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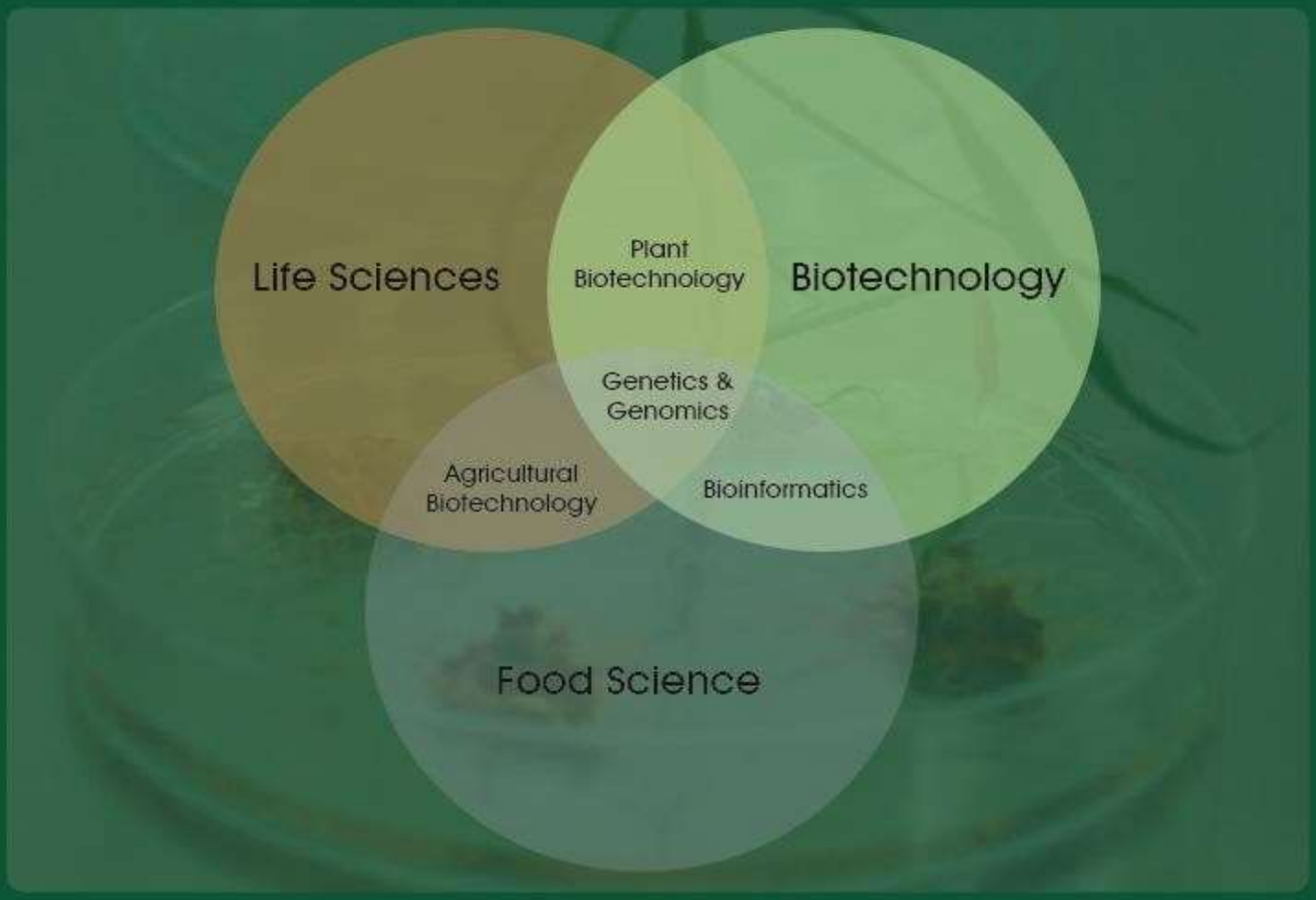

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## Editörden;

**Değerli okurlar ve yazarlar,**

"International Journal of Life Sciences and Biotechnology" olarak dergimizin onuncu sayısını yayın hayatına sunmaktan mutluluk ve onur duyuyoruz. "International Journal of Life Sciences and Biotechnology" dergisi araştırma- geliştirme ve uygulama ilkeleri baz alınarak yayınlanan uluslararası hakemli açık erişimli akademik bir elektronik dergidir.

"International Journal of Life Sciences and Biotechnology" dergisi Yaşam Bilimleri, Biyoloji, Biyoteknoloji, Biyomühendislik, Ziraat Bilimleri, Gıda Biyoteknolojisi ve Genetik alanlarındaki ilgili araştırmacılara, kurum ve kuruluşlara teorik ve pratik uygulamalarda katkı sağlamayı, tarafsızlık ve bilim etiği ilkelerine bağlı kalarak çözüm temelli, yenilikçi ve katma değeri olan çalışmalara odaklanan, günceli ve geleceği tartışan çalışmaların yayınlanmasını hedeflemektedir.

Bu düşüncelerle 2021 yılı ikinci sayısını yayınladığımız "International Journal of Life Sciences and Biotechnology" dergisini, makaleleri ile onurlandıran akademisyenlere, Fikir / Görüş / Öneri / Katkı ve Eleştirileri ile değerlendirme süreçlerine katkılarından dolayı hakem ve yayın kurullarında yer alan kıymetli bilim insanlarına yürekten teşekkür ediyoruz. Bir sonraki sayıda görüşmek ümidiyle…

**15.08. 2021**
**Editör**
**Dr. Öğrt. Üyesi Yilmaz KAYA**

**From The Editor;**

**Dear Readers and Authors,**

As “International Journal of Life Sciences and Biotechnology”, we are pleased and honored to present the 10th issue of the journal. "International Journal of Life Sciences and Biotechnology" is an international double peer-reviewed open access academic journal published on the basis of research-development and code of practice.

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

With these thoughts, We are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in the second issue of 2021 "International Journal of Life Sciences and Biotechnology". Hope to see you in the next issue…

**15. 08. 2021**

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## İçindekiler/ Contents

### Research Articles/ Araştırma Makaleleri



### Review Articles / Derleme Makaleler

**Research article**

Ahmed, H., A. Mariod, and T. A. A. Hammoda, The Chronic Toxicity Studies of Camellia Seed Oil Containing Tea Saponins on Mice Blood and Organs. International Journal of Life Sciences and Biotechnology, 2021. 4(2): p. 178- 191. DOI: 10.38001/ijlsb.807980

# The Chronic Toxicity Studies of Camellia Seed Oil Containing Tea Saponins on Mice Blood and Organs

**Hadeel O. A. Ahmed[1], Abdalbasit A. Mariod[2,3*], Tarig A. A. Hammoda[4d]**

**ABSTRACT**

Tea saponins are normal non-ionic surfactants with surface-dynamic (surfactant) properties, isolated from the *Camellia oleifera* seed. These saponins are more prominent than 10% of the camellia seed and are used as a natural tenside that is thoughtful to the environment. Camellia seed oil containing tea saponins was used as the main material. Three experimental groups were used: Low, Medium, and High groups with different doses. 30 days feeding in mice experiment. Mice blood and organs were used to analyze the chronic toxicity among the experimental rats in 30 d and olive oil used as a control group. The study showed that, there were significant differences in some index and there were no vital changes in the other indicators. The analysis of the results indicates that, camellia oil containing tea saponins have some toxic effects in functions of viscera, peripheral blood in mice. The results provides a theoretical basis for the utilization and safety of camellia oil. The chronic toxicity of camellia oil containing tea saponin was not strong, feeding on mice in a high dose (255 mg/kg·bw low tea saponin/oil) for 30 days each viscera and other features and growth have no significant impacts.

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blood,
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hemogram,
histology

## Introduction

Camellia oil coming from Camellia oleifera, it's a natural, edible oil in China; it has been considered as a very old edible vegetable oil in southern China, contains high unsaturated fatty acids, polyphenols, vitamin E and carotene. FAO recommended it as a healthcare plant oil [1, 2], with similar properties as olive oil, can also be used as cooking oil [3]. Tea saponin was frequently disposed of with the oil cake or used as manure in the conventional oil preparation techniques. Tea saponin is viewed as a solid frothing, emulsifying,

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scattering and wetting agent, with various therapeutic uses. It has also been broadly utilized in various fields [4] such as in food, chemical, industrial, agricultural and construction materials, metallurgy and metal processing [5, 6.] Generally, saponins are amphipathic glycosides amassed phenomenologically by the cleanser like foam they give when shaken in watery arrangements, and fundamentally by having, in any event, one hydrophilic glycoside moieties got together with a lipophilic triterpene or steroid derivative [7]. It has a very high bioactive components. Previous research showed that tea saponinfoaming ability is better than fine soap [8]. The structure of tea saponin is shown in Figure1, with a molecular weight of 1222.54 [9]. This study aims to investigate the impact of camellia seed oil containing tea saponins on mice blood and organs.

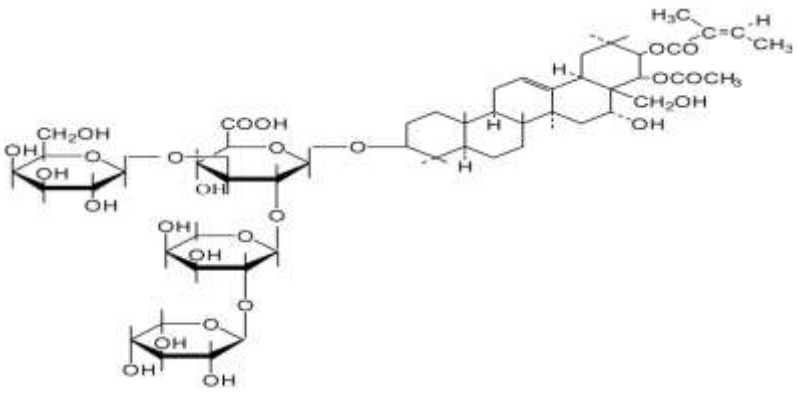

**Fig 1** Structures of saponins isolated from leaves of the tea plant (*Camellia sinensis var. sinensis*) [10].

## Materials and Methods

### The experimental drug

96% of tea saponin of purple reagent was obtained from a factory in Shanghai, China; Qiagen integral protein, albumin kit, cereal third transaminase kits, aspirate aminotransferase kits, triglyceride kits, and total cholesterol kits all were obtained from Nanjing Science and Technology Co., China.

**The experimental animal**

60 healthy male Kunming mice of five weeks old were obtained, weighing 25 to 30 g specific pathogen free (SPF) were obtained from the Center for Disease Control (CDC), Hubei province, China, were used. Mice were fed with tea oil containing tea saponin for 30 days; were housed in a room with temperature (18 to 24ºC), and relative humidity (40% to 60%); all the experiments were done at College of Food Science and Technology; Huazhong University; Wuhan City, Hubei Provence.

**Experimental instrument**

Optical microscope (EX20) Ningbosyunny Instruments Co., Ltd., Bench top high speed refrigerated centrifuge (H1850R) Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Microplate Reader (51119200), America Thermo Scientific Company and Electronic balance BS224S Sartorius analytical instrument (Beijing) Co., Ltd.

**Test's methods**

***Animal and dose***

Mice were separated into six cages, every cage containing 10. They were stamped, weighed, and fed with essential feed, with versatile feeding and observed for seven days. The mice were then arbitrarily partitioned into six groups of 10 mice each. Standard saline (control A), olive oil (control B) as control groups, tea saponin/water, 25 mg/kg b.w. (C), low, 255 mg/kg b.w. (D), medium, 382.5 mg/kg b. w. (E) and high, 510 mg/kg b.w. (F) dose group. All animal treatments were performed according to the standards and methodology illustrated in the National Institutes of Health Guide on the Care and Use of Laboratory Animals [11, 12]. The study was endorsed and inspected by Institutional Animal Welfare and Research, Ethics Committee guidelines of Huazhong Agricultural University, Wuhan, China (Approval number: 31273519).

All mice were fed *ad libitum*; they were given an intra-gastric administration every morning at 8:00–9:00 once a day for 30 days. Control A was given standard saline; control B was given olive oil (0.01 mL each). The other groups were given the test samples (tea oil containing tea saponin) 28, 255, 382.5, and 510 mg/kg.bw, respectively. The mice were weighed every week and the activity of the mice, feeding, presence of poisoning and death were observed every day, and drug dose was balanced by body weight.

***Blood measurement***

Method of Abliz et al. [13] was adopted with some modifications. Mice treated for 30 days, and fasted one day before the end of the trial. On the last day, the mice weighed and fixed. The blood was collected from their eyes. The blood was allowed to remain at room temperature for 0.5 hr, later was centrifuged at 3000 rpm for 10 min. Serum was pipetted for serum lipid concentration estimation, the serum blood was determined using the automatic blood cell analyzer [14-19].

***Mice organs measurement***

After blood collection, the liver, spleen, and kidney immediately taken, and every each organ, washed with super cold saline; blotted on filter paper and weighed, the organ's indexes were indicated by the accompanying formula:

$$\text{Organ index\%} = \left(\frac{\text{organ weight(g)}}{\text{animal weight(g)}}\right) * 100$$

The organs washed and dried, and three examples of every one taken. These were rapidly placed into glass vials loaded up with formaldehyde 4%, cooled to 4°C, for histopathological examination [12]. Serum was utilized in biochemical assays. Triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) were determined using TG kit, TC kit, HDL kit and LDL kit (Beijing wantai DRD CO., LTD., Beijing, PR China) [20].

**Statistical analysis**

The analysis of variance (ANOVA) was used to compare the means. Analyses were carried out using IBM® SPSS® Statistics V25 statistical software to compare treatment groups, and observations at $P<0.05$ were considered statistically significant.

## Results

**The general status and body weight of mice**

Daily changes in mice were observed before and after lavage signs; drinking water, and feeding, activities. Fine lavage tea saponin/camellia oil for each group of mice, and olive oil, movement is not active, to eat and drink is compared with the normal group and tea

saponin/water are generally low, and lavage mice before and after the change is not obvious, the entire process of experimental mice were no poisoning or death situation.

All the mice were given same diet throughout the study, and there was difference between the different treatment groups in dose. In the process of experiment, tea saponin dose/camellia oil, olive oil group and normal group, tea saponin/aqueous solution are in the trend of steady growth. The body weight of mice and rats weight growth stage before the experiment, the normal growth conform to animals, each dose group mice weight there was no significant difference in comparison with other groups.

**Hemogram and histological results**

From Table 1 it can be observed that, during the period of feeding, according to the records of each food intake in mice, it was observed that the tea saponin/camellia oil food utilization rate of each dose group compared with normal group found that, there was no significant difference.

Table 2 and 3, showed the experimental results of tea saponin in each dose group compared with the control. The LYM, MONO, NEU, and EO figures of the treated samples were statistically ($P > 0.05$) not different from the control (Table 2), the result of each index was within the normal range. In the same manner WBC, and HGB treated samples were statistically ($P > 0.05$) not different from the control, while RBC, and PLT figures of the treated samples were statistically ($P > 0.05$) different from the control (Table 3). The effects of tea saponins of camellia oil on blood lipids in mice are shown in Table 4. In comparison with control group A, TC and TG were the important index reflecting the body lipid metabolism. Table 5 shows the effect of tea saponins from camellia oil on Alanine transaminase (ALT), Aspartate transaminase AST, and Total protein (TP) in serum of mice for 30 days to assay the toxicity. Table 6 shows the weight/body of the liver, spleen, and kidney ascertained; and compared according to the experimental results. Figure 1 -3, shows no adverse effects of camellia oil containing tea saponin of all mice organ's kidney, liver and spleen physiology compared with control groups.

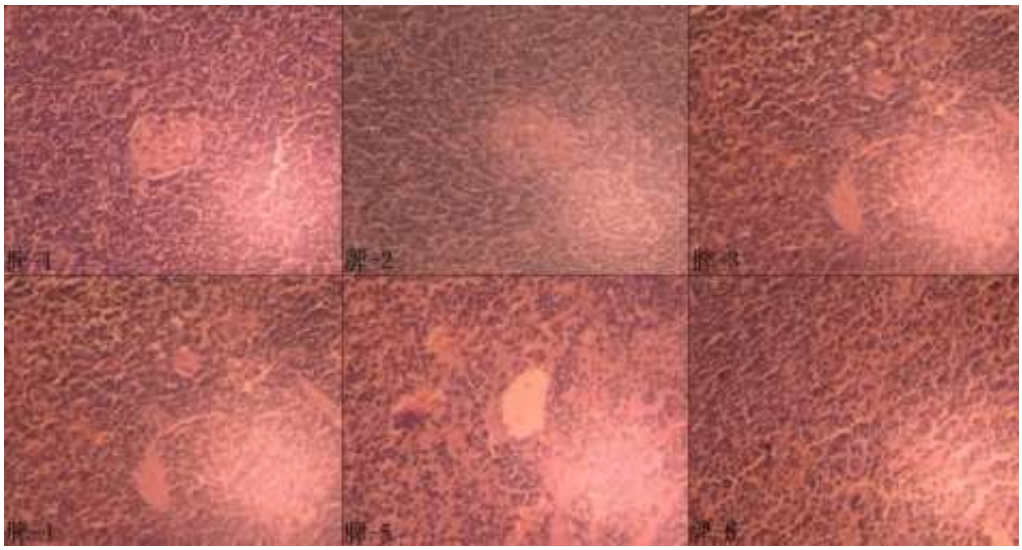

**Fig 2.** Histological observation of kidney from mice feed tea saponins of camellia oil. *Where are: 1: Normal control; 2: TS/ aqueous solution; 3: Low dosage of TS/ camellia oily solution ; 4: Medium dosage of TS/ camellia oily solution ; 5: High dosage of TS/ camellia oily solution ; 6:Olive oil* **.**

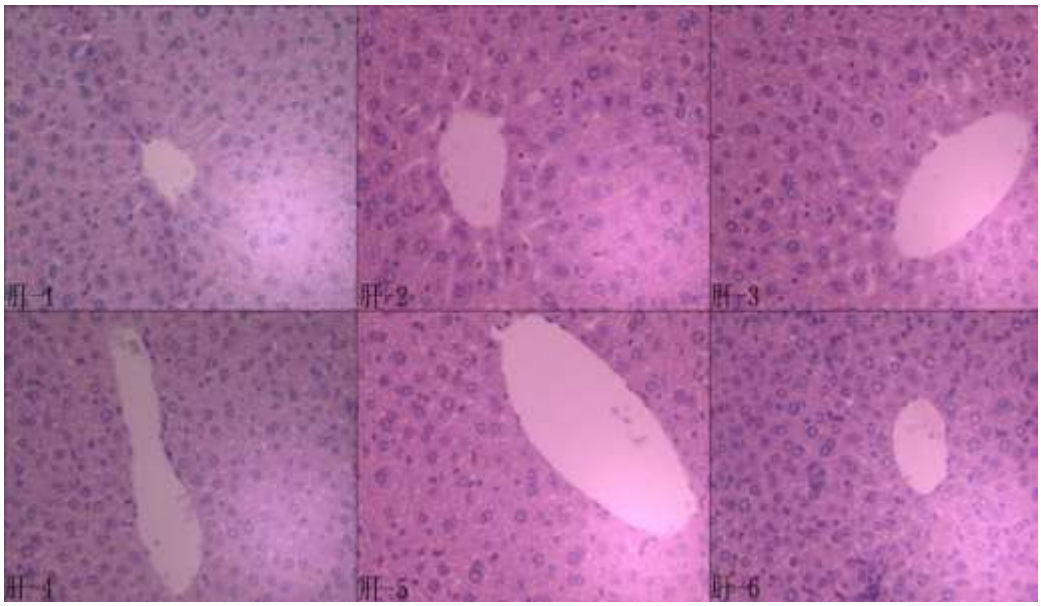

**Fig 3.** Histological observation of Liver from mice feed tea saponins of camellia oil. Where are: 1: Normal control; 2: TS/ aqueous solution; 3: Low dosage of TS/ camellia oily solution; 4: Medium dosage of TS/ camellia oily solution; 5: High dosage of TS/ camellia oily solution; 6:Olive oil .

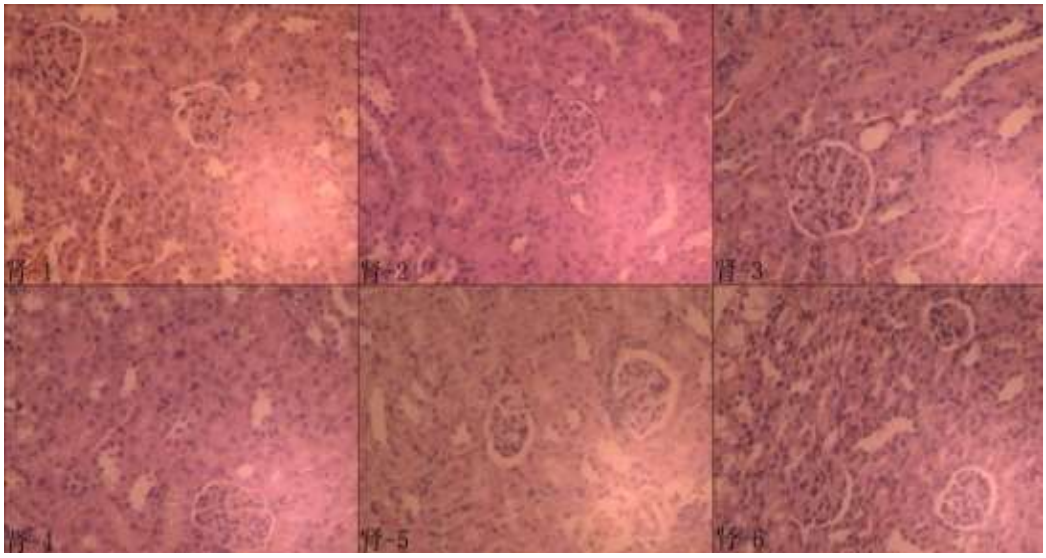

**Fig 3.** Histological observation of Spleen from mice feed tea saponins of camellia oil. Where are: 1: Normal control; 2: TS/ aqueous solution; 3: Low dosage of TS/ camellia oily solution ; 4: Medium dosage of TS/ camellia oily solution ; 5: High dosage of TS/ camellia oily solution.

## Discussion

### Body weight and food utilization

From Table 1, the weight of mice was increased each day after tea oil saponin treatment. No changes in eating, drinking and exploratory behavior have seen in the mice treated with tea oil saponin. The body weight plot demonstrated that, the weights of the mice were decreased and the tea oil saponin-treated groups were differ significantly when compared with control groups ($p>0.05$). This proposed the tea oil saponin did not make any unfriendly impact to the body weight of the mice. Nevertheless, the body weight of tea oil saponin-treated groups did not differ significantly when compared with each other ($p > 0.05$). The observed body weight loss in experimental groups may be due to appetite affected by the tea oil saponin, leading to decreased food consumption. The feed utilization ratio in groups treated with tea oil saponin (255, 382.5 and 510 mg/kg) decreased significantly when compared to the control group ($p<0.05$). Tea oil saponin had no effect on organ tissue histopathology compared to control groups

**Table 1** Effects of tea saponins in camellia oil on food utilization ratio in mice

| Group and dosage | Weight changes (g) | Food utilization ratio (%) |
|---|---|---|
| Control A | 4.34[a] | 14.42[a] |
| Olive oil control B | 0.42[b] | 7.74[b] |
| 28 mg/kg·bw tea saponin/water | 3.76[c] | 11.48[c] |
| 255 mg/kg·bw low tea saponin/oil | 3.11[d] | 8.86[d] |
| 382.5 mg/kg·bw med. tea saponin/oil | 3.01[d] | 8.04[d] |
| 510 mg/kg·bw high tea saponin/oil | 3.22[d] | 8.19[d] |

Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately.The numbers with different letters in columns represent the data has significant difference from control group

## Peripheral hemogram

In Tables 2 ad 3, each dose group inside the treated groups, there were no significant differences, which can be concluded that, camellia oil containing tea saponin has no effect on lymphocytes in mice. However, all the haematological values in all treated groups were still within the normal range and the alteration had no clinical significance. These results are comparable with our recent results [11], where tea oil saponin fed to mice for 90 days showed no significant difference on histopathological values when compared with control groups.

Table 2 Hematological parameters (peripheral hemogram) of mice treated orally with different doses of tea saponins in camellia oil for 30 days

| Group and dosage | LYM (109/L) | MONO (109/L） | NEU（109/L） | EO(109/L） |
|---|---|---|---|---|
| Control A | 1.76±0.26 [a] | 0.01±0.01 [a] | 0.11±0.01[a] | 0.02±0.01[a] |
| Olive oil control B | 2.14±1.51 [a] | 0.37±0.17 [b] | 0.22±0.17[b] | 0.02±0.02[a] |
| 28 mg/kg·bw tea saponin/water | 2.19±0.07 [a] | 0.01±0.01[a] | 0.13±0.03[c] | 0.01±0.01[b] |
| 255 mg/kg·bw low tea saponin/oil | 2.19±0.62 [a] | 0.03±0.02[a] | 0.15±0.06[c] | 0.01±0.01 [b] |
| 382.5 mg/kg·bw med. tea saponin/oil | 2.34±0.68[b] | 0.02±0.02[a] | 0.13±0.11[c] | 0.02±0.01[a] |
| 510 mg/kg·bw high tea saponin/oil | 2.25±0.21[a] | 0.01±0.00[a] | 0.14±0.02[c] | 0.02±0.01[a] |

Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately, LYM: lymphocytes, EO: eosinophils, NEU: neutrophils, BA: basophiles, MONO: monocytes. The numbers with different letters in columns represent the data has significant difference from control group

**Table 3** Effects of tea saponins in camellia oil on peripheral hemogram in mice

| Group and dosage | WBC (109/L) | RBC(1012/L) | PLT(109/L) | HGB (g/L) |
|---|---|---|---|---|
| Control A | 2.13±0.29[a] | 8.51±0.36[a] | 809.00±59.65[a] | 149.75±1.89 [a] |
| Olive oil control B | 3.90±0.07[b] | 9.21±0.20[b] | 534.50±338.18[b] | 146.00±0.00[a] |
| 28 mg/kg·bw tea saponin/water | 2.33±0.06 [a] | 8.60±0.27 [a] | 820.33±15.89[c] | 149.60±2.51[a] |
| 255 mg/kg·bw low tea saponin/oil | 2.20±0.14 [a] | 9.18±0.51[b] | 793.00±69.46[d] | 155.50±9.47[b] |
| 382.5 mg/kg·bw med. tea saponin/oil | 2.37±0.15 [a] | 8.97±0.38[c] | 749.67±35.70[e] | 151.40±5.55[a] |
| 510 mg/kg·bw high tea saponin/oil | 2.25±0.07 [a] | 9.39±0.12[d] | 698.50±16.26[f] | 150.50±3.54[a] |

Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately. WBC: white blood corpuscles RBC: red blood corpuscles, HGB: hemoglobin, PLT: blood platelet count. The numbers with different letters in columns represent the data has significant difference from control group

## Serum lipids

Table 4. in comparison with control group A, TC and TG were the important index reflecting the body lipid metabolism. All measured parameters were dose dependent and has not significant impact, which can explain the effect of camellia oil containing tea saponin on mice lipid metabolism. These results are in good agreement with Ahmed et al. [11] who extracted tea saponin using different techniques and studied its effect on mice lipid metabolism, they reported the same findings. Results showed lipid profile total cholesterol had decreased in various degrees, and significant differences were recorded between dosed and control groups. However, TG did not reveal any significant alteration.

**Table 4** Effects of tea saponin in camellia oil on blood lipids of mice

| Group and dosage | TC（mmol/L） | TG (mmol/L) |
|---|---|---|
| Control A | 1.92±0.05[a] | 0.25±0.10 |
| Olive oil control B | 1.96±0.03[b] | 0.25±0.05 |
| 28 mg/kg·bw tea saponin/water | 1.88±0.05[c] | 0.23±0.20 |
| 255 mg/kg·bw low tea saponin/oil | 1.90±0.04[d] | 0.25±0.10 |
| 382.5 mg/kg·bw med. tea saponin/oil | 1.89±0.05[e] | 0.24±0.08 |
| 510 mg/kg·bw high tea saponin/oil | 1.92±0.05[a] | 0.25±0.02 |

Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately TC: total cholesterol, TG: total triglyceride. The numbers with different letters in columns represent the data has significant difference from control group.

**Serum AST, ALT and TP**

From Table 5 it was clear that, the doses of 28, 255, 382.5 and 510 mg/kg/bw increased the ALT, and AST, levels in serum compared with the two control groups; while there these doses decreased the TP levels in serum compared with the two control groups; olive oil group and normal control group of extremely significant differences ($P < 0.01$). This result is in good agreement with our previous results [11] which, demonstrated that tea oil saponin increased ALT, AST activities. But olive oil group is not the main research object of this experiment; the experiment set olive oil group is just making a reference of grease, but its special circumstances, worthy of our attention and discussion. Mice in the serum total protein determination of TP, according to the results of olive oil group the low, middle and high dose group was significantly lower than control water ($P < 0.01$), due to the special control of total protein of olive oil. Generally, it was a little higher in the treated groups.

**Table 5** Effects of tea saponins in camellia oil on ALT, AST and TP in mice

| Group and dosage | ALT(mmol/L) | AST (mmol/L) | TP (mmol/L) |
|---|---|---|---|
| Control A | 14.36±1.09$^{f}$ | 64.39±0.99$^{f}$ | 2.07±0.07$^{e}$ |
| Olive oil control B | 8.16±0.40$^{a}$ | 152.44±0.34$^{a}$ | 1.48±0.04$^{a}$ |
| 28 mg/kg·bw tea saponin/water | 14.81±0.75$^{d}$ | 65.12±0.04$^{d}$ | 1.65±0.03c$^{a}$ |
| 255 mg/kg·bw low tea saponin/oil | 14.43±0.48$^{e}$ | 64.55±0.10$^{ef}$ | 1.76±0.02$^{cd}$ |
| 382.5 mg/kg·bw med. tea saponin/oil | 14.78±0.31$^{c}$ | 66.19±0.14$^{bc}$ | 1.60±0.03$^{bc}$ |
| 510 mg/kg·bw high tea saponin/oil | 14.40±0.5$^{ab}$ | 67.19±0.09$^{ab}$ | 1.36±0.04b$^{a}$ |

Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately,ALT: Alanine transaminase, AST: Aspartate transaminase, TP: Total protein. The numbers with different letters in columns represent the data has significant difference from control group.

## Organ indexes

Following 30 days of administrations, the mice sacrificed and the significant organs were gathered. The organ indexes (organ weight/body weight) of the liver, spleen, and kidney ascertained; and compared according to the experimental results (Table 5). The outcome demonstrated that the tea oil saponin had undeniable impact on the organ indexes. No significant difference was detected between the control and treated groups on kidney, and spleen but on liver significant differences were detected between two control groups and treated groups. Livers were bigger in all groups compared with other organs. These findings disagreed with the results of Yoshikawa et al. [21] who studied the inhibitory activity of camellia saponins extracted, from the seeds of *Camellia japonica* on mice, they reported no obvious changes in the body weight, organ index and biochemical parameters.

**Table 6** Effects of tea saponins in camellia oil on visceral index in mice

| Group and dosage | Liver-index (mg/g) | Renal index (mg/g) | Spleen-index (mg/g) |
|---|---|---|---|
| Control A | 44.03±0.19[a] | 12.23±0.09[a] | 3.78±0.03[a] |
| Olive oil control B | 38.35±0.22[b] | 9.86±0.02[a] | 4.48±0.02[a] |
| 28 mg/kg·bw tea saponin/water | 40.84±0.21[c] | 11.28±0.04[a] | 3.50±0.02[a] |
| 255 mg/kg·bw low tea saponin/oil | 43.29±0.14[d] | 10.37±0.03[ad] | 3.43±0.02[b] |
| 382.5 mg/kg·bw med. tea saponin/oil | 40.00±0.20[ce] | 10.46±0.03[ac] | 3.16±0.04[cd] |
| 510 mg/kg·bw high tea saponin/oil | 40.00±0.16[ce] | 10.92±0.04[ab] | 3.19±0.01[c] |

Data are expressed as mean ± SD and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately. The numbers with different letters in columns represent the data has significant difference from control group

## Mice organs histopathological results (kidney, liver, spleen)

Anatomy of mice organs (liver, kidney, spleen and heart) was observed by the naked-eye; it was found that, no differences between the control groups and doses groups concerning edema, hyperplasia, and atrophy lesions, with bright-color appearance, as the normal organs and without any hitopathological changes. Finally, based on the corresponding viscera, mainly by the formalin preserved and dyeing embedding tissue section using the optical microscope in each slice observation, above for the observations.

Figure1 shows kidney histopathological tissue in Kunming mice, the performance of kidney tissues in the doses groups was roughly same as control groups with no significant difference, where there was clear renal tubular structure, complete epithelium; no crystals within the lumen, also no any inflammatory cells. Figure 2 shows liver pathological tissue in Kunming mice, the figure showed an ordinary liver tissue, no changes in the appearance of the controls and dose groups. There were big nucleus, neat veins closely surrounding cells tissue, and regular lobular, beside no cavitations, no abnormal accumulation or precipitation. Generally, the dose groups showed a normal liver tissue, fatty degeneration also not found in the liver [16] and no toxic effects. Figure 3 shows spleen histopathological tissue in Kunming mice, the figure showed tissue morphology; all groups were characterized by clear organizational structure, some big volume and irregular polygonal cells, scattered distribution in the edge area of the spleen cells, red pulp spleen sonne and lymph follicle germinal center can be clearly seen. All treatment groups compared with control groups showed as normal, and there was no significant difference.

So as the general results, camellia oil containing tea saponin of all mice organ's kidney, liver and spleen physiology without adverse effects.

The study indicates that, the appearance of the mouse performance is normal, but in food, intake and body weight changes exist certain differences. The weight of mice showed normal speed growth, prove that camellia oil containing tea saponin have little impact on weight and food utilization in mice. AST and ALT in each group were no significant differences between groups; so that tea saponin in camellia oil didn't damage liver cells, so its non-toxic . TC and TG is one of the important indices for detecting small body lipid metabolism. The results prove that camellia oil containing tea saponin in mice don't disruptive effects of lipid metabolism. Sung et al [22] investigated the effects of saponin extracted from *Asparagus cochinchinensis* on the weights of eight organs and seven urine factors. Their results indicated no significant differences in the weights of any of the organs, while significant alterations were observed in ALT, AST, and LDH.

In conclusion, the results of the present study suggest that feeding on mice in high dose 30 days each viscera and other features and growth are not a significant impact. This study confirmed no toxicity for tea saponin when using tea saponin less than 255mg/kg.bw and it was dose-dependent. Furthermore, these findings provide vital information regarding the histopathological studies which, revealed that, all treatment groups compared with control groups showed no significant difference.

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**Research article**

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# Investigating the Effect of Several Palm Oil Mill Effluent (POME) Dilutions on Biomass And Specific Growth Rate of *C. sorokiniana*

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**ABSTRACT**

Palm Oil Mill Effluent (POME) contained high amount of nutrients necessary to support the growth of microalgae. However, high amount of turbidity was identified as one of the important factor affecting the successful cultivation of microalgae in POME. In this study, *Chlorella sorokiniana* was cultivated in POME, under different dilutions of 80, 60, 40 and 20% (v/v) with distilled water. Standard methods were used for the analysis of the wastewater parameters such as nitrate, phosphate, turbidity, pH, total carbohydrate, phenols and heavy metal. The results indicated that, *C. sorokiniana* grew well on both sterilized and unsterilized condition. Result of the batch experiment showed that, POME turbidity of 45 NTU produce the maximum specific growth rate of 0.14 $d^{-1}$, which correspond to highest biomass production of 4.06±0.16 g/L in sterilized POME. Whereas in unsterilized POME, turbidity of 45 NTU produces specific growth rate of 0.2 $d^{-1}$ which correspond to biomass production of 5.94±0.12 g/L in unsterilized POME. Biomass production of 5.88±0.31 was obtained when cultured in control experiment. This study concluded that 80% dilution was identified as the optimal medium for enhance biomass production of algae in POME which could add value for sustainable bioenergy production.

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## Introduction

Malaysia is a palm oil producing country accounting for about 41% world palm oil trade making it the second largest producer of palm oil in the world [1]. Due to increase in palm oil production of about 16.3 million tonnes in a year, large volume of palm oil mill effluent (POME) of about 2.5 to 3.5 tonne are generated [2]. POME contains large amount of chemical oxygen demand (COD), Biochemical oxygen demand (BOD), nitrate, phosphate

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and turbidity. However, these pollutants are dangerous to human and aquatic life especially when they are discharge into the environment in an untreated form [3].

Currently, there was a developing interest in the biotechnological exploitation of POME for the production of value added materials [4] which is due to its ability to contain nutrient (nitrogen and phosphorus), lipid, phenolic compounds, heavy metals and carbohydrate. One of these biotechnological areas involved the utilization of POME as suitable growth medium for the cultivation of microalgae for biofuel production [5]. This is of economic important because, using wastewater as medium for culturing microalgae will directly eradicate the need of synthetic medium and therefore this may reduce the cost of production.

