japanese quail (Coturnix japonica). Ecotoxicology, 20: 1–8.
[56] Hussain, R., Mahmood, F., Khan, A., Javed, M. T., Rehan, S., Mehdi, T. (2012): Cellular and biochemical effects induced by atrazine on blood of male Japanese quail (Coturnix Japonica). Pesticides Biochemistry and Physiology, 103: 38–42.
[57] Mahboob, S., Al-Balwai, H. F. A., Al-Misned, F., Ahmad, Z. (2014): Investigation on thegenotoxicity of mercuric chloride to freshwater Clarias gariepinus. Pakisthan Veterinary Journal, 34(1):100-103.
[58] Simoniello, M. F., Gigena, F., Poletta, G., Loteste, A., Kleinsorge, E., Campana, M., Scagnetti, J., Parma, M. J. (2009): Alkaline comet assay for genotoxic effect detection in neotropical fish Prochilodus lineatus (Pisces, Curimatidae). Bulletin of Environmental Contamination Toxicology, 83:155–158.
[59] Ansari, R. A., Rahman, S., Kaur, M., Anjum, S., Raisuddin, S. (2011): In vivo cytogenetic and oxidative stress-inducing effects of cypermethrin in freshwater fish Channa punctata Bloch. Ecotoxicology and Environmental Safety,74 (1):150-156.
[60] Rana, R. (2016): Chromosomal abnormalities in Channa Punctatus exposed to Cypermethrin. International Journal of Engineering Science and Computing, 6: 1825-1826.
[61] Rakesh, P. (2018): Cypermethrin exposed chromosomal aberrations of Indian major carp. International Research Journal, 7(8): 16-20.
[62] Kumar, R., Nagpure, N. S., Kushwaha, B. et al. (2010): Investigation of the genotoxicity of Malathion to freshwater Teleost fish Channa punctatus (Bloch) using the micronucleus test and comet assay. Archieves of Environmental Contamination Toxicology, 58 (1): 123-130.
[63] Parveen, N., Shadab, G. G. H. A. (2011): Evaluation of micronuclei and haematological profiles as genotoxic assays in Channa punctatus exposed to Malathion. Intlernational Journal of Science and Nature, 2(3): 625- 631.
[64] Schmidt, W. (1975): The micronucleus test. Mutation Research, 31 (1): 9-15.
[65] Korte, A., Wager, H. M., Obe, G. (1981): Simultaneous exposure of Chinese hamsters to ethanol and cigarette smoke: Cytogenetic Aspects. Toxicology, 20: 237-246.
[66] Basler, A. (1982): SCEs in lymphocytes of rats after exposure in vivo to cigarette smoke or to cyclophosphamide. Mutation Research/Genetic Toxicology, 102 (2):137-143.
[67] Kar, R. N., Khan, K. A., Sethi, N. (1989): Genotoxicity studies on mice after short term inhalation exposure to methyl isocyanate. Cytobios, 59: 167-176.
[68] Kirsch Volders, M., Sofuni, T., Aardema, M., Albertini, S., Fenech M. et al. (2003): Report from the in vitro micronucleus assay working group. Mutation Research, 540 (2): 153-163.
[69] Heddle, J. A., Cimino, M. C., Hayashi, M., Romagna, F., Shelby, M. D., Tucker, J. D., Vanparys, Ph., Mac Gregor, J. T. (1991): Micronuclei as an index of cytogenetic damage: past, present, and future. Environmental and Molecular Mutagenesis, 18 (4): 277–291.
[70] Heddle, J. A., Salmone, M. F. (1981): Chromosomal aberrations and bone marrow toxicity. Environmental Health Perspectives. 39: 23-27.
[71] Landolt, M. L., Kocan, R. M. (1983): Fish cell cytogenetics: a measure of genotoxic effects of environmental pollutants. In: J. O. Nriagu (Ed.), Aquatic Toxicology, Wiley, New York, pp. 335–352.
[72] Mersch, J., Beauvais, M. N. (1997): The micronucleus assay in freshwater mussel, Dreissena polymorpha to in situ monitor genotoxicity in freshwater environments. Mutation Research, 393 (1-2): 141-149.
[73] Hose, J. E., Cross, J. N., Smith, S. G., Diehl, D. (1987): Elevated circulating erythrocyte micronuclei in fishes from contaminated sites of southern California. Marine Environmental Research, 22: 167–176.

[74] Gronlund, W. D., Chan, S. L., Mc Cain, B. B., Clark, R. C., Meyers, M. S., Stein, J. E., Brown, D. W., Landahl, J. T., Krahn, M. M., Varnasi, U. (1991): Multidisciplinary assessment of pollution at three sites in Long Island Sound. Estuaries, 14: 299–305.

[75] Hughes, J. B., Herbert, A. T. (1991): Erythrocyte micronuclei in winter flounder (Pseudopleuronectes americanus): result of fieldsurveys during 1980–1988 from Virginia to Nova Scotia and in Long Island Sound. Archieves of Environment Contamination Toxicology, 20: 474–479.

[76] Hooftman, R. N., de Raat, W. K. (1982): Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mud minnow Umbra pygmaea by ethylmethansulphonate. Mutation Research, 104 (1-3): 147–152.