Microalgae are micro plant capable of growing in wastewater containing nitrogen and phosphorus [6, 7]. Various researches have been shown that, the biomass of wastewater grown algae has enormous important. For instance, A report by [6] indicated that, microalgae biomass can use for the production of animal feed. The cultivation of Chlorella sp. in anaerobic digested dairy manure effluent for the production of lipid was conducted by [7]. The result of the study showed that, the biomass of microalgae contains total lipid content of 13.7%. Kang and his co-workers [8] also reported the cultivation of *Haematococcus pluvialis* in piggery wastewater, and the result showed that, large amonut of astaxanthin was produce from the microalgal biomass after treament. Similarly, [9] reported the succesfull cultivation of Nannochloropsis in POME coupled together with recovery of high amount polyunsaturated fatty acid.

Considering the above mention important of algal biomass, the cultivation of algae will benefit immensely from the economic point of view. Despite the existence of report on utilization of POME for microalgae production, Literature have indicated that different species of microalgae varies in terms of their capacity to growth in stress environment like wastewater. Our paper emphasized specifically on *C. sorokiniana* strain because literature reported that this species have strong cell wall making it the most robust among the families of *Chlorella* and most exploitation has not been given to it in relation to its growth in POME. Since POME is dark brown in color which can potentially affect photosynthesis by algae [10], then we came up with dilutions of the POME as an adaptive mechanism in

order to study the effect of POME turbidity on the increase in biomass and speicific growth rate of *Chlorella sorokiniana*. Various dilutions of 80. 60, 40, and 20% (v/v) was used inorder to determined the optimal condition for enhance biomass production in POME.

## Materials and Methods

### Sample collection

POME was collected at the facultative anaerobic pond (FAP) from local palm oil mill industry (Kilang Sawit Bukit Besar, Johor Bahru Malaysia). Due to its turbid characteristic, it was left to settle for four hours before being filtered using Whatman filter paper. POME was sterilized by autoclaving at 121$^{o}$C for 20 minutes and later placed in a laminar flow under the effect of UV sterilization for 15 minutes in order to obtained sterile filtrate. For the purpose of preservation, POME was refrigerated at 4$^{o}$C to prevent biodegradation.

### Algae and Culture Condition

A pure strain of *C. sorokiniana* was obtained from algae culture collection center at University of Texas, Austin Texas USA. The culture was maintained on Proteose media at 29$^{o}$C. The composition of Proteose media consist of $NaNO_3$ (25 g/L), $CaCl_2.2H_2O$ (2.5 g/L), $MgSO_4.7H_2O$ (7.5 g/L), $K_2HPO_4$ (7.5 g/L), $KH_2PO_4$ (17.5 g/L), NaCl (2.5 g/L), Urea (1.5 g/L). pH of the media was kept at 7. The experiments were conducted in a batch system. 1 L flasks were used for the algae cultivation. Before the commencement of the experiments, about 20% volume of algae was inoculated in 250 mL Proteose media (composition as shown above) for 21 days. This is to ensure that, *C. sorokiniana* acclimatize well before inoculation in POME. The justification of using *C. sorokiniana* for these experiments was due to its robust nature and ability to grow under limited photoheterotrophic making it one of the most tolerant microalgae to grow in a strange environment.

### Characterization of POME

Various wastewater parameters such as nitrate, phosphate, phenols and ammonium were determined following the Hatch DR 6000$^{TM}$ Spectrophotometric Manual (DR/6000, Hatch Co. Ltd. Tokyo 2008)**.**

**Determination of wastewater parameters**

Nitrate and phosphate were specifically determined using cadmium reduction method and an acid hydrolysable method respectively while ammonium and phenols were determined using Nessler and 4-Aminoantipyrine methods [11, 12]. Total carbohydrate was determined using phenol sulphuric acid assay [13]. Suitable dilutions were made for the high concentration and the final results were computed by multiplying the dilution factor. 5 mL microalgae suspension was taken from each Erlenmeyer flask to measure the nutrient removal throughout the experiments. The samples were centrifuged at 4,000 rpm for 15 min and afterward, the supernatants were analyzed for nitrate, phosphate, ammonium and phenols based on the HACH DR 5000 spectrophotometer manual [12].

**Determination of Turbidity**

A volume of 10 mL sample was taken and pours in a turbidity vial, afterward, it was then analyzed in turbidity using turbidity meter (model; 2100Q).

**Determination of pH**

pH was determined using the portable pH meter according to the standard methods of APHA, [14].

**Determination of COD**

Ten mL of POME was taken and then centrifuged at 4000 rpm for 15 minutes. The supernatant was mixed thoroughly using vortex and was placed in a preheated digester block at 1,500°C. Following the next 2 h, the sample was withdrawn and measured using spectrophotometer.

**Determination of Heavy Metals Ions**

To determine the concentration of heavy metals in POME, 10 mL of POME was acid digested using 2.5 mL concentrated nitric acid (65% $HNO_3$) and 0.8 mL of hydrochloric acid (HCL) at 180$^{o}$C for 20 min in a microwave oven (model; Bergh of *Speed wave 4*). Afterward, the digested samples were taken out, allowed to cool, filtered and diluted with distilled water. The sample was then analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) according to the method portrayed by [15]. All the data were analyzed using Microsoft excel 2010.

**Experimental Setup**

The experiments were conducted in a batch system using 1 L Erlenmeyer flask. At the inception of the experiment, 20% (v/v) of pre-cultured microalgae cell was cultivated in sterilized POME initially diluted to 80, 60, 40, and 20% (v/v) of sterilized and unsterilized POME with distilled water. Cultivation of algae in proteose medium was used as the control experiment. This initial inoculum concentration was kept constant throughout the experiment. The experiment was repeated three times and the average values of the replicate results were used for analysis. Various level of turbidly was monitored during the growth of the microalgae and the specific growth rate was determined for the individual dilutions. The cultivation condition was maintained by a continuous supply of carbon dioxide ($CO_2$) for mixing purpose using an air pump. The pH was maintained at 7 by the addition of 1 M sodium hydroxide (NaOH) and 2 M HCL. Fluorescence lamps (3000 lux) were used as a source of illumination for 12 h (Day: Night) interval. The experiment was conducted at temperature 25-30$^{o}$C for 15 days.

**Determination of Biomass Concentration**

Algal biomass was determined using cell dry weight (CDW) based on the method described by [4]. 20 mL of algal culture was centrifuged at 4000 rpm for 15 min. The supernatant was discarded and the pellet was washed three times with distilled water. The pellets were dried at 70$^{o}$C in a hot air oven until constant weight was achieved. Biomass ~~was~~ determined in terms of cell dry weight per volume of culture (g/L).

**Determination of Specific Growth Rate**

The specific growth rate was calculated by the equation;

$$\mu = \frac{1}{t} In\left(\frac{Xm}{Xo}\right) \qquad \textbf{eq. (1)}$$

Where $X_o$ and $X_m$ are the initial and final concentration of biomass respectively, and t is the duration of the batch run.

## Result and Discussion

The anaerobic digested POME was characterized and the initial values of various parameters were summarized in Table 1. It can be seen that, POME despite it treatment via anaerobic digestion, still contain high amount of nutrients. This nutrient has the potential to

support the successful growth of microalgae as this study demonstrates. Sample inoculation set and POME dilution can be found in Figure 1.

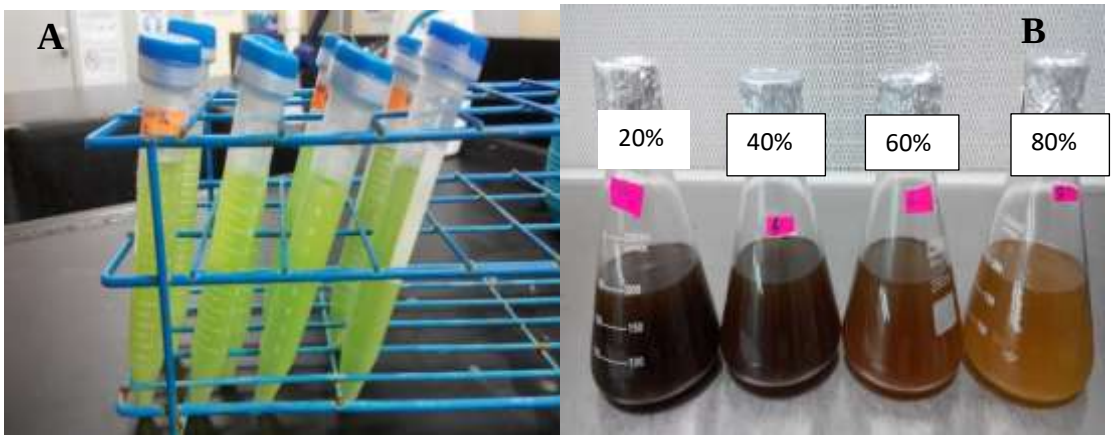


**Fig 1** Pictures of sample inoculation (**A**) and POME dilutions (**B**)

**Table 1** Characteristic of anaerobic digested POME

| **General Characteristics** | **POME[a]** | **Malaysian discharge limits[b]** |
|---|---|---|
| pH | 6.5 | 5-9 |
| COD | 2100 | - |
| TSS | 3200 | 400 |
| Nitrate | 181 | - |
| Phosphate | 131 | - |
| Ammonium | 245 | - |
| Phenols | 295 | 0.5 |
| Total carbohydrate | 211 | |
| Turbidity | 420 | |
| **Heavy metals** | | |
| $Zn^{2+}$ | 0.79 | 1 |
| $Fe^{2+}$ | 118.1 | 5 |
| $Mn^{2+}$ | 9.2 | 1 |

[a] All parameters unit is in mg/L except pH and turbidity (NTU). [b] Reference [16]

Algal growth is affected by several factors, which include nutrients, light, temperature, and inoculum size. Among these, inoculum size was identified as the most important factor affecting the growth of microalgae and its nutrient removal ability. For instance, [7] conducted an experiment using *Chlorella* sp. in dairy wastewater and reported that an optimal initial cell density of $1.2 \times 10^7$ cells $mL^{-1}$ is sufficient to overcome shading effect factor thereby allowing better growth and nutrients removal. In this study, an optimal initial size of the inoculum was reported to be $1.2 \times 10^6$. While the inoculum size reported from this study was lower than that reported by [7], still satisfactory treatment was achieved. This could be due to the difference of the substrate used for the cultivation and the variation in the substrate dilution factor.

The correlation between 9 day specific growth rate of *C. sorokiniana* and turbidity strength of the four dilutions under both sterilized and unsterilized POME was plotted in Figure 1 and 2**.** The aim of this analysis was to investigate whether turbidity could be an important factor affecting the growth of algae in POME. The low specific growth rate relates well with high turbidity in both the two conditions. The results showed that the increase in POME dilution decreases the specific growth rate of microalgae and vice versa. The lowest turbidity of 45 NTU at 80% dilution produces the maximum specific growth rate of 0.14 $d^{-1}$ in sterilized POME, whereas the highest turbidity of 98 NTU at 20% dilution produces the lowest specific growth rate of 0.07 $d^{-1}$ (Fig 2).

It was however noted that, the control experiment gave specific growth of 0.19 $d^{-1}$ which is relatively higher than the rate produced by POME dilution.

In the case of unsterilized condition; the minimum turbidity of 45 NTU at 80% dilution produces a maximum specific growth rate of 0.2 $d^{-1}$ whereas the maximum turbidity of 98 NTU at 20% dilution produces the lowest specific growth rate of 0.07 $d^{-1}$ (Fig 3). The $R^2$ values for sterilized and unsterilized POME were found to be above 0.90 indicating the accuracy of the graphs. Moreover, the specific growth rate of control (0.19) was found to produce almost similar growth rate of 2 in 80% dilutions.

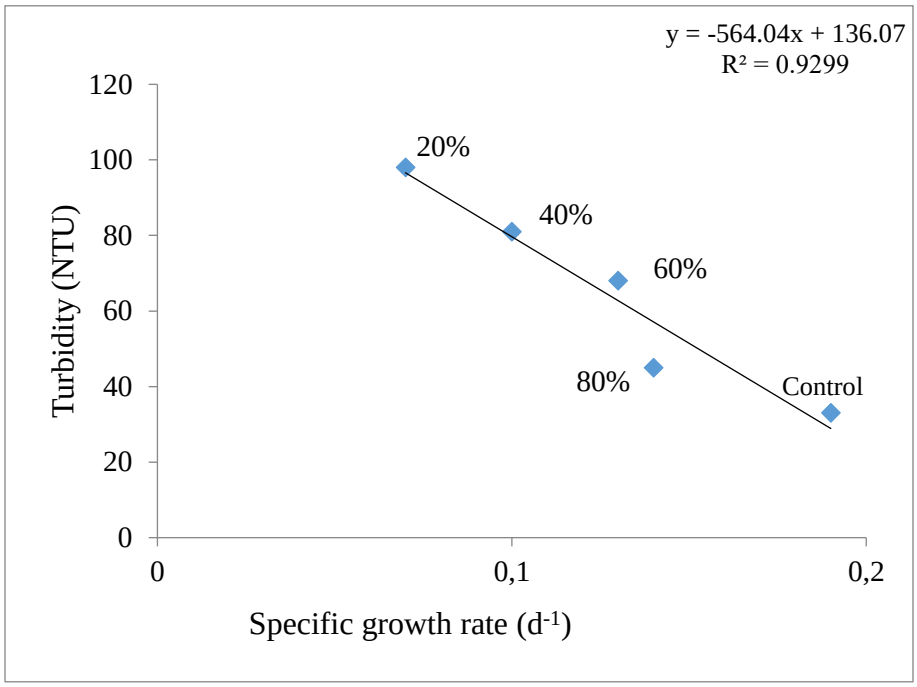


**Fig 2** Relationship between sample turbidity and specific growth rate in the first 9 days under various dilutions of sterilized POME

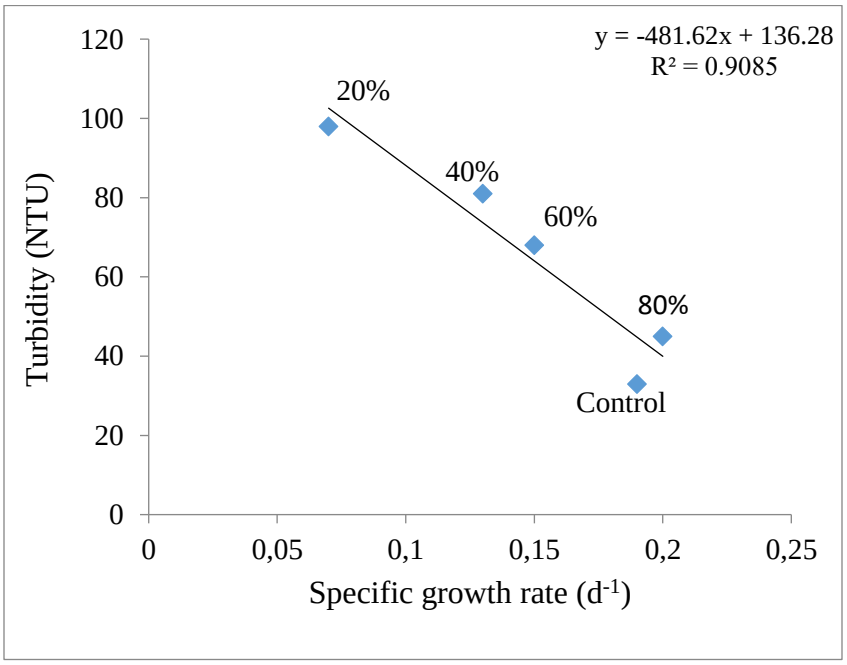


**Fig 3** Relationship between sample turbidity and specific growth rate in the first 9 days under various dilution of unsterilized POME

It was noted that, unsterilized POME support maximum specific growth rate of microalgae than sterilized which could be due to the presence of bacteria that could assist the microalgae growth during aerobic degradation [4]. During the growth of microalgae in unsterilized POME, aerobic bacteria degrade the organic substrate leading to the production of carbon dioxide which will then be utilized by microalgae for photosynthesis. The bacteria utilized the oxygen produce by microalgae as by-product of photosynthesis for their growth [4].

High turbidity in POME is usually caused by the presence of freely suspended material such as fine particles, colour organic compounds and impurities [11]. Also, report indicated that occurrence of certain chemical reaction between tannic acid and other substrates at the point of refluxing (steam sterilization) have been confirmed to be responsible for the darkening of POME. These materials brought darkening of POME which prevent sufficient light penetration thereby creating a shading effect and further affect chlorophylls formation. Since POME was diluted and filtered in the case of sterilized POME, the high turbidity in 20% and 40% was as result of the presence of disintegrating organic matter remains. For unsterilized POME, the turbidity could result from the presence of a microorganism in the medium.

The Dark colour of POME can also cause *C. sorokiniana* to switch its growth mode from autotrophic to the heterotrophic or mixotrophic condition [4]. This mode of growth occurs especially when growing in a medium of minimal dark coloration (such as 40% or 20%). *C. sorokiniana* can utilize organic carbon substrates for both heterotrophic and mixotrophic growth [17]. Mixotrophic growth mode occur when microalgae utilized light and organic carbon as a source of carbon while heterotrophic growth means that, organic carbon is the only source of carbon for the growth of microalgae. A report by [18] reported that microalgae produce a high amount of biomass when growing under mixotrophic condition than other mode of growth.

The quantity of the biomass accumulation under different concentrations of sterilized and unsterilized POME was showed in Table 3. The rate of biomass accumulation in 80, 60, 40 and 20% of sterilized POME was found to be 4.06, 2.66, 2.65 and 2.36 g/L respectively. Furthermore, when compared with control, biomass accumulation of 5.88 g/L was

obtained. While under unsterilized condition the various biomass productions in all the dilutions was found to be 5.94, 5.43, 3.59 and 3.16 g/L respectively with the control experiment providing the highest amount of biomass. Overall the dilutions, 80% stimulate the highest biomass production and therefore can be designated as the optimal concentration of POME dilution for maximum biomass production using *C. sorokiniana*. One of the factors responsible for the variation in the biomass concentration is the nutrient strength since POME was diluted to different percentages. The high amount of biomass production under unsterilized POME was due to the symbiotic activities of algae with bacteria which favoured high biomass production [19]. Low biomass production in low diluted POME could be due to the presence of some toxic substance such as phenols and organic acid [20, 21].

**Table 2** Effect of different POME dilutions on biomass production

| **POME dilutions** | **Biomass production (g/L)** | |
|---|---|---|
| | **Sterilized POME** | **Unsterilized POME** |
| 80 | 4.06±0.16 | 5.94±0.12 |
| 60 | 2.66±0.13 | 5.43±0.16 |
| 40 | 2.65±0.28 | 3.59±0.22 |
| 20 | 2.36±0.21 | 3.16±0.22 |
| Control | 5.88±0.31 | 5.88±0.31 |

The growth of *C. sorokiniana* was poor in 20% POME as indicated by low specific growth rate of $0.07d^{-1}$ (sterilized and unsterilized POME) and low biomass production of 2.36±0.21 (sterilized) and 3.16±0.22 (unsterilized). This could be due to the low photosynthetic activities of algae resulted from limited light penetration. The high turbidity value of 20% dilution presented in Fig 1 and 2 further confirmed the assertion that efficient photosynthetic activities did not take place. Algae growth in high dilution (60 and 80%)

were reported to better because high dilution cause reduction in the concentration of toxic substances and also increases the clarity of the medium given the chance for maximum light penetration as well as efficient photosynthetic activities to occur. This could be the reason why *C. sorokiniana* were able to produce high biomass of 4.06±0.16 (sterilized) and 5.94±0.12 (unsterilized) as indicated by high specific growth of 0.14 (sterilized) and 0.2 (unsterilized) POME. Efficient photosynthesis was observed in control experiment as the medium is not cloudy giving it the ability to induce better algae growth. Moreover, the amount of biomass produces in unsterilized POME was relatively close with biomass produce under control experiment. This could be due the presence bacteria growing in unsterilized which concomitantly increase the biomass concentration. Results of this study suggested that dilution of high strength wastewater could be employed as an adaptive mechanism toward reduce wastewater pollutant and allowing better light penetration for the application of algae. This research can contribute in finding an optimal dilution of POME for enhance cultivation of *C. sorokiniana* for the production of value added substances as the darkening characteristics of POME affect algae growth.

## Conclusion

*C. sorokiniana* was successfully cultivated in different dilutions of sterilized and unsterilized POME and 80% (v/v) was reported to produce higher specific growth rate and biomass production. Dilutions strategy was observed to support the growth of algae in POME. Moreover, advance pre-treatment techniques can be incorporated in order to reduce the effect of other inhibitory substances in POME.

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**Research article**

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# Cadmium stress in barley seedlings: Accumulation, growth, anatomy and physiology

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**ABSTRACT**

Heavy metal stress has marked effects on some growth parameters, physiology, anatomy, and genetics of plants. Among heavy metals, cadmium (Cd) is an extremely toxic one and effects living organisms at even low concentrations. The presence of Cd in air, water and soil and its accumulation in plants create significant negations such as cancer, renal failure, cardiovascular and musculoskeletal diseases in humans when taken from direct and indirect ways. The defense mechanism of the plants which is responsible from stress tolerance can be investigated to improve crop yield under Cd stress. Numerous studies have shown negative effects in plants exposed to Cd. Therefore, in this study, 0 (for control), 50, 100, 200 and 400 µM (for experimental groups) $CdCl_2$ were applied to barley (*Hordeum vulgare* L.) plants and some growth, development, physiological and anatomical parameters were measured. As a result, it has been observed that barley plants can manage stress in terms of some parameters under low Cd stress conditions, however, they are negatively affected at all Cd concentrations to a certain extent. In addition, it was observed that barley plants were adversely affected by high levels of Cd stress, although they maintained their vitality throughout the experiment.

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## Introduction

Cadmium (Cd) is a heavy metal with an atomic number 48 and atomic weight 112.41 g/mol, has the density value of 7.86 g/cm$^3$, boiling point of 767°C and melting point of 321°C. It is a soft, workable and silver color metal which cannot be found alone in nature [1-3].

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Cd is released into the environment in various amounts, both from anthropogenic and natural sources such as volcanic eruptions, parent rocks weathering, wind-blown dust, forest fires and sea sprays [4-7]. Highest contribution to environmental Cd pollution arise form anthropogenic sources such as combustion of fossil fuel, use of phosphate fertilizers, mining, smelting and cement industries, metal ore processing, metallurgical works, sewage sludge and municipal wastes and reach almost 90% of total Cd emissions [8, 9]. In addition, it is frequently used in pigments, plastics, alloys, batteries and solar panels to increase corrosion resistance. In addition to all these, Cd can also be found in polyvinyl chloride (PVC) materials, engine oil, fungicides, vulcanized polymers and textile leach solutions [10]. Industrial processes and traffic emissions are the main responsible factors of Cd pollution in urbanized areas and soils [11]. Cd has a wide usage area (67%) in the production of Cd electroplates to be used in nickel-Cd batteries [7, 12]. While phosphate fertilizers are considered to be the major source of Cd pollution in agricultural soils, the use of contaminated manure may also result in Cd contamination/pollution in soils [7, 13].

Among phytotoxic heavy metals, Cd has a considerable importance due to high water solubility of its salts, high mobility, and significant toxic effects even at low concentrations. Features causing mutagenesis and carcinogenesis together with its high accumulation capacity in plants tissues are the main factors of the rigorous toxicity of Cd [3, 14-16]. All these properties mentioned made Cd one of the most dangerous pollutants in agricultural soils, which has a high potential to participate in the food chain of living things [17, 18] and negatively effects human health [19]. Cd is on the priority list of harmful substances by many environmental non-governmental organizations and the United States Environmental Protection Commission [20].

Although plants can regulate their metabolism under relatively higher amounts of Cd without getting adversely affected (compared to animals), excessive amounts of Cd cause negative effects on plants [21]. Above a critical threshold concentration, obvious signs of cadmium toxicity, such as brownish roots, growth retardation, chlorosis, necrosis, and even death were observed in many plant species [22-24]. Accumulation of Cd in plant tissues can intensely affects lots of physiological processes, such as the interference of respiration and photosynthesis [25], transport and absorption of mineral nutrients [26-28]. Also, diminished

water balance [29], and disturbed carbohydrate metabolism [30] are seen after Cd treatments. Cd exposure in plants, especially at high concentrations, may result in a series of toxicity-related deteriorations in metabolism, such as lipid peroxidation, enzyme inhibition, the formation of reactive oxygen species (ROS) altering the gene and protein expression and even cause DNA damage [3, 31-33].

Cd uptake in plants usually takes place from soil solutions through the root system. Therefore, the amount of Cd taken up by plants is directly related to the Cd concentration in the soil solution [34, 35]. Cd, along with many other heavy metals uses ion transporters such as $Ca^{2+}$, $Fe^{2+}$ and $Mn^{2+}$ to enter into the root system from the soil solution [36]. The amount of cadmium that plants take from the soil is affected by the pH range of the soil, salinity, humus content and the types of crops grown. Cd regulatory limit of agricultural soils is reported to be 100 mg $kg^{-1}$ soil [37]. Moreover, primary processes such as transport efficiency of the xylem sap, uptake ability of the plant roots and final relocation within the seeds of the plants have an effect on the Cd uptake and accumulation capacity of the [3, 7].

Plant roots play a key role in absorbing heavy metals as well as water and essential nutrients. Additionally, a small portion of Cd in the soil system is adsorbed by the surface of plant roots. Therefore, the increase in the surface area of plant roots extends the plant's exposure time to toxic heavy metal and results in higher concentrations of Cd accumulation in various plant parts [38-40]. Once Cd enters into the plant roots, then transportation occurs, either through apoplastic or symplastic pathways. However, Cd from different complexes formed with various ligands such as organic acids and phyto-chelators mainly moves into the root vacuoles and/or nuclei [7, 16, 41]. Cd is mainly transported by xylem from the roots to upper plant parts such as shoots and leaves, while plasmalemma and Casparian strips also support xylem for upward metal transport within plant tissues [36, 42, 43]. The transport of Cd from roots to various plant parts is carried out by both passive (transpiration) and active (ion transport channels) mechanisms [41, 44]. However, the amount of translocated Cd depends on both the concentration of Cd in the lower parts of the plant, and the concentration of all other nutrients in the cell sap. The uptake of Cd from the soil by plants causes toxic effects on various physiological and morphological processes, adversely affecting plant growth and development [7].

Plants have developed some natural defense systems, such as using enzymatic and non-enzymatic antioxidants, osmolyte production, and synthesizing chelating agents, to protect their metabolic processes against stress caused by metals. Plant species tolerate heavy metal stress at varying rates depending on the variety of plant and the type of heavy metal [45-48]. Metal-binding ligands play important roles in plant metabolism as taking part in heavy-metal detoxification with naturally occurring ligands such as amino acids, organic acids, peptides and polypeptides [49, 50]. Phytochelatins (PCs) are glutathione-derived peptides [51] and they are responsible in decreasing free metal concentration in plant tissues [52], they support plants defense mechanism [53] and protect plant tissues from heavy metal damage. PCs support cellular detoxification by forming stable complexes with metal ions and reduce the adverse effects of heavy metal stress [54]. Metallothioneins (MTs) presumably function similar to PCS [50]. Chelators such as PCs and MTs, which have cysteine sulfhydryl groups in their structures, allow storage in the vacuole and cell wall by binding to heavy metals and forming stable complexes [3, 55].

Barley (*Hordeum vulgare* L.) is an important annual cereal species belonging to the Poaceae family such as wheat, rice and corn. It is the fourth important annual cereal product of the Poaceae family after wheat, rice and corn. In addition to being consumed as animal feed, barley is consumed by itself or mixed with other grains in the form of porridge, breakfast foods, sattu (roasted barley) and chapattis, and is also used in malt fermentation [56]. According to (USDA, 2020), barley production in Kyrgyzstan were in a period of rapid increase especially after 2014 and in 2020, it was reached 500 hundred metric tons (MT) with a Growth Rate of 13.64 compared to previous years [57]. However, recent studies conducted with plants in Kyrgyzstan showed that Cd concentrations are higher than normal limits in many plants [58-60]. Therefore, in this study, the effects of Cd on growth, development, some anatomical and physiological parameters in barley, which is increasingly important for Kyrgyzstan, were investigated by applying Cd exposures at different concentrations (50, 100, 200 and 400μM).

## Materials and Methods

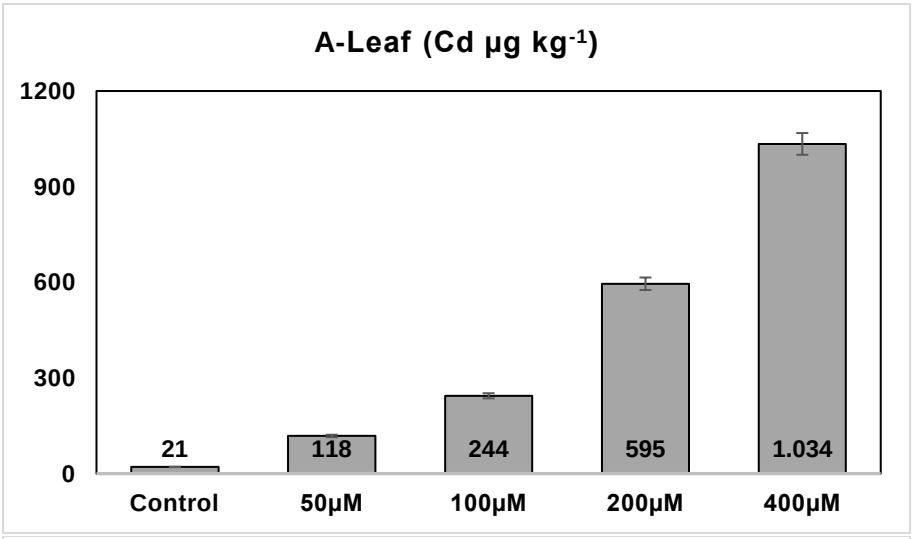
In this study, Alta variety belonging to barley (*Hordeum vulgare* L.) plant were obtained from "Kyrgyzstan State Plant Genetic Resources Center". Seeds of Alta barley variety were washed under tap water for 1 hour, and then germinated for 14 days in standard pots with 100 g of standard soil. During the germination period, the seeds were watered with the full-strength Hoagland nutrient solution [61]. Plant samples were grown under 5000 μmol $m^{-2}$ $s^{-1}$ fluorescent light, in a relative humidity percentage of 45-50% at 24 ± 2°C. During 14 days of germination, 16 hours light and 8 hours darkness/day of photoperiod conditions were applied to each group of 10 replicated seedlings. After the germination period, each of the experimental groups were watered with 20 ml Hoagland's solution containing 0, 50, 100, 200 and 400 μM $CdCl_2$ once per two days for 45 days in above mentioned growth conditions.

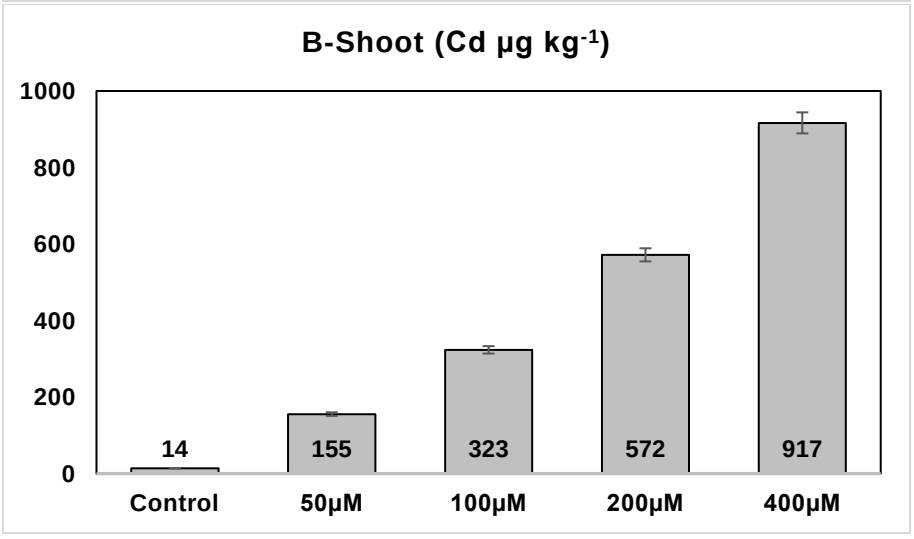
At the end of 45-days of vegetation period, leaves, shoots and roots of the harvested seedlings were oven-dried at 80ºC for 48 h. Plant parts which reached to constant weight were milled in a micro-hammer cutter and passed through a 1.5-mm sieve. For the determination of Cd accumulation level, 0.5 g sample of each plant part was placed in a Teflon vessel and 8 ml of 65% nitric acid ($HNO_3$) was added. Samples were digested at 160°C by using a microwave oven (CEM Mars 5). After cooling, digested samples were filtered through Whatman filter paper, and diluted to 50 ml with ultra-pure water. Cd concentrations were measured by an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES - PerkinElmer Optima 7000dv).

Parameters such as length and width of the lower and upper leaves, shoot length, and total chlorophyll amounts, were measured using calipers, millimetric rulers and chlorophyll content meter (Opti-Sciences Inc., CCM-300). In addition, stomata numbers of abaxial leaf surfaces (for each $1mm^2$ area) of control and experimental groups were counted using an Olympus digital microscope. For this purpose, cross sections were taken from the lower surfaces of the leaves using a sharp razor blade and preparations were examined directly under the microscope.

## Result and Discussion

In this study, the amount of Cd accumulated in the roots, shoots and leaves of barley plants treated with various concentrations (0, 50, 100, 200 and 400 µM) of $CdCl_2$ during the 45-day experiment period was measured by an ICP-OES instrument. In addition, the length and width of the lower and upper leaves, shoot length and total chlorophyll amounts in the lower and upper leaves were measured. As a result of the experiment; cadmium accumulation values (in µg $kg^{-1}$) were 34, 282, 410, 1178 and 1427 in the root, 14, 155, 323, 572 and 917 in the shoot and 21, 118, 224, 595 and 1034 in the leaves of the control (0) and experimental (50, 100, 200 and 400 µM $CdCl_2$ treated) groups, respectively (Figure 1, A-C). Accordingly, the accumulation was in the order of root>leaf>shoot.



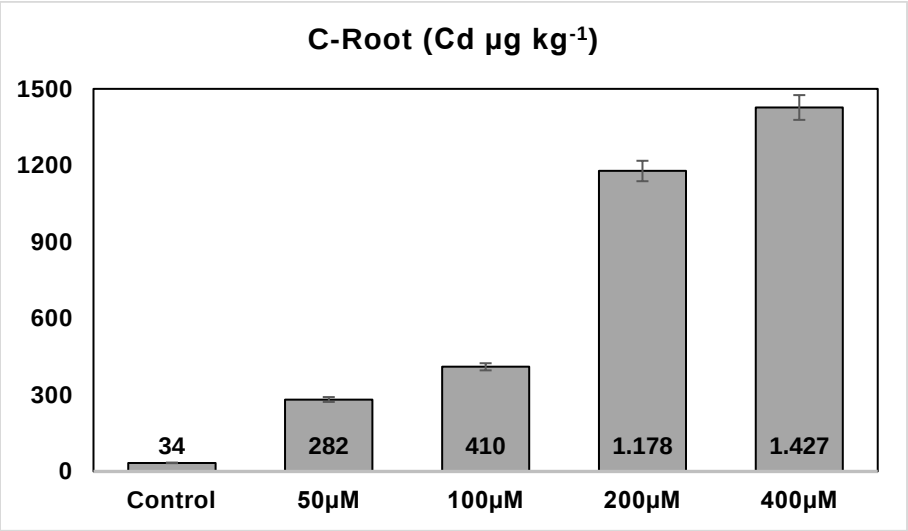



**Fig 1** Cd accumulation values (µg $kg^{-1}$) in **(A)** leaf, **(B)** shoot and **(C)** root of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM).

In a similar study, *Bromus sterilis* plant was exposed to Cd stress at concentrations of 40 and 120 mg $kg^{-1}$, and Cd accumulations (in mg $kg^{-1}$) were determined as 114 and 463 in roots, and 65 and 75 in shoots [62]. The same researchers also observed Cd accumulation levels of 189 and 495 (roots) and 14 and 29 (shoots) after 40 and 160 mg $kg^{-1}$ Cd applications in *Vicia sativa* plant. Furthermore, in the same research, Cd accumulations in *Apium graveolense* plant (after 40 and 120 mg $kg^{-1}$ Cd applications) were obtained between 220 and 300 (root) and 16 to 40 (shoots). In another study, *Paspalum atratum* cv. Reyan plants were exposed to Cd at a concentration of 8 mg $kg^{-1}$ and Cd accumulations were measured as 349 (root) and 46 (shoot) [63]. In a similar study, *Salix caprea* treated with 0.5 mg $L^{-1}$ Cd, and Cd accumulations were 400 in roots and 340 in leaves [64]. On the other hand, in *Cynara cardunculus* plants treated with 2100 mg $kg^{-1}$ Cd, and accumulation ranges were 10-280 (roots) and 10-260 (shoots) [65]. In 2 different studies conducted with *Zea mays*, the applied Cd concentrations were 2-100 mg $kg^{-1}$ [65] and 40-160 mg $kg^{-1}$ [62], and the accumulations were as 10-500 (roots), 5-130 (shoots) [65] and 199-243 (roots), 57-60 (shoots) respectively [62].

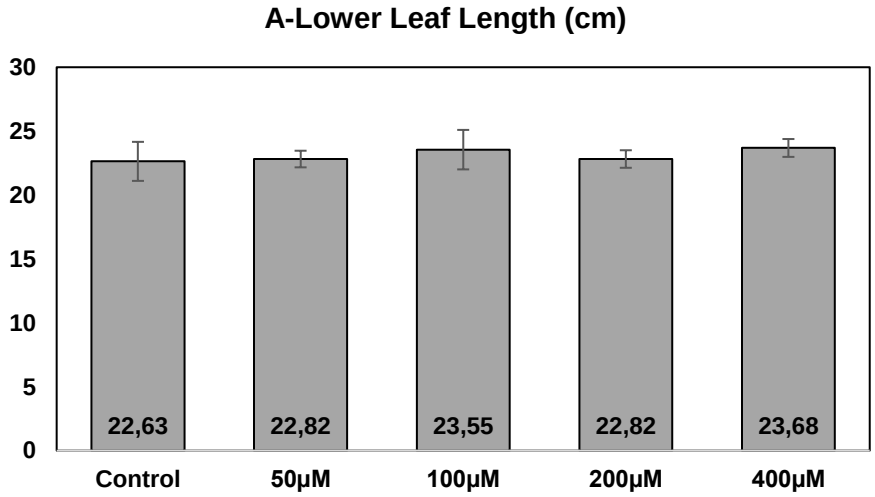
Di Baccio et al. (2014) applied lower (54.3 mg $kg^{-1}$) and higher (163 mg $kg^{-1}$) concentrations of Cd to poplar (*Populus x canadensis*) clones. Accumulation levels at lower Cd

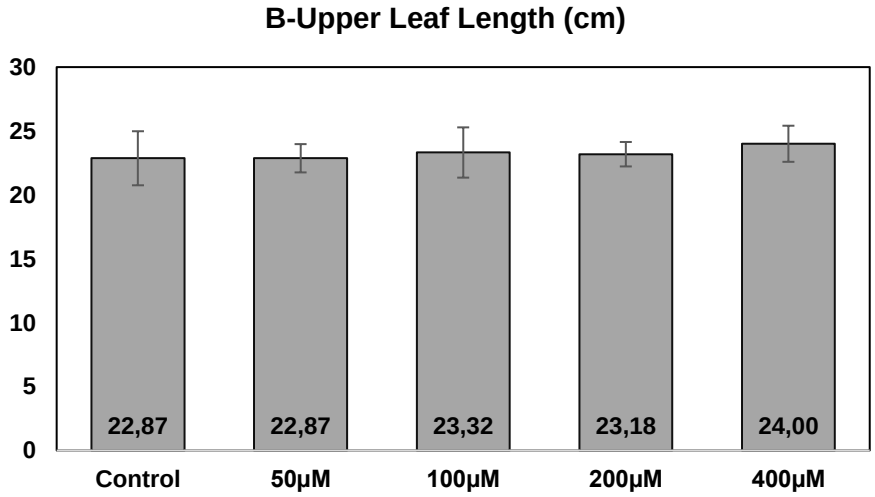
concentrations were determined as 7.6 (leaves), 4.6 (shoots) and 57 (roots), while at higher Cd concentrations accumulation values were measured as 12.7 (leaves), 15 (shoots) and 80 (roots) [66]. In another study, conducted with Kalanchoe plants, Cd accumulations (in mg $ml^{-1}$) increased from 0.629 to 3.164 in leaves, from 0.460 to 2.890 in shoots and from 1.327 to 5.178 in roots after 0-400 μM Cd applications [22]. In another study, the degree of Cd uptake from soil into vegetable species was given in the order of French beans, beetroots, radishes, peas, carrots, broccoli < potatoes, tomatoes, zucchini, and sweet corn < onions, leeks, parsnips, < turnips < cabbage, kale < lettuce, and spinach [18].

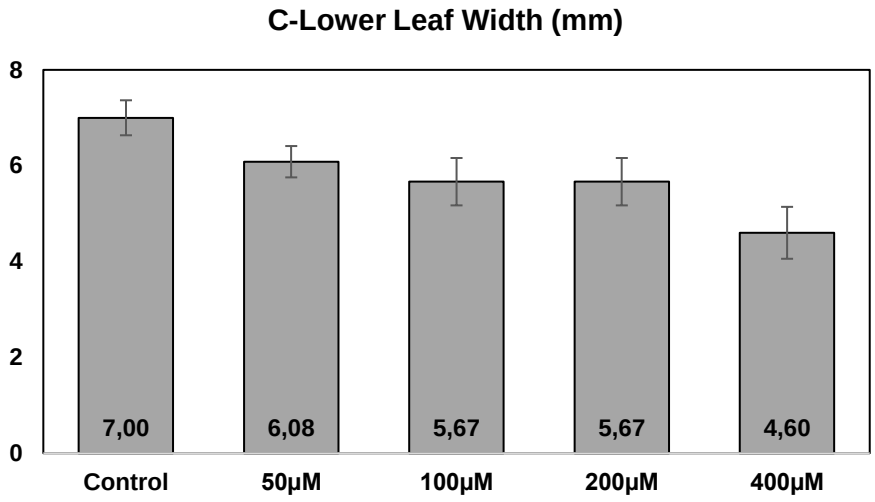
"Soil-related parameters such as pH, temperature, cation exchange capacity and particle size play an important role in the degree of Cd uptake. In addition, physiological parameters of the plants such as total root surface area, transpiration and root exudation rate are also effective in the mobilization of Cd in plant tissues. Cd accumulates predominantly in the roots in most plant species and is transferred to the shoots in very low concentrations [67, 68].

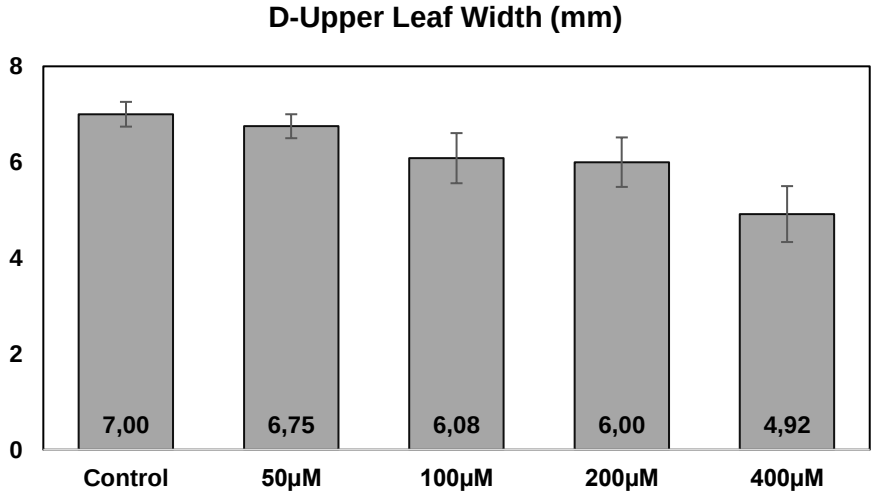
As a result of the above-mentioned Cd applications (0, 50, 100, 200 and 400 μM $CdCl_2$), the lower leaf lengths (in cm) were measured as 22.63, 22.82, 24.42, 22.82 and 23.68, while the upper leaf lengths were 22.87, 22.87, 23.32, 23.18 and 24.00, respectively (Figure 2, A and B). These results indicate that the leaf length slightly increases in barley under the effect of Cd stress. It was observed that the largest increase was 1.05 cm in lower leaves and 1.13 cm in upper leaves in the plants exposed to 400 μM $CdCl_2$. When the widths of the lower and upper leaves were examined, it was observed that the widths of lower leaves (in mm) were 7.00, 6.08, 5.67, 5.67 and 4.60, while the widths of upper leaves were 7.00, 6.75, 6.08, 6.00 and 4.92 in applied Cd concentrations, respectively (Figure 2, C and D). The greatest reduction in leaf width was observed as 2.4 mm in the lower leaves and 2.08 mm in the upper leaves in the application of 400 μM $CdCl_2$. These findings suggest that, although there was not a considerable change in leaf length, leaf width decreased significantly with increasing Cd concentrations. As a result, it can be said that Cd has a reducing effect on the leaf width in the barley plant. In this study, it was determined that the shoot length values (in cm) measured at the end of the experiment periods were 13.57, 12.03, 11.15, 10.75 and 8.65, respectively (Figure 2, E). This finding indicates that the shoot length of the barley plant is

negatively affected by the Cd stress and decreased inversely with increasing concentration. When the total chlorophyll values of the lower and upper leaves were examined, it was seen that the total amounts of chlorophyll in the lower leaves were 6.18, 3.40, 3.35, 3.01 and 2.91, while in the upper leaves were 8.55, 3.84, 3.66, 3.24 and 2.97, respectively (Figure 3, A and B). This result indicates that the total amount of chlorophyll of the barley plant was negatively affected by the Cd element and decreased inversely with increasing Cd concentrations.




C-Lower Leaf Width (mm)
8
6
4
2
0
7,00
6,08
5,67
5,67
4,60
Control
50µM
100µM
200µM
400µM


D-Upper Leaf Width (mm)
8
6
4
2
0
7,00
6,75
6,08
6,00
4,92
Control
50µM
100µM
200µM
400µM

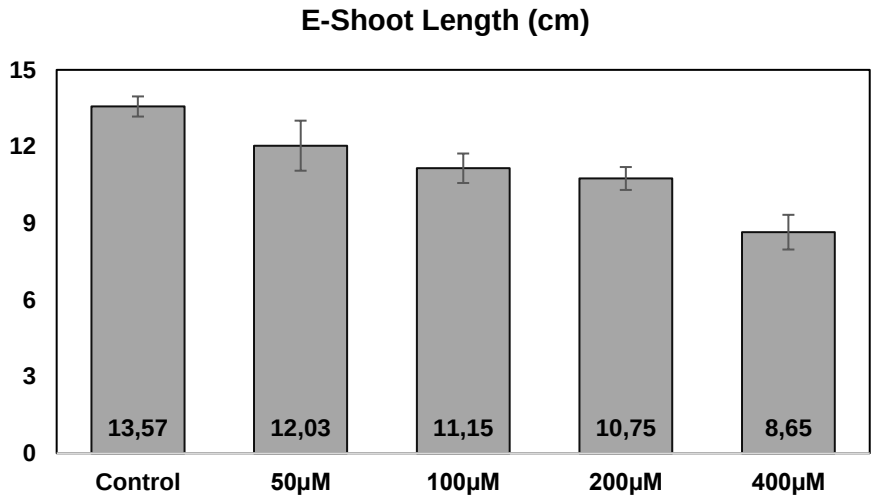
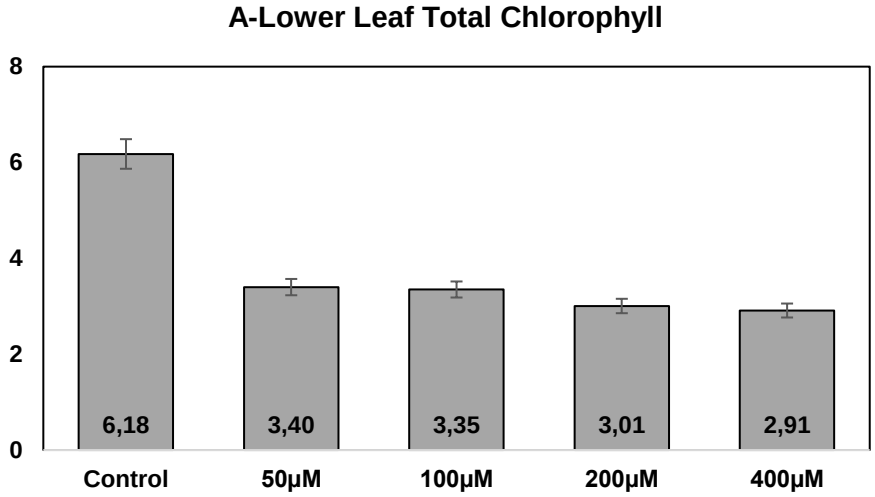



**Fig 2** Some growth parameters of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM); **(A)** lower leaf length, **(B)** upper leaf length, **(C)** lower leaf width, **(D)** upper leaf width, and **(E)** shoot length.



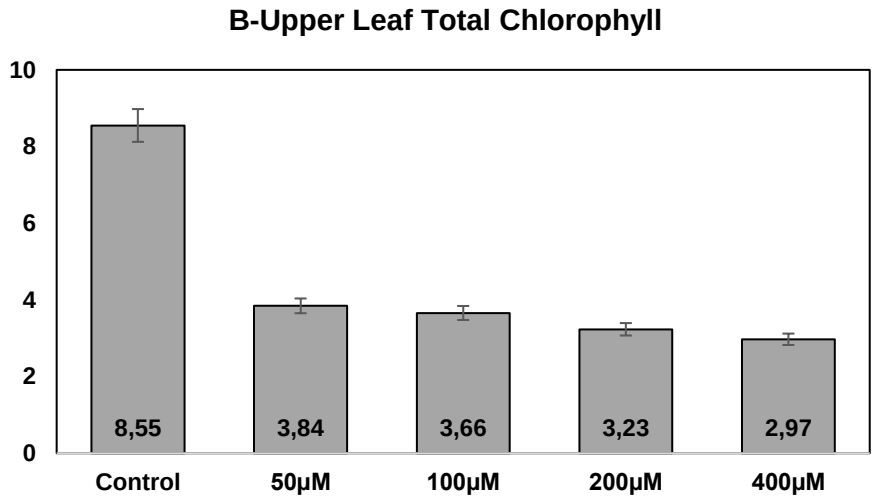



**Fig 3** Total chlorophyll amounts of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM); **(A)** lower leaf and **(B)** upper leaf.

In a similar study, a decrease was observed in fresh and dry weights of roots and shoots as well as biomass, growth rate and chlorophyll content of *Tagetes patula* plant exposed to 10, 25 and 50 µM Cd stress [69]. A gradual decrease in photosynthetic pigmentation, chlorophyll *a*, *b* and total chlorophyll ratios were observed in *Groenlandia densa* (0-20 mg $L^{-1}$ Cd application) [70]. In addition to the decreasing plant growth parameters in *Solanum tuberosum* plant treated with 1, 5, and 25 mg $kg^{-1}$ Cd [71] and *Triticum aestivum* plant treated with 200 mg $kg^{-1}$ Cd, reductions in chlorophyll (*a* and *b*) and photosynthetic pigment contents were also observed [72].

Li et al. (2013) observed a decrease in root and shoot lengths of two kenaf (*Hibiscus cannabinus* L.) cultivars treated with 20-120 µM Cd, as well as a decrease in root and shoot biomass [73]. In *Brassica napus* treated with 10-50 µM Cd, no adverse effects were observed up to 20 µM Cd concentration (compared to the control group), but especially at higher concentrations decreases were observed in plant height, leaf area and leaf number, root length, root diameter, root surface, root tip number and root volume [74]. Ozyigit et al. (2016) observed significant decreases in growth parameters and chlorophyll concentrations of kalanchoe (*Kalanchoe daigremontiana*) plants after 60 days of Cd (0, 50, 100, 200 and 400 µM) application. The reduction rates were ~40.57% for chlorophyll *a*, ~37.63% for

chlorophyll *b*, ~20.58% for chlorophyll *a*/*b*, and ~36.27% for total chlorophyll [22]. From the studies mentioned above, it is clearly seen that different responses (mostly negative) occur in plants depending on the type, concentration and duration of the applied Cd stress.

In another part of our study, transverse sections were taken from the lower surfaces of the leaves (Figure 4, A-E), and the stomata of the abaxial leaf surfaces (per 1mm$^2$ area) of the control and experimental groups were counted. As a result of the 45-day 0 (control), 50, 100, 200 and 400 μM $CdCl_2$ applications, the average number of stomata in 1 mm$^2$ surface area was examined. It was observed that the stoma number of 61.88 in the control was changed into 64.38, 59.25, 58.13 and 57.63 in the experimental groups, respectively (Figure 5).

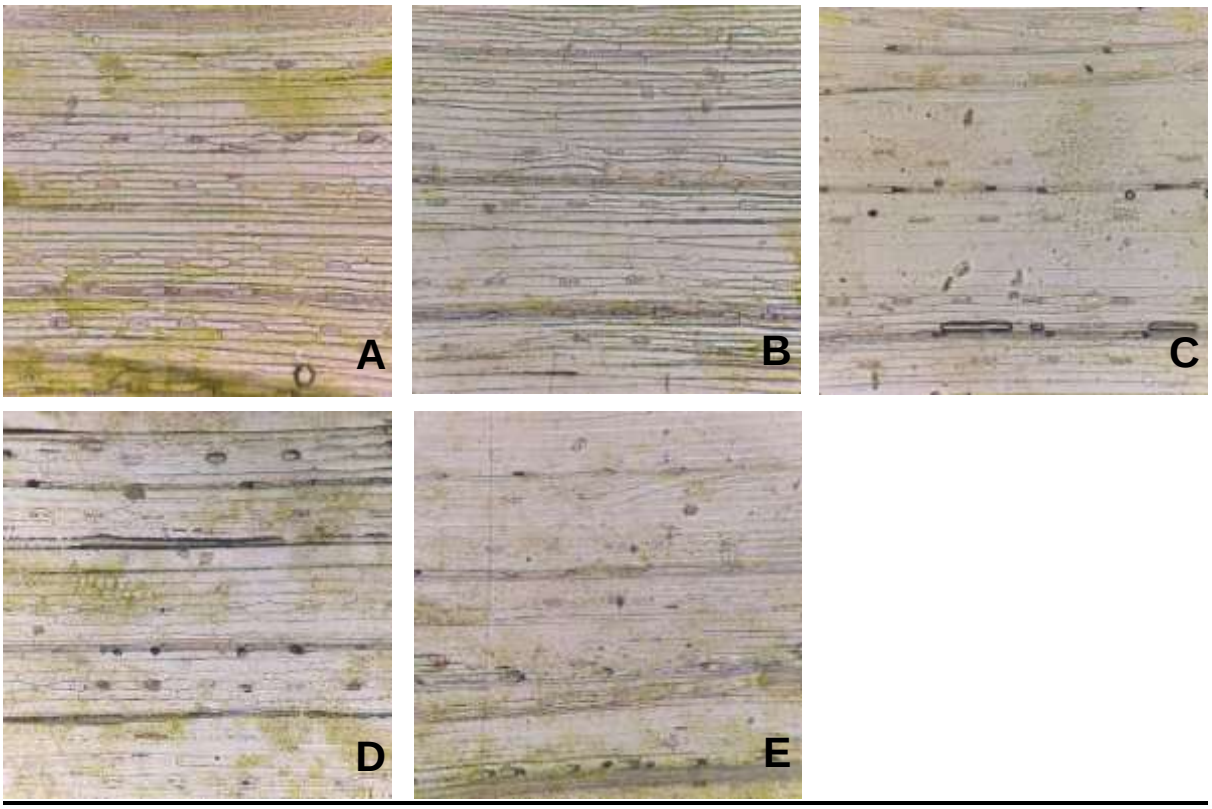



**Fig 4** Anatomical images of abaxial leaf surfaces (1 mm$^2$) of barley treated with four different concentrations of $CdCl_2$ **(A)** 0 control, **(B)** 50, **(C)** 100, **(D)** 200 and **(E)** 400 μM.

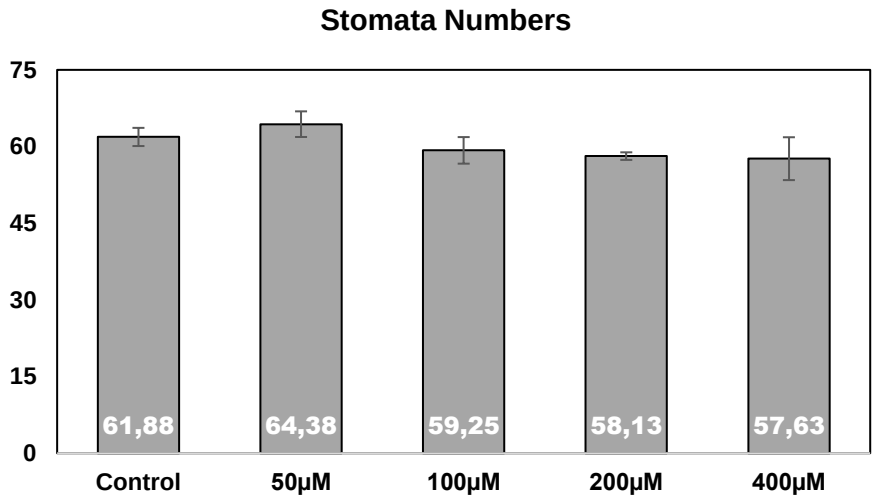



**Fig 5** Stomata numbers of $CdCl_2$ treated barley plants in different concentrations (0, 50, 100, 200 and 400 µM).

The results showed that 50 µM Cd stress caused a small increase in the number of stomata, but at other Cd concentrations, the stomata numbers decreased inversely related to increasing Cd concentration. This result shows that the barley plant increases stomata to manage lower concentrations of Cd stress, but this mechanism does not work at higher Cd concentrations. In a similar study conducted with 0, 1, 5, 25 mg $kg^{-1}$ Cd applied barley plant, stomata numbers on the adaxial and the abaxial leaf surfaces (in 1 $mm^2$ area) were calculated as 59, 53, 59, 60 and 51, 58, 63, 46 respectively [75]. Similar to ours, researchers observed a fluctuating change due to the applied Cd concentrations instead of a sharp decrease. Stomata numbers of the lemon balm plant exposed to 0, 10, 20, 30 mg kg Cd concentrations were determined as 3.5, 2.6, 1.6, and 0.8 for adaxial and 5.8, 4.5, 3.3, and 1.6 for abaxial leaf surfaces, respectively [76]. Stomata number increased from 189.86 to 291 in *Biscutella auriculata* leaf exposed to 0 and 125 µM Cd concentrations [77]. Similarly, in our study, it was observed that 50 µM Cd stress caused an increase in the number of stomata, but this increase occurred at a lower rate. In *Cenchrus ciliaris* leaf, stomata numbers in 0, 30, 60 mg $L^{-1}$ Cd applications were determined as 80.25, 69.12 and 60.23 respectively [78]. Stomata numbers in *Trigonella foenum graecum* Linn. plant exposed to 0, 5, 15, 30, 50 µg $g^{-1}$ Cd concentrations were determined as 33.20, 29.21, 27.20, 25.19 and 22.17, respectively [79]. In *Cajanus cajan* plant exposed to 0, 5, 10, 15, 25 and 50 µg $g^{-1}$ Cd concentrations, stomata numbers were

determined as 26.40, 20.64, 19.04, 18.60, 18.40, and 17.20, respectively [80]. When the above studies and the obtained data from our study are evaluated together, it is concluded that the plants show different responses depending on the species, the severity and duration of the Cd stress applied. In our study, while a slight increase was observed in the number of stomata in lower (50 μM) Cd application, there was a slight decrease in applications at other concentrations. However, when compared to some other studies, they have emerged with sharper increases and decreases in stomata numbers. All these findings were the results of different stress responses that vary from plant to plant.

Cadmium is one of the most toxic metals that prevail in agricultural soils, and negatively affects plant growth. Different sources increase the Cd concentrations in the soil day by day, and this causes water and nutritional imbalance in agricultural soils. On the one hand, the stress caused by Cd pollution causes contamination in agricultural products, and adversely affects the growth and development processes of plants by affecting the physiological and molecular mechanisms resulted with plant productivity decrease. Today, the most suitable option can be applied is to obtain new tolerant/resistant agricultural varieties using classical or biotechnological methods that can grow in contaminated soils, and to manage the nutritional imbalance in the soil in order to maintain the quantity and quality of the crops grown. According to the findings of our study and previous studies, plants (except some hyperaccumulator species) are negatively affected by Cd. Although field crops manage low concentrations of Cd, they are adversely affected by Cd above a certain concentration. Future studies on this subject should be aimed at producing safe products in contaminated soils. It is obvious that applying a single method will not be sufficient enough to increase the product yield and production quality in Cd contaminated soil. A diverse and integrated approach will be developing strategies to reduce Cd uptake and migration into the food chain by agricultural crops. Use of combined approaches such as biotechnological studies, soil amendments, plant rotation and metal immobilization, can help with sustainable agriculture and food security in metal contaminated areas worldwide.

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# *Alkanna strigosa* Boiss. & Hohen. (Boraginaceae) Kökünden Doğal Boya Eldesi

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**ÖZET**

Bu çalışmada kullanılan Havacıva otu doğal bitkilerden biri olup, *Alkanna* cinsine ait olup Boraginaceae familyasına bağlıdır. *Alkanna strigosa* Boiss. & Hohen.bitkisi bölgede Havaco veya Havaciva otu olarak bilinmektedir. Bu çalışmada, 2017 yılında Türkiye için yeni kayıt olarak belirlenen *Alkanna strigosa* bitkisinin kökünden elde edilen doğal boyalar verilmiştir. Kurutulmuş ve yaş Havaciva otu kullanılarak yapılan araştırmada toplam 9 adet boyama yapılmıştır. Mordansız yaptığımız boyama işleminde füme rengi elde edilmiştir. Mordanlama yöntemi ile kahverengi, açık kahverengi, koyu kahverengi, yeşil, açık yeşil, sütlü kahve ve açık toprak renkleri elde edilmiştir. Bu çalışma ile doğal boyamacılığın bölgede tekrar canlanması ve gelecek kuşaklara aktarılması amaçlanmıştır. Bu araştırma, bölgede ilk olması açısından önem arz etmektedir.

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**ANAHTAR KELİMELER**
Bitkisel boyacılık,
Havacıva otu,
Şanlıurfa

# Natural Dye From Root of *Alkanna strigosa* Boiss. & Hohen. (Boraginaceae)

**ABSTRACT**

Havacıva grass used in this study is one of the natural plants and belongs to the genus *Alkanna* and belongs to the family of Boraginaceae. *Alkanna strigosa* Boiss. & Hohen. plant is known as Havaco or Havaciva grass in the region. In this study, natural dyes derived from the root of *Alkanna strigosa* plant, which was introduced as a new record for Turkey in 2017, is given. As a result of this study, different colors with and without mordant were obtained. A total of 9 dyeings were made in the research using dried and wet Havaciva grass. Smoked color was obtained in the painting process we made without mordant. Brown, light brown, dark brown, green, light green, milky brown and light earth colors were obtained by the method of mordant. With this study, it is aimed to revive the natural dyeing in the region and transfer it to the next generations. This research is important for being the first in the region.

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## Giriş

Bitkisel boyamacılık insanlık tarihi kadar eskidir. Tarihte ilk kez Hindistan ve Mezoptamya'da başladığı bilinmektedir. 1850'li yıllarda Kimyacı William Henry Perkin, sentetik boya maddelerini geliştirdiğinden dolayı bitkisel boyamacılık kültürü yavaşlamıştır [1-2]. Bu sebeple, 19. Yüzyıldan itibaren doğal boyamacılık azalmış ancak sentetik boyarmaddelerin kanserojen etkileri nedeniyle doğal boyamacılık yeniden önem kazanmaya başlamıştır. Özellikle kilim ve halıcılık sektöründe kök boyamacılık yeniden ilgi görmeye devam etmiştir. Bitkisel boyacılık aynı zamanda kozmetik ve gıdaların renklendirilmesinde de kullanılmaktadır [1-4]. Bitkisel boyacılıkta bitkiler genelde yabani olarak yetişmekte, bunların kökü, kabuğu, yaprağı, meyvesi, tohumu ve çiçeği kullanılabilmektedir. Bitkilerin bu kısımları genelde antioksidan ve antimikrobiyal özellikler de taşımaktadırlar [2].

Türkiye florasında çok sayıda bitki boyamacılıkta kullanılmakta, bunlar arasında en yaygın olanları ise Zerdeçal, Mürver, Sütleğen, Zeytin, Aspir, Nar, Kayısı, Ceviz, Siyah havuç, Ihlamur, Gelincik, Şekerciboyası, Soğan, Böğürtlen, Porsuk, Doğu çınarı, Sumak, Ayva ve Safran'dır [1].

Bu çalışmada kullanılan Havacıva otu doğal bitkilerden biri olup, *Alkanna* cinsine aittir. *Alkanna* cinsi Boraginaceae familyasına ait ve dünya genelinde 50 kadar türü bulunmaktadır. Türkiye'de ise 36 tür ve türaltı taksonlarla beraber 42 taksonu mevcuttur. Bu taksonların 37'si ülkemiz için endemik olup, endemizm oranı %87'dir. Cinsin dağılışı genelde Akdeniz ve İran-Turan bölgesidir [5].

Bu araştırmada kullandığımız *Alkanna strigosa* yörede "Havaco" olarak bilinir ve 2017 yılında Türkiye için yeni kayıt bir bitki türüdür. Ülkemiz dışında Suriye'de de yetişmektedir [5]. Çok yıllık, tabanda çok gövdeli, kök morumsu renktedir. Şekil 1'de görüldüğü gibi masmavi çiçekleri ve gösterişli yapısıyla aynı zamanda güzel bir süs bitkisidir. *Alkanna strigosa'*ya çok benzeyen *Alkanna tinctoria* bitkisinin köklerinden elde edilen boya, eczacılık endüstrisinde, gıda boyası endüstrisinde, merhem, dudak boyası ve yağların boyanmasında kullanılır [6-7]. Yörede kökünden elde edilen boya, tereyağı ve zeytin yağı ile karıştırılarak yanık ve yara tedavisinde merhem olarak kullanılır [8].

*Alkanna strig*osa bitkisinin kökünden elde edilen boya ile alakalı direkt bir çalışmaya rastlanmamıştır. Ancak, boya bitkileri ile alakalı literatüre [1-56] arasında yer verilmiştir.

Amacımız, yeni keşfedilen *Alkanna strigosa* (Havaciva otu) bitkisinin kökü ile doğal boyalar elde etmektir. Bu çalışma ile unutulan doğal boyamacılığın bölgede tekrar hatırlanması ve gelecek kuşaklara aktarılması amaçlanmıştır. Çalışma, bölgede ilk olması açısından önem arz etmektedir.

## Materyal ve Yöntem

### Materyal

Çalışma materyali olarak; Şanlıurfa'da doğal olarak yetişen *Alkanna strigosa* (Havaciva otu) bitkisinin kökü, yün, yün iplik, alüminyum şap, demir şapı, bakır şapı, bakır sülfat ve oksalik asit kullanılmıştır. Havaciva otu kökü 2016-2017 yılları arasında Şanlıurfa'nın farklı lokalitelerinden toplanmıştır (Tablo 1, Şekil 1). Bitkinin kökleri çekiçle, kök kabukları havanda iyice ezilerek toz haline getirilmiştir (Şekil 2 ve Şekil 3).

**Tablo 1** Çalışma materyali olan *Alkanna strigosa*'nın toplandığı lokaliteler

| **Araştırma materyali** | **Lokaliteler** | **Toplayıcı no** |
|---|---|---|
| ***Alkanna strigosa*** | C7 Şanlıurfa: Yenice, step, 600 m, 19.04.2015 | Balos 4000 |
| | C7 Şanlıurfa: Maşuk, step, 640 m, 16.05.2015 | Balos 4001 |
| | C7 Şanlıurfa:Karaköprü, step, 720 m, 19.05. 2016 | Balos 4002 |
| | C7 Şanlıurfa: Germuş, step, 600 m, 20.05 2016 | Balos 4003 |
| | C7 Şanlıurfa: Badıllı, step, 650 m, 15.06.2016 | Balos 4004 |
| | C7 Şanlıurfa: Göbeklitepe, step, 600 m, 16.06.2016 | Balos 4005 |

**Şekil 1** *Alkanna strigosa*'nın genel görüntüsü

**Şekil 2** *Alkanna strigosa*'nın köklerinin genel görünümü

**Şekil 3** Bitki kökünün A) havanda ezilmesi B) havanda toz haline getirilmesi

## Metod

Mordan, boyama işleminde renk sabitleştirici olarak kullanılan maddelere denir. Mordanlar boyanacak malzemeyi boya alacak duruma getirerek önemli rol oynarlar. Havaciva otunun yaş ve kurutulmuş örnekleri kullanılarak beş farklı mordan kullanıldı, mordansız yöntem de kullanılarak, ön ve son mordanlama ile beraber toplam 9 adet boya elde edildi.

**Ekstraktın hazırlanması**

Mordansız boyama için yün iplikler, boyama işlemi öncesi suyla nemlendirilmiştir. Bitkinin kökünden ipliğin ağırlığına göre %100 oranında alınmış, 1'e 50 oranında su içerisine konulmuş ve 1 saat kaynatılmıştır. Kaynama sonunda filtre ile süzülmüştür.

**Mordansız boyama**

Ekstraktın içine nemlendirilmiş iplikler eklenmiş ve 90 $^0$C derece sıcaklıkta 1 saat kaynatılmış, boyanan iplikler soğutulup yıkanmış ve açık havada kurumaya bırakılmıştır. Bu işlem sonunda füme rengi elde edilmiştir (Şekil 4).

**Mordanlı boyama**

Ön mordanlanma için alüminyum şap, demir şapı, bakır şapı, bakır sülfat ve oksalik asit gibi mordanlar ön işlemlerde tek tek kullanılmıştır. Mordanlar %3 oranında, 1'e 50 oranında ılık suda eritilmiş ve nemli yün ipliği mordanlı su içerisine konulmuş, daha sonra 1 saat kaynatılmıştır. Daha sonra boyanmış olan iplikler soğumaya bırakılmış, soğuk su ile durulması sağlanmış ve açık havada kurutulmuştur.

Son mordanlama için önceden hazırlanmış ekstrakt içerisine nemlendirilmiş iplik konularak 1 saat kaynatılmıştır. Daha sonra boyanmış yün iplik 1 saat süre ile % 3'lük alüminyum şapı, bakır sülfat ve demir sülfat mordanlarıyla tek tek işlem görmüştür. Yün iplikleri daha sonra mordanlı su içerisinden çıkarılmış, soğumaya bırakılmış, bol su ile durulanmış ve açık havada kurutulmaya bırakılmıştır.

**Elde edilen renklerin adlandırılması**

Elde edilen renklerin adlandırılması için doğal aydınlatmalı bir ortamda boyalı yün ipliği örnekleri beyaz zemin üzerinde esas alınarak yapılmıştır [47].

**Işık haslığı, sürtünme haslığı ve su damlası haslığı tayini**

Renklerin ışık haslığı tayini [48-49], sürtünme haslığı tayini [48-49] ve su damlası haslığı tayini [50-51] ilgili literatüre uygun verilmiştir.

## Bulgular

Havaciva bitkisinden mordanlama yöntemi ile elde edilen renkler Tablo 2 ve Şekil 4'de verilmiştir.

**Tablo 2** Havaciva bitkisinden mordanlama yöntemi ile elde edilen renkler

| Mordanlama yöntemi | | | Elde edilen renkler |
|---|---|---|---|
| Alüminyum Şapı | Ön mordanlama | 1 saat | Kahverengi |
| | Son mordanlama | 1 saat | Açık kahverengi |
| Bakır Sülfat | Ön mordanlama | 1 saat | Yeşil |
| | Son mordanlama | 1 saat | Açık yeşil |
| Demir Sülfat | Ön mordanlama | 1 saat | Sütlü kahve |
| | Son mordanlama | 1 saat | Açık toprak |
| Oksalik Asit | Ön mordanlama | 1 saat | Koyu kahverengi |
| | Son mordanlama | 1 saat | Açık kahverengi |
| | Mordansız | 1 saat | Füme rengi |

**Şekil 4** Boyamanın aşamaları ve elde edilen renkler

Işık haslığı, sürtünme haslığı ve su damlası haslığı tayinlerine ait elde edilen değerler Tablo 3'te verilmiştir.