[77] Schultz, N., Norrgren, L., Gawe, J., Johannisson, A., Medhage, O. (1993): Micronucleus frequency in circulating erythrocytes from rainbow trout (Oncorhynchus mykiss) subject to radiation, an image analysis and flow cytometric study. Comparative Biochemistry and Physiology, 105: 207–211.

[78] Ayllon, F., Garcia-Vasquez, E. (2000): Induction of micronuclei and other nuclear abnormalities in European minnow Phoxinus phoxinus and mollie Poecilia latipinna: an assessment of the fish micronucleus test. Mutation Research, 467 (2): 177–186.

[79] Al Sabti, K. (1986): Clastogenic effect of five carcinogenic mutagenic chemicals on the cells of the common carp Cyprinus carpio L. Comparative Biochemistry and Physiology, 85 (1):5–9.

[80] Nepomuceno, J. C., Ferrari, I., Spano, M. A., Centeno, A. J. (1997): Detection of micronuclei in peripheral erythrocytes of Cyprinus carpio exposed to metallic mercury. Environmental and Molecular Mutagenesis, 30 (3): 293–297.

[81] Gustavino, B., Scornajenghi, K. A., Minssi, S., Ciccoti, E. (2001): Micronuclei induced in erythrocyte of Cyprinus carpio (Teleostei, Pisces) by Xrays and colchicine. Mutation Research, 494: 151-159.

[82] Bombail, V., Gordon, E., Batty, J. (2001): Application of the comet and micronucleus assays to butterfish (Pholis gunnellus) erythrocytes from the Firth of Forth, Scotland. Chemosphere, 44 (3): 383–392.

[83] de Flora, S. L., Vigano, F., Agostini, A. D., Camoirano, M., Bagnasco, C., Bennecelli, F., Melodia, F., Arillo, A. (1993): Multiple genotoxicity biomarkers in fish exposed in-situ to polluted river water. Mutation Research, 319: 167–177.

[84] Minissi, S., Ciccoti, E., Rizzoni, M. (1995): Micronucleus test in erythrocytes of Barbus plebejus (Teleostei, Pisces) from two natural environments: A biossay for the in situ detection of mutagens in freshwater. Mutation Research/Genetic Toxicology, 367 (4):245-251.

[85] Vigano, L., Camoirano, A., Izzotti, A. A., Agostini, F. D., Polesello, S., Francisci, C., De Flora, S. (2002): Mutagenicity of sediments along the Po river and genotoxicity biomarkers in fish from polluted areas. Mutation Research, 515:125–134.

[86] Remirez, A., Saldanha, P. H. (2002): Micronuclei investigation of alcoholic patients with oral carcinomas. Genetics and Molecular Research, 1 (3): 246-260.

[87] Fenech, M., Holland, N., Chang, W. P., Zeiger, E., Bonassi, S. (1999): The human micronucleus project Dan international collaborative study on the use of micronucleus technique for measuring DNA damages in humans. Mutation Research, 428: 271-283.

[88] Norppa, H., Falck, G. C. M. (2003): What do human micronuclei contain? Mutagenesis, 18 (3): 221-233.

[89] Porto, J. I. R., Araujo, C. S. O., Feldberg, E. (2005): Mutagenic effects of mercury pollution as revealed by micronucleus test on three Amazonian fish species. Environmental Research, 97 (3): 87-92.

[90] Pantaleao, S. de M., Alcantara, A. V., Alves, P. J., Spano, M. A. (2006): The piscine micronucleus test to assess the impact of pollution on the Japaratuba River. Brazil Environmental and Molecular Mutagenesis, 47: 219-224.