**Tablo 3** Sürtünme, ışık ve su damlası haslığı değerleri

| Mordanlama yöntemi | | Süre | Sürtünme haslığı [53-54] | Işık haslığı | Su damlası haslığı |
|---|---|---|---|---|---|
| Alüminyum Şapı | Ön mordanlama | 1 saat | 3 | 4 | 3-4 |
| | Son mordanlama | 1 saat | 3-4 | 4 | 4 |
| Bakır Sülfat | Ön mordanlama | 1saat | 3 | 4 | 3-4 |

| | | | | | |
|---|---|---|---|---|---|
| | Son mordanlama | 1saat | 3-4 | 4 | 4 |
| Demir Sülfat | Ön mordanlama | 1 saat | 3 | 4 | 3-4 |
| | Son mordanlama | 1 saat | 2-3 | 4 | 3-4 |
| Oksalik Asit | Ön mordanlama | 1 saat | 3 | 4 | 4 |
| | Son mordanlama | 1 saat | 3 | 4 | 4 |
| | Mordansız | 1 saat | 3 | 3 | 3 |

## Bulgular ve Tartışma

Araştırma kapsamında yörede doğal olarak yetişen *Alkanna strigosa* bitkisinin yöresel ve bilimsel isimleri, ülkemizdeki yayılış alanları ve habitat durumları, uygulanan boyama yöntemleri ve boyamalar sonucunda elde edilen renklerin değerleri belirlenmiştir. Kurutulmuş ve yaş Havaciva otu kullanılarak yapılan araştırmada toplam 9 adet boyama yapılmıştır. Mordansız yaptığımız boyama işleminde füme rengi elde edilmiştir. Mordanlama yöntemi ile kahverengi, açık kahverengi, koyu kahverengi, yeşil, açık yeşil, sütlü kahve ve açık toprak renkleri elde edilmiştir.

Çalışmamıza en yakın yapılan araştırma Kayabaşı ve ark. [52] tarafından *Alkanna tinctoria* bitkisinden elde edilen renklerdir. Kayabaşı ve ark. [52] tarafından mordanlama yöntemi ile sonucu elde edilen bulguları Tablo 4 karşılaştırılmalı olarak verilmiştir. Tablo 4 incelendiğinde her iki çalışmada da benzer sonuçlara varıldığı görülmüştür. Kayabaşı ve ark. [52] tarafından kullanılmayan Oksalik asit mordanı bu çalışmada kullanılmış olup açık ve koyu kahve renkleri elde edilmiştir.

Şanlıurfa'da geçmişte insanların çoğunun doğal bitki boyamalarını kullandıkları ve çeşitli renkte boyalar elde ettikleri bilinmektedir. Bunun için şap, demir sülfat, bakır sülfat, kireç, tuz, kül ve limon tuzu gibi maddeler ile yün ipleri boyamışlardı. Sayılan bu maddeler boya maddeleri ve yün lifleri arasında bir köprü görevi görür [50-51]. Ayrıca, renk tonlarını değiştirirler. Günümüzde yok olma tehlikesi ile karşı karşıya kalan bu yöntemlerin tekrar hatırlanması amacıyla belediyeler ve üniversiteler tarafından projelendirilmelidir. Bir tarım kenti olan Şanlıurfa'da boya bitkileri yetiştiriciliği yapılarak yeni iş sektörleri oluşturularak ülke ekonomisine katkı sağlanabilir. Ülkemizin pamuk üretimin yarısından fazlasını üreten bu kentte iplik ve kumaş fabrikalarının olmasının yanında açılacak doğal boya fabrikaları ile tekstil sektörü hız kazanmalıdır.

Yapılan araştırmalar sonucu antimikrobiyal özelliğe sahip olduğu bilinen *Alkanna strigosa* bitkisinin [55] tarımının arttırılması ve halk arasında bilinçli kullanımının yaygınlaştırılması sağlanmalıdır.

Ülkemizden bilim dünyasına yeni tanılan *Alkanna strigosa* bitkisinin köklerinden elde edilen doğal boya eldesi ilk kez bu çalışma ile ortaya konmuştur.

**Tablo 4** Daha önce yapılmış çalışmalarla karşılaştırma

| Mordanlama Yöntemi | | | Elde edilen renkler (Bizim çalışmamız) | Kayabaşı ve ark. [52] tarafından elde edilen renkler |
|---|---|---|---|---|
| Alüminyum Şapı | Ön mordanlama | 1 saat | Kahverengi | Açık toprak |
| | Son mordanlama | 1 saat | Açık kahverengi | Bej |
| Bakır Sülfat | Ön mordanlama | 1 saat | Yeşil | Kuru meşe yaprağı |
| | Son mordanlama | 1 saat | Açık yeşil | Açık nil yeşili |
| Demir Sülfat | Ön mordanlama | 1 saat | Sütlü kahve | Sütlü kahve |
| | Son mordanlama | 1 saat | Açık toprak | Açık kahve |
| Oksalik Asit | Ön mordanlama | 1 saat | Koyu kahverengi | - |
| | Son mordanlama | 1 saat | Açık kahverengi | - |
| | Mordansız | 1 saat | Füme rengi | - |

Gelecekte bu bitkinin kültüre alınması, etnobotanik özelliklerinin detaylı olarak ortaya konulması ve sanayide kullanılması önerilmektedir. Nitekim daha önce başka *Berberis* cinsi ile alakalı yapılan benzer çalışmalarda [56] da ekonomiye kazandırılmasının yararlı olacağı belirtilmektedir.

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**Research article**

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# The effects of cadmium on growth, some anatomical and physiological parameters of wheat (*Triticum aestiv*um L.)

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**ABSTRACT**

Nowadays, increased population and traffic density, together with the development of industry, caused increasing levels of heavy metals releasing to the environment, and environmental pollution has reached its highest level worldwide. Chemical products, fertilizers, industrial dyes, construction materials, silver dental fillings and vaccines are some of the well-known sources of heavy metals exposed the environment. Toxic heavy metals can normally be present in body parts of living things at very low levels, but at higher concentrations they can show toxic effects depending on species and duration. Among heavy metals, cadmium is one of the most harmful ones to the environment, humans, animals and plants, and can be toxic even at low concentrations. Thus in this study, Cd was applied to the wheat (*Triticum aestivum* L.) plants grown in Kyrgyzstan in different concentrations (0, 50, 100, 200 and 400 µM for experimental groups) and in addition to accumulations in different plant parts, some growth, development, physiological and anatomic parameters were measured. As a result, it was observed that wheat plants were affected by all Cd concentrations, although they were able to manage lower stress in terms of some parameters. It was also seen that plants were negatively affected by higher levels of Cd stress, although remained alive throughout the experimental period.

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*T. aestivum*

## Introduction

Metals, with a relatively higher atomic mass and specific gravity greater than 5 g/cm$^3$ are called “heavy metals”. More than sixty elements are considered as heavy metals and these metals are located in a part of the periodic table called “transition elements” [1-3]. Heavy metals are important environmental pollutants, many of which can be toxic even at a very low concentrations [4].

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In parallel with the recent increasing world population growth, industrial developments and heavy traffic in cities, heavy metal release into the biosphere caused pollution, and this problem is dramatically increasing day by day [5, 6]. The main factors causing the spread of heavy metals to the environment could be by natural and/or anthropogenic sources such as volcanic activities, industrial activities, motor vehicle exhausts, paints, mineral deposits and mining sites, fertilizers and chemicals used in agriculture [2, 7, 8].

Although mining of heavy metals and their usage in different industrial processes are caused a problem called "heavy metal pollution", they are important raw materials in industry and required for socio-economic development. Nevertheless, they are toxic and therefore a potential threat to human health and ecosystems [9-11]. They are major threats to living organisms, as they tend to be bioaccumulated and do not degrade easily in nature, and as mentioned above, some of them can be toxic even at low concentrations [4, 12].

Uptake of metal ions by plants involves; (1) attachment of metal ions to the root surface, (2) uptake into the roots and (3) translocation to the body through mass flow and diffusion. Uptake of substances from the soil is provided by metal chelating molecules secreted from the roots into the rhizosphere, metal reductase enzyme and proton release [13]. The heavy metals uptaken from soil by the root systems of plants are then transported to the aboveground parts by passing into xylem with the effect of transpiration power [14, 15]. The metals reaching up to the leaf are distributed in leaf cells via apoplastic and symplastic ways, due to the binding of the metals to the chelators such as phytochelatins (PC) and metallothioneins (MT) [16]. These chelators contain large numbers of cysteine sulfhydryl groups, and after binding to the heavy metals, they can form them stable complexes and help for the storage in the vacuole and cell wall [2, 16]. Nevertheless, the success of these mechanisms varies from one plant species to another. While the plants classified as "hyperaccumulator" are more successful in this regard as taking certain heavy metals and accumulate them in their bodies without showing any symptoms, some plant species, which are not successful in these mechanisms are negatively affected from heavy metals biologically, and toxicity symptoms appear in their bodies [17, 18].

In plants exposed to high concentrations of heavy metals, many physiological events such as germination, growth and development, protein synthesis, enzyme activity, transpiration,

stomatal movements, water uptake, photosynthesis, membrane stability are negatively affected [19-21].

Cd, which in the group 2B of the periodic table, is one of the most dangerous heavy metal pollutants in the ecosystem, and a highly toxic element for living things [9, 22]. The importance of Cd as an environmental pollutant has become more evident, especially in recent years. Cd can be found naturally in the world, as well as it can spread to the environment as a result of human activities such as phosphorus fertilizers, sewage wastes and atmospheric deposits [23, 24]. These factors increase the importance of Cd as a pollutant and nowadays, plants, humans and animals, especially living in urban and/or industrial areas are constantly exposed to Cd and are being affected adversely even its small concentrations [2, 4].

The plants exposed to Cd, especially in higher concentrations, show toxicity-related symptoms such as generation of reactive oxygen species (ROS) resulted lipid peroxidation, enzyme inactivation and DNA damage [25, 26], reduced photosynthesis [27], inhibited respiration and gas exchange [28, 29] and cell proliferation [30], diminished water balance [31], and disturbed carbohydrate [32] and nutrient uptake metabolisms [33] resulting in visible symptoms such as chlorosis, necrosis, root blackening, stunting and general reductions in biomass production [34-36]. Due to the above effects, Cd causes a decrease in yield and quality, especially in field crops.

Poaceae family member wheat *Triticum aestivum* L. is one of the most important cereal crops in the world as well as in Kyrgyzstan. Wheat and wheat-derived products provide high amounts of energy due to their high carbohydrate content. They also contain substantial levels of protein, lipid, B1 and niacin and therefore together with rice, it is the primer food source for the world [37]. Recent studies conducted with plants in Kyrgyzstan showed that Cd concentrations are higher than normal limits in many plants [38-40]. For this reason, in this study, the effects of Cd on growth, development, some anatomic and physiological parameters were investigated in wheat plants grown under different levels (50, 100, 200 and 400 μM) of Cd exposures.

## Materials and Methods

### Plant Material

Wheat is a plant belonging to the genus Triticum from the Poaceae family. It is a self-pollinating, one-year monocot plant, with fringed roots and caryopsis type fruit [41]. Wheat can be grouped in three categories, according to the number of chromosomes as; 14 (diploid), 28 (tetraploid) and 42 (hexaploid). The basic set of chromosomes is 7, and the above designations signify 2×7 (2n), 4×7 (4n), and 6×7 (6n) respectively [42]. In this study, Intensivnaya variety belonging to wheat (T. aestivum L.) plant, which has been widely cultivated in Kyrgyzstan was obtained from "Kyrgyzstan State Plant Genetic Resources Center" and used as experiment material.

### Plant Sampling and Germination

The seeds of wheat cultivar Intensivnaya were washed with tap water for 1 hours and then germinated in standard pots containing 100 g soils in growth conditions for 14 days and watered with full strength Hoagland nutrient solution [43]. Plants were grown under 5000 μmol $m^{-2}$ $s^{-1}$ fluorescent lights with photoperiod of 16 hours light and 8 hours dark period, at temperature of 24 ± 2°C and relative humidity 45-50%. After 14 days of germination, each of the experimental groups of 10 replicated seedlings were watered with 20 ml Hoagland's solution containing 0, 50, 100, 200 and 400 μM $CdCl_2$ at two-day intervals for 45 days.

### Measurements

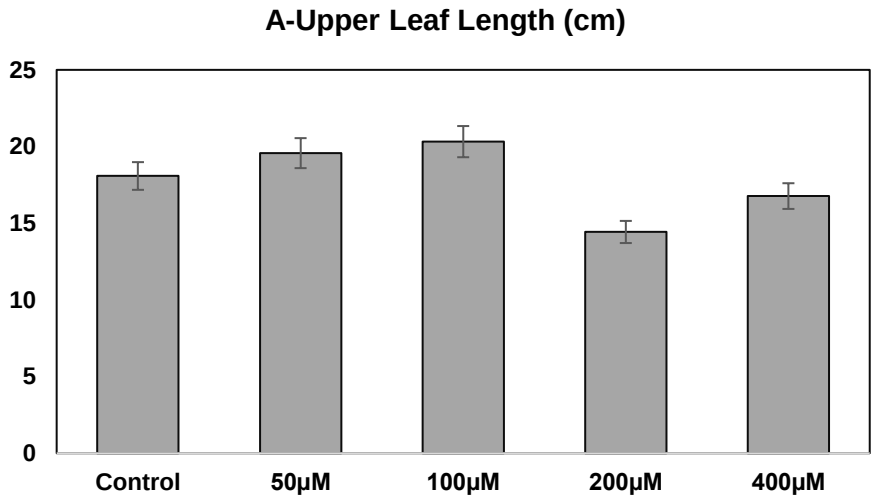
Seedlings were harvested at the end of 45 days experiment period. Parameters such as length and width of the upper and lower leaves, shoot length, and total chlorophyll amounts, were measured using calipers, millimetric rulers and chlorophyll content meter (Opti-Sciences Inc. CCM-300, USA). In addition, stomata and epidermal hair numbers of abaxial leaf surfaces (for each $1mm^2$ area) of control and experimental groups were counted using a digital microscope (Olympus, DSX510). For this purpose, cross sections were taken from the lower surfaces of the leaves using a sharp razor blade and preparations were examined directly under the microscope.

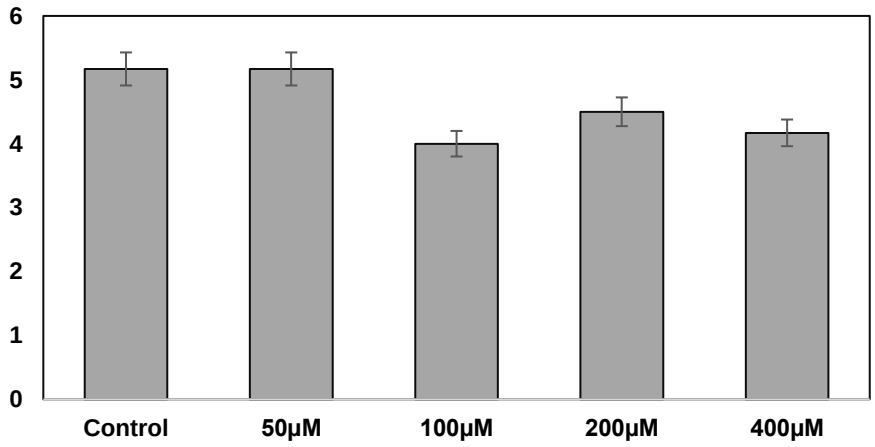
For measurement of Cd accumulation, leaves, shoots and roots were separated and oven-dried for 48 h at 80°C, milled in a micro-hammer cutter and fed through a 1.5-mm sieve. 0.5

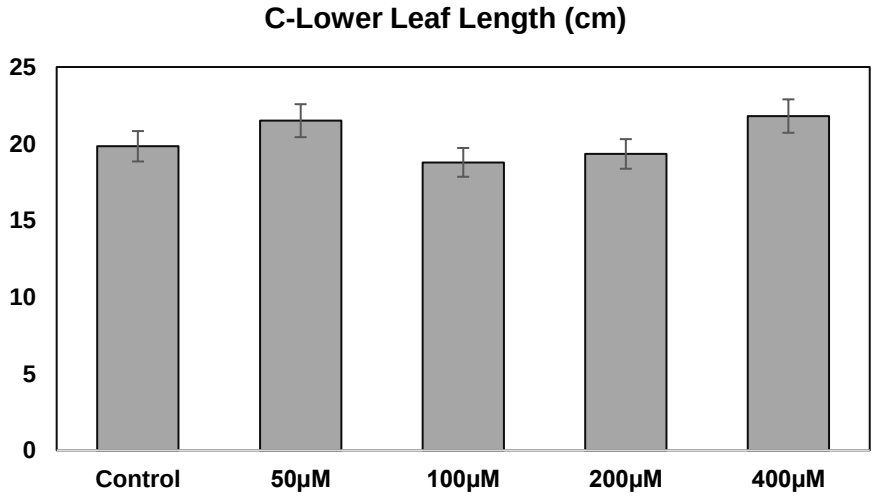
g of plant samples were placed in Teflon vessels and then 8 ml of 65% nitric acid ($HNO_3$) was added. Samples were mineralized in a microwave oven. After cooling, the samples were filtered with Whatman filters, and diluted to 50 ml with ultra-pure water. Cd concentrations were measured by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; PerkinElmer Optima7000DV).

## Result and Discussion

In this study, leaf length, leaf width, shoot length and total chlorophyll amounts (for upper and lower leaves) were analyzed in wheat seedlings in response to different $CdCl_2$ concentrations (0, 50, 100, 200 and 400 μM). According to our results, it was observed that length of upper leaves (in cm) increased up to (19.57 and 20.32) at concentrations of 50 and 100 μM Cd, and decreased down to (14.43 and 16.77) at 200 and 400 μM compared to the control (18.08) (Figure 1-A). Upper leaf width remained the same (5.17) with control group in 50 μM Cd application, whereas decreased (4.00, 4.50 and 4.17) at 100, 200 and 400 μM compared to the control (Figure 1-B). Lower leaf length increased up to (21.50 and 21.80) at 50 and 400 μM Cd applications, and decreased down to (18.78 and 19.33) at 100 and 200 μM compared to the control (19.83) (Figure 1-C). In addition, it was observed that the lower leaf width increased (4.83) at 50 μM Cd application and decreased (3.83, 3.78 and 3.67) at 100, 200 and 400 μM compared to the control (4.33) (Figure 1-D). In this case, it can be said that Cd stress above a certain degree has a reducing effect on the leaf width in wheat. Although there were some fluctuations, it was observed that shoot length was decreased down to (16.57, 12.20, 13.02 and8.52) in all applied Cd concentrations compared to the control (18.95) (Figure 1-E).


A-Upper Leaf Length (cm)
25
20
15
10
5
0
Control
50µM
100µM
200µM
400µM


B-Upper Leaf Width (cm)
6
5
4
3
2
1
0
Control
50µM
100µM
200µM
400µM


C-Lower Leaf Length (cm)
25
20
15
10
5
0
Control
50µM
100µM
200µM
400µM

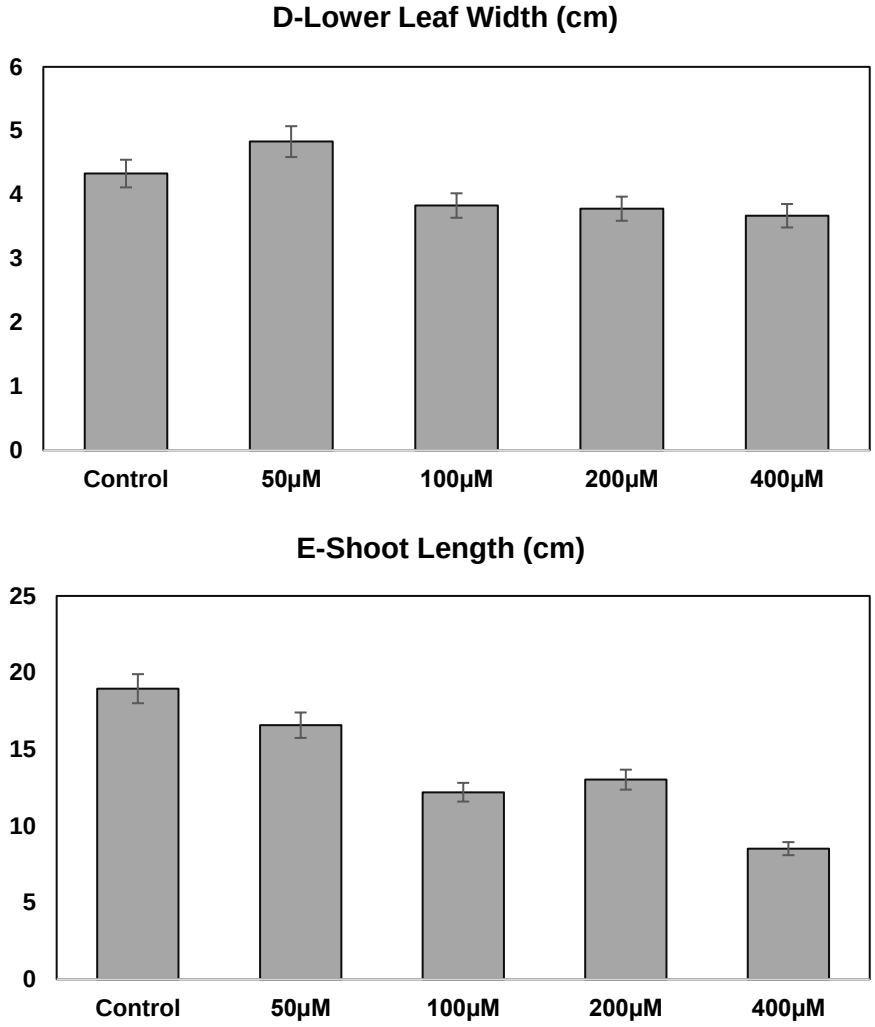



**Fig 1** Some growth parameters of $CdCl_2$ treated wheat plants in different concentrations (0, 50, 100, 200 and 400 µM). **(A)** Upper leaf length and **(B)** width, **(C)** Lower leaf length and **(D)** width, and **(E)** Shoot length.

The total chlorophyll contents in the lower and upper leaves were measured using a chlorophyll content meter (CCM-300) (Figures 2-A and B). Accordingly, it was observed that total chlorophyll values (in mg $m^{-2}$) were increased in both upper (2.09, 2.04 and 1.99) and lower (2.11, 2.58 and 1.82) leaves at 50, 100 and 200 µM Cd applications compared to the control (1.75 for upper and 1.70 for lower leaves), but this value was lower (1.67 for upper and 1.63 for lower leaves) than that of the control at higher Cd concentrations (400 µM).

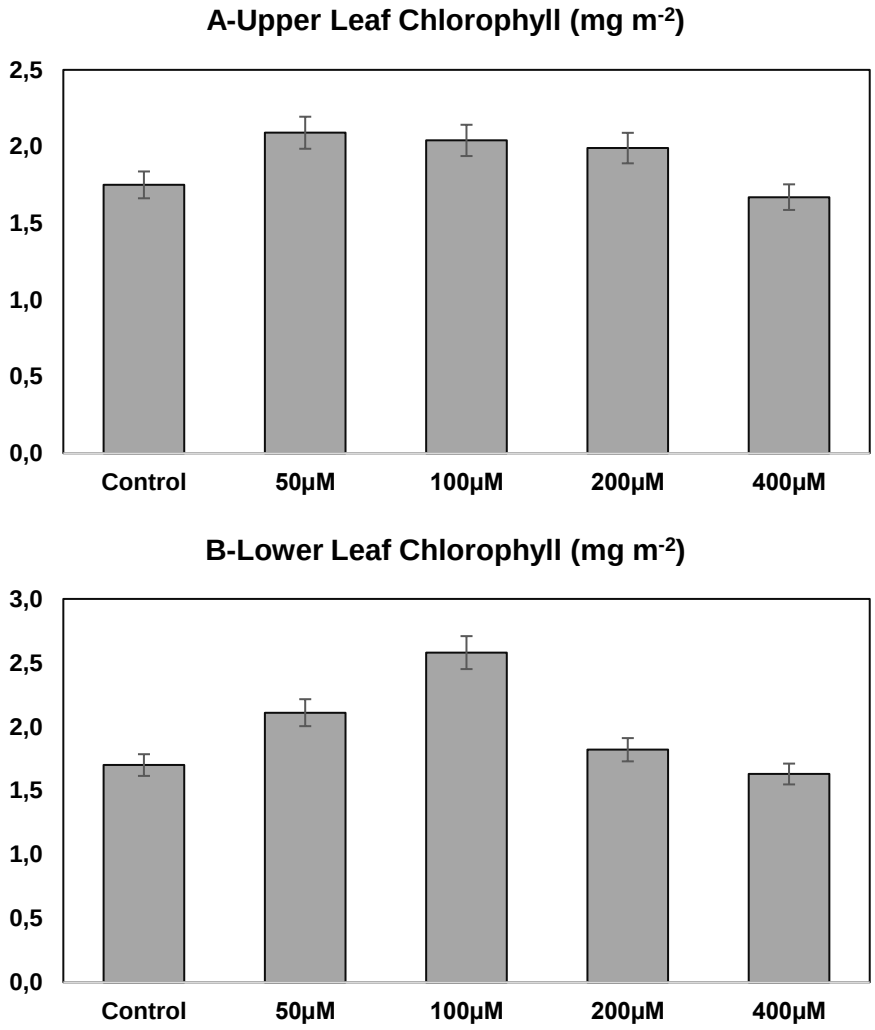



**Fig 2** Total chlorophyll amounts of $CdCl_2$ treated wheat plants in different concentrations (0, 50, 100, 200 and 400 µM). **(A)** Upper leaf and **(B)** Lower leaf.

Cross sections were taken from the lower surfaces of the leaves, and stomata and epidermal hair numbers of abaxial leaf surfaces (for each $1mm^2$ area) of control and experimental groups were counted (Figure 3).

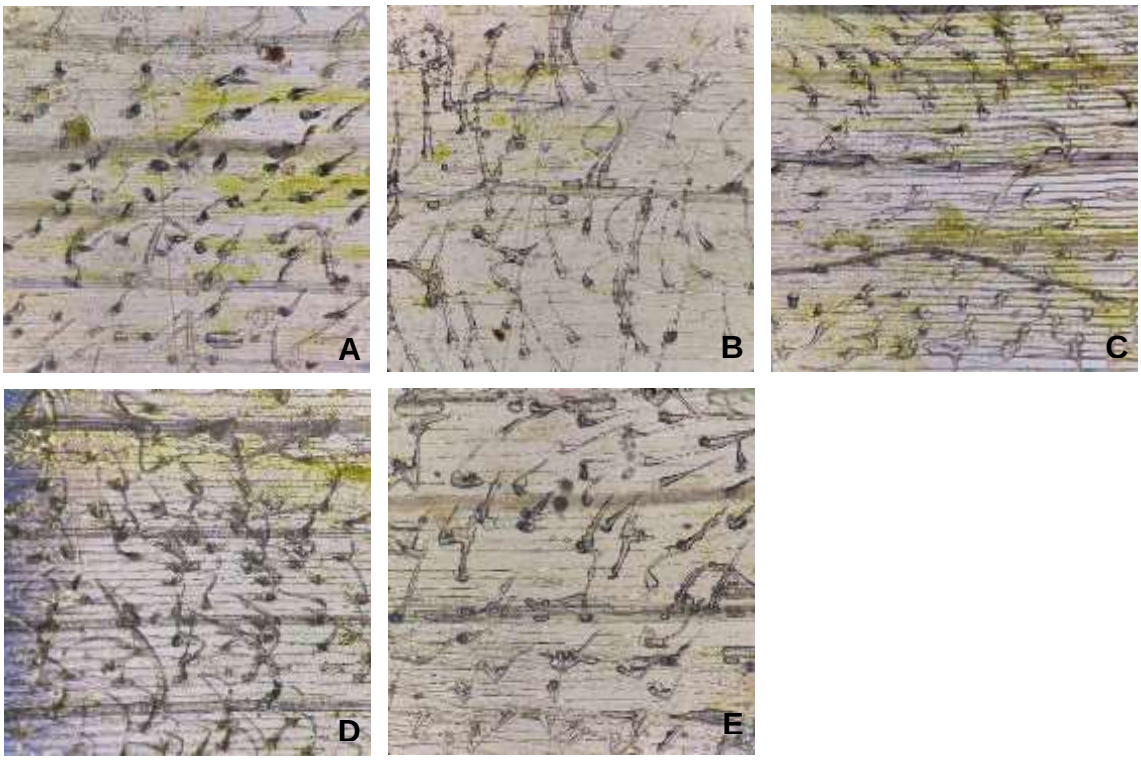



**Fig 3** Anatomical view of abaxial leaf surfaces (1mm$^2$) treated with four different concentrations of $CdCl_2$. **(A)** 0 for control, **(B)** 50, **(C)** 100, **(D)** 200 and **(E)** 400 µM.

It was observed that stomata of wheat plants were negatively affected by Cd stress, and the stomata numbers on 1mm$^2$ surface area was 15.75 in control, and this value decreased to 13.00, 12.63, 7.75 and 3.20 at 50, 100, 200 and 400 µM Cd applications, respectively (Figure 4-A). However, the reduction was the lowest in 50 µM Cd application. Changes in the number of epidermal hairs on a 1 mm$^2$ surface area of leaves under Cd stress showed that, as a response of plants, the number of epidermal hairs were increased parallel to increasing Cd stress as; 83.00 for 50 µM, 103.14 for 100 µM, 99.60 for 200 µM. Conversely, the number of epidermal hairs was quite lower compared to the control (57.29) at 400 µM Cd application (Figure 4-B).

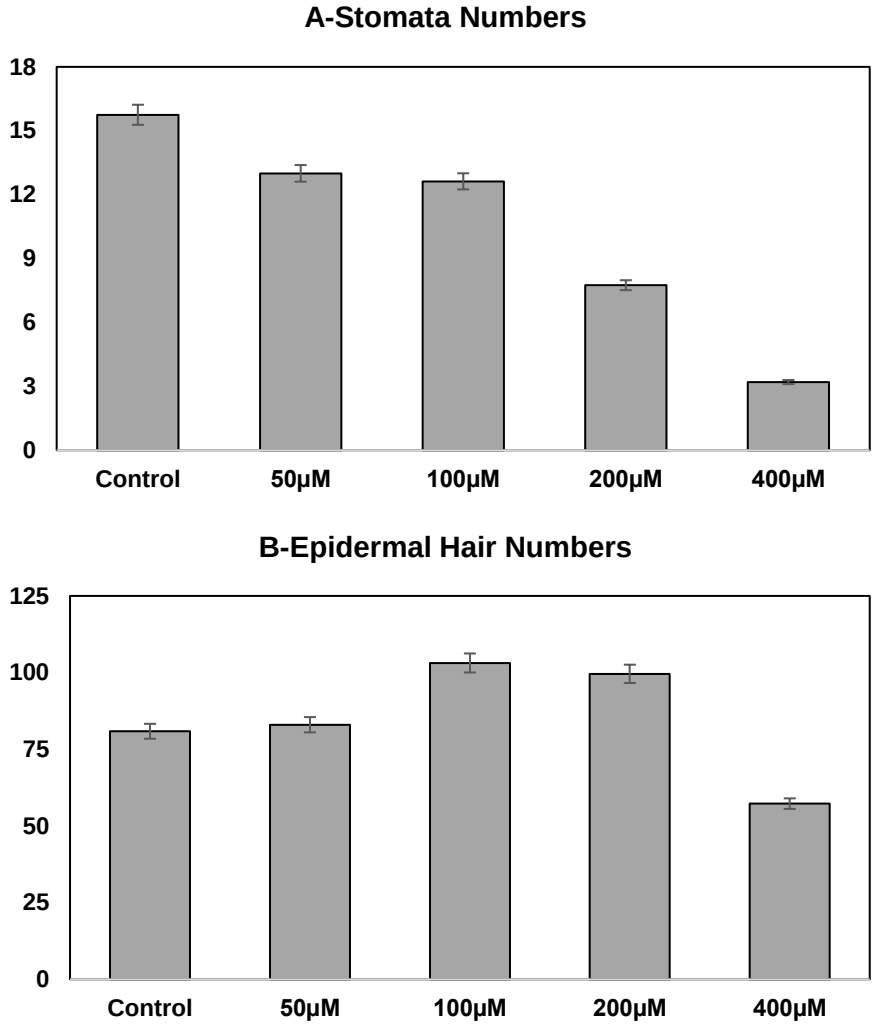



**Fig 4 (A)** Stomata and **(B)** Epidermal hair numbers of $CdCl_2$ treated wheat plants in different concentrations (0, 50, 100, 200 and 400 µM).

In a similar study, inhibited biomass production, decreased chlorophyll and carotenoid concentrations were observed in sunflower (*Helianthus annuus*) seedlings exposed to 20 µM Cd [44]. Another study indicated a decrease in root/shoot length in 7 days stressed (0, 50 or 200 µM Cd) Indian mustard (*Brassica juncea*) [45]. Also, reduced chlorophyll and carotenoid contents were observed in the same study. Inhibited relative growth and significant reduction in biomass and leaf area were observed in an aquatic fern (*Ceratopteris pteridoides*) at 20 and 40 µM Cd applications [46]. In another study conducted with cotton (*Gossypium*

*hirsutum*), growth inhibition, decreased plant height, reduced chlorophyll content, decreased biomass and leaf area were observed at 25, 50, and 100 μM Cd applications [47].

In *Groenlandia densa*, a gradual decrease in photosynethic pigmentation, chlorophyll *a* and *b*, and total chlorophyll ratios were observed in concentrations of 0-20 mg $L^{-1}$ Cd [48]. 20-120 μM Cd were applied to two kenaf (*Hibiscus cannabinus* L.) varieties and reduction in the root and shoot lengths, root and shoot biomass were observed, while cv. Fuhong 1991 was essentially unaffected under the 20 μM treatment compared with control [49]. In 10-50 μM Cd treated *Brassica napus*, a reduction was observed in plant height, root length, leaf area and number of leaves, root diameter, root surface area, number of root tips, and root volume especially in higher concentrations [50]. 10, 25, and 50 μM Cd stress was applied to *Tagetes patula* and reductions in fresh and dry weights of roots/shoots, in biomass, growth rate, and chlorophyll content were observed [51]. In *Urtica pilulifera*, root and shoot lengths were gradually decreased from 13.83 cm (control) to 10.24 cm and 7.48 cm in roots and from 7.3 (control) to 4.8 cm and 3.7 cm in shoots after the application of 100 and 200 μM Cd [52]. Ozyigit et al., (2016) observed considerable reductions in chlorophyll concentrations of kalanchoe (*Kalanchoe daigremontiana*) plants after 60 days of Cd application at different levels (0, 50, 100, 200 and 400 μM) in comparison with control group. The reduction rates were ~40.57% for chlorophyll *a*, ~37.63% for chlorophyll *b*, ~20.58% for chlorophyll *a*/*b* and ~36.27% for total chlorophyll [34].

Furthermore, reduced seed germination [53], reduced growth and photosynthesis parameters [54], negatively induced oxidative stress [54], reduced photosynthesis and chlorophyll contents [56], detrimental effects on growth and physiological process [57, 58], reduction in growth and antioxidant system activity of seedlings [59], negative effect on photosynthesis and chlorophyll fluorescence [60], disturbance in roots proteins [61], negative effects in growth, oxidative stress and antioxidant system in seedlings [62], reduced glutathione reductase activity and isoforms in leaves and roots [63], were observed in wheat plants, which were treated to Cd in different concentrations.

According to similar studies on stoma and epidermal hairs (for 1 $mm^2$ leaf surface); in *Trigonella foenum graecum* Linn., similarly to our results, number of stomata was reduced from 33.20 (control) to 29.21, 27.20, 25.19, 22.17 and in contrary to the results we obtained

in our study, number of epidermal hair was reduced from 10.07 (control) to 9.92, 8.80, 7.80, 6.20 after application of 5, 15, 30, 50 μg/g Cd respectively [64]. In another study, stomata numbers of lemon balm (*Melissa officinalis*) were reduced from 3.5 to 2.6, 1.6, 0.8 in abaxial leaves and from 5.8 to 4.5, 3.3, 1.6 in adaxial leaves in 10, 20, 30 mg/kg Cd applications [65]. In *Cenchrus ciliaris* number of stomata reduced from 80.25 to 69.12 and 60.23 in 30 and 60 mg/kg Cd applications, respectively [66]. Furthermore, in *Cajanus cajan*, stomata numbers were reduced from 26.40 to 20.64, 19.04, 18.60, 18.40, 17.20 in 5, 10, 15, 25, 50 μg/g Cd applications respectively [67]. Lately, in *Biscutella auriculata*, stomata numbers were increased from 189.86 to 291, and epidermal numbers (like our study) from 22.37den 34.64, after the applications of 0 and 125 μM Cd [68]. This could be a response for survival of the plant in applied Cd concentrations, with increased stomata and epidermal hair numbers even when the plant is under stress. Also, related to species, and applied Cd concentrations the numbers of both stomata and epidermal hairs could be increased and decreased.

It is obviously seen from above studies that, there are different responses in plants depending on the adequacy of the defense system. Exact plant response may change from one species to another depending on the duration and concentration of the applied Cd stress. As seen in our study, it has been observed that plants can manage with Cd stress in lower concentrations, but cannot achieve this after a certain concentration level. After a certain stress level, decreases have been started in growth and development parameters.