[91] Fenech, M., Chang, W. P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeigere (2003): Human Micronucleus Project. "HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research, 534 (1-2): 65-75.
[92] Udroiu, I. (2006): The micronucleus test in piscine erythrocytes. Aquatic Toxicology, 79(2): 201–204
[93] Kirsch Volders, M., Matenca, R. A., Roelants, M., Treamp, A., Zeigar, E. et al., (2006): The effect of GSIM 1 and GSTT 1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo. Cancer Epidemiology, 17: 379-386.
[94] Bolognesi, C., Perrone, E., Roggieri, P., Pampanin, D. M., Sciutto, A. (2006): Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquatic Toxicology, 78(Suppl 1):93-98.
[95] Ergene, S., Cavas, T., Celik, A., Koleli, N., Kaya, F., Karahan, A. (2007): Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution. Ecotoxicology, 16:385-391.
[96] Saleh, K., Sarhan, M. A. A. (2007): Clastogenic analysis of chicken farms using micronucleus test in peripheral blood. Journal of Applied Science Research, 3: 1646–1649.
[97] Fagr, A., El-Shehawi, A. M., Seehy, M. A. (2008): Micronucleus test in fish genome: a sensitive monitor for aquatic pollution. African Journal of Biotechnology, 7: 606-612.
[98] Cavas, T., Ergene-Gozukara S. (2005): Micronucleus test in fish cells: a bioassay for in situ monitoring of genotoxic pollution in the marine environment. Environmental and Molecular Mutagenesis, 46: 64–70.
[99] Isani, G., Andreani, G., Cocchioni, F., Fedeli, D., Carpene, E., Falcioni, G. (2009): Cadmium accumulation and biochemical responses in Sparus aurata following sub-lethal Cd exposure. Ecotoxicology and Environmental Safety, 72: 224-230.
[100] Bolognesi, C., Hayashi, M. (2011): Micronucleus assay in aquatic animals. Mutagenesis, 26: 205–21.
[101] Bucker, A., Carvalho, M., Conceicao, M., Alves-Gomes, J. (2012): Micronucleus test and comet assay in erythrocytes of the Amazonian electric fish Apteronotus bonapartii exposed to benzene. Journal of Brazilian Society of Ecotoxicology, 7: 65-73.
[102] Saotome, K., Hayashi, M. (2003): Application of a sea urchin micronucleus assay to monitor aquatic pollution: influence of sample osmolality. Mutagenesis, 18 (1):73-76.
[103] Obiakor, M. O., Okonkwo, J. C., Nnabude, P. C., Ezeonyejiaku, C. D. (2012): Eco-genotoxicology: Micronucleus assay in fish erythrocytes as in situ aquatic pollution biomarker: a review. Journal of Animal Science Advances, 2: 123-133.
[104] Souza, T. S., Fontanetti, C. S. (2006): Micronucleus test and observation of nuclear alterations in erythrocytes of Nile Tilapia exposed to waters affected by refinery effluent. Mutation Research, 605: 87-93.
[105] Della Torre, C., Buonocore, F., Frenzilli, G., Corsolini, S., Brunelli, A., Guidi, P., et al. (2015): Influence of titanium dioxide nanoparticles on 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin bioconcentration and toxicity in the marine fish European Sea Bass (Dicentrarchus labrax). Environmental Pollution, 196: 185-193.
[106] Bhatnagar, A., Yadav, A. S., Cheema, N. (2016): Genotoxic effects of chlorpyrifos in freshwater fish Cirrhinus mrigala using micronucleus assay. Advances in Biology, Volume 2016: 1-6.
[107] Tahir M. M., Senthilkumar, C. S., Akhtar, S., Ganesh, N. (2011): Micronuclei as an evidence of DNA damage in freshwater catfish Heteropneustes fossilis (Bloch) exposed to synthetic sindoor. ARPN Journal of Agricultural and Biological Science, 6 (5): 41-44.
[108] Rocha, C. A. M., Santos, R. A., Bahia, M. D. O., Cunha, L. A. J. D., Ribeiro, H. F., Burbano, R. M. R. (2009): The micronucleus assay in fish species as an important tool for

xenobiotic exposure risk assessment-A brief review and an example using Neotropical fish exposed to Methyl mercury. Review of Fisheries Science, 17(4): 478–484.

[109] Bahari, I. B., Noor, F. M., Daud, N. M. (1994): Micro nucleated erythrocytes as an assay to assess actions by physical and chemical genotoxic agents in Clarias gariepinus. Mutation Research, 313:1-5.

[110] Sandra, S., Cicotti, E., Rizzoni, M. (1996): Micronucleus test in erythrocytes of Barbus plebejus (Teleostei, Pisces) from two natural environments; a bioassay for the in- situ detection of mutagens in freshwaters. Mutation Research, 367: 141-149.

[111] Svobodova, Z., Flaj Shan, M., Vykusova, B., Machova, J. (1997). The effect of long term therapeutic batch of malachite green on common carp (Cyprinus carpio). Acta Veterinaria Brno Acta Vet Brno. 66 (2): 111-117.

[112] Campana, M. A., Panzeri, A. M., Moreno, V. O., Dulout, F. M. (1999): Genotoxic evaluation of the parathyroid lambda- cyclothrin using the micronucleus test in erythrocytes of the fish Cheirdon interrtuptus interrtuptus. Mutation Research Genetics Toxicology Environmental Mutation, 438 (2):155-161.

[113] de Lemos, C. T., Rodel, P. M., Terra, N. R., Erdtmann, B. (2001): Evaluation of basal micronucleus frequency and hexavalent chromium effects in fish erythrocytes. Environmental Toxicology Chemistry, 20: 1320-1324.

[114] Ale, E., Fenocchio, A. S., Pastori, M. C., Ribeiro, C. O., Cestari, M. M., Zacharzewski, C. (2004): Evaluation of the effects of $(NO_3)_2Pb$ on Oreochromis niloticus (Pisces, Cichlidae) by means of cytogenetic techniques. Cytologia, 69(4): 453-458.

[115] Ferraro, M., Fenocchio, A., Mantovani, M., Ribeiro, C., Cestari, M. (2004): Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish H. malabaricus as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. Genetics and Molecular Biology, 27:103–107.

[116] Farah, M. A., Ateeq, B., Ahmad, W. (2006): Anti mutagenic effect of neem leaves extract in freshwater fish, Channa punctatus evaluated by cytogenetic tests. Science of The Total Environ., 364 (13):200-214.

[117] Jiraungkoorskul, W., Kosai, P., Sahaphong, S., Kirtputra, P., Chawlab, J., Charucharoen, S. (2007): Evaluation of micronucleus test's sensitivity in freshwater fish species. Research Journal of Environmental Science, 1(2): 56-63.

[118] Ali, D., Nagpure, N. S., Kumar, S., Kumar, R., Kushwaha, B. (2008): Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish Channa punctatus (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere, 71:1823-1831.

[119] Galindo, T. P., Moreira, L. M. (2009): Evaluation of genotoxicity using the micronucleus assay and nuclear abnormalities in the tropical sea fish Bathygobius soporator (Valenciennes, 1837) (Teleostei, Gobiidae). Genetics and Molecular Biology, 32 (2): 394-398.