When the average Cd accumulation values (μg/kg) that gave the above obtained changes were examined it can be seen that the Cd accumulation value of 21 in control groups of the roots, reached to 171, 322, 1076 and 1447 in 50, 100, 200 and 400 μM Cd applications, respectively; 11 in the control groups of the shoots became 74, 177, 560 and 952 in the experimental groups, and 15 in the leaves of control groups became 135, 282, 812 and 1094 in the experimental groups, respectively (Figure 5 A-B and C). Accordingly, the accumulations were seen as root>leaf>shoot.

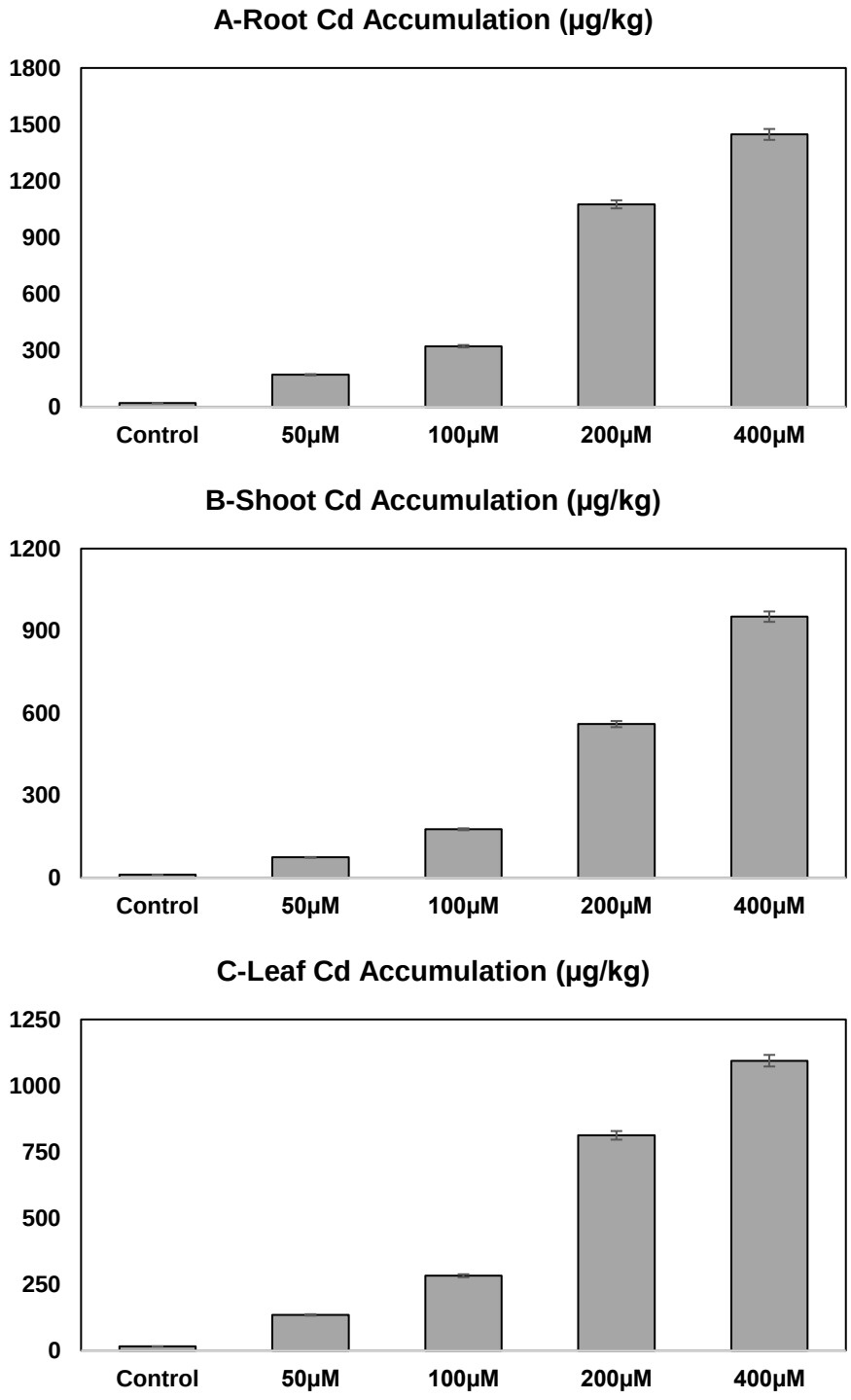



**Fig 5** Cd accumulation values (µg/kg) in **(A)** roots, **(B)** shoots and **(C)** leaves of $CdCl_2$ treated wheat plants in different concentrations (0, 50, 100, 200 and 400 µM).

In a similar study, Di Baccio et al., (2014) applied lower (54.3 mg/kg) and higher (163 mg/kg) Cd concentrations to poplar (*Populus* x *canadensis*) clones and their Cd accumulations in plant parts (in mg/kg) were 7.6 (leaves), 4.6 (shoots), 57 (roots) for lower and 12.7 (leaves), 15 (shoots), 80 (roots) for higher Cd applications [69]. In *Apium graveolens*, Cd accumulations (in mg/kg) were between 220 and 300 (roots) and 16 and 40 (shoots) in 40-120 mg/kg Cd applications for 35-50 days [70], while in *Paspalum atratum* cv. Reyan accumulations (in mg/kg) were 349 (roots) 46 (shoots) in 8 mg/kg Cd applications [71]. In another study conducted with kalanchoe plants, Cd accumulations (in mg/ml) were increased from 0.629 to 3.164 in leaves, 0.460 to 2.890 in shoots, and 1.327 to 5.178 in roots after 0 to 400 μM Cd applications. This means that the increment levels were found to be ~5.03 fold in leaves, ~6.28 fold in shoots and ~3.90 fold in roots [34].

Roots' uptake ability, xylem sap's transporting efficiency, and ultimate re-translocation within plant seeds are main factors for Cd accumulation efficiency in plants [72, 73]. Plant roots play a key role for the uptake and accumulation of mineral nutrients together with water and heavy metals as well as Cd. Also, NRAMPs (natural resistance associated macrophage proteins) play an important role in Cd transport across the cell membrane [74, 75]. Translocation of Cd from roots to aerial parts, occurs both passive (transpiration) and active (ion channels) mechanisms. On the contrary, the ability of Cd uptake and accumulation also depend on plant type, species and genotype [2, 73, 76].

In this study, the wheat plant showed an increasing Cd accumulation parallel with the increasing Cd applications in its organs. Cd accumulation was the highest in the roots and then in the leaves in all applied concentrations. In addition, the plant was affected by all concentrations and represented different responses. Also, there have been positive changes in some parameters, especially in the 50 μM application. This has shown that the plant can manage lower level of Cd stress by activating its defense mechanisms in order to survive, growth and development. Nevertheless, this situation did not work in higher Cd concentrations, especially in applications of 400 μM. Almost all parameters were adversely affected by the application of 400 μM.

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# Genotypic differences in aluminum tolerance of cowpea accessions utilizing germination parameters

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**ABSTRACT**

One of the major factors which limit the productivity of cowpea on acid soils is aluminum toxicity. Reliable methods for identifying genetic variation for its tolerance is indispensable. Genetic variability for aluminum tolerance in 10 accessions of cowpea were studied in the laboratory. Fifteen seeds of each accession were sown in sterilized petri dishes containing filter papers and 5 ml of $AlCl_3$ at four levels (0, 50, 100 and 200 µm) and replicated three times in a completely randomised design (CRD). Petri dishes were incubated under room temperature in the dark for 48 hours. After 48 hours, they were exposed to photoperiod of 12 hr. / 12 hr. (day/night) at room temperature for another 48 hours. At day four after sowing, data were collected on percentage germination, number of roots per shoot, fresh weight of shoot, root length and hypocotyl length and fresh weight of shoot. Data were subjected to statistical analysis and accessions were arranged on their tolerance to aluminum stress by means of tolerance indices. Analysis of variance revealed significant effect of accessions on all parameters. Treatment was significant for all excluding percentage germination. Treatment by accession was significant for number of roots and root length. The observed genetic variation in cowpea for aluminum stress could be exploited by hybridisation to establish tolerant lines. Selection based on high heritability and GAM in percentage germination, hypocotyl length and number of roots in cowpea under aluminum stress can be exploited for selection.

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## Introduction

In the nutrition and cropping systems of tropical and subtropical countries, the role cowpea plays can never be over stressed. Protein content of cowpea revolves around 25 percent; it is a fast growing crop with tremendous capacity to control erosions through ground surface cover and at the same time fixes atmospheric nitrogen in soil for soil improvement [1]. It can tolerate soils of diverse pH range compared to other legumes [2], with its productivity nonetheless limited by many factors.

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One of the major factors which limit the productivity of cowpea on acid soils is aluminum toxicity. Exposure to micromolar concentrations in soil solution can rapidly hamper root elongation and consequently hinder the uptake of water and nutrients, thus causing substantial decrease of crop production on acid soils [3, 4]. Acid soils are toxic to plants for their nutritional disorder, deficiency, or restriction of vital nutrients such as phosphorus, calcium and magnesium. Toxicity resulting from aluminum, manganese and activity of hydrogen also play an important role in soil toxicity [5].

Plants lay open to aluminum stress have been shown to display distinct root elongation inhibition very early within hours or even minutes of exposure [6]. Plants grown in the fields find it hard to explore the soil for nutrients and moisture if the subsoil is acidic, making the plants to experience drought stress after only a few days of lack of rainfall. In addition to the reduced root system, plants suffering from aluminum stress have reduced shoot growth and cause a decline in crop yield. Aluminum toxicity hampers cell division in root tips and lateral roots, escalates cell wall inflexibility by cross linking pectins, decreases DNA replication, decreases phosphorus fixation in soil and on root surfaces, drops root respiration, disturbs enzyme action governing sugar phosphorylation and deposition of cell wall polysaccharides. Aluminum is available in all soils, but its toxicity becomes apparent only in acidic situations in which plant-toxic form, $Al^{3+}$ predominates [7].

Acid soils occupy nearly 17 million hectares in Nigeria, making up 18% of the total land area [8], and about 35% of the world's arable land [9], and about 50% of the possible cultivated land [4, 10, 11, 12]. Soils of the humid tropics, especially the whole lands of the South Eastern Nigeria are acidic [13, 14, 15], due to high precipitations (1500 mm and above) in these regions which lead to the leaching of substantial amounts of exchangeable bases from soil surfaces [16]. Since there is a direct correlation between soil acidity and aluminum toxicity, liming of the soil could be adopted to raise the pH level to enhance plant growth and development under aluminum stress [17]. However, the practice of agriculture in developing countries has been mainly relegated to subsistence levels, hence it is unwise both economically and logistically to adopt liming of soil by the resource poor farmers [10, 16, 18]. Excess liming also have negative consequences, such as leaching of soil minerals causing Manganese (Mn) deficiencies in soils as well as acquisition by plants and Phosphorus

(P) deficiencies in soils [18]. Availability of higher aluminum tolerant cultivars of cowpea to farmers will contribute positively at ensuring food security, enhanced nourishment and also protect the soils against the negative effects of over liming.

Crop plants have adopted different mechanisms of tolerating toxic level of aluminum in acidic soils, these mechanisms include exclusion and internal control which are governed by the expression of multiple genes [4, 12]. Toxicity sensitivity is genotype-specific in cowpea germplasm. Understanding the physiological mechanisms of tolerance is very cogent in the improvement programs of the adapted genotypes [12]. However, improving the genetic and physiological tolerance of crop species to aluminum stress has been quiet challenging [4]. Genotypes of cowpea from Brazil were shown to possess different tolerance levels to acidic Alumihaplic Acrisol (pH 3.8) under phosphorus limiting conditions [19]. Genotypic differences in the performance of some cowpea genotypes on acid soils might be related with variation in the expression of aluminum tolerance mechanisms, especially under limited phosphorus conditions [20, 21]. Kushwala [22] reported significant genotypic differences for aluminum tolerance among twenty accessions of cowpea for important quantitative and physiological traits. Aluminum stress was found to decrease the yield and protein content of the seeds. Aluminum tolerance of eight cultivars of cowpea was studied at early growth and maturity by [23], and it was reported that both genotype and genotype x aluminum was significant for the growth and yield traits. Traits such as plant height at late growth stage, biomass yield and pod weight were all enhanced by aluminum stress, while plant height at earlier growth stage, nodulation and number of pods per plant were subdued by aluminum stress.

Impairment of root growth occurred only in the condition of exposing about 2 – 3 mm of the apical root to aluminum stress in maize, while other parts of the root if in contact with aluminum produced no effect on root growth [24]. Stubby and brittle roots proceeding aluminum exposure indicate that cytoskeleton may the point where toxicity of aluminum activities lies [25]. Aluminum tolerant maize genotypes have been found to excrete much citric acid from roots apices in response to aluminum stress compared to genotypes susceptible to aluminum [26]. Phosphorus deficiency with increased aluminum stress led to increased citrate exudation in a genotype of maize [27]. A study by [28] on genotypic

differences for aluminum toxicity among fourteen inbred lines of maize for seedling root variation in hydroponics, employing tolerance membership index on actual root length, relative root length and root length response classified genotypes into three classes of highly tolerant, tolerant and intermediate. High heritability of the root traits indicated possibility of successful breeding programme for aluminum stress if selection is done among the maize genotypes. Root traits also displayed high expected genetic gain which proved that root traits are best for screening for tolerance in maize. A population of $F_{2:3}$ hybrids derived from the crosses between a tolerant maize parent and other susceptible lines of Kenyan maize showed high significant genotypic variability for relative net root growth in nutrient solution with heritability for aluminum tolerance reaching 97% [10]. However, information regarding estimates of genetic variations of germination and root traits under aluminum stress is limited in cowpea.

Germination parameters in hydroponics and petri dishes under laboratory conditions have been used successfully to screen for aluminum tolerance in many crop species including cowpea [21], maize [28], wheat [29], *Vigna* species [30], alfalfa [31], rice [32] and soy bean [33]. These were in a bid to avoid the hassles associated with field experiments since correlated responses have been achieved in many instances between field and laboratory performances among crops evaluated under aluminum stress [28].

The present study objectives were to screen for aluminum tolerance in cowpea using a simple and fast laboratory procedure and to pinpoint the best germination traits for which selection could be effective.

## Materials and Methods

### Procedure

The study was set up at the laboratory in the Department of Plant Science and Biotechnology, Adekunle Ajasin University, Akungba-Akoko, Nigeria in July, 2016. Seeds of the 10 accessions were screened for tolerance to aluminum by employing the procedure of [34] and [35] with some modifications. A factorial experiment (10 x 4) was employed; seeds of uniform size were surface sterilized in sodium hypochlorite (NaClO) solution 5% (v/v) for 1 hr. and rinsed in distilled water five times. The seeds were sown in sterilized petri dishes

containing two pieces of sterilized filter paper and 5 ml of aluminum chloride ($AlCl_3$) at four levels (0, 50, 100 and 200 μm). Fifteen seeds of each accession were placed on the filter paper in each petri dish and replicated three times for each treatment in a Completely Randomized Design (CRD). Petri dishes were incubated under room temperature in the dark for 48 hours. After 48 hours, they were exposed to photoperiod of 12 hr. / 12 hr. (day/night) at room temperature for another 48 hours. The 10 accessions are: TVu-199, TVu-207, TVu-218, TVu-235, TVu-236, TVu-241, IT98K-205-8, IT98-555-1, TVu-4886 and TVu-9256 coded as AC01, AC02, AC03, AC04, AC05, AC06, AC07, AC08, AC09 and AC10 respectively. Details of the accessions are described in [1].

**Data gathering and analyses**

At four days after sowing, data were collected on percentage germination, number of roots per shoot, root length, hypocotyl length and fresh weight of shoot. Data for were run through analysis of variance (ANOVA) using the Generalized Linear Model (GLM) procedure of the Statistical Package for Social Science (SPSS) version 20 (SPSS Inc., Chicago IL) [36]. Combination of duncan multiple range test (DMRT) and least significant difference (LSD) ($P \leq 0.05$ levels of significance) were adopted for mean separation where appropriate. Accessions were arranged on their tolerance to aluminum stress by means of tolerance indices (TI) according to [37], where the averages of other treatments were compared against that of control. The data on tolerance indices were run through cluster analysis with Palaeontological Statistic Software (PAST version 3.01) [38]. Estimates of heritability for each treatment was done with Plant Breeding Tools (PBTools version 1.4) [39], while the combined estimates of genetic parameters were done according to [28]. Pearson correlation coefficients were calculated using SPSS version 20, to determine the level of associations among all measured parameters. The data were also subjected to Principal Component (PC) analyses adopting the PAST.

## Results

### Variability among accessions of cowpea for germination parameters under aluminum stress

Results from analysis of variance (ANOVA) revealed that the mean square values for effect of accession was significant ($P \leq 0.05$) for all measured germination parameters. Aluminum effect was significant for all germination parameters excluding percentage germination. Accession x treatment effect was also significant for number of roots per seedling and root length, but not significant for percentage germination, hypocotyl length and fresh weight of shoots. The most variable traits among the germination parameters under aluminum stress included root length (CV = 50.16%) and hypocotyl length (CV = 34.44%), while the least variable trait was percentage germination (CV = 20.23%), (Table 1).

Effects of accession and aluminum treatments on germination parameters are shown in Table 2. The highest value for germination (91.65%) was obtained in AC06, while the lowest value (51.65%) was obtained in AC01. Number of roots per seedling was highest (15.45) in AC02, while the least (6.30) was obtained in AC01. Hypocotyl length was highest (7.41 cm) in AC10, while AC02 had the least (2.92 cm). Root length was highest (1.48 cm) in AC06, while the least (0.61 cm) was obtained in AC09. The highest value (0.31 g) for fresh weight of shoot was obtained in AC06, while the least (0.19 g) was obtained in AC02. For effect of treatment, aluminum enhanced all germination parameters except in percentage germination, with the differences not significant in all except in fresh weight of shoots.

Effects of aluminum x accession on germination parameters of accessions of cowpea are presented in Table 3. Germination was generally inhibited in accessions AC01, AC03, AC04, AC05 and AC07, while it was enhanced in AC06, AC08 and AC09. Heritability estimate was high for all treatments with the highest value (79.00%) obtained in 50 µm of aluminum treatment, while the least (60.00%) was obtained in 100 µm of aluminum treatment. Number of roots per seedling was mostly enhanced above that of control by 50 µm and 100 µm, while 200 µm mostly inhibited number of roots in most accessions. Heritability for number of roots trait ranged from moderate to high, with the lowest (57.00%) in 100 µm, however the highest (84.00%) was obtained in the control. Hypocotyl length was majorly inhibited by 50 µm in

AC01, AC02, AC03, AC04 and AC05, while other treatments majorly enhance the hypocotyl length across accessions. Heritability estimate was high in all treatments, and it ranged between 75.00% in 100 µm and 87.00% in 200 µm aluminum treatment. Root length was enhanced above that of the control by 50 µm in most accessions, whereas 100 µm and 200 µm played the role inhibiting the root length compared to the control in most accessions. Heritability estimate was low (30.00%) in 50 µm of aluminum treatment, moderate (57.00%) in 100 µm and 200 µm of aluminum treatments and high (61.00%) in control treatment. Fresh shoot weight was majorly enhanced above that of control by aluminum treatment in almost all accessions, with the estimate of heritability ranging from low (0.00%) in 50 µm to moderate, 50.00% in 100 µm.

**Table 1** Mean square values of accession, treatment and accession by treatment interaction of accessions of cowpea under aluminum stress in the laboratory

| Source of Variation | DF | PG (%) | NR | HYPL (cm) | RL (cm) | FW (g) |
|---|---|---|---|---|---|---|
| **Accession** | 9 | 2506.38** | 113.67** | 30.17** | 1.25** | 0.02** |
| **Treatment** | 3 | $83.92^{ns}$ | 16.28** | 5.09** | 0.2** | 0.03** |
| **Accession x treatment** | 27 | $63.23^{ns}$ | 28.54** | $2.47^{ns}$ | 0.42** | $0.01^{ns}$ |
| **Error** | 80 | 251.78 | 14.03 | 3.22 | 0.31 | 0.01 |
| **CV (%)** | | 20.23 | 30.58 | 34.44 | 50.16 | 40.00 |

**: Significant at $P \leq 0.05$; DF: Degree of freedom; PG: Percentage germination; NR: Number of roots; HYPL: Hypocotyl length; RL: Root length; FW: Fresh weight of shoot; CV: Coefficient of variation.

**Table 2** Effects of accession and aluminum treatment on germination parameters of accessions of cowpea under aluminum stress

| Accession | PG (%) | NR | HYPL (cm) | RL (cm) | FW (g) |
|---|---|---|---|---|---|
| **AC01** | 51.65$^{a}$ | 6.30$^{a}$ | 3.41$^{a}$ | 0.67$^{a}$ | 0.22$^{ab}$ |
| **AC02** | 55.00$^{a}$ | 15.45$^{e}$ | 2.92$^{a}$ | 1.45$^{c}$ | 0.19$^{a}$ |
| **AC03** | 78.31$^{bc}$ | 13.74d$^{e}$ | 5.56$^{b}$ | 1.37$^{c}$ | 0.20$^{ab}$ |
| **AC04** | 79.97$^{bc}$ | 12.68cd$^{e}$ | 6.42$^{bc}$ | 1.05$^{abc}$ | 0.28$^{ab}$ |
| **AC05** | 83.87$^{bc}$ | 11.43$^{bcd}$ | 5.50$^{b}$ | 1.04$^{abc}$ | 0.26$^{ab}$ |
| **AC06** | 91.65$^{c}$ | 14.19$^{de}$ | 7.16$^{c}$ | 1.48$^{c}$ | 0.31$^{b}$ |
| **AC07** | 74.43$^{b}$ | 9.65b$^{c}$ | 3.83$^{a}$ | 0.79$^{ab}$ | 0.25$^{ab}$ |
| **AC08** | 89.98$^{c}$ | 14.91$^{e}$ | 5.87$^{bc}$ | 1.24$^{bc}$ | 0.25$^{ab}$ |
| **AC09** | 88.31$^{bc}$ | 9.00a$^{b}$ | 3.99$^{a}$ | 0.61$^{a}$ | 0.27$^{ab}$ |
| **AC10** | 91.08$^{c}$ | 15.15$^{e}$ | 7.41$^{c}$ | 1.34$^{c}$ | 0.28$^{ab}$ |
| **±SE** | 4.58 | 1.08 | 0.52 | 0.16 | 0.03 |
| **Treatment** | | | | | |
| **Control** | 79.76$^{a}$ | 11.37$^{a}$ | 4.66$^{a}$ | 0.99$^{a}$ | 0.21$^{a}$ |
| **50µm** | 79.09$^{a}$ | 12.89$^{a}$ | 5.13$^{a}$ | 1.13$^{a}$ | 0.27$^{ab}$ |
| **100µm** | 75.98$^{a}$ | 12.82$^{a}$ | 5.51$^{a}$ | 1.19$^{a}$ | 0.25$^{ab}$ |
| **200µm** | 78.87$^{a}$ | 11.91$^{a}$ | 5.54$^{a}$ | 1.09$^{a}$ | 0.29$^{b}$ |
| **±SE** | 2.89 | 0.68 | 0.33 | 0.10 | 0.02 |

Means followed by similar alphabets in the same column are not significantly different from one another at $P \leq 0.05$ using Duncan Multiple Range Test (DMRT). SE: Standard error of means; PG: Percentage germination; NR: Number of roots; HYPL: Hypocotyl length; RL: Root length; FW: Fresh weight of shoot.

**Table 3** Effects of accession and aluminum interaction on germination parameters of accessions of cowpea under aluminum stress

| Treatment | AC01 | AC02 | AC03 | AC04 | AC05 | AC06 | AC07 | AC08 | AC09 | AC10 | $H^2B$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Percentage germination (%)** | | | | | | | | | | | |
| **Control** | 55.53[a] | 53.33[a] | 86.63[a] | 86.63[a] | 91.07[a] | 88.87[a] | 77.77[a] | 86.63[a] | 80.00[a] | 91.10[a] | 69.00 |
| **50µm** | 53.30[a] | 53.33[a] | 80.00[a] | 75.53[a] | 84.43[a] | 93.30[a] | 75.53[a] | 88.87[a] | 93.30[a] | 93.30[a] | 79.00 |
| **100µm** | 48.87[a] | 60.00[a] | 66.63[a] | 75.53[a] | 77.77[a] | 91.10[a] | 68.87[a] | 91.10[a] | 91.07[a] | 88.87[a] | 60.00 |
| **200µm** | 48.90[a] | 53.33[a] | 79.97[a] | 82.17[a] | 82.20[a] | 93.33[a] | 75.57[a] | 93.30[a] | 88.87[a] | 91.07[a] | 66.00 |
| **±SE** (9.16) | | | | | | | | | | | |
| **LSD** (NS) | | | | | | | | | | | |
| **Number of roots** | | | | | | | | | | | |
| **Control** | 7.55[a] | 11.42[a] | 16.73[b] | 13.27[a] | 11.62[a] | 13.60[a] | 11.80[a] | 13.40[a] | 2.66[a] | 14.33[a] | 84.00 |
| **50µm** | 5.17[a] | 11.53[a] | 15.53[b] | 12.23[a] | 12.04[a] | 17.07[a] | 9.47[a] | 17.27[a] | 12.00[b] | 16.67[a] | 71.00 |
| **100µm** | 4.25[a] | 19.07[b] | 12.47[ab] | 13.87[a] | 11.37[a] | 13.82[a] | 10.40[a] | 16.07[a] | 14.20[b] | 12.67[a] | 57.00 |
| **200µm** | 8.23[a] | 19.77[b] | 10.23[a] | 11.33[a] | 10.70[a] | 12.27[a] | 6.93[a] | 12.90[a] | 9.80[b] | 16.93[a] | 70.00 |
| **±SE** (2.16) | | | | | | | | | | | |
| **LSD** (5.07) | | | | | | | | | | | |
| **Hypocotyl length (cm)** | | | | | | | | | | | |
| **Control** | 4.07[a] | 2.83[a] | 5.56[a] | 6.01[a] | 4.58[a] | 4.87[a] | 3.44[a] | 6.05[a] | 2.23[a] | 6.96[a] | 86.00 |
| **50µm** | 3.30[a] | 2.51[a] | 4.97[a] | 5.94[a] | 4.48[a] | 7.00[ab] | 3.93[a] | 6.81[a] | 4.47[a] | 7.84[a] | 82.00 |
| **100µm** | 3.37[a] | 3.31[a] | 6.48[a] | 7.33[a] | 7.25[b] | 7.48[b] | 4.20[a] | 4.85[a] | 4.34[a] | 6.49[a] | 75.00 |
| **200µm** | 2.91[a] | 3.03[a] | 5.22[a] | 6.39[a] | 5.71[ab] | 9.30[b] | 3.77[a] | 5.77[a] | 4.96[a] | 8.36[a] | 87.00 |
| **±SE** (1.04) | | | | | | | | | | | |
| **LSD** (2.43) | | | | | | | | | | | |

**Table 4** cont'd

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Root length (cm)** | | | | | | | | | | | |
| **Control** | 0.48$^{a}$ | 1.45$^{a}$ | 1.47$^{a}$ | 0.81$^{a}$ | 1.53$^{b}$ | 0.91$^{a}$ | 1.13$^{b}$ | 0.95$^{a}$ | 1.67$^{b}$ | 1.27$^{a}$ | 61.00 |
| **50µm** | 0.33$^{a}$ | 1.61$^{a}$ | 1.65$^{a}$ | 1.32$^{a}$ | 0.67$^{a}$ | 1.70$^{ab}$ | 0.75$^{ab}$ | 1.17$^{a}$ | 0.77$^{a}$ | 1.34$^{a}$ | 30.00 |
| **100µm** | 0.24$^{a}$ | 1.49$^{a}$ | 1.22$^{a}$ | 1.30$^{a}$ | 0.99$^{ab}$ | 1.97$^{b}$ | 0.97$^{ab}$ | 1.59$^{a}$ | 0.96$^{ab}$ | 1.18$^{a}$ | 57.00 |
| **200µm** | 1.63$^{b}$ | 1.26$^{a}$ | 1.13$^{a}$ | 0.78$^{a}$ | 0.99$^{ab}$ | 1.35$^{ab}$ | 0.34$^{a}$ | 1.25$^{a}$ | 0.70$^{a}$ | 1.57$^{a}$ | 57.00 |
| **±SE** (0.32) | | | | | | | | | | | |
| **LSD** (0.75) | | | | | | | | | | | |
| **Fresh weight of shoot (g)** | | | | | | | | | | | |
| **Control** | 0.17$^{a}$ | 0.18$^{a}$ | 0.26$^{a}$ | 0.21$^{a}$ | 0.24$^{a}$ | 0.31$^{a}$ | 0.19$^{a}$ | 0.14$^{a}$ | 0.11$^{a}$ | 0.27$^{a}$ | 27.00 |
| **50µm** | 0.31$^{a}$ | 0.19$^{a}$ | 0.24$^{a}$ | 0.27$^{ab}$ | 0.25$^{a}$ | 0.30$^{a}$ | 0.23$^{a}$ | 0.28$^{ab}$ | 0.30$^{b}$ | 0.30$^{a}$ | 0.00 |
| **100µm** | 0.13$^{a}$ | 0.18$^{a}$ | 0.14$^{a}$ | 0.37$^{b}$ | 0.27$^{a}$ | 0.30$^{a}$ | 0.23$^{a}$ | 0.26$^{ab}$ | 0.32$^{b}$ | 0.28$^{a}$ | 50.00 |
| **200µm** | 0.26$^{a}$ | 0.19$^{a}$ | 0.17$^{a}$ | 0.26$^{ab}$ | 0.29$^{a}$ | 0.31$^{a}$ | 0.33$^{a}$ | 0.31$^{b}$ | 0.35$^{b}$ | 0.29$^{a}$ | 39.00 |
| **±SE** (0.07) | | | | | | | | | | | |
| **LSD** (0.15) | | | | | | | | | | | |

Means followed by similar alphabets in the same column are not significantly different from one another at $P \leq 0.05$. NS: Non-significant; SE: Standard error of means; LSD: Least significant difference; $H^2B$: Heritability.

**Correlation and estimates of genetic parameters of germination traits of cowpea under aluminum stress**

Strong associations among germination parameters were experienced among all traits in this study, except between percentage germination and root length (0.089), and root length and fresh weight (0.119) (Table 4). Estimates of variances, Genotypic and Phenotypic coefficients (GCV and PCV) and Broad sense heritability ($H^2B$) of germination parameters of cowpea exposed to aluminum stress are presented in Table 5. GCV and PCV were high for all traits. In fresh shoot weight, the lowest (21.91%) GCV was obtained while in hypocotyl length, the highest (57.52%). Highest PCV (70.94%) was obtained in root length, while the lowest (40.39%) was obtained in percentage germination. Heritability in the broad sense ranged from low to high among parameters. Heritability was lowest (23.08%) in fresh weight of shoot, while the highest heritability (73.61%) was obtained in hypocotyl length.

**Table 4** Pearson correlation of germination traits of accessions of cowpea under aluminum stress

| | **PG (%)** | **NR** | **HYPL (cm)** | **RL (cm)** | **FW (g)** |
|---|---|---|---|---|---|
| **PG** | 1 | 0.226* | 0.571** | 0.089 | 0.407** |
| **NR** | | 1 | 0.319** | 0.600** | 0.217* |
| **HYPL** | | | 1 | 0.204* | 0.361** |
| **RL** | | | | 1 | 0.119 |
| **FW** | | | | | 1 |

**: Significant at $P \leq 0.01$; *: Significant at $P \leq 0.05$; PG: Percentage germination; NR: Number of roots; HYPL: Hypocotyl length; RL: Root length; FW: Fresh weight of shoot.

**Table 5** Estimates of genetic parameters of germination traits of accessions of cowpea under aluminum stress

| **Trait** | **GM** | **GV** | **PV** | **GCV (%)** | **PCV (%)** | **$H^2B$ (%)** |
|---|---|---|---|---|---|---|
| **PG** | 78.42 | 751.53 | 1003.06 | 34.96 | 40.39 | 74.92 |
| **NR** | 12.25 | 33.21 | 47.24 | 47.04 | 56.11 | 70.59 |
| **HYPL** | 5.21 | 8.98 | 12.2 | 57.52 | 67.04 | 73.61 |
| **RL** | 1.11 | 0.31 | 0.62 | 50.16 | 70.94 | 50.00 |
| **FW** | 0.25 | 0.003 | 0.013 | 21.91 | 45.61 | 23.08 |

GM: Grand mean; GV: Genotypic variance; PV: Phenotypic variance; GCV: Genotypic coefficient of variation; PCV: Phenotypic coefficient of variation; $H^2B$: Heritability in the broad sense; PG: Percentage germination; NR: Number of roots; HYPL: Hypocotyl length; RL: Root length; FW: Fresh weight of shoot.

**Principal Component and bi-plot profiling of cowpea parameters under aluminum stress**

The Principal Components centered on germination parameters under aluminum stress are presented in Table 6. Five Principal Components axis were extracted for all parameters out of which the first (eigen-values greater than 1.00) accounted for 89.29% of the total variation. The first PC axis accounted for 58.66% of the total variation with all traits having high loadings and making positive contributions to the total variation. The second PC accounted for 30.63% of the total variation with traits such as percentage germination (0.36) and fresh weight (0.55) with high positive contributions, root length (-0.55) and number of roots (-0.49) were negative contributors. Bi-plot of all the parameters under aluminum stress based on Principal Components 1 and 2 is presented in Figure 1. The bi-plot resulted in four major groups for the ten accessions under aluminum influence. Group I consisted of two accessions (AC03 and AC08), group II had four (AC10, AC06, AC04 and AC05), group III had three (AC09, AC01 and AC07) however, group IV had one (AC02). Accessions in group I and II were the most tolerant to aluminum toxicity and they were positively correlated with all the germination parameters. Accessions in group III were the sensitive accessions, while the one in group IV was the slightly sensitive one. These accessions were not strongly correlated with any of the germination parameters. Parameters like fresh weight, percentage germination and hypocotyl length were strongly correlated (angle < $90^0$), while parameters like number of roots and root length were also strongly correlated (angle < $90^0$). All traits were regarded as aluminum tolerant traits.

**Table 6** Principal Component analysis of germination parameters of accessions of cowpea under aluminum stress

| | **Principal Components** | |
|---|---|---|
| | **PC1** | **PC2** |
| **Eigen-value** | 2.93 | 1.53 |
| **Cumulative eigen-value** | 2.93 | 4.46 |
| **Variability (%)** | 58.66 | 30.63 |
| **Cumulative variability** | 58.66 | 89.29 |
| **Variables** | **PC1** | **PC2** |
| **Percentage germination (%)** | **0.46** | **0.36** |
| **Number of roots** | **0.45** | **-0.49** |
| **Hypocotyl length (cm)** | **0.54** | 0.15 |
| **Root length (cm)** | **0.41** | **-0.55** |
| **Fresh weight (g)** | **0.36** | **0.55** |

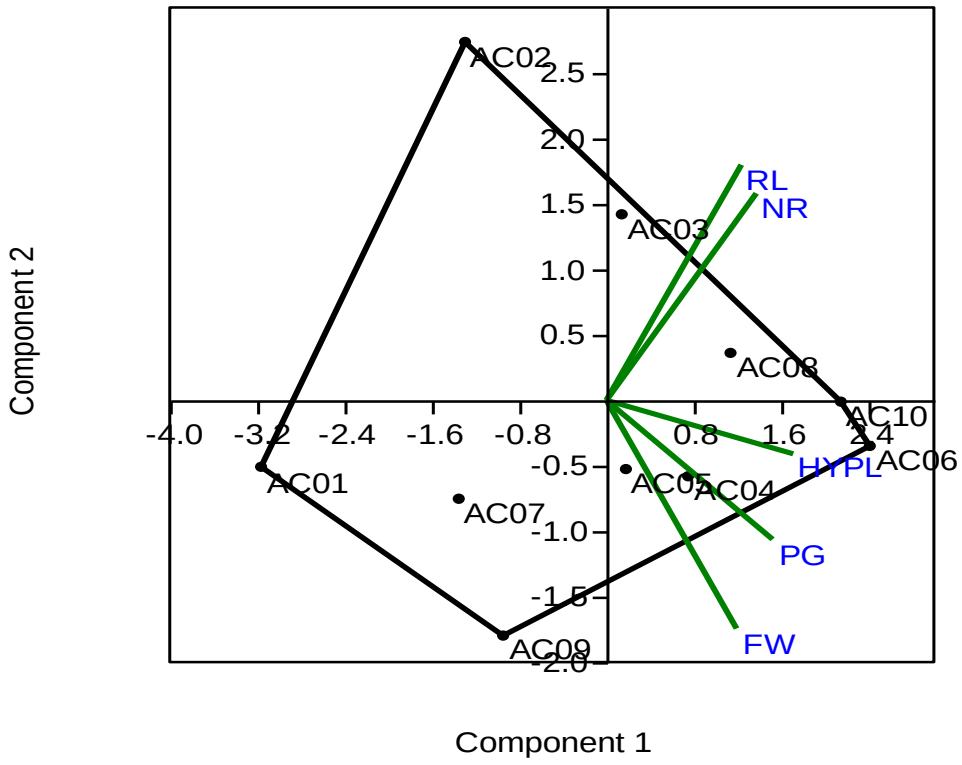


**Fig 1** Bi plot of germination parameters of accessions of cowpea under aluminum stress

**Aluminum tolerance indices based on germination parameters of accessions of cowpea**

Table 7 presents the results of aluminum tolerance indices based on cowpea germination parameters. Nine groups were extracted from this based on similar mean index; groups 1 to 5 are those with mean indices ranging between 1.17 and 1.69 (AC05, AC04, AC08, AC03, AC06, and AC10). Groups 6 to 9 consisted of accessions with indices of between 0.51 and 0.92 (AC01, AC09, AC07 and AC02). Accessions in group 1 and 2 were the highly tolerant accessions, accessions in groups 3, 4 and 5 were moderately tolerant accessions, and accessions in groups 6, 7 and 8 were susceptible accessions, while the accession in group 9 was the highly susceptible accession.

**Cluster analysis based on aluminum tolerance indices of germination parameters of accessions of cowpea under aluminum stress**

A dendrogram of four major clusters was formed by the aluminum tolerance indices based on germination parameters of cowpea accessions (Figure 2). Cluster I consisted of two accessions (AC10 and AC06), highly tolerant accessions. Cluster II consisted of four accessions (AC05, AC04, AC08 and AC03), moderately tolerant accessions. Clusters III consisted of one susceptible accession (AC02), and IV consisted of two susceptible and one highly susceptible accessions (AC09, AC01 and AC07).

**Table 7** Aluminum tolerance indices for germination parameters of accessions of cowpea under aluminum stress

| Accession | PG (%) | NR | HYPL (cm) | RL (cm) | FW (g) | Mean | Rank |
|---|---|---|---|---|---|---|---|
| **AC01** | 0.44 | 0.33 | 0.6 | 0.26 | 0.92 | 0.51 | 9 |
| **AC02** | 0.47 | 1.42 | 0.38 | 1.55 | 0.78 | 0.92 | 6 |
| **AC03** | 1.03 | 1.57 | 1.42 | 1.44 | 1.10 | 1.31 | 3 |
| **AC04** | 1.06 | 1.22 | 1.81 | 0.67 | 1.46 | 1.24 | 4 |
| **AC05** | 1.17 | 0.98 | 1.23 | 0.99 | 1.50 | 1.17 | 5 |
| **AC06** | 1.29 | 1.44 | 1.78 | 1.12 | 2.17 | 1.56 | 2 |
| **AC07** | 0.90 | 0.78 | 0.63 | 0.57 | 1.16 | 0.81 | 7 |
| **AC08** | 1.24 | 1.52 | 1.62 | 0.93 | 0.92 | 1.25 | 4 |
| **AC09** | 1.15 | 0.24 | 0.47 | 0.99 | 0.82 | 0.73 | 8 |
| **AC10** | 1.30 | 1.63 | 2.42 | 1.27 | 1.81 | 1.69 | 1 |

PG: Percentage germination; NRT: Number of roots; HYPL: Hypocotyl length; RL: Root length; FWS: Fresh weight of shoots.

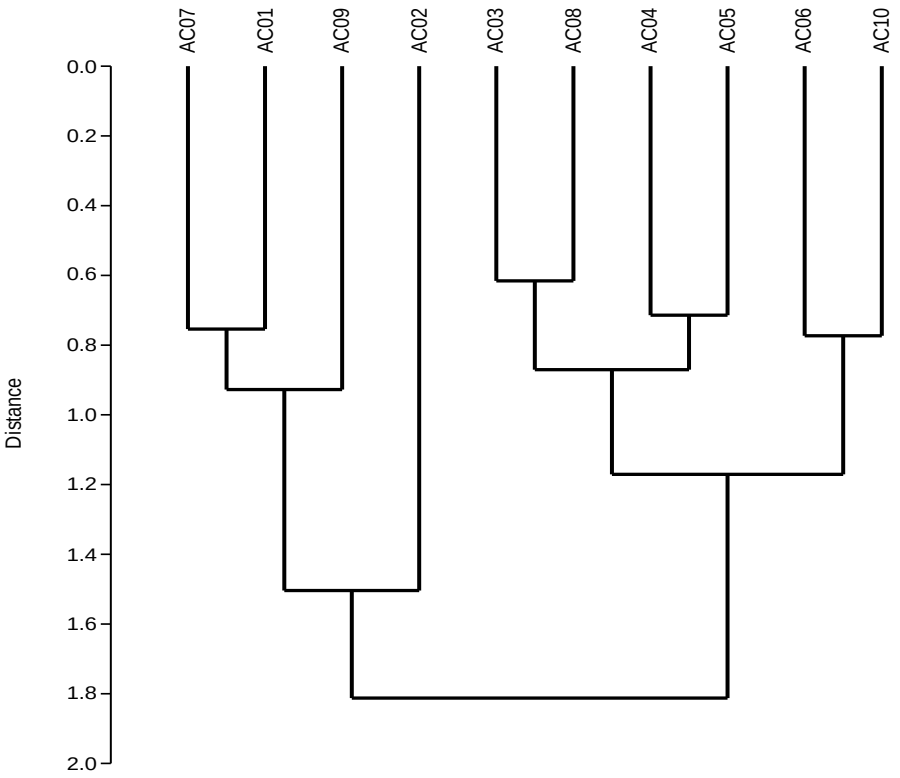


**Fig 2** Dendrogram (Euclidean distance) based on aluminum tolerance indices of germination parameters of accessions of cowpea under aluminum stres

## Discussion

Analysis of variance revealed significant effect of accession on all germination parameters. This indicated sufficient level of variations among the accessions involved. Aluminum treatment was significant for all parameters except for percentage germination. Aluminium and accession interaction was also significant for all parameters except for percentage germination, hypocotyl length and fresh weight of shoot. These were in agreement with the findings of [30] who reported no significant effect of aluminum treatment on germination of *Vigna radiata* and *Vigna sinensis*, but with significant effect on shoot and root growth. Five of the accessions (AC01, AC03, AC04, AC05, and AC07) had higher germination percentage under control than under aluminum stress, while germination under control was lower for the remaining accessions, indicating stimulatory effects. The number of roots in AC02 and AC09

increased significantly; the hypocotyl length increased significantly in AC06, the root length decreased significantly in AC01, while others had no significant changes in all of these parameters. These were in accordance with the findings of [30] who reported the observation of combination of inhibition symptoms and promotive effect of aluminum stress in *Vigna* species. Rout [7] reported that aluminum had no effect on seed germination, but promoted new root development and seedling establishment, while root growth was found to be more susceptible to aluminum stress than shoot in maize [3] and in wheat [40]. Low dosage of aluminum was also found to promote germination in *Vigna radiata* [41].

All germination parameters were observed to be negatively affected by aluminum in alfafa cultivars [35], in contrast, all germination parameters were generally enhanced in cowpea in this study, except for percentage germination which was generally reduced by treatment. Tolerance indices were able to group accessions into different classes of tolerance: AC10, AC06 and AC03 were highly tolerant; AC04, AC05, AC08 and AC02 were tolerant; AC07 and AC09 were moderately sensitive; whereas AC01 was highly sensitive. The dendrogram based on tolerance indices of germination parameters divided accessions into four key clusters. Cluster I clearly separated the most tolerant accessions (AC10 and AC06), while cluster IV clearly separated the most susceptible accessions (AC01, AC09 and AC07).

Target improvement for aluminum tolerance will depend on existence of sufficient genetic variability and identification of traits that are correlated under stress. Findings from many workers suggest that many approaches could be adopted in breeding for aluminum tolerance and identification of germane traits should be the focus of each breeding program [10, 28, 32, 42]. In this study, percentage germination was positively correlated with number of roots, hypocotyl length and fresh weight of shoot. Number of roots was positively correlated with hypocotyl length, root length and fresh weight of shoot. The root length and fresh weight of shoot were positively associated with hypocotyl length. This indicated that good germination will results in positive increment with all positively correlated traits; this was reflected in AC06 and AC10 with very good germination and subsequent superiority for number of roots, root length and hypocotyl length under aluminum stress. These accessions will be useful for aluminum tolerance breeding program. This agrees with the findings of [35]. GCV and PCV were very close in this study, suggesting strong genetic effect for most traits [43]. In control

treatment of most traits, heritability was individually higher. Heritability was high for all traits apart from root length and fresh weight of shoot, meanwhile heritability was moderate for root length. Therefore, aluminum tolerant accessions can be selected based on percentage germination, hypocotyl length and number of roots in cowpea. This is similar to the findings of [28] in maize screened under aluminum stress.

The PCA can be used to identify the most powerful traits, therefore, first two PCs with Eigen values greater than 1 were used to ascertain important traits. All important traits with positive loadings greater than 0.30 in PC1 and PC2 were considered as best. Bi-plot was used for inter-relationships between the traits, and all traits were identified to have selection potential. These traits had high heritability except for fresh weight which had very low heritability. Bi-plot clearly separated highly tolerant (AC06 and AC10) accessions, tolerant (AC03, AC04, AC05 and AC08) accessions, and moderately susceptible accessions (AC09, AC07 and AC02) and the highly susceptible accession (AC01) from one another. Fresh weight of shoot, percentage germination and hypocotyl length were highly positively correlated, while number of roots and root length were also highly correlated according to the bi-plot. The most tolerant accessions were the only vertex accessions corresponding to the important traits sector. PCA and cluster analysis have been used to group genotypes of maize under aluminum stress [28].

## Conclusion

The observed genetic variation in aluminum stressed cowpea accessions could be exploited by hybridisation to establish tolerant lines. High heritability and GAM observed in percentage germination, hypocotyl length and number of roots in cowpea can be exploited for selection. Consequently, selection for these traits would result in genetic gain and breeding progress. Crosses involving the tolerant AC10 and AC06 with the sensitive AC01 and AC09 would contribute positively to improvement programs for aluminum tolerance. Also, this level of genetic variability in the present accessions can be exploited in molecular breeding programs especially in generating population of hybrids for QTL mapping for aluminum tolerance.

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**Araştırma makalesi / Research article**

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# Orta Karadeniz Bölgesinden Toplanan Dactylis glomerata, Festuca arundinacea, Trifolium repens Genotiplerinin Çekirdek DNA İçeriklerinin Belirlenmesi ve Ploidi Analiziyle Tür Teşhisinde Kullanılması

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**ÖZET**

Bu çalışma, Orta Karadeniz Bölgesi doğal florasından toplanan domuz ayrığı (*Dactylis glomerata* ssp.), kamışsı yumak (*Festuca arundinaceae* ssp.) ve ak üçgül (*Trifolium repens* ssp.) popülasyonlarının çekirdek DNA içeriklerinin flow sitometri yöntemiyle belirlenmesi ve ploidi analizi ile tür teşhisinde kullanılması amacıyla yürütülmüştür. Proje kapsamında ak üçgülden mera tipi 80, domuz ayrığından ot ve mera tipi 80 ve kamışsı yumaktan ot, mera ve çim tipi 130 adet genotip kullanılmıştır. Analizlerde tarlada yetişmekte olan bitkilerden alınan taze yaprak dokuları kullanılmıştır. Floresan boya olarak ise propidium iodide kullanılmıştır. İnternal standard, *Dactylis glomerata ve Trifolium repens* genotiplerinde *Vicia sativa*, *Festuca arundinecea* genotiplerinde ise *Hordeum vulgare* kullanılmıştır. Elde edilen sonuçlara göre araştırmada kullanılan *Dactylis glomerata* genotiplerinin ortalama 2C çekirdek DNA içeriği 9.12 pg (8.32 pg-9.66 pg); *Trifolium repens* genotiplerinin ortalama 2C çekirdek DNA içeriği 2.33 pg (2.22 pg-2.44 pg); *Festuca arundinecea* genotiplerinin ortalama 2C çekirdek DNA içeriği 17.47 pg (17.02 pg-17.98 pg) arasında değiştiği belirlenmiştir. Çekirdek DNA içerikleri ile genotiplerin ploidi düzeylerini ilişkilendirmek amacıyla yapılan kromozom sayımlarında domuz ayrığı genotiplerinin 2n=4x=28, ak üçgül genotiplerinin 2n=4x=32 ve kamışsı yumak genotiplerinin 2n= 6x=42 kromozoma sahip oldukları belirlenmiştir. Elde edilen bu sonuçlara göre çalışmada kullanılan *Dactylis* ve *Trifolium* genotiplerinin tetraploid, *Festuca* genotiplerinin ise hekzaploid olduğu saptanmıştır. Ayrıca çalışmada kullanılan tüm genotipler çekirdek DNA içeriklerinin de yardımıyla taksonomik olarak teşhis edilmiş ve *Trifolium repens* ssp.'nin *Trifolium repens var. repens*, *Dactylis glomerata* ssp.'nin *Dactylis glomerata subsp. glomerata* ve *Festuca arundineceae* ssp.'nin de *Festuca arundinacea subsp. arundinacea* olduğu ve genotiplerin içerisinde başka türlere ait hiç bir genotipin bulunmadığı belirlenmiştir.

**MAKALE GEÇMİŞİ**
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**Kabul**
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**ANAHTAR KELİMELER**
*Festuca arundinecea*, *Dactylis glomerata*, *Trifolium repens*, Flow sitometri, Ploidi

# Use in Species Identification by Ploidy Analysis and Determination of Core DNA Contents of genotypes of *Dactylis glomerata, Festuca arundinacea* and *Trifolium repens* Collected from the Central Black Sea Region

**ABSTRACT**

This study was carried out for the purpose to the determination of the nuclear DNA contents by flow cytometry method and its use in species identification with ploidy analysis of the populations of *Dactylis glomerata*, *Festuca arundinaceae* and *Trifolium repens* collected from the natural flora of the Central Black Sea Region. Pasture type 80 from *T. repens*, hay and pasture type 80 from *D. glomerata* and grass, pasture and hay type 130 from *F. arundinaceae* genotypes were used. Fresh leaf tissues from plants growing in the field were used in the analysis. Propidium iodide was used as fluorescent dye. Used *Hordeum vulgare* in *Festuca arundinecea* genotypes and *Vicia sativa* in *Dactylis glomerata* and *Trifolium repens* genotypes as internal standard. According to the results obtained average 2C core DNA content of *Dactylis glomerata* genotypes 9.12 pg (8.32 pg-9.66 pg); average 2C core DNA content of *Trifolium repens* genotypes 2.33 pg (2.22 pg-2.44 pg); average 2C core DNA content of *Festuca arundinecea* genotypes 17.47 pg (17.02 pg-17.98 pg) was determined. Chromosome counts were performed to correlate the core DNA contents with the ploidy levels of genotypes. 2n = 4x = 28 of *D. glomerata* genotypes and 2n = 4x = 32 of *T. repens* genotypes and 2n = 6x = 42 of *F. arundinaceae* genotypes was determined to have chromosomes. According to these results, tetraploid of *Dactylis* and *Trifolium* genotypes and hekzaploid of *Festuca* genotypes were determined to be. In addition, all the genotypes used in the study were taxonomically diagnosed with the help of the core DNA contents and *Trifolium repens var. repens* of *Trifolium repens* ssp., *Dactylis glomerata subsp. glomerata* of *Dactylis glomerata* ssp. and *Festuca arundinacea subsp. arundinacea of Festuca arundineceae* ssp. was founded. It was determined that genotypes did not contain any genotypes of other species.

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## Giriş

Ülkemizin uzun yıllardır çözülemeyen en ciddi tarımsal sorunlarından birisi de, kaliteli kaba yem üretiminin yetersiz oluşudur. Bu durum hayvansal gıda üretim girdilerinin artmasıyla birlikte hayvansal ürün fiyatlarının halkımızın alım gücünün üzerinde gerçekleşmesine sebep olmaktadır. Diğer taraftan küçük üreticiler zarar ettiklerinden hayvanlarını kesime sevk ederek üretim faaliyetlerini durdurmak zorunda kalmaktadırlar. Bu nedenle ülkemizde canlı hayvan ve et ithalatı son yıllarda artmıştır.

Ülkemizde kaliteli kaba yem üretimini artırmanın en önemli yolları; meraların ıslah edilerek üretim kapasitelerinin artırılması ve tarla tarımı içerisinde yem bitkileri ekiliş alanlarının artırılmasıdır. Mera alanlarının ıslah edilmesi ve yem bitkisi ekiliş alanlarının genişletilmesiyle birlikte farklı bölgeler için yüksek verim ve kaliteye sahip farklı yem bitkisi türlerine/çeşitlerine olan talep artacaktır. Ancak ülkemizde tescilli yerli ve milli yem bitkisi tür ve çeşidi sayısı son derece azdır. Bu nedenle ülkemizin farklı bölgeleri

için lokal şartlara uygun yüksek verim ve kaliteye sahip yem bitkisi çeşitlerinin geliştirilmesine yönelik ıslah çalışmalarına hız verilmesi gerekmektedir.

Türkiye'de olduğu gibi, Orta Karadeniz Bölgesi meralarında da yıllardan beri sürdürülen aşırı otlatma ve bilinçsiz kullanım, çayır-mera vejetasyonlarında arzulanan türlerin yok olmasına ve biyolojik çeşitliliğin hızlı bir şekilde daralmasına yol açmaktadır. Ayrıca, bölgenin kıyı kesiminde gittikçe artan yapılaşma sorunu, hem iç hem de kıyı kesim taban meralarında yapılan çim kesme işlemi meraların doğal yapısını bozmaktadır. Zaten hayvanlarımızın ihtiyacı olan kaliteli kaba yemin, ihtiyacı karşılama oranı da yetersizdir [2] . Hem çayır-meraların iyileştirilmesi hem de kaliteli kaba yem üretimi için uygun yem bitkisi türlerinin toplanması, sınıflandırılması ve ıslah projelerine alt yapı oluşturması bakımından gen havuzunun genişletilmesi gerekmektedir [3].

Yabani bitki formları, yeni çeşitlerin geliştirilmesinde kullanılacak en değerli gen kaynaklarıdır. Orta Karadeniz Bölgesi doğal florası, başta yem bitkileri olmak üzere, bitki türleri ve tür içi çeşitlilik yönünden oldukça zengindir [3]. Orta Karadeniz Bölgesi'nde yem bitkileri tarımında ve çayır-mera ıslahında kullanılabilecek doğal vejetasyondan seçilip çoğaltılmış ve tescil edilmiş (Albayrak, İlkadım ve Sultan 1919 hariç) herhangi bir çeşit bulunmamaktadır.

Yem bitkisi çeşitlerinin geliştirilmesinde hedef bölgede yetişmekte olan ekotipler son derece büyük bir öneme sahiptir. Bu ekotipler bölgede uzun yıllardır doğal olarak yetişmekte olduklarından bölgeye iyi bir şekilde adapte olmuşlardır. Bundan dolayı lokal ekotipler, yeni çeşitlerin geliştirilmesinde kullanılabilecek en değerli gen kaynaklarıdır. Fakat yem bitkisi türlerinde ploidy oldukça değişken olduğundan, aynı tür içerisinde bile diploid-decaploid arasında değişim gösterebilmektedir. Bu nedenle doğadan yeni toplanmış olan yem bitkisi genetik kaynaklarının bir ıslah programına dâhil edilmeden önce ploidy düzeyleri mutlaka belirlenmelidir. Aksi takdirde uyuşmazlık kaynaklı ciddi sorunlar ortaya çıkabilmekte ve ıslah projelerinin başarıya ulaşmasının önünde çok ciddi bir engel oluşturmaktadır [12].

Bitkilerin sahip olduğu tüm kromozomlar hücre çekirdeğinde bulunduğundan, çekirdek DNA miktarı ile ploidi düzeyi arasında sıkı bir doğrusal ilişki vardır. Bu nedenle çekirdek DNA içeriği esasına göre ploidi analizi giderek yaygınlaşmaktadır. Önceleri bitkilerde çekirdek DNA miktarları feulgen mikrospektrofotometri ile belirlenmekteydi. Ancak, son

yıllarda, kolaylığı, hızı ve güvenilirliğinden dolayı flow sitometri ploidi analizlerinde tercih edilen bir metot olmuş ve başarıyla kullanılmaya başlanmıştır [12].

Günümüzde en hassas ve güvenilir metot olan flow sitometri yöntemi ile 290 ekotipin/genotipin çekirdek DNA içerikleri ve ploidi düzeyleri ilk defa belirlenmiştir. Ploidi analizi esnasında flow sitometri ile genotiplerin belirlenen çekirdek DNA içerikleri taksonomik teşhislerinin teyidinde de kullanılmış ve genotipler arasında başka türlere ait bitkilerin bulunup bulunmadığı kontrol edilmiştir. Elde edilen sonuçlar, bu ekotiplerin bitki ıslahı programlarına entegrasyonunu kolaylaştıracak ve hızlandıracaktır.

## Materyal ve Yöntem

Samsun Karadeniz Tarımsal Araştırma Enstitüsü Ambarkörü deneme istasyonunda bulunan kamışsı yumak, domuz ayrığı ve ak üçgül genotipleri Tekirdağ Namık Kemal Üniversitesi, Ziraat Fakültesi, Tarla Bitkileri Bölümü, Bitki Genetiği ve Sitogenetiği Laboratuvarına gönderilerek ploidi seviyelerine bakılmış ve kromozom sayımları yapılmıştır.

Daha önce yürütmüş olduğumuz TAGEM /TA/00/11/01/009 nolu ve "Karadeniz Bölgesi Yem Bitkileri Araştırmaları Projesi" kapsamında Samsun, Ordu, Tokat, Amasya ve Sinop illerinden *Dactylis glomerata, Festuca arundinecea* ve *Trifolium repens* türleri toplanmıştır. Toplam 229 populasyon içerisinden üstün performansları nedeniyle seçilmiş olan 80 adet ak üçgül, 80 adet domuz ayrığı ve 130 adet kamışsı yumak olmak üzere toplam da 290 adet genotipin yaprak dokusu kullanılmıştır. Aynı zamanda her türe ait 5'er adet genotipin kök ucu materyal olarak kullanılmıştır.

**Yöntem**

Kamışsı yumak ve domuz ayrığının 70x70 cm, ak üçgül'ün ise 100x100 cm olduğu araştırma alanı killi tınlı, organik madde içeriği orta (%1.23), fosfor içeriği çok düşük (2.00 ppm), kireç içeriği (%6.8) orta, tuz (0.027 µmhos/cm) tuzsuz, potasyum içeriği (55 ppm) yetersiz ve 7.35 pH değerinde olduğu ve hafif alkali bir yapıya sahip olduğu tespit edilmiştir [1].

Toprak analiz sonuçları dikkate alınarak ak üçgüle tesis yılında dekara 5 kg N, 8 kg $P_2O_5$; buğdaygillere ise tesis yılında 5 kg N ve 8 kg $P_2O_5$, 2. ve 3. yıllarda ise erken ilkbaharda 8 kg N olacak şekilde gübreleme yapılmıştır. Temmuz-Ağustos aylarında bitkilerin kuraklık stresini azaltmak için 2-3 kez çok az miktarda yağmurlama sulama yapılmış ve

etkili maddesi Lambda-cyhalothrin olan ilaçla yılda 2 kez mayıs böceğine karşı ilaçlanmıştır. Gerektiğinde yabancı ot mücadelesi yapılmıştır.

**Flow sitometri ile çekirdek DNA analizi (pg)**

Çekirdek DNA analizi için örnekler, Tuna ve ark. (2016) tarafından açıklanan protokol takip edilerek hazırlanmış ve Tekirdağ Ziraat Fakültesi, Tarla Bitkileri Bölümü, Bitki Genetiği ve Sitogenetiği Laboratuvarında bulunan Partec marka flow sitometri cihazı ile belirlenmiştir.

**Yaprak örneklerinin transfer protokolü**

Üç türe ait toplam 290 yaprak örneği Samsun Karadeniz Tarımsal Araştırma Enstitüsü Ambarköprü deneme arazisinden alınarak Tekirdağ Ziraat Fakültesi, Tarla Bitkileri Bölümüne aşağıdaki protokole uyularak gönderilmiştir.

1-Genç ve sağlıklı bitkilerden 50-60 mg ağırlığında yaprak örnekleri alınmıştır,

2-Petri kabına sığacak şekilde 2 tabaka halinde filtre kağıdı hazırlanmıştır,

3-Filtre kağıtlarının arasına yaprak numunesi konulmuştur,

4-Saf su ile petri kabındaki örnek ıslatıp, fazla su dökülmüştür,

5-Petri kabının etrafı iyice kapatılıp, üzerine etiket yapıştırılmış ve numuneyi tanımlayıcı kod verilmiştir,

6-Örnekler, içinde buz kalıplarının olduğu yalıtımlı kutu içerisine konulmuş ve transfer edilmiştir.

Çekirdek DNA analizi 2019 bahar aylarında tarlada yetişmekte olan bitkilerden elde edilen taze yaprak dokuları kullanılarak gerçekleştirilmiştir. Ak üçgül'den 80, domuz ayrığı'ndan 80 ve kamışsı yumak'tan 130 genotip analiz edilmiştir.

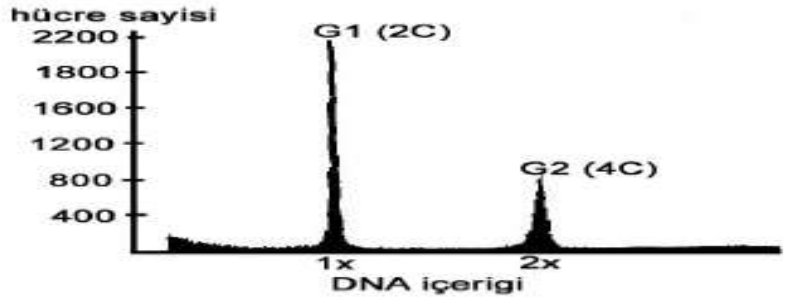
Analizlerde, *Dactylis glomerata* ssp. ve *Trifolium repens* ssp. için standart olarak 2C çekirdek DNA içeriği tarafımızdan 3.65 pg olarak hesaplanmış olan yaygın fiğ (*Vicia sativa*), *Festuca arundinaceae* ssp. için ise standart olarak 2C çekirdek DNA içeriği yine tarafımızdan 10.65 pg olarak hesaplanmış olan arpa bitkisi (*Hordeum vulgare* L.) kullanılmıştır.

**Çekirdek DNA izolasyonu için protokol**

1. Yaklaşık olarak 0.5 $cm^2$ büyüklüğünde taze yaprak dokusu petri kabına konulmuş ve üzerine 500 µl Exraction Buffer ilave edilmiştir.

2. Yaprak dokusu keskin jilet ile 30-60 saniye süresince küçük parçalara ayrılana kadar parçalanmıştır. Bu şekilde hazırlanmış örnek, petri kabı içerisinde hafifçe 10-15 saniye kadar çalkalanmıştır.
3. Çalkalama işleminden sonra 40 saniye kadar petri kabında bekletilen örnek, Partec marka 50 µl CellTrics filtre ile süzülerek tüp içerisine transfer edilmiştir.
4. Tüp içerisine daha önce hazırlanmış 2 ml staining solüyon ilave edilerek hazırlanan örnek ışıksız bir ortamda 30-60 dakika inkübe edilmiştir. Bu sürenin sonunda örnekler flow sitometri cihazı kullanılarak analiz edilmiştir.

Protokolü takip ederek muamele edilmiş bitki dokusu hücreleri mekanik olarak birbirinden ayrılmış, hücre çekirdekleri serbest kalmış, çekirdek zarı buffer tarafından içerdiği bazı kimyasal maddeler ile tahriş edilmiş ve çekirdek zarı üzerinde açıklıklar (delik) oluşmuştur. Propidium iodide bu açıklıklardan yararlanarak çekirdek içerisine girmiş ve nükleik asitlere bağlanmıştır. Çekirdek DNA içeriği arttıkça çekirdek içerisine giren ve bağlanan PI miktarı da aynı oranda artmıştır. Örneklerin içerisinde bulunan hücre çekirdekleri, analiz sırasında lazer ışığı önünden geçerken içerdiği PI miktarı (dolaylı olarak DNA içeriği) ile doğru orantılı olarak florosan ışığı yaymıştır. Yayılan florosanlar cihazın içerisinde bulunan ilgili bölümlerde bir dizi işlemden geçtikten sonra dijital değerlere dönüşmüş ve bilgisayar monitörüne histogram olarak yansımıştır ( Şekil 1).




**Şekil 1** Flow sitometri cihazındaki histogram görüntüsü

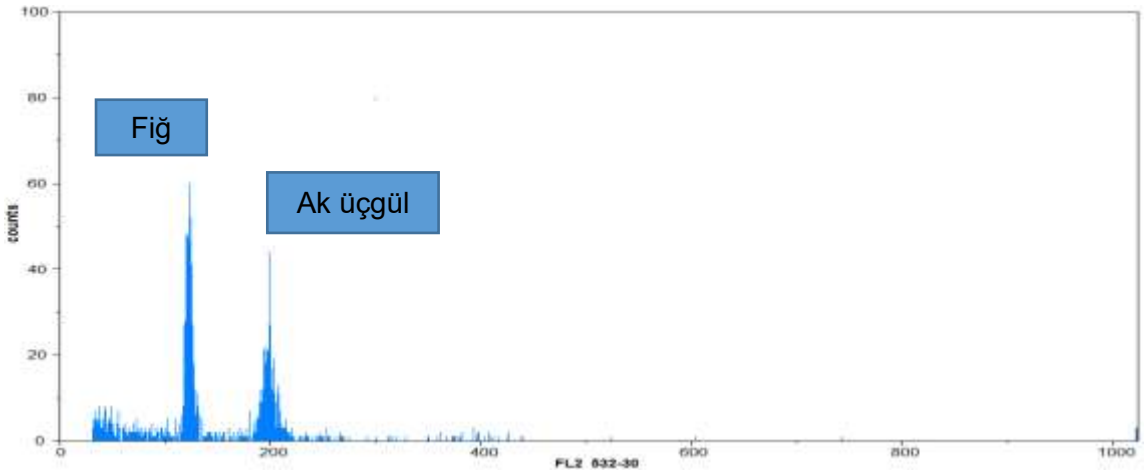
Histogramın dikey ekseni; analiz edilen hücre sayısını, yatay ekseni ise; analiz edilen örneklerin florosan yoğunluğunu göstermektedir. Yatay eksenin sağına doğru gittikçe florosan yoğunluğu dolayısıyla DNA içeriği artmaktadır.

Flow sitometri ile yapılan rutin çekirdek DNA analizlerinde her örnek için yaklaşık 10000 çekirdeğin DNA içeriği belirlenir ve ortalaması analiz edilen örneğin çekirdek DNA içeriği olarak sunulur. Hassas bir analiz için histogram üzerinde bulunan pikler mümkün

olduğunca ince ve uzun olmalıdır. Piklerin şekli flow sitometri cihazını kalibre ederek ve örneği dikkatli hazırlayarak daha kaliteli bir hale getirilebilir.

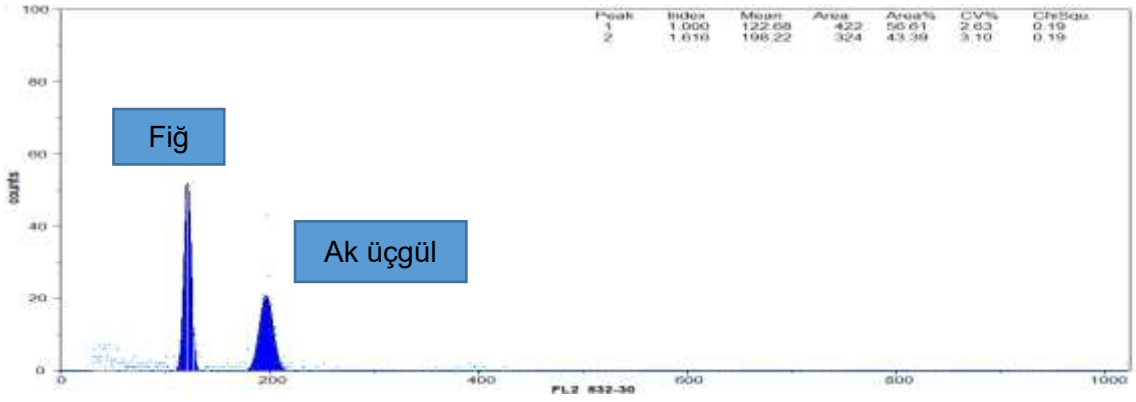
**Flow sitometri ile ak üçgülün çekirdek DNA içeriğinin ölçülmesi ve mutlak değerinin hesaplanması**

Ak üçgül genotiplerinin çekirdek DNA içeriklerinin belirlenmesi; ak üçgül ve standart olarak kullanılan fiğ bitkisine ait G1 piklerinin florosan yoğunlukları kıyaslanarak yapılmıştır. Yukarıda açıklandığı gibi hazırlanmış bir örnek flow sitometri cihazı ile analiz edildiğinde aşağıdaki flow histogramı elde edilmiştir (Şekil 2).




**Şekil 2** Ak üçgül'ün flow sitometri cihazındaki histogram görüntüsü

Cihazın sahip olduğu flow max programı kullanılarak histogram analiz edildiğinde aşağıdaki histograma (Şekil 3) dönüşmektedir.




**Şekil 3** Ak üçgülün flow histogramın flow max programı ile analiz edilmesi sonrasındaki görünüşü

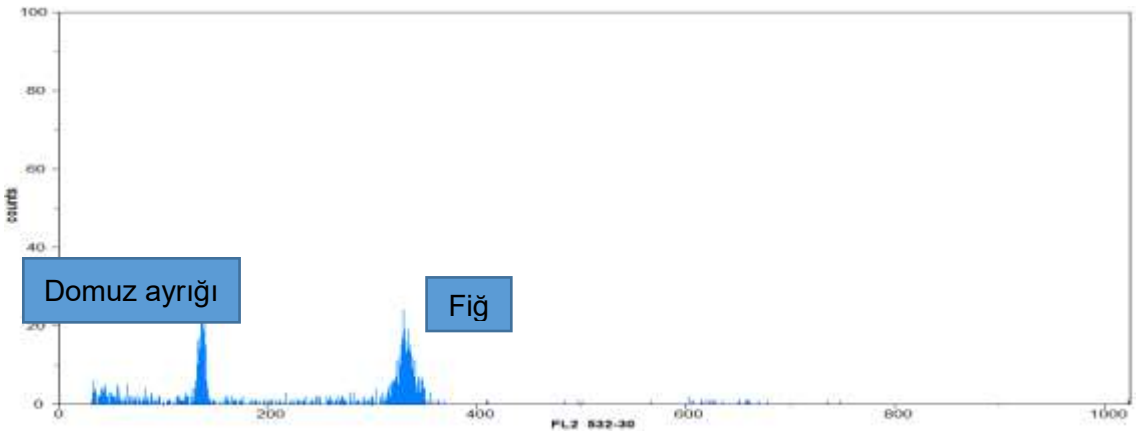
Çalışma kapsamında analiz edilmiş olan her örnek bitkinin ve kullanılan standardın G1 piklerinin florosan yoğunluklarına ait değerler (histogramın sağ üst köşesinde yer alan mean değerleri) kullanılarak aşağıdaki formül aracılığıyla örneklerin çekirdek DNA içerikleri pikogram olarak hesaplanmıştır.

$$\text{Çekirdek DNA miktarı} = \frac{\begin{array}{c}\text{DNA miktarı bilinmeyen türe ait}\\ \text{florasan yoğunluğu}\\ \text{(G1 pikinin değeri)}\end{array}}{\begin{array}{c}\text{Standarda ait}\\ \text{örneğin florasan yoğunluğu}\\ \text{(G1 pikinin değeri)}\end{array}} \times \text{standardın DNA içeriği}$$

Denkleme göre bir genotipe ait çekirdek DNA miktarı 2.25 pg olarak bulunmuştur. Diğer genotiplerinde çekirdek DNA miktarları bu formülle tek tek hesap edilmiş, ortalama çekirdek DNA miktarı tespit edilmiştir.