[120] Candioti, J. V., Soloneski, S., Larramendy, M. L. (2010): Genotoxic and cytotoxic effects of the formulated insecticide Aficida on Cnesterodon decemmaculatus (Jenyns, 1842) (Pisces: Poeciliidae). Mutation Research, 703: 180-186.

[121] Ahmed, M. K., Habibullah-Al-Mamun, M., Hossain, M. A., Arif, M., Parvin, E., Akter, M. S., Khan, M. S., Islam, M. M. (2011): Assessing the genotoxic potentials of arsenic in tilapia (Oreochromis mossambicus) using alkaline comet assay and micronucleus test. Chemosphere, 84: 143-149.

[122] Ghisi, N., Ramsdorf, W. A., Ferraro, M. V. M. et al. (2011): Evaluation of genotoxicity in Rhamdia quelen (Pisces, Siluriformes) after sub-chronic contamination with Fipronil. Environmental Monitoring Assessment, 180 (1-4): 589-599.

[123] Guner, U., Dilek, F., Muranl, G. (2011): Micronucleus Test, Nuclear Abnormalities and Accumulation of Cu and Cd on Gambusia affinis (Baird & Girard, 1853). Turkish Journal of Fisheries and Aquatic Science, 11: 615-622.

[124] Ansoar-Rodríguez, Y., Christofoletti, C., Marcato, A., Correia, J., Bueno, O., Malaspina, O., Fontanetti, C. (2015): Genotoxic potential of the insecticide Imidacloprid in a non-target organism (Oreochromis niloticus-Pisces). Journal of Environmental Protection, 6: 1360-1367.
[125] Al-Sabti, K., Kurelec, B. (1985). Induction of chromosomal aberrations in the mussel, Mytilus galloprovincialis. Bulletin of Environmental Contamination Toxicology, 35: 660-665.
[126] Kumari, S. A., Ramkumaran, S. (2006): Chromosomal aberrations in Channa punctatus from Hussainsagar Lake, Hyderabad (A. P). Indian Journal of fisheries, 53(3): 359-362.
[127] Hafez, A. M. (2009): Mugil cephalus Genome: A sensitive monitor for genotoxicity and cytotoxicity in aquatic environment. Australian Journal of Basic and Applied Science, 3(3): 2176-2187.
[128] Mahmoud, A., Mohamed, Z., Yossif, G., Sharafeldin, K. (2010): Cytogenetical studies on some River Nile species from polluted and nonpolluted aquatic habitats. Egyptian Academy Journal of Biological Science, 2(1): 1-8.
[129] Rose, M.H., Sudhakar, K., Sudha, P.N. (2010): Effect of water pollutants on the freshwater fish, Hypophthalmicthys molitrix. The Ecoscan, 4(1): 31-35.
[130] Carrasco, K. R., Tiburi, K. L., Myers, M. S. (1990): Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Canadian Journal of Fisheries and Aquatic Science, 47 (11): 2123-2136.
[131] Matter, E. E, ELserafy, S. S., Zowail, M. E. M., Awwad, M. H. (1992): Genotoxic effect of carbamyl insecticide (sevin) on the grass carp Ctenopharygodan idella (VAL). Egyptian Journal of Histology, 15 (1): 9-17.
[132] Das, P., John, G. (1999): Induction of sister chromatid exchanges and chromosome aberrations in vivo in Etroplus suratensis (Bloch) following exposure to organophosphorus pesticides. Toxicology Letters, 104: 111-116.
[133] Anitha, B., Chandra, N., Gopinath, P., Durairaj, G. (2000): Genotoxicity evaluation of heat shock in gold fish (Carassius auratus). Mutation Research/Genetic Toxicology Environmental Mutagenesis, 469(1):1-8.
[134] Ouseph, A., Sundarsanam, D., Nainar, A. M., Gandheeswari, P. (2000): Frequency of chromosomal aberration in fish inhabiting polluted ecosystem. Pollution Research Paper, 19 (1):123-128.
[135] Mahrous, K., Abdou, H. (2001): Cytogenetical studies on some Nile fish. Journal of Egyptian German Society of Zoological Histological Histochemistry and Genetics, 36:133-144.
[136] Lopez-Poleza, S. (2004): Evaluation of methyl mercury (CH_3Hg^+) effects in Hoplias malabaricus through the frequency of chromosome aberrations and of the micronucleus test and comet assay. Master's Dissertation. University of Parana, Brazil (2004).
[137] Cestari, M. M., Lemos, P. M. M., Ribeiro, C. A. O., Costa, J. R. M. A., Pelletier, E., Ferraro, M. V. M., Mantovani, M. S., Fenocchi, A. S. (2004): Genetic damage induced by trophic doses of lead in the neotropical fish Hoplias malabaricus (Characiformes, Erythrinidae) as revealed by the comet assay and chromosomal aberrations. Genetics and Molecular Biology, 27(2):
[138] Chandra, P., Khuda-Bukhsh, A. R. (2004): Genotoxic effects of cadmium chloride and azadirachtin treated singly and in combination in fish. Journal of Ecotoxicology and Environmental Safety, 58: 194-201.
[139] Gadhia, P. K., Gadhia, M., Georje, S., Vinod, K. R., Pithawala, M. (2008): Induction of chromosomal aberrations in mitotic chromosomes of fish Boleophthalmus dussumieri after exposure in vivo to antineoplastics Bleomycin, Mitomycin-C and Doxorubicin. Indian Journal of Science and Technology, 1 (7): 1-6.
[140] Chaurasia, O. P., Kumari, C. (2007): Genotoxic effect of ground water salts rich in fluoride. Cytologia, 72(2):141-144.

[141] Palikova, M., Rabova, M., Krejci, R., Navratil, S., Blaha L. (2007): Chromosomal aberrations in early embryos of weather fish (Misgurnus fossilis L.) exposed to crude Cyanobacterial extract and semi purified compound of Microcystins-a Pilot Study. Acta Veterinary Brno., 76: S55-S60.
[142] Mohamed, M. M., EL-Fiky, S. A., Soheir, Y. M., Abeer, A. I. (2008): Cytogenetic studies on the effect of copper sulfate and lead acetate pollution on Oreochromis niloticus fish. Asian Journal of Cell Biology, 3: 51-60.
[143] Rita, A. J. J., Milton, J. M. C. (2008): Karyomorphological analysis of the fresh water cichlid Oreochromis mossambicus (Peter) exposed to carbamate pesticide methomyl (Lannate). Journal of Advanced Zoology, 29(1):57-61.
[144] Kaur, H., Kalotra, R, Walia, G. K., Handa, D. (2013): Genotoxic effects of dyeing industry effluent on a freshwater fish, Cirrhinus mrigala by chromosomal aberration test. International Journal of Pharmacy and Biological Science, 3 (1): 423-431.
[145] Yadav, A. S., Bhatnagar, A., Kaur, M. (2013): Aberrations in the chromosomes of Cirrhinus mrigala (Hamilton) upon exposure to Butachlor. Iranian Journal of Toxicology, 7 (21): 857-865.
[146] Promsid, P., Neeratanaphan, L., Supiwong, W., Sriuttha, M., Tanomtong, A. (2015): Chromosomal aberration of snakehead fish (Channa striata) in affected reservoir by Leachate with Lead and Mercury contamination. International Journal of Environmental Research, 9(3):897-906.
[147] Tengjaroenkul, B., Intamat, S., Boonmee, S., Neeratanaphan, L. (2017): Chromosomal aberration assessment of silver rasbora fish (Rasbora tornieri) living near gold mine area with heavy metal contamination. Human Ecology Risk Assessment: An International Journal, 23 (5): 1140-1152.
[148] Abd Ali, M. A., Mohammed, M. H., Sadeq, M. K. (2018): In vitro chromosomal aberration frequency by electro fishing on Poecilia latipinna (Sailfin Molly) fishes in Southern of Iraq. American Journal of Molecular Biology, 8: 109-118.
[149] Tripathi, N., Bajpai, S., Tripathi, M. (2009): Genotoxic alterations induced by fluoride in Asian catfish, Carias batrachus (Linn.). Research Report Fluoride, 42(4):292–296.
[150] Ostling, O., Johanson, K. J. (1984): Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells. Biochemistry and Biophysics Research Communication, 123: 291-298.
[151] Singh, N. P., Mc Coy, M. T., Tice, R. R., Schneider, E. L. (1988): A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research, 75:184-191.
[152] Park, J. K, Lee, H. H, Choi, I. S., Park, S. D. (1991): Accumulation of polyclic aromatic hydrocarbon-induced single-strand breaks is attributed to slower rejoining process by DNA polymerase inhibitor, cystosine arabinoside in CHO-Kl. Life Science, 48:1255-1261.
[153] Mullenders, L., van Zeeland, A., Natarajan, A. (1987): The localization of ultraviolet induced excision repair in the nucleus and the distribution of repair events in higher order chromatin loops in mammalian cells. Journal of Cell Science Supplements, 6:243–262.
[154] Padrangi, R., Petras, M., Ralph, S., Vrzoc, M. (1995): Alkaline single cell gel (comet) assay and genotoxicity monitoring using bullheads and carp. Environmental and Molecular Mutagenesis, 26:345–356.
[155] Mitchelmore, C. L., Chipman, J. K. (1998): Detection of DNA strand breaks in brown trout (Salmo trutta) hepatocytes and blood cells using the single cell gel electrophoresis (Comet) assay. Aquaculture Toxicology, 41:161–182.
[156] Belpaeme, K., Delbeke, K., Zhu, L., Kirsh-Volders, M. (1996): Cytogenetic studies of PCB77 on brown trout (Salmo trutta fario) using the micronucleus test and the alkaline comet assay. Mutagenesis, 11:485–492.