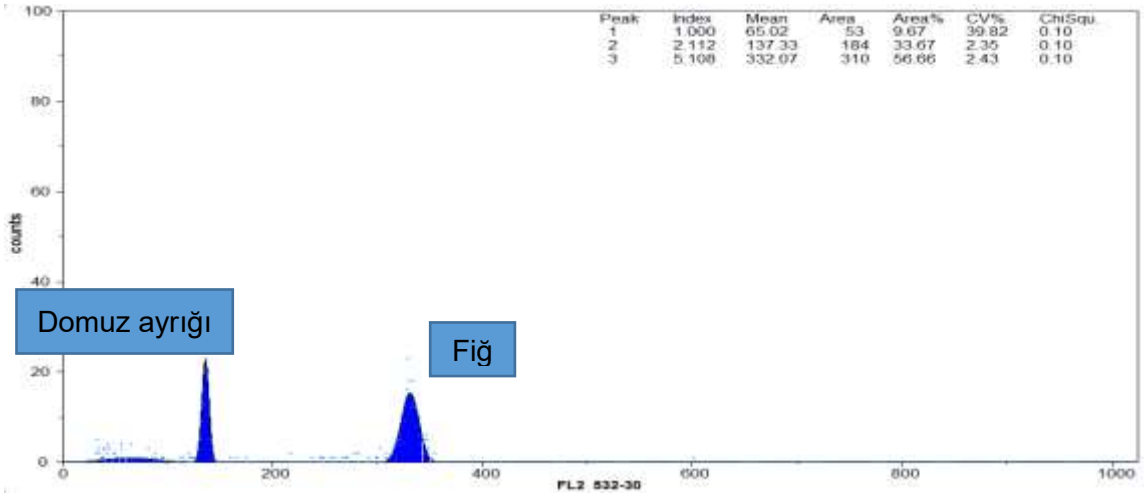
**Flow sitometri ile domuz ayrığının çekirdek DNA içeriğinin ölçülmesi ve mutlak değerinin hesaplanması**

Domuz ayrığı genotiplerinin çekirdek DNA içeriklerinin belirlenmesi; domuz ayrığı ve standart olarak kullanılan fiğ bitkisine ait G1 piklerinin florosan yoğunlukları kıyaslanarak yapılmıştır. Yukarıda açıklandığı gibi hazırlanmış bir örnek flow sitometri cihazı ile analiz edildiğinde aşağıdaki flow histogramı elde edilmiştir (Şekil 4.).




**Şekil 4** Domuz ayrığının flow sitometri cihazındaki histogram görüntüsü

Cihazın sahip olduğu flow max programı kullanılarak histogram analiz edildiğinde histogram aşağıdaki histograma (Şekil 5.) dönüşmektedir.



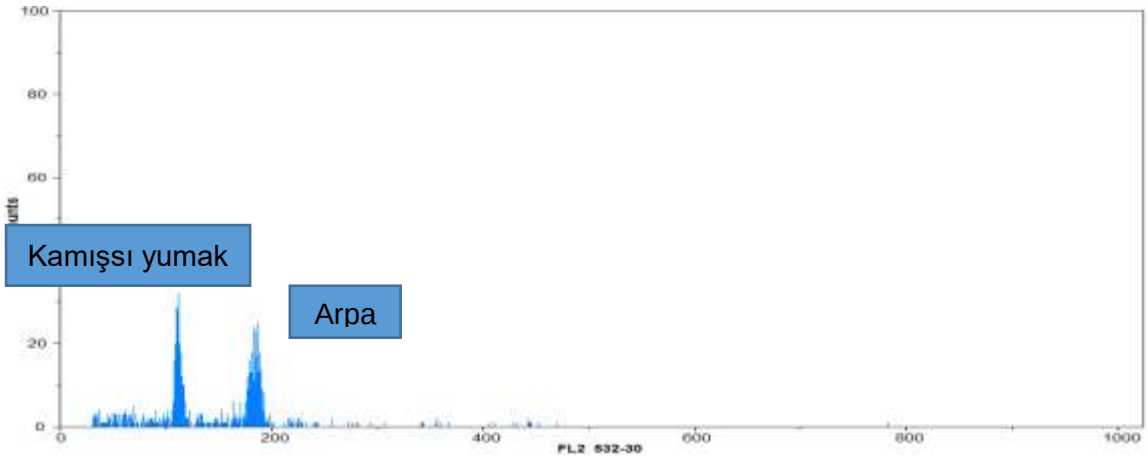

**Şekil 5** Domuz ayrığında flow histogramın flow max programı ile analiz edilmesi sonrasındaki görünüşü

Çalışma kapsamında analiz edilmiş olan genotiplerin ve kullanılan standardın piklerine ait florosan yoğunluk miktarları, histogramda verilmiştir. Verilen mean değerleri kullanılarak "Çekirdek DNA Miktarı" nı veren formül vasıtasıyla, örneklerin çekirdek DNA içerikleri hesaplanmıştır.

Denkleme göre bir genotipe ait çekirdek DNA miktarı 8.83 pg olarak bulunmuştur. Diğer genotiplerinde çekirdek DNA miktarları bu formülle tek tek hesap edilmiş, ortalama çekirdek DNA miktarı tespit edilmiştir.

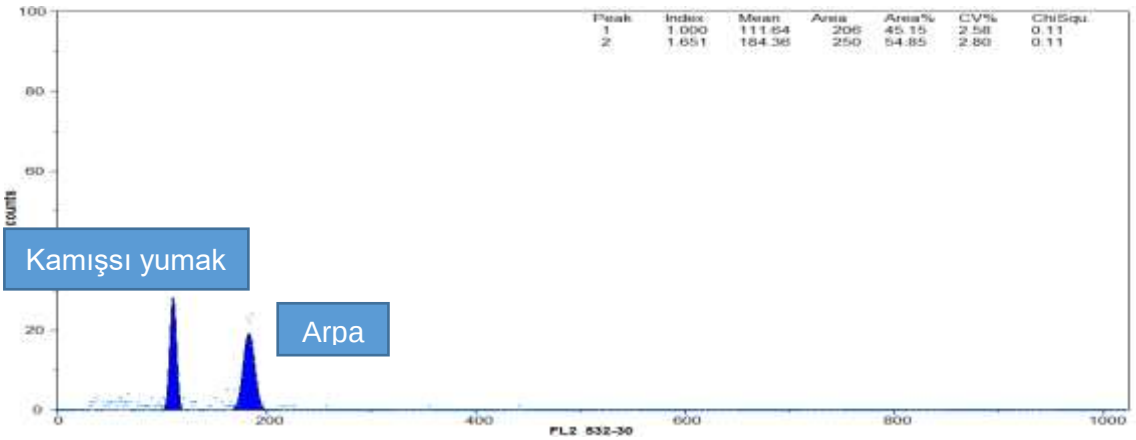
**Flow sitometri ile kamışsı yumağın çekirdek DNA içeriğinin ölçülmesi ve mutlak değerinin hesaplanması**

Kamışsı yumak genotiplerinin çekirdek DNA içeriklerinin belirlenmesi; kamışsı yumak ve standart olarak kullanılan arpa bitkisine ait G1 piklerinin florosan yoğunlukları kıyaslanarak yapılmıştır. Yukarıda açıklandığı gibi hazırlanmış bir örnek flow sitometri cihazı ile analiz edildiğinde aşağıdaki flow histogramı elde edilmiştir (Şekil 6).




**Şekil 6** Kamışsı yumağın flow sitometri cihazındaki histogram görüntüsü

Cihazın sahip olduğu flow max programı kullanılarak histogram analiz edildiğinde aşağıdaki histograma (Şekil 7) dönüşmektedir.




**Şekil 7** Kamışsı yumağın flow histogramın flow max programı ile analiz edilmesi sonrasındaki görünüşü

Bu çalışma ile analiz edilmiş olan genotiplerin ve kullanılan standardın G1 piklerinin florosan yoğunluk miktarları, histogramın sağ üst köşesinde verilmiştir. Verilen mean değerleri kullanılarak "Çekirdek DNA Miktarı" nı veren formül vasıtasıyla, genotiplerin çekirdek DNA miktarları hesaplanmıştır.

Denkleme göre bir genotipe ait çekirdek DNA miktarı 17.58 pg olarak bulunmuştur. Diğer genotiplerinde çekirdek DNA miktarları bu formülle tek tek hesap edilmiş, ortalama çekirdek DNA miktarı tespit edilmiştir.

**Verilerin değerlendirilmesi**

Ak üçgülden mera tipi 80, domuz ayrığından ot tipi 40, mera tipi 40 ve kamışsı yumaktan ot tipi 40, mera tipi 40 ve çim tipi 50 adet olmak üzeretoplam 290 genotip, tür içinde mera, ot ve çim tipi olma özelliğine göre ayrı ayrı tanımlayıcı istatistiğe tabi tutulmuştur. Excel 2016 tanımlayıcı istatistik programı [13] kullanılarak çekirdek DNA içeriklerinin ortalaması, en küçük ve en büyük değerleri ve değişim katsayıları hesaplanmıştır. Ayrıca veriler sadece ilgili olduğu grubun özelliklerini göstereceğinden "ortalama±standart sapma" hesaplanmıştır [6].

**Çekirdek DNA içeriği ile ploidi düzeyinin ilişkilendirilmesi**

Ak üçgül, domuz ayrığı ve kamışsı yumak genotiplerinin çekirdek DNA içerikleri ile ploidi düzeyleri için her türe ait genotiplerden 5'er adet mitotik kromozomlar, mikroskop altında sayılarak ilişkilendirilmiştir.

Kromozom sayımları Samsun Karadeniz Tarımsal Araştırma Enstitüsü Ambarköprü deneme alanından alınarak Tekirdağ Ziraat Fakültesi Tarla Bitkileri bölümüne gönderilen klon materyallerden elde edilen kök ucu meristem dokuları kullanılarak hazırlanan preparatlar üzerinde bulunan, iyi dağılmış ve düzgün morfolojiye sahip kromozomlar sayılarak yapılmıştır.

**Mitoz kromozomlarının sayılması**

Kromozom sayımları kök ucu meristem dokuları kullanılarak Feulgen metoduna göre hazırlanmış olan preparatların ışık mikroskobu altında incelenmesiyle yapılmıştır [6].

**Bitki kök uçlarının eldesi**

Kök ucu hasadı saksılarda yetiştirilmekte olan ergin bitkilerden bahar aylarında sabah erken saatlerde (9:00-10:00) yapılmış ve sadece saksıların dibinde bulunan beyaz görünümlü hızlı büyüyen kök uçları hasat edilmiştir.

**İlk işlem**

Hasat edilen kök uçları 24 saat soğuk su (+4 $^{0}$C) ile muamele edilmiştir.

**Materyalin tespiti**

24 saat soğuk su muamelesinden sonra kök uçları 3:1 alkol: asetik asit solusyonunda tespit edilmiş ve kullanılana kadar -20 $^{0}$C de muhafaza edilmiştir.

**Hidroliz**

Kök uçları 1N HCL ile 60 ºC de hidroliz edilmiştir. Hidrolizin süresi 12 ile 20 dakika arasında olup, türe göre değişmektedir.

**Feulgen boyaması**

Kök uçları hidrolizden sonra, 60-90 dakika Feulgen'de bekletilmiştir. Boyama sonunda kök uçlarının 1–2 mm'lik meristem bölgelerinin koyu viyole rengine boyandığı görülmüştür.

**Preparatların hazırlanması**

Kök uçlarının koyu viyole rengine boyanan kısımları jilet ile kesilerek lam üzerine alınmış ve bistürü ucu ile ezmek suretiyle lam üzerine yayılmıştır. Dağılmış olan meristem dokusu üzerine 1 damla asetokarmin damlatılarak üzerine lamel kapatılmıştır. Düz bir zemin üzerinde lamelin üzerine başparmak ile bastırıldıktan sonra slayt mikroskop altında incelenmiştir.

**Fotoğraf çekimi**

Hazırlanan slaytlar Olympus marka BX 51 model mikroskobuna yerleştirildikten sonra, hücrelerin fotoğrafları 10 x 100 = 1000 kez büyütülerek spot marka Rt Slider model CCD dijital kamera ile çekilmiştir. Morfolojisi düzgün, iyi dağılmış ve kromozom sayısı tam olan hücrelerin kromozomları sayılmış ve fotoğrafları çekilmiştir.

**Taksonomik teşhisler**

Çekirdek DNA analizi sonuçlarına göre her tür taksonomik olarak incelenmiş ve teşhis edilmiştir. Saksılara dikilen bitki örnekleri çiçeklenme dönemlerinde teşhise konu olan kısımları dikkate alınarak herbaryum materyali haline getirilmiş, Avrupa ve Sibirya Florasında (Davis 1985) verilen ilgili cins anahtarları kullanılarak teşhisleri gerçekleştirilmiştir.

## Bulgular ve Tartışma

### Bulgular

Çalışmamızda Orta Karadeniz bölgesinden toplanmış olan *Trifolium repens, Dactyis glomerata ve Festuca arundinacea* türlerine ait toplam 290 genotipin flow sitometri ile çekirdek DNA miktarları ilk defa başarıyla belirlenmiştir. Kamışsı yumak ot, mera ve çim tipi genotiplerin çekirdek DNA miktarları yakın çıktığı için sadece kamışsı yumak başlığı altında incelenmiştir. Benzer şekilde domuz ayrığı ve ak üçgülde de çekirdek DNA miktarları yakın çıktığı için ak üçgül ve domuz ayrığı başlığı altında aşağıda incelenmiştir.

**Ak üçgül (*Trifolium repens)* Çekirdek DNA Miktarları**

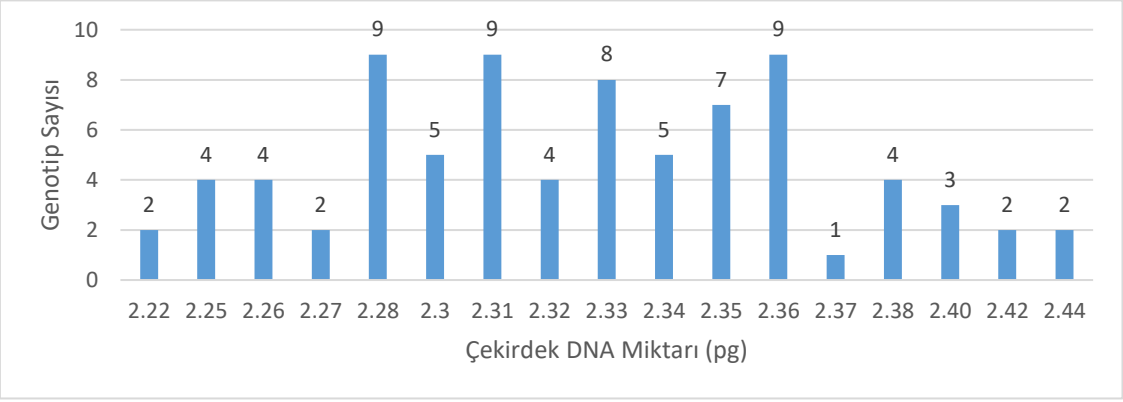
80 adet ak üçgül genotipi analiz edilmiştir. Elde edilen sonuçlara göre *Trifolium repens* genotiplerinin ortalama çekirdek DNA içeriği 2.33 pg, çekirdek DNA içeriklerinin 2.22-2.44 pg arasında değiştiği, standart sapma değerinin 2.33±0.046 ve değişim katsayısının % 2 olduğu belirlenmiştir. Standart sapma değerinin çok küçük olması, ak üçgül genotiplerinin çekirdek DNA miktarlarının birbirlerine çok yakın olduğunu ve dolayısıyla ploidi düzeylerinin aynı olacağı anlamına gelmektedir.

Tanımlayıcı istatistik neticesinde çekirdek DNA içeriklerinin ortalaması, en küçük ve en büyük değeleri, standart sapmaları ve değişim katsayıları hesaplanmıştır (Tablo 1).

**Tablo 1.** *Trifolium repens* genotiplerin pikogram olarak tanımlayıcı istatistik değerleri

| **Tanımlayıcı İstatistik Değerleri** | **Pg** |
|---|---|
| Ortalama Çekirdek DNA Miktarı | 2.33 |
| Standart Sapma | 0.046 |
| Çekirdek DNA içeriği En Küçük | 2.22 |
| Çekirdek DNA içeriği En Büyük | 2.44 |
| Adi Fiğ (standart) Ortalama Çekirdek DNA Miktarı | 3.65 |
| Genotip Adedi (n) | 80 |
| D.K (Değişim Katsayısı) (%) | 2 |

80 adet *Trifolium repens* mera tipi genotipin, çekirdek DNA miktarına karşılık gelen genotip sayısını gösteren sonuç Şekil 8.'de verilmiştir.




**Şekil 8** *Trifolium repens* genotip sayılarına karşılık gelen çekirdek DNA miktarları

38 adet genotipin 2.22-2.32 pg, 8 adet genotipin 2.33 pg ve 34 adet genotipin ise 2.34-2.44 pg arasında değiştiği Şekil 8'de görülmektedir.

*Trifolium repens* ve standart olarak kullanılan *Vicia sativa* bitkilerine ait G1 piklerinin birbirine göre pozisyonları Şekil 2'de, G1 piklerinin flow sitometri paket programı tanımlanmış hali ise Şekil 3'te verilmiştir.

**Domuz ayrığı (*Dactylis glomerata*) Çekirdek DNA Miktarları**

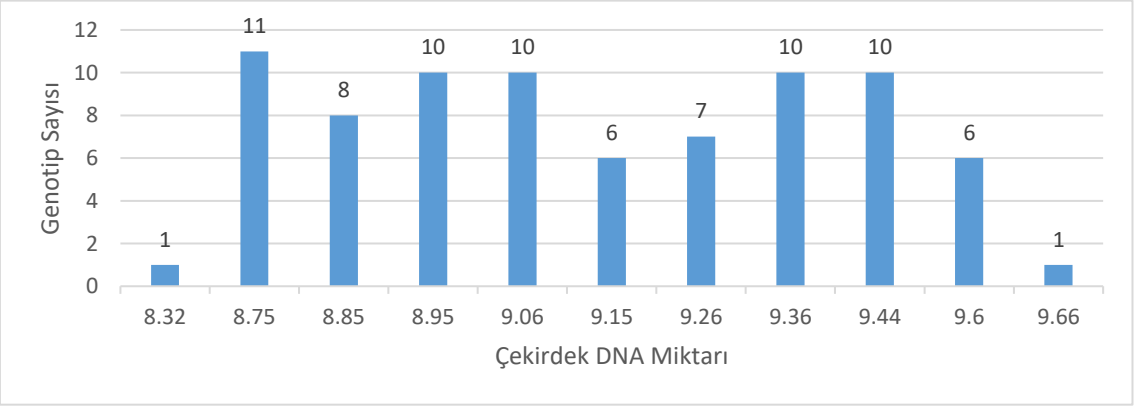
80 adet domuz ayrığı genotipi kullanılmıştır. *Dactylis glomerata* genotiplerinin ortalama çekirdek DNA içeriği 9.12 pg, çekirdek DNA içeriklerinin 8.32-9.66 pg arasında değiştiği, standart sapma değerinin 9.12±0.28 ve değişim katsayısının % 3.1 olduğu tespit edilmiştir. Standart sapma değerinin çok az değişmesi domuz ayrığı genotiplerinin çekirdek DNA miktarının çok yakın olduğunu ve ploidi düzeylerinin aynı olacağı anlamına gelmektedir.

Tanımlayıcı istatistik neticesinde çekirdek DNA içeriklerinin ortalaması, en küçük ve en büyük değeleri, standart sapmaları ve değişim katsayıları hesaplanmıştır (Tablo 2).

**Tablo 2.** *Dactylis glomerata* genotiplerin pikogram olarak tanımlayıcı istatistik değerleri

| **Tanımlayıcı İstatistik Değerleri** | **Pg** |
|---|---|
| Ortalama Çekirdek DNA Miktarı | 9.12 |
| Standart Sapma | 0.28 |
| Çekirdek DNA içeriği En Küçük | 8.32 |
| Çekirdek DNA içeriği En Büyük | 9.66 |
| Adi Fiğ (standart) Ortalama Çekirdek DNA Miktarı | 3.65 |
| Genotip Adedi (n) | 80 |
| D.K (Değişim Katsayısı) (%) | 3.1 |

80 adet *Dactylis glomerata* ot ve mera tipi genotipin, çekirdek DNA miktarına karşılık gelen genotip sayısını gösteren sonuç Şekil 9'da verilmiştir.




**Şekil 9** *Dactylis glomerata* çekirdek DNA miktarına karşılık gelen genotip sayıları

1 adet genotipin 8.32 pg, 78 adet genotipin 8.75-9.6 pg arasında ve 1 adet genotipin ise 9.66 pg olduğu Şekil 9'da görülmektedir.

*Dactylis glomerata* ve standart olarak kullanılan *Vicia sativa* bitkisine ait G1 piklerinin birbirine göre pozisyonları Şekil 4'te, G1 piklerinin flow sitometri paket programı tanımlanmış hali ise Şekil 5'te verilmiştir.

**Kamışsı yumak (*Festuca arundinaceae*) Çekirdek DNA Miktarları**

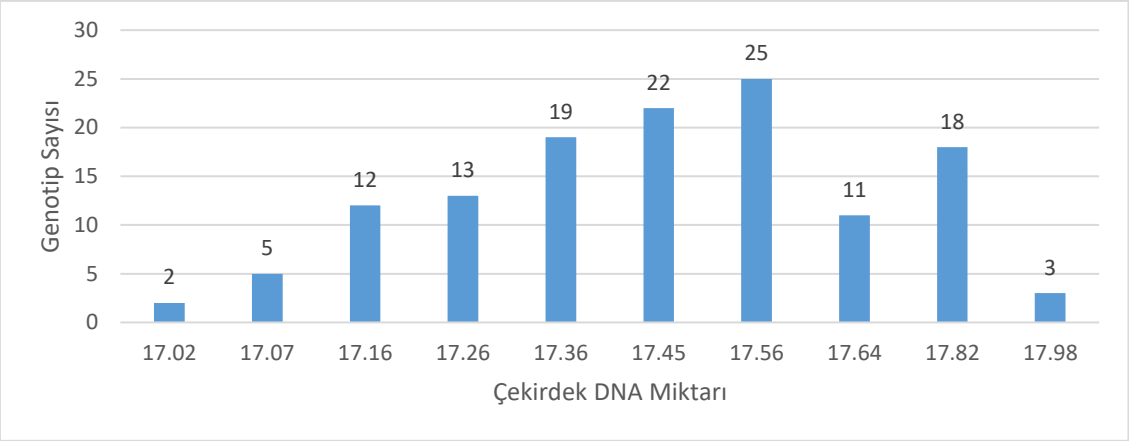
130 adet kamışsı yumak genotipi analiz edilmiştir. Elde edilen sonuçlara göre *Festuca arundinaceae* genotiplerinin ortalama çekirdek DNA içeriği 17.47 pg, çekirdek DNA içeriklerinin 17.02-17.98 pg arasında değiştiği, standart sapma değerinin 17.47±0.23 ve değişim katsayısının % 1.3 olduğu belirlenmiştir. Standart sapma değerinin çok az değişmesi kamışsı yumak genotiplerinin çekirdek DNA miktarının çok yakın olduğunu ve ploidi düzeylerinin aynı olacağı anlamına gelmektedir.

Tanımlayıcı istatistik neticesinde çekirdek DNA içeriklerinin ortalaması, en küçük ve en büyük değeleri, standart sapmaları ve değişim katsayıları hesaplanmıştır (Tablo 3).

**Tablo 3.** Kamışsı yumak genotiplerin pikogram olarak tanımlayıcı istatistik değerleri

| **Tanımlayıcı İstatistik Değerleri** | **Pg** |
|---|---|
| Ortalama Çekirdek DNA Miktarı | 17.47 |
| Standart Sapma | 0.23 |
| Çekirdek DNA içeriği En Küçük | 17.02 |
| Çekirdek DNA içeriği En Büyük | 17.98 |
| Arpa (standart) Ortalama Çekirdek DNA Miktarı | 10.65 |
| Genotip Adedi (n) | 130 |
| D.K (Değişim Katsayısı) (%) | 1.3 |

130 adet kamışsı yumak genotipinin, çekirdek DNA miktarına karşılık gelen genotip sayılarını gösteren sonuç Şekil 10'da verilmiştir.



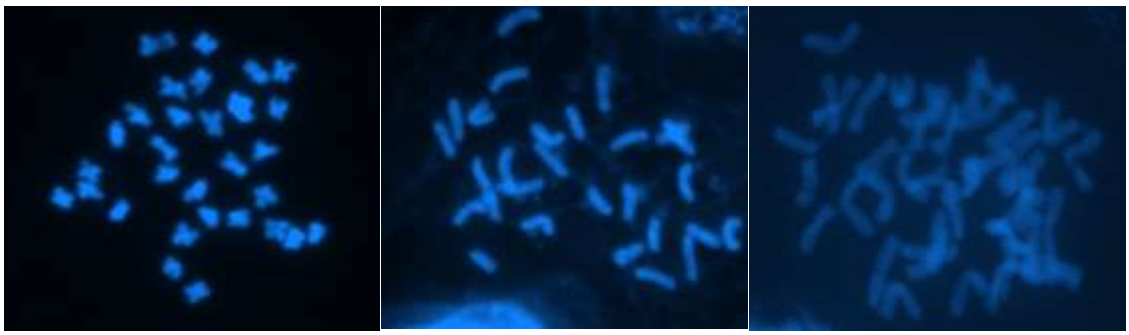

**Şekil 10** Kamışsı yumak genotip sayılarına karşılık gelen çekirdek dna miktarları

51 adet genotipin 17.02-17.36 pg, 22 adet genotipin 17.45 pg ve 57 adet genotipin 17.56-17.98 pg arasında değiştiği görülmektedir.

*Festuca arundinaceae* ve standart olarak kullanılan *Hordeum vulgare* bitkisine ait G1 piklerinin birbirine göre pozisyonları Şekil 6'da, G1 piklerinin flow sitometri paket programı ile tanımlanmış hali ise Şekil 7'de verilmiştir.

**Çekirdek DNA içeriği ile ploidi düzeyinin ilişkilendirilmesi**

Çalışmamızda analiz edilmiş 80 adet ak üçgül, 80 adet domuz ayrığı ve 130 adet kamışsı yumak genotiplerinin çekirdek DNA içerikleri ile ploidi düzeyleri, tür bazında ayrı ayrı ilişkilendirilmiştir. Bu amaçla yapılan kromozom sayımlarında domuz ayrığı genotiplerinin 2n=4x=28, ak üçgül genotiplerinin 2n=4x=32 ve kamışsı yumak genotiplerinin 2n=6x=42 kromozoma sahip oldukları belirlenmiştir. Elde edilen bu sonuçlara göre çalışmada kullanılan *Dactylis* ve *Trifolium* genotiplerinin tetraploid, *Festuca* genotiplerinin ise hekzaploid olduğu saptanmıştır. Genotiplere ait mitoz kromozomlarının fotoğrafları 10 x 100 = 1000 kez büyütülerek Şekil 11'de verilmiştir.



**Şekil 11** *Trifolium repens* (sol) Tetraploid (2n=4x=32), *Dactylis glomerata* (orta) Tetraploid (2n=4x=28), *Festuca arundinaceae* Hekzaploid (2n=6x=42) bitkilerine ait mitoz kromozomlarının görünüşü

**Türlerin taksonomik teşhisleri**

Çekirdek DNA analizi sonuçlarına göre her tür içerisinden 5'er adet genotip taksonomik olarak incelenmiş ve teşhis edilmiştir. Yapılan taksonomik değerlendirmelerde tüm ak üçgül genotiplerinin *Trifolium repens var. repens*, tüm domuz ayrığı genotiplerinin *Dactylis glomerata subsp. glomerata* ve tüm kamışsı yumak genotiplerinin *Festuca arundinacea subsp. arundinacea* oduğu tespit edilmiştir.

## Tartışma

**Ak üçgül (*Trifolium repens)***

80 adet ak üçgül genotipinin ortalama çekirdek DNA içeriğinin birbirine yakın olduğu tespit edilmiştir. Sitolojik incelemeler neticesinde, çekirdek DNA içerikleri 2.22 pg/2C ile 2.44 pg/2C (Tablo 1) arasında yer alan genotiplerin, 2n=4x=32 kromozom sayısına sahip tetraploid bitkiler oldukları belirlenmiştir (Şekil 11). Yapılan taksonomik değerlendirmelerde de genotiplerin, *Trifolium repens var. repens* olduğu teşhis edilmiştir. Araştırmalar neticesinde; Avrasya, Afrika ve Amerika orijinli önemli agronomik değere sahip 31 üçgül türünde çekirdek DNA içeriğinin 0.688 pg (*T. ligusticum*) -7.375 pg (*T. burchellianum*) arasında değiştiği bulunmuştur. *Trifolium* türlerinin çoğunun diploid, çok azının tetraploid ve çok nadir olarakta yüksek ploidi seviyeleri görülmüştür. En yaygın temel kromozom sayısı (türlerin % 80'i) x = 8 olarak tespit edilmiştir [15]. Güney Amerika ve Avrasya orijinli 6 adet *Trifolium* türünde flow sitometri yöntemiyle çekirdek DNA miktarını *T. argentinense, T. polymorphum, T. pratense, T. repens, T. riograndense ve T. medium* türlerinde 0.91 pg-8.58 pg arasında bulmuşlardır [8].

Bu çalışmadan elde edilen sonuçların, daha önce yapılmış olan çalışmaların sonuçlarıyla benzerlik gösterdiği tespit edilmiştir. Kullanılan bitki türleri, kullanılan standartlar, toplanan yer ve yükseltilerin aynı olmamasından dolayı bazı farklılıkların olduğu anlaşılmaktadır.

**Domuz ayrığı (*Dactylis glomerata*)**

80 adet domuz ayrığı genotipinin ortalama çekirdek DNA içerikleri birbirine yakın olup, 8.32 pg/2C ile 9.66 pg/2C arasında değişmektedir (Tablo 2).

Sitolojik incelemeler neticesinde, birbirine yakın çekirdek DNA içeriklerine sahip genotiplerin, 2n=4x=28 kromozom sayısına sahip tetraploid bitkiler oldukları belirlenmiştir (Şekil 11). Taksonomik değerlendirmelerde ise genotiplerin *Dactylis glomerata subsp. glomerata* olduğu teşhis edilmiştir.

Yapılan araştırmalarda; İngiltere'de doğadan toplanan 18 domuz ayrığı popülasyonlarının 17'sini tetraploid (2n=4x=28), 1 tanesi diploid (2n=2x=14) olduğunu, DNA C içeriği olarak 17 tetraploid popülasyonun %28,7'sinin 4,35-5,60 pg arasında değiştiği diploid olanınsa 3,3 pg olduğu [4], İtalya ve Fransa' nın bazı kısımlarında Domuz ayrığının 8 doğal popülasyonuyla yaptıkları araştırmada, yükseklikle DNA C değerleri arasında negatif bir ilişki olduğunu [7], Trakya bölgesi doğal florasından toplanan 57 domuz ayrığı populasyonlarında flow sitometri yöntemiyle çekirdek DNA içeriklerini 9.24 pg/2C-9.9 pg/2C arasında ve ploidi seviyesini de 2n=4x=28 tetraploid olarak [12], Kuzey Slovenya'daki Alplerin güneydoğu kesiminde farklı yüksekliklerde büyüyen beş doğal *Dactylis glomerata* L. (Poaceae) popülasyonunu incelemiş, Ortalama 2C DNA değeri, 8.6 pg, *D. glomerata* subsp. *glomerata* tetraploid (2n=4x =28) olduğunu tespit etmişlerdir [14].

Araştırma sonuçlarının, daha önce yapılmış olan çalışmaların sonuçları ile yakın olduğu görülmektedir. Farklılıkların, kullanılan bitki türleri, kullanılan standartlar, toplanan yer ve yükseltilerden kaynaklanabileceği düşünülmektedir.

**Kamışsı yumak (*Festuca arundinaceae*)**

130 adet kamışsı yumak genotipinin ortalama çekirdek DNA içerikleri birbirine yakın olup, 17.02 pg/2C ile 17.98 pg/2C arasında değişmektedir (Tablo 3).

Yapılan sitolojik incelemelerde, belirtilen miktarlar arasında yer alan genotiplerin, 2n=6x=42 kromozom sayısına sahip hekzaploid bitkiler oldukları belirlenmiştir (Şekil 11). Taksonomik değerlendirmelerde ise genotiplerin *Festuca arundinacea subsp. arundinacea* olduğu teşhis edilmiştir.

Bilimsel çalışmalarda; Değişik Avrupa ülkelerinden toplanan *Festuca* türlerine ait 205 örnekte flow sitometri yöntemi kullanılarak ploidi seviyelerini *F. alpestris 2n =2 x; F. amethystina 2n =4 x; F. billyi 2n =6 x; F. brevipila 2n =6 x; F. carnuntina 2n =6 x; F. cinerea 2n =4 x; F. degenii 2n =4 x; F. duernsteinensis 2n =4 x; F. duvalii 2n =4 x; F. gracilior 2n =2 x; F. lemanii 2n =6 x; F. ovina subsp. guestfalica 2n =4 x; cf.F. ovina × F. pallens 2n =2 x; F. pallens 2n =2 x,3 x,4 x; F. psammophila 2n =2 x; F.*

*pseudodalmatica 2n =4 x; F. pseudovina 2n =2 x; F. rupicola 2n =6 x; F. stricta 2n =6 x; F. vaginata subsp. dominii 2n =2 x; F. vaginata subsp. vaginata 2n =2 x; F. vaginata × F. valesiaca 2n =2 x; F. valesiaca 2n =2x; F. versicolor subsp. versicolor 2n =2x; F. wagneri 2n =4x* olduğunu[9], 101 *Festuca* taksonu ve 14 yakın akrabasının çekirdek DNA içeriklerini flow sitometri ile belirlemiş ve cinsin içerisinde 2C çekirdek DNA içeriğinin 3.88 pg (*F. arvensis*) ile 24.08 pg (*F. gamisansii*) arasında değiştiğini ve ploidi düzeyi diploid (2n=2x=14) ile decaploid (2n=10x=70) arasında olduğunu [10], Doğu Anadolu Bölgesi dağlık bölgelerinden toplanmış olan *Festuca arundinaceae* sp. popülasyonlarında ortalama çekirdek DNA miktarlarını 4.51 pg/2C ile 15.03 pg/2C arasında ve ploidi düzeylerini diploid ile octoploid (2n=14, 28, 42, ve 56) arasında değiştiğini tespit etmişlerdir [12].

Araştırmalar neticesinde elde edilen sonuçların, önceden yapılmış olan bazı çalışmaların sonuçlarıyla örtüştüğü bazılarıyla da örtüşmediği tespit edilmiştir. Örtüşmemesinin sebepleri, kullanılan bitki türleri, kullanılan standartlar, genotiplerin toplandığı yer ve yükseltilerin farklı olması olabilir.

## Sonuç ve Öneriler

TAGEM projesi olan "Karadeniz Bölgesi Yem Bitkileri Islahı" projesi kapsamında sentetik varyete ıslahı yönteminin "kaynak popülasyon" aşamasında morfolojik ve tarımsal karakterizasyonla 2 buğdaygil türüne ait 210 adet ve 1 baklagil türüne ait 80 adet yem bitkisi genotipi seçilmiştir. Seçilen bu genotipler, TÜBİTAK 1002 projesinde kullanılmıştır. Bu çalışma yukarıda belirtilen genotiplerde çekirdek DNA içeriklerinin flow sitometri yöntemiyle belirlenmesi ve ploidi analizi ile tür teşhisinde kullanılması amacıyla yürütülmüştür.

Elde edilen sonuçlara göre, araştırmada kullanılan *Dactylis glomerata* genotiplerinin ortalama 2C çekirdek DNA içeriği 9.12 pg (8.32 pg-9.66 pg); *Trifolium repens* genotiplerinin ortalama 2C çekirdek DNA içeriği 2.33 pg (2.22 pg-2.44 pg); *Festuca arundinecea* genotiplerinin ortalama 2C çekirdek DNA içeriği 17.47 pg (17.02 pg-17.98 pg) arasında değiştiği belirlenmiştir. Çekirdek DNA içerikleri ile genotiplerin ploidi düzeylerini ilişkilendirmek amacıyla yapılan kromozom sayımlarında domuz ayrığı genotiplerinin 2n=4x=28, ak üçgül genotiplerinin 2n=4x=32 ve kamışsı yumak genotiplerinin 2n= 6x=42 kromozoma sahip oldukları belirlenmiştir. Elde edilen bu

sonuçlara göre çalışmada kullanılan *Dactylis* ve *Trifolium* genotipleri tetraploid iken *Festuca* genotiplerinin ise hekzaploid olduğu saptanmıştır. Ayrıca çalışmada kullanılan tüm genotipler çekirdek DNA içeriklerinin de yardımıyla taksonomik olarak teşhis edilmiş ve ak üçgülün *Trifolium repens* var. *repens,* domuz ayrığının *Dactylis glomerata* subsp. *glomerata* ve kamışsı yumağın da *Festuca arundinacea* subsp. *arundinacea* olduğu ve genotiplerin içerisinde başka türlere ait hiç bir bitkinin bulunmadığı belirlenmiştir.