[157] Nacci, D. E., Cayula, S., Jackim, E. (1996): Detection of DNA damage in individual cells from marine organisms using the single cell gel assay. Aquaculture Toxicology, 35: 197–210.
[158] Kleinjans, J. C. S., Van Schooten, F. J. (2002): Ecogenotoxicology: the evolving field. Environmental Toxicology and Pharmacology, 11: 173–179.
[159] Belpaeme, K, Cooreman, K., Kirsch-Volders, M. (1998): Development and validation of the in vivo alkaline comet assay for detecting genomic damage in marine flatfish. Mutation Research/ Genetics Toxicology and Environmental Mutagenesis, 415:167–184.
[160] Gontijo, A. M. M. C., Barreto, R. E., Speit, G., Reyes, V. A. V., Volpato, G. L., Salvadori, D.M.F. (2003): Anesthesia of fish with benzocaine does not interfere with comet assay results. Mutation Research, 534:165–172.
[161] Lee, R., Steinert, S. (2003): Use of the single cell gel electrophoresis /Comet assay for detecting DNA damage in aquatic (Marine and Freshwater) animals. Mutation Research/ Reviews in Mutation Research, 544: 43-64.
[162] Moller, P. (2005): Genotoxicity of environmental agents assessed by the alkaline comet assay. Basic and Clinical Pharmacology and Toxicology, 96:1–42.
[163] Moller, P. (2006): The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. Basic and Clinical Pharmacology and Toxicology, 98:336–345.
[164] Vanzella, T. P., Colus Martinez, C. B., M. S. I. (2007): Genotoxic and mutagenic effects of diesel oil water soluble fraction on a neotropical fish species. Mutation Research, 631:36–43.
[165] Frenzilli, G., Nigro, M., Lyons, B. P. (2009): The comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research, 681:80–92.
[166] de Campos Ventura, B., de Angelis, D., Marin-Morales, M. A. (2008): Mutagenic and genotoxic effects of the Atrazine herbicide in Oreochromis niloticus (Perciformes: Cichlidae) detected by the micronuclei test and the comet assay. Pesticides Biochemistry and Physiology, 90:42–51.
[167] Toyoizumi, T., Deguchi, Y., Masuda, S., Kinae, N. (2008): Genotoxicity and estrogenic activity of 3, 3'-dinitrobisphenol A in gold fish. Bioscience, Biotechnology and Biochemistry, 72: 2118-2123.
[168] Cavalcante, D. G., Martinez, C. B., Sofia, S. H. (2008): Genotoxic effects of round up on the fish Prochilodus lineatus. Mutation Research, 655: 41-46.
[169] Caliani, I., Porcelloni, S., Mori, G., Frenzilli, G., Ferraro, M., Marsili, L., Casini, S., Fossi, M. C. (2009): Genotoxic effects of produced waters in mosquito fish (Gambusia affinis). Ecotoxicology, 18:75-80
[170] Petridis, P., Jha, A. N., Langston, W. J. (2009): Measurements of the genotoxic potential of (xeno-) oestrogens in the bivalve mollusc Scrobicularia plana using comet assay. Aquaculture Toxicology, 94: 8-15.
[171] Binelli, A., Cogni, D., Parolini, M., Riva, C., Provini, A. (2009): In vivo experiments for the evaluation of genotoxic and cytotoxic effects of triclosan in Zebra mussel hemocytes. Aquaculture Toxicology, 91: 238-244.
[172] de Andrade, V. M., de Freitas, T., da Silva, J. (2004): Comet assay using mullet. (Mugil sp.) and sea catfish (Netuma sp.) erythroyites for the detection of genotoxic pollutants in aquatic environment. Mutation Research, 560:57–67.
[173] Russo, C., Rocco, L., Morescalchi, M., Stingo, V. (2004): Assessment of environmental stress by the micronucleus test and the comet assay on the genome of teleost populations from two natural environments. Ecotoxicology and Environmental Safety, 57:168–174.
[174] Bucker, A., Carvalho, W., Alves-Gomes, J. (2006): Evaluation of mutagenicity and genotoxicity in Eingenmannia virescens (Teleostei: Gymnotiformes) exposed to benzene. Acta Amazonica, 36: 357–230.
[175] Klaude, M., Eriksson, S., Nygren, J., Ahnstrom, G. (1996): The comet assay: Mechanisms and technical considerations. Mutation Research, 363:89-96.

[176] Brendler Schwaab, S., Hartman, A., Pfuhler, S., Speit, G. (2005): The in vivo comet assay: use and status in genotoxicity testing. Mutagenesis, 20:245–254.
[177] Muid, K. A., Shahjahan, R. M., Begum, R., Begum, R. A. (2012): Zinc phosphide induced DNA damage in the blood cells of Gallus sp. using comet DNA assay. American Journal of Agricultural and Biological Science, 7: 82-87.
[178] Nagrani, N., Janaki Devi, V., Kumaraguru, A. K. (2012): Identification of DNA damage in marine fish Therapon jarbua by comet assay technique. Journal of Environmental Biology, 33: 699-703.
[179] Buschini, A., Martino, A., Gustavino, B., Monfrinotti, M., Poli, P., Rossi, C., Santoro, M., Dorr, A. J. M., Rizzoni, M. (2004): Comet assay and micronucleus test in circulating erythrocytes of Cyprinus carpio specimens exposed in situ to lake waters treated with disinfectants for potabilization. Mutation Research, 557: 119–129.
[180] Wirzinger, G., Weltje, L., Gercken, J., Sordyl, H. (2007): Genotoxic damage in field-collected three-spined sticklebacks (Gasterosteus aculeatus L.): A suitable biomonitoring tool? Mutation Research, 628: 19–30.
[181] Christofoletti, C. A., David, J. A. O., Fontanetti, C.S. (2008): Application of the comet assay in erythrocytes of Oreochromis niloticus (Pisces): A methodological comparison**.** Genetics and Molecular Biology, 32(1): 155–158.
[182] Mitkovska, V. I., Dimitrov, H. A., Chassovnikarova, T. G. (2017): In vitrogenotoxicity and cytotoxicity assessment of allowable concentrations ofnickel and lead: comet assay and nuclear abnormalities in acridine orangestained erythrocytes of common carp (Cyprinus carpio L.) Acta Zoologica Bulgarica, 8: 47–56.
[183] Arunachalam, K. D., Annamalai, S. K., Kuruva, J. K. (2013): In-Vivo evaluation of hexavalent chromium induced DNA damage by alkaline comet Assay and oxidative stress in Catla catla. American Journal of Environmental Science, 9 (6): 470-482.
[184] Moorthy, M. V., Moorthy, P. B. (1994): Analysis of sister chromatid exchange, micronucleus and chromosomal aberrations frequencies in rodents exposed to mosquito coil smoke by inhalation route. Toxicology Letters, 70:357-362.
[185] Tripathy, S. K. (1996): Evaluation of genotoxic effects of inhalation of petroleum vapour on pulmonary alveolar macrophase chromosomes of rats. In: M Phil. dissertation thesis submitted to Sambalpur University in Life Sciences. Orissa, India, Pp: 18.
[186] Bajpayee, M., Pandey, A. K., Parmar, D., Marthur, N., Seth, P. K., Dhawan, A. (2005). Comet assay responses in human lymphocytes are not influenced by the menstrual cycle: a case study in healthy Indian females. Mutation Research, 565:163–172.
[187] Talapatra, S. N., Banerjee, S. K. (2007): Detection of micronucleus and abnormal nucleus in erythrocytes from the gill and kidney of Labeo bata cultivated in sewage-fed fish farms. Food Chemistry Toxicology, 45: 210-215.
[188] Sarangi, P. K. (2012): Micronucleus assay: a sensitive indicator for aquatic pollution. International Journal of Research in Bio Science, 1 (2): 32-37.
[189] Kousar, S., Javed, M. (2015): Studies on induction of nuclear abnormalities in peripheral blood erythrocytes of fish exposed to copper. Turkish Journal of Fisheries and Aquatic Science, 15(4): 879-886.
[190] Manna, G. K., Banerjee, G., Gupta, S. (1985): Micronucleus test in the peripheral erythrocytes of the exotic fish Oreochromis mossambica. The Nucleus, 28 (3): 176-179.
[191] Al Sabti, K. (1986 b): Comparative micronucleated erythrocyte cells induction in three cyprinids by five carcinogenic, mutagenic chemicals. Cytobios, 47: 147-154.
[192] Manna, G. K., Biswas, S. (1986): Micronucleus test in four species of fishes treated with the bacterium Pseudomonas aeruginosa. National Academy of Science Letters, 9 (6): 189-191.
[193] Manna, G. K., Sadhukhan, A. (1986): Use of cells of gills and kidney of tilapia fish in micronucleus test (MNT). Current Science, 55 (10): 498-501.

[194] Manna, G. K. (1989): Cytogenetical assays of some toxicants using fish as a model. In: Harmful Effects of Common Environmental Toxicants, by Om Prasad (ed.), Zoology Deptt. University of Allahabad, Allahabad. Pp. 65-74.
[195] Tripathy, A. P. (1993): Induction of micronuclei in the peripheral blood of fish exposed to paper mill effluent in natural and laboratory conditions. M.Phil dissertation submitted to Sambalpur University, Orissa, India in Environmental Sciences, 1993: Pp. 25.
[196] Sahoo, S. N., Bhuyan, S. P. (2002): Evaluation of genotoxic potential of an insecticide (Sevin ®) in fish in vivo test system. In: Proc. Fifth Indian Fisheries Forum. Published by AFSIB, Mangalore and AoA, Bhubaneswar, India: pp. 219-221.
[197] Farah, M. A., Ateeq, B., Ali, M. N., Ahmad, W. (2003): Evaluation of genotoxicity of PCP and 2,4-dichlorophenoxyacetic acid by micronucleus test in fresh water fish Channa punctatus (Bloch). Environmental Toxicology and Pharmacology, 54 (1): 25–29.
[198] Velmurugan, B., Ambrose, T., Selvanayagam, M. (2006): Genotoxic evaluation of lambda-cyhalothrin in Mystus gulio. Journal of Environmental Biology, 27(2): 247-250.
[199] Yadav, K. K., Trivedi, S. P. (2006): Evaluation of genotoxic potential of chromium (VI) in Channa punctata fish in terms of chromosomal aberrations. Asian Pacific Journal of Cancer Prevention, 7: 472-476.
[200] Sharma, S., Nagpure, N. S., Kumar, R. et al. (2007): Studies on the genotoxicity of Endosulfan in different tissues of fresh water fish Mystus vittatus using the Comet assay. Archieves of Environmental Contamination Toxicology, 53 (4): 617-623.
[201] Malla, T. M., Ganesh, N. (2009): Cytogenetic and tissue toxicity by synthetic sindoor in freshwater catfish Heteropneustes fossilis. Biomed Pharma Journal, 2 (1): 77-81.
[202] Yadav, K. K., Trivedi, S. P. (2009): Chromosomal aberrations in a fish Channa punctata after in vivo exposure to three heavy metals. Mutation Research/Genetics Toxicology and Environmental Mutagenesis, 678(1):7-12.
[203] Nwani, C. D., Lakra, W. S., Nagpure, N. S., Kumar, R., Kushwaha, B., Srivastava, S. K. (2010): Mutagenic and genotoxic effects of carbosulfan in freshwater fish Channa punctatus (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food and Chemical Toxicology, 48 (1):202–208.
[204] Saxena, K. K., Chaudhari, R. (2010): Study of chromosomal abnormalities in C. punctatus expose to fenvalerate. Journal of Applied and Natural Sciience, 2: 70-73.
[205] Yadav, A. S., Bhatnagar, A., Kaur, M. (2010): Assessment of genotoxic effects of Butachlor in fresh water fish, Cirrhinus mrigala (Hamilton). Research Journal of Environmental Toxicology, 4 (4): 223-230.
[206] Mohanty, G., Mohanty, J., Nayak, A. K., Mohanty, S. and Dutta, S. K. (2011). Application of Comet assay in the study of DNA damage and recovery in rohu (Labeo rohita) fingerlings after an exposure to Phorate an Organophosphate pesticide. Ecotoxicology, 20: 283-292.
[207] Nwani, C. D., Nagpure, N. S., Kumar, R., Kushwahab, B., Kumar, P., Lakra, W. S. (2011): Mutagenic and genotoxic assessment of Atrazine based herbicide to freshwater fish Channa punctatus (Bloch) using micronucleus test and single cell gel electrophoresis. Environmental Toxicology and Pharmacology, 31 (2):314-322.
[208] Kushwaha, B., Pandey, S., Sharma, S., Srivastava, R., Kumar, R., Nagpure, N. S., Dabas, A., Srivastava, S. K. (2012): In situ assessment of genotoxic and mutagenic potential of polluted river water in Channa punctatus and Mystus vittatus. International Aquaculture Research, 4 (16): 1-11.
[209] Patowary, K., Hazarika, N. S., Goswami, M. (2012): Studies on the toxic impact of arsenic on some enzymes and chromosomes of Channa punctatus. The Clarion, 1: 148-153.
[210] Parveen, N., Shadab, G. G. H. A. (2012): Cytogenetic evaluation of cadmium chloride on Channa punctatus. Journal of Environmental Biology, 33 (3): 663-666.PMID: 23029919
[211] Pavan, K., Ravindra, K., Nagpure, N. S., Nautiyal, P., Dabas, A., Kushwaha, B., Lakra, W. S. (2012): Genotoxic and mutagenic assessment of hexavalent chromium in fish

following in vivo chronic exposure. Human Ecology Risk Assessment: An International Journal, 18(4):855-870.

[212] Gadhave, P. D., Brar, R. S., Banga, H. S., Dhawan, A. (2014). λ- cyhalothrin induced genotoxicity in freshwater fish Labeo rohita. Veterinary World, 7(6):412-415.

[213] Ismail, M., Qaiser, M. K., Ali, R., Ali, T (2014): Genotoxicity of chlorpyrifos in freshwater fish Labeo rohita using alkalines single-cell gel electrophoresis (Comet) assay. Drugs and Chemicals Toxicology, 37 (4):466-471.

[214] Marques, A., Guilherme, S., Gaivao, I., Santos, M. A., Pacheco, M. (2014): Progression of DNA damage induced by a Glyphosate based herbicide in fish (Anguilla anguilla) upon exposure and post exposure periods, Insights into the mechanisms of genotoxicity and DNA repair. Comparative Biochemistry and Physiology, Part C, 166: 126-133.

[215] Chaudhari, R., Saxena, K. K. (2015): Evaluation of Bifenthrin genotoxicity on Channa punctatus. International Journal of Pure and Applied Zoology, 3 (4): 297-301.

[216] Nagpure, N. S., Srivastava, R., Ravindra, K., Kushwaha, B., Srivastava, S. K., Pavan, K., Dabas, A. (2015): Assessment of genotoxic and mutagenic potential of hexavalent chromium in the freshwater fish Labeo rohita (Hamilton, 1822). Drugs and Chemicals Toxicology, 38 (1):9-15.

[217] Srivasatava, P., Singh, A. (2015): Evidence of micronuclei in fish blood as a biomarker of genotoxicity due to surface run off agricultural fungicide (Propiconazole). Journal of Toxicology and Environmental Health Sciences, 7 (1): 4-8.

[218] Tripathi, M., Mishra, R. P., Girdoniya, V. (2015): Study of the genotoxic effects of polluted water of certain ponds of Narsinghpur in the R.B.Cs. of the fish Labeo rohita by applying micronucleus assay. International Journal of Pharmaceutical Biological Archieves, 6(2): 9- 13.

[219] Rajan, A. P., Anandan, S. (2017): Investigation of carcinogenic and mutagenic property of food color using cat fish Clarias batrachus by using alkaline single-cell gel electrophoresis (COMET) assay and micronucleus assay. International Journal of Medical Research and Pharmaceutical Science, 4 (7): 29-34.

[220] Hussain, B., Sultana, T., Sultana, S., Masoud, M. S., Ahmed, Z., Mahboob, S. (2018). Fish eco-genotoxicology: Comet and micronucleus assay in fish erythrocytes as in situ biomarker of freshwater pollution. Saudi Journal of Biological Science, 25(2):393-398.

[221] Tasneem, S., Yasmeen, R. (2018): Evaluation of genotoxicity by comet assay (single-cell gel electrophoresis) in tissues of the fish Cyprinus carpio during sub-lethal exposure to Karanjin. Journal of Basic and Applied Zoology, 79 (19): 1-13.