Elde edilen sonuçlar ile türlere ait genotipler arasında herhangi bir karışıklığın olmadığı tespit edilmiştir. Eğer karışıklık olsaydı ıslahın en başında türler içerisindeki genotipler, negatif seleksiyona tabi tutulacaktı. Türlere ait genotiplerde karışıklığın çıkmamasının sebebi, "kaynak popülasyon" içerisinden morfolojik ve tarımsal karakterizasyon ile ümitvar genotiplerin seçilmiş olması olabilir. Yine de her zaman bitkilerin fenotipine bakıp genotipi anlaşılamaz. Çünkü doğal floradan toplanan popülasyonlar, farklı tür ve ploidi düzeyine sahip bitkileri içerebilir. Bu karışıklığı gidermek için flow sitometri yöntemi özellikle morfolojik olarak birbirine çok benzeyen yem bitkilerinin teşhisinde ve ploidi düzeylerinin belirlenmesinde son derece güvenilir, hassas, kolay ve hızlı bir metottur.

Bu araştırmada, ıslah programlarında kullanmak amacıyla Orta Karadeniz Bölgesi doğal florasından toplanmış *Festuca arundinace, Dactylis glomerata,* ve *Trifolium repens* genotiplerinin çekirdek DNA içerikleri flow sitometri ile ilk defa belirlenmiş ve ploidi düzeyleri, populasyonların teşhislerinde kullanılmıştır. Karadeniz bölgesinde çalışılmış öncü çalışmalardan biridir ve bu alanda çalışan araştırıcılar için referans niteliğinde örnek olabilir.

**Teşekkür**
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**Review article**

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# *Lactobacillus acidophilus* and Non-Digestible Carbohydrates: A Review

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**ABSTRACT**

In the recent years, lactic acid bacteria species such as *Lactobacillus* are considering one of the important species of probiotics used in the food processing sector to produce fermented products. It plays a significant role for the transformation and preservation of food. Besides, there is a huge exploration of new molecules that promote health and exhibit potential for technological applications such as non-digestible carbohydrates. The non-digestible carbohydrates provide various health benefits such as balancing and sustaining the microbiota in the intestine and increasing the production of short chain fatty acids (SCFA). The aim of this review is to discuss some aspects of non-digestible carbohydrates as an enhancer for the growth of probiotics. These compounds can help in improving many characteristics of food such as sensory and textural properties.

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## Introduction

Probiotics are live microorganisms and play an important role in the digestive system by keeping the gut healthy by balancing the beneficial microflora [1]. In last two decades, the use of probiotics has been increased significantly because of probiotic's ability in conferring many health benefits to human's digestive system like protecting the host from different harmful microorganisms and making the immune system stronger. Non-digestible carbohydrates are dietary fibers (prebiotics) and cannot be digested and absorbed by the small intestine. Prebiotic is defined as non-digestible food which helps in improving and balancing the growth of the beneficial microflora in the digestive system. Plants are considering the common source of these non-digestible carbohydrates and contain a mixture of polysaccharides which are the integral components in the cell wall of the plants [2]. Many

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researchers proved the ability of prebiotics (non-digestible carbohydrates) in inhibiting the attachment of pathogens to epithelial cells and it is depending on the secretion of the peptides which are responsible in enhancing the absorption of minerals, stimulating the appetite, and preventing from dangerous diseases. In 1995, the term "synbiotic" was introduced by Gibson and Roberfroid which referred to a combination of both probiotics and prebiotics [3]. Consuming synbiotic can stimulate and activate the metabolism of a physiological intestinal microbiota [4]. As a result, using synbiotic can help in overcoming problems related to the survival of probiotics in the gastrointestinal tract. The combination of both probiotics and prebiotics as a single product can lead to great influence compared of using probiotic or prebiotic alone [5]. Thus, current studies are focusing on studying and selecting carbohydrates components which can be utilized by probiotic strains in high levels to produce synbiotic products to enhance the growth of beneficial bacteria in the gastrointestinal tract.

## Probiotics

In the recent years, many scientists explored about the broad advantages of using functional foods as a sustainable product for the health of the beneficial bacteria in the digestive system. Nowadays, lactic acid bacteria (LAB) species are widely used in food industries to produce fermented products. For a long time, many countries use LAB species in food processing industries to produce fermented milk. Species of probiotics are playing a big role in the colonization of the mucosal surface for the gastrointestinal tracts of humans [6]. In many years ago, researchers been working on the isolation of LAB naturally to be used as starter culture to produce fermented food [7]. LAB provide antimicrobial activities against food borne pathogens based on the production of different inhibitory mechanisms like organic acids, hydrogen peroxides, diacetyl and ammonia [8]. Probiotics can stand the harsh conditions such as the salivary enzymes, the acidic environment of the stomach, bile acids and salts that present in the gastrointestinal tract [9]. Probiotics exhibit many health benefits to the host if they are provided in adequate amounts. Studies indicated that probiotic bacteria can regulate the health of the gastrointestinal tract and vagina. Moreover, probiotics provide some essential mechanisms such as the regulation of the intrinsic defenses by increasing the antimicrobial peptide production through paneth cells and the mucin production through

goblet cells, and degradation of the luminal for both antigens and toxins. Probiotics was firstly isolated from many food products [10]. The common species of probiotics are *Bacteriodes, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Ruminococcus, Saccharomyces* and *Streptococcus* [11]. Many studies showed that probiotics can provide positive effects and play an effective role in fighting some of the gastrointestinal diseases such as irritable bowel syndrome, elimination of *Helicobacter pylori*, inflammatory bowel disease, and allergic disease. Also, several clinical studies proved the efficiency of probiotics in treating some diseases like obesity, insulin resistance syndrome, diabetes type 2, and non-alcoholic fatty liver disease [5]. In addition, the common strains of Gram-positive bacteria and yeast used in probiotic products are the genus of *Bacillus* and the genus of *Saccharomyces* respectively [12]. In the few years, there was a significant increase in the production of dairy products that contain probiotic bacteria like fermented milk, baby food, cheese, and ice cream. Commonly, probiotic food produced via fermentation process using fruits, vegetables, and cereals as substrates. The effectiveness of the consumed probiotic food products is depending on the number of active cells that been consumed by the individuals. Thus, increasing shelf-life of probiotics must be maintained during the production of probiotics foods. The addition of *Bifidobacterium* and *Lactobacillus acidophilus* to probiotic foods was reported to increase the nonspecific immune phagocytic activity of the circulating blood granulocytes. A study showed that the intake of yogurt containing a probiotic strain can help in stimulating the production of cytokines by blood mononuclear cells [13].

## *Lactobacillus acidophilus* in food industries

*Lactobacillus acidophilus* is a type of lactic acid bacteria which is capable of fermenting sugars and yield lactic acid as a final product. *L. acidophilus* is a homofermentative, Gram-positive, rod in shape bacteria. This strain can exhibit many useful benefits such as providing thermostability, maintaining the growth activity at a wide pH range, and offering a strong inhibition actions against spoilage of food and pathogenic bacteria. These properties make *L. acidophilus* an important class of bio-preservatives and widely used in many fermented food products. Table 1 shows some common examples of *L. acidophilus* fermented products. Consumption of *L. acidophilus* can help in decreasing the blood cholesterol, reducing the

risk of mutagenicity and carcinogenicity and it can help in decreasing constipation, diarrhea and lactose intolerance. In addition, for a long-time *L. acidophilus* has been introduced into many dairy products, commonly yoghurt and sweet *acidophilus* milk by inoculating *L. acidophilus* into the milk [14]. Furthermore, the mechanism of *L. acidophilus* makes it a good competitor, and able to adhere easily to epithelial cells of the intestine and enhance the immunity and it is identified as nontoxic strain [15]. Moreover**,** there is no difference between *acidophilus* milk and non-fermented but from a nutritional perspective, consuming *acidophilus* milk is more beneficial especially for those who have less lactase (lactose-digesting enzyme). Meanwhile, lactose can be hydrolyzed by *β*-galactosidase in *L. acidophilus*. Thus, it is important to increase the shelf-life of *L. acidophilus* in the fermented products by providing efficient substrates to be utilized fully as an energy source. Besides, final pH and storage temperature are critical factors to be consider for the stability determination and the survival of *L. acidophilus* [8].

**Table 1** Examples of common available Lactobacillus acidophilus products [15]

| **Meat-based products** | **Dairy products** | **Non-dairy products** |
|---|---|---|
| Sausage | *acidophilus* milk | Soy milk |
| Salami | Yogurt | Boza |
| Ground beef | Miru-miru | Fermented tomato juice |
| Dry fermented pork loins | Kefir | Kombucha |

## Non-digestible carbohydrates

The use of prebiotics particularly dietary fibers (non-digestible carbohydrates) attracted many scientists. The dietary fibers can be metabolized by the intestinal microbiota and this will result in the production of short-chain fatty acids. Besides, the mechanism of non-digestible carbohydrates during fermentation can offer an anti-inflammatory and immunomodulatory abilities to treat many pathological conditions [16]. The non-digestible carbohydrates are dietary fibers which are not digested and absorbed by human's small intestine such as cereals, legumes, fruits and vegetables. The categorization of dietary fibers is mainly based on sources, solubility and fermentation properties. Many studies highlighted

about the importance of using non-digestible carbohydrates because of their advantages to human's health such as lowering the blood cholesterol and enhancing the function of the large bowel. Non-digestible carbohydrates are comprising of a carbohydrate polymers which are the components of plant cell walls such as cellulose and hemicelluloses [17]. Moreover, non-digestible carbohydrates are used as energy source for the growth of probiotics based on fermentation in order to stimulate the microbiotas activities and keep the host healthy [18]. Non-digestible carbohydrates can be found in many natural sources such as asparagus, sugar beet, garlic, grains and onion. Generally, non-digestible carbohydrates can be synthesized by different reactions such as hydrolysis, isomerization and fructosyltransfer [19]. The use of non-digestible carbohydrates can help in improving the quality of final products based on the sensory properties, texture, and physicochemical characteristics [20]. Prebiotics (non-digestible carbohydrates) can also regulate obesity by the expression of intestinal anorectic hormones by reducing the number of orogenic hormones [21].

## Sweet potato

Sweet potato (*Ipomoea batatas*) belongs to *Convolvulaceae* family (*Morning glory*). It is one of the important agricultural crops. It supplied with dietary fibers and vitamins and one of the best favourable products because of the various properties it has such as its versatility and its strong resistance to different environmental conditions [22]. In general, the bioactive compounds of sweet potato are metabolized steadily in intestine where there are variety of different probiotic floras, hence these floras are helping in releasing and transforming bioactive compounds and yield bioavailability through fermentation. An analysis was carried and it showed that few individuals were having problems related to digestion and were not able to digest the nutrients in sweet potato. Thus, fermentation of this nutrients by *L. acidophilus* is believed to act as a pre-digestion stage; helping in increasing free form bioactive compounds of the sweet potato and increase the bioavailability by reducing the content of sugar [23]. Since sweet potato is comprising of many carbohydrates, this make it a good substrate to be fermented by *L. acidophilus*. Hence, this leads in releasing high levels of bound bioactive compounds during fermentation along with the formation of free secondary metabolites [24]. Previously, it was reported that sweet potato has the potency to act as prebiotic source and utilized by *Lactobacillus* species based on *in vitro* fermentation

[25]. The amount of dietary fibers in sweet potato are not constant and subjected to the genetic conditions of the crops and the analytical methods used. The main compounds of carbohydrates source in sweet potato root are sucrose, maltose, and glucose and these compounds give the sweetness taste for the sweet potato root [26]. Furthermore, studies on monkey kidney cell (CV-1) indicated that fermented sweet potato can inhibit pheochromocytoma derived cancer cell (PC-12) along with the amount of cytotoxicity to normal [24]. It helps in bringing up a novel technique for the utilization of sweet potato by *L. acidophilus* to improve the anticancer capabilities needed for health.

## Yam

Yam which known as (*Dioscorea spp.*) is one of the high demanding food recently and has a significant role for small and marginal rural families in the world [27]. There are a lot of yam processed products such as pounded yam, boiled yam, fufu, amala, and elubo. Yam has high contents of sugars which are sucrose, glucose, fructose, and maltose. All these sugars are very suitable to be used in microbial fermentation. Furthermore, it also has amounts of polyphenols, allantoin, and diosgenin which contribute many health benefits. For instance, Allantoin is used to protect the skin, while diosgenin provides benefits like antitumor and lipid metabolism [28]. In addition, *Dioscorea* tubers have many nutritious benefits compared to other root crops [29]. Yam is rich with carbohydrates and it is a good source of prebiotics and has bioactive components; if it is administered into probiotic products it will help in increasing and balancing the growth of the microbiota in human's gut [30]. In addition, it contains prebiotic properties since it is rich with polysaccharides; this make the dietary fibers in yam resistant to the hydrolysis of the human digestive enzymes present in the intestine which are specific for glycosidic bonds as shown in Figure1.

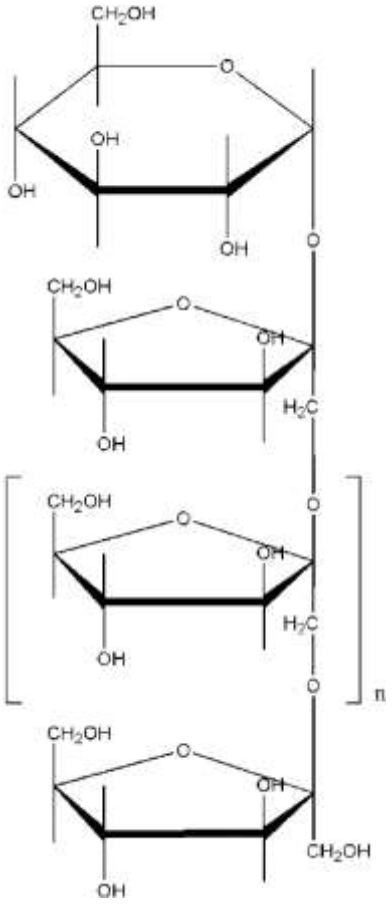


**Fig 1** Structure of inulin, a linear fructosyl polymer linked by *β*-(2,1) bonds [31]

In addition, *Lactobacillus* was capable to ferment yam as energy source *in vitro* and the level of lactic acid produced was 8.1 g/L after 6 h of fermentation [32]. Yam possess many benefits to individuals and it is very useful in the pharmaceutical industries to produce dietary supplements and some cosmetic products. It can also regulate the production of sex steroid hormones [33].

## Barley

Barley (*Hordeum vulgare L*.) is believe to be one of the most important cereal in the world beside maize, rice, and wheat. Barley can adapt easily to any environmental conditions. The great increase of barley use in food area is because of the functional components that barley has which is the *β*-glucans which contribute to many health benefits such as lowering the

level of blood cholesterol and control the glycemic index [34]. The *β*-Glucans in barley forms the water-soluble fraction of many cereals and are stored in the cell walls of the aleurone and subaleurone layer of barley [35]. Figure 2 shows the structure of *β*-glucans in barley.

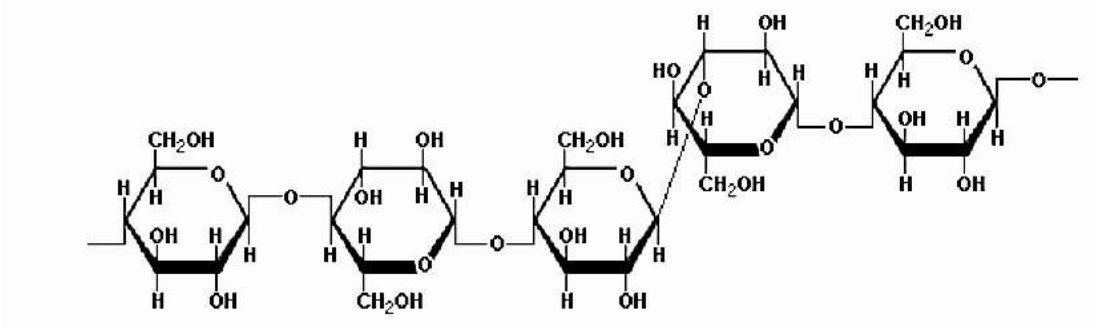

**Fig 2** The structure of *β*-glucans in barley [36]

It was reported that fermentation of barley exhibit a good enhancement for food's quality. Based on previous studies, results showed that fermentation of barley with *Lactobacillus* species possess a great influence on the level of phenolics and *β*-glucan compounds [37,38]. Moreover, the *β*-glucan in barley, was characterized by groups of contiguous (1 → 4)-*β*-linkages and isolated (1 → 3)-*β*-linkages and it was reported that there were positive effects recorded on the health of animals and humans, and this showed the ability of barley in stimulating and modulating the beneficial bacteria in the immune system, pathogen inhibition, anti-tumor and anti-cancer activity, and reduce cholesterol levels [39]. Some of the *in vitro* studies have shown that (1→3) (1→6)-*β*-glucans can improve the activity of macrophages, besides activate the antimicrobial activity for both mononuclear cells and neutrophils and this result helps in making the immune system much stronger due to the increase of pro-inflammatory cytokine levels and chemokine [40].

## Garlic

Garlic (*Allium sativum*) is mainly used as a treatment and as a preventer from diseases across the world [41]. Garlic can promote many benefits and in the past it was used in ancient Egypt as a therapeutic plant to cure some diseases. Garlic allicin is an antibiotic and antifungal which gives the specific smell in garlic. The chemical structure of allicin is shown in Figure 3 [42].

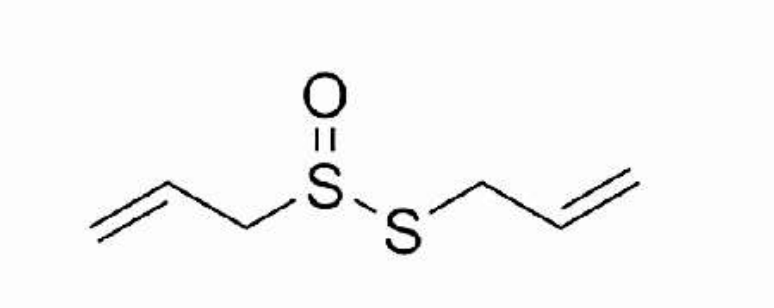

**Fig 3** The chemical structure of allicin [43]

Based on a previous study [42], it was reported that the addition of garlic as a prebiotic source for lactic acid bacteria can enhance its growth and contribute a significant role to improve the growth and activity for some strains in the human's gut. The effectiveness of the antibacterial activity in garlic makes it important in fighting various kinds of Gram-negative or -positive bacteria [44]. Moreover, *Staphylococcus*, *Mycobacteria*, and *Proteus* species were recognized to have high sensitivity towards the antibacterial activity present in garlic, hence this will help in fighting pathogenic bacteria if garlic administered in high levels [45].

## Banana

Banana which known as (*Musa acuminata*) is a common consumed fruit in the world and it is rich with vitamin B6, vitamin C, potassium and resistance starch; whereby individuals not able to digest it in the small intestine thus, it can pass to the large intestine and it produces short chain fatty acids, carbon dioxide, methane and hydrogen in it. The produced fatty acids offer health benefits to human such as reducing the pH of the colon, protect the gut from colon cancer, inhibit the formation of any carcinogenic amines, and enhance the process of feces discharging [46]. For prebiotic properties banana is consisting of 60 to 80% of non-digestible carbohydrates [47,48]. These facts make banana a good source of prebiotic for *Lactobacilli* species. Based on the previous research [49], the addition of banana powder to certain species of gut microbiota was capable to regulate the microflora in the intestine and produced different short-chain fatty acids (SCFAs) through *in vitro* fermentation process after 24 h. Figure 4 demonstrates the chemical structure of the SCFAs produced.

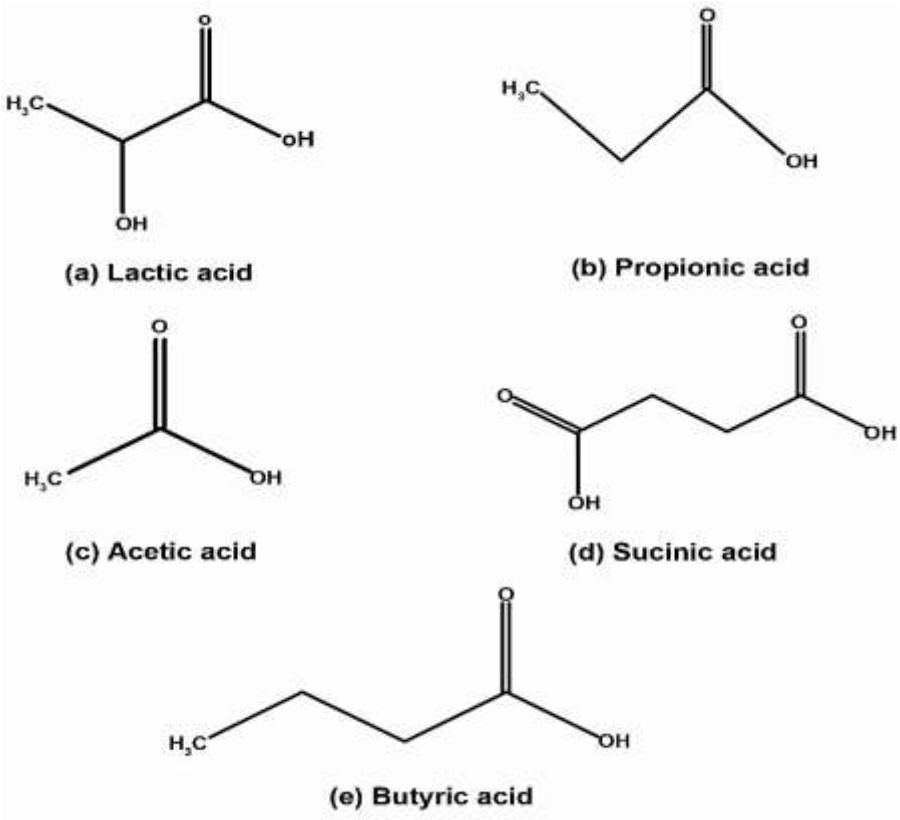


**Fig 4** The chemical structures of SCFA [50]

## Current trends of probiotics and prebiotics

Nowadays, functional food is receiving greater attention by many people in the world. This attention is due to the advances in food technology particularly in food processing sector which offered many positive effects in the manufacturing of processed products that are associated with health benefits. However, some of the functional ingredients can alter the properties of the products such as the texture of food. In addition, the use of probiotics strains and prebiotics is increasing and many studies focusing on producing functional products with better textural properties and able to contribute positive effects to human health [51]. Consumers now are more cautious in purchasing their food products and more knowledgeable about the unhealthy properties or ingredients in food like artificial additives. Therefore, they are trying to look for products that contain more natural and healthy features [52]. In addition, the production of functional foods is offering many benefits like fighting

against common health disorders specifically the chronic diseases such as obesity, cancer, diabetes, and cardiovascular problems [51]. The use of probiotics and prebiotics in the processing of functional ingredients is believed to provides many health benefits and alters the food texture to makes it more safely to consume and beneficial to enhance the sensorial pleasant of the products [52]. Texture in food production is important as it is a sensory characteristic for food products and it plays a role for the perception and enjoyment in terms of food quality. The addition of prebiotics mainly non-digestible carbohydrates into the ingredients of food products can alter the structure of the food because of the fibers content in which can change the textural properties depending on the degree of polymerization (dp) and the chain length, since it is important for the solubility of fibers in water and interaction with other food compounds such as proteins [53]. Moreover, some probiotics strains can change the food texture as well, depending on the production of exopolysaccharides by lactic acid bacteria. The studies that contributed in texture changes by the addition of probiotics can be seen in Table 2. Generally, for the texture alteration of food products it is important to choose strains and prebiotics which are effective for fermentation process such as the use of *L. acidophilus* strain which is commonly used in many food industries.

**Table 2** Effects of probiotics in the texture of food products

| **Study** | **Probiotic strain** | **Food product** | **Effects** |
|---|---|---|---|
| [54] | *Lactobacillus rhamnosus* | Cream cheese | -Influence the firmness due to the acid development and proteolysis activity. |
| [55] | Exopolysaccharide producing lactic acid bacteria | Carrot puree | -Observed texture was associated with the types of produced EPS. -The fermentation using different bacteria strains resulted in changing the product texture. |
| [56] | *Lactobacillus casei 01* | Ice cream | -The ice cream produced had a lower fat destabilization and a huge increase was reported in viscosity, however, the melting time was reduced. |
| [57] | *Lactobacillus rhamnosus GG Bifidobacterium breve* | Panela cheese | -The cheese produced showed greater consumer acceptance based on the compactness, hardness, moisture, and softness characteristics. |

Furthermore, in the past few years, functional food products that contain both prebiotics and probiotics (synbiotic) were highly demanded. Food industries nowadays are trying to develop non-dairy synbiotic products due to the various health benefits that they can provide such as enhancing the growth of beneficial bacteria in the gut. Thus, the addition of probiotic strains into fruit matrices which are rich in carbohydrates and dietary fibers can contribute to provide health benefits to the host. For dairy products, a problem is always occurring; which is the low survival rate of the probiotic bacteria used during storage however, many studies reported about the overcoming of this problem by the addition of non-digestible carbohydrates along with probiotics strains; because non-digestible carbohydrates contain high amounts of fibers which can be utilized by bacteria in order to increase its growth and shelf-life. Consumption of synbiotic food can help in modifying the microbiota composition and metabolic activities in the gut. In addition, it is reported that synbiotic can help in increasing the levels of *Lactobacilli* and *Bifidobacteria*, boosting the functions of the liver, helping in the prevention of bacterial translocation, and reducing susceptibility to pathogens in surgical patients [58]. As synbiotic is a combination of probiotics and prebiotics, therefore it can provide both prebiotic and probiotic effects. Probiotics help in improving the growth of beneficial bacteria, while prebiotics stimulate the growth of a specific group of bacteria present in the gastrointestinal tract. The use of synbiotic is extremely recommended since probiotic without its prebiotic food cannot survive longer in the digestive system. Accordingly, probiotics need an efficient source of prebiotic to be used for their growth and this will result in gaining better intolerance for oxygen, low pH, and temperature. Combining both prebiotics and probiotics into synergy will result in increasing the number of beneficial bacteria [59].

## Future Prospect

The current trends dictated by the increase of interest towards purchasing healthier food which result in offering an opportunity for the use of novel, economy and technological matrices for the improvement of the functional products [60]. These potential heterogeneous matrices are held to be opened to innovate and need evaluation for both efficacy and safety

to ensure the consumer's acceptance. The notable feature in the marketing strategy will be based on the exclusive health claims and the consumer's understanding [61].

## Conclusion

The use of probiotics in the last years has received a great attention to produce fermented products that contribute and offer many benefits to the health. This review has highlighted about some of non-digestible carbohydrates to be used as carbon sources and enhancer to promote the growth of *Lactobacillus acidophilus* and other LAB strains.

**Abbreviations**
LAB: lactic acid bacteria; dp: degree of polymerization; SCFA: short chain fatty acids; h: hours

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**Review article**

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# Human Activities' Impacts on Cave Microbial Diversity: Perspectives for Cave Microbial Diversity Conservation

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**ABSTRACT**

Microorganisms are distributed everywhere even in extreme environments such as caves. The underground surfaces are minerals rich and the life in there is found to be related to both biotic and abiotic factors. Because the cycle of these minerals is insured by the chemolithotrophs living in there. In addition, caves are considered to be important reservoirs of bioactive compounds. However, caves are entered for different reasons. Some of the cavers are of scientific research, some of them are for recreational reasons like sports and simple cave visits. All these activities are thought to impacts the visible mat and invisible colonies of microorganisms through mechanical force or importing exogenous microorganisms. This study summarizes the different impacts which can be caused by the different human activities in caves. Finally, perspectives of the conservation of the cave microbial structure are suggested for the further uses of caves.

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## Introduction

Caves are natural underground surfaces formed under geological, chemical, and biological factors. They are classified in different ways among them the type of rocks and their dissolution ways for the karst caves [1]. Characterized by extreme conditions accounting for high humidity, low stable temperature, restricted nutrients, darkness, and low oxygen, these natural environments contain numerous extreme organisms understudying [2]. Studying cave microbiological characteristics is a challenge for microbiologists because of the difficulties in the accession of the ecosystem, the living conditions which are difficult to be adapted in a laboratory environment as well as the multiple types of caves in the world. Nevertheless, they

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represent an important microbiological studying area, principally due to the adaptation of such microorganisms to the limited resources like light and others.

Caves are used for different objectives such as tourism, sport, scientific investigations, and others. Some caves are epigenic (opened to the earth's surface) and animals can have the opportunities to enter them, water from the rain or other sources can also drip on them. All these activities can impact the cave ecosystems from their abiotic and biotic factors [3]. However, it is also suggested that a limited human interference does not cause an important change in the cave structure and inhabitant populations [2].

Early before 1997, microbiologists started to study microbiology in cave environments [4]. Before the development of molecular dependent methods, these studies were multiplying slowly and only a few microorganisms were identified. After 1997, the apparition of molecular-based methods has boosted cave microbiology and numerous cave microbial importance are identified [4]. Studies found that cave ecosystems could be large reservoirs of drugs, enzymes, fertilizers as well as several human welfare compounds [5]. Therefore, the conservation of cave structure is a challenge that should be tried.

Understanding the cave activities and their impacts on the cave structure and the life in a cave is the main aim of this study. This study provides an overview of the caving impacts on cave ecosystems especially the microbial structure of caves. It also highlights some of the cave conservation protocols and gives further perspectives for cave protection. This review should be of interest mainly to scientists of different fields such as microbiologists, geologists, engineers, and ecologists. Besides, cavers, and institutions/ministries in charge of country environment protection and tourism, are also concerned.

## Cave Microorganisms Inside and Outside the Cave Environments

Caves can be defined as natural openings below the earth's surface that extend beyond the twilight zone and large enough to admit a human being [1, 6]. Cave formations can be through mechanical force, differential erosion and scour, volcanic eruption, rock dissolution, or glacier melting [1]. It is reported that the most types of caves are those forming from calcareous rock dissolution and lava tubes. In caves, secondary mineral deposits formed from a primary mineral by a physicochemical reaction are known as speleothems; one speleothem

can be composed of different minerals or unconsolidated materials [6]. Behind these speleogenesis processes (natural cave formation processes), diverse microorganisms are thought to contribute to the formation and development of caves by different redox reactions even if some of the identified cave microorganisms are still under investigation for their active role in a cave. Microorganisms play an important role in dissolution and precipitation processes which result in the deposition of speleothems and other cave fabrics. They, by their metabolisms, participate in the redox reactions taken inside the caves during the formation of caves and cave structures.

Precipitation of carbonate is well observed in the formation of most caves and cave structures speleothems [6]. This process was thought to be only of abiotic origins. However, the *in vitro* studied through different karsts revealed the biogenic role played by microorganisms [7, 8]. The precipitation of carbonate is mainly depending on the pH of the environment; microorganisms can modify the pH of the living environment through their metabolic activities [8, 9]. This is due to the affinity for calcium/magnesium ions present in the involved microorganisms. Microorganisms take energy from the nitrogenated organic materials, and the adsorption of $Ca^{+2}$ ions together with the production of $CO_2$ and $NH_4^+$ ions are involved with an increase of the pH. The supersaturation of carbonates on the cell or hyphae surface induces their precipitation [6, 8]. Bacteria identified in invitro studies from cave walls and speleothems that involve such precipitation are members of *Proteobacteria* such as *Pseudomonas sp.* and *Acinetobacter*, *Actinobacteria* such as *Streptomyces* and *Kocuria sp.*, and *Firmicutes* such as *Sporosarcina sp.* and *Bacillus sp.* [7, 8, 10, 11]. Precipitation of carbonate is observed in cave walls like in Altamira and Tito Bustillo caves in Spain, in speleothems including moonmilk deposits in Cervo Cave, Italy, and the stalactite formation in Botovskaya Cave in Siberia [8, 9, 12, 13]. Other bacteria and microorganisms were isolated or identified through metagenomic analyses from such carbonate precipitation cave formation areas are still under investigation for their role in these environments.

Cave and cave structures can also be formed or be under mineral dissolution processes. Limestone, dolomite, and gypsum rocks can be dissolved by the action of underground water or the stream dripping on rocks. The weak sulfuric acid, which is one of the most implicated acids in speleogenesis, has been reported to cause the dissolution of Limestone and

precipitation of gypsum which is more soluble in water [6]. The sulfuric acid formation is thought to be originated from sulfur-oxidizing bacteria present in the sulfur-rich cave environment [14, 15]. The mechanism of this process in the cave bacteria still under investigation but studies state that the sulfur-oxidizing bacteria like *Thiobacillus spp.* and *Thiothrix* both members of *Proteobacteria*, isolated from different caves such as the Lower Kane Cave, can oxidize $H_2S$ completely to sulfate during their metabolic activities [6, 16]. The dominance of bacterial group belonging to *Epsilonproteobacteria* in the Lower Cave in Wyoming was observed [16]. The authors added that even these bacteria remained unculturable, other culturable bacteria belonging to this class were characterized by sulfur/sulfide oxidation. Other studies of the chemical reactions in caves that involve microorganisms, also include iron-oxides, nitrification, ammonification and, manganese oxides [6,17,18]. All these precipitation/dissolution processes involve primary the chemolithotroph bacteria, fungi, and sometimes archaea from different phylum and species, acting in a biofilm structure [12, 15, 18].

Microbiologists are most attracted by the adaptation of microorganisms in the extreme environmental conditions of the caves. Different studies revealed the application and the possibility of the application of cave microorganisms for human welfare. Antimicrobial activities are observed in bacteria and fungi isolated from different caves. The antimicrobial substances which can be isolated from these microorganisms, far from the human impacts, could be favorable in the actual drug-resistant world problem. The most antimicrobial compound producers are *Streptomyces* sp., belonging to the phylum *Actinobacteria* [5, 20]. These bacterial members are mainly studied in caves and favorable results were obtained [20, 21, 22, 23]. Peptide A12-C, cervimycins A-D, undecyprodigiosin, and xiakemycin A are some of known antimicrobial and anticancer active products isolated respectively from bacterial strains from Cueva de Los Murciélagos in Spain, Grotta dei Cervi in Italy, Miroc Mountain Cave in Serbia, and a karst cave in Chongqing city in China [21]. Besides the medicine potentials, cave microorganisms were observed with a construction capacity. As previously seen, some bacteria from cave walls and speleothems are calcifying organisms. These microorganisms were determined to be used in the construction of bio-concretes, crack healing in concretes, and soil bioremediation. In addition, diverse enzyme activities revealed

in cave isolates can serve industrial uses with low energy spending [11, 22, 24, 25]. Wastes bioremediation potentials were detected also in cave isolates [5, 25].

## Cave Activities (Caving)

We can define caving as the entrance to cave environments for different reasons. Studies show that even in the Paleolithic period, humans have used these environments [21]. They were used for different uses such as housing, obtention of minerals, protection against weather phenomena, and interment [21]. Using the cave as burial chambers was a common human practice over the world. The Easter Island Anthropological Expedition revealed the uses of the caves located on the south coast of this island as human burial chambers [26]. Another study in the Adıyaman province of Turkey shown the presence of tombs as a part of the complex cave of Necropolis [27]. Authors have also found some proofs shown the use of this system of caves as a storehouse and other economic tasks. In Africa, where caves have been considered sacred places, it is found that ritual and religious practices were common in these underground surfaces. In Zanzibar island, the Kumbi limestone cave are one of the most destroyed natural environments. Human skeletons were found inside that cave and is the proof that cave was also used as a human burial chamber in this part of Tanzania [28]. On other hand, studies revealed that caves were thought to be used for treasure hiding by pirates and others [29]. Thereby, caves were the targets of treasure hunting. It is the case of the Yarımburgaz Caves in Turkey which had inhabiting by the people of 'Homo Erectus' 400 thousand years ago [29, 30]. Similarly, the Ayub Cave in the Philippines was severely damaged by illegal diggers who were treasure hunters [31].

Nowadays, caving or cave activities are improved since cavers are for different reasons. However, even those named as vocational cavers, who enter caves for their professional tasks, all cave activities can turn to recreational caving [32]. Depending on the caving distance and caving reasons, cavers can spend some hours or days inside caves. Therefore, the installation of some camps inside caves can be imperative for cavers. It is revealed that cavers started to establish camps inside caves or near the entrance of the cave in the early of 40s by US cavers the National Speleological Society, Inc. [33]. In general, as deeper, they go camping remains inevitable. Because after exhaustion of cavers, it is difficult to return

upstairs the same day. Sometimes, as the deeper they go, they should avoid handling heavy bags, so they should have a camping site to leave somethings like exceed foods and drinks [33]. However, for the conservation of cave natural quality and avoid cave diversity disturbance, cavers should be careful in front of the camping site as well as the hole cave.

The natural formations of the subterrestrial surface contribute to the history of different regions. Even if their environments are considered as magics and attractive, caves remain hardly accessible by most scientists. For mapping caves and providing more information about them, cavers enter with high curiosity to discover the inside magical world of these underground openings. The features of caves provided by cavers can facilitate to answering questions and solving problems in archaeology, geology, geomicrobiology, paleontology, hydrology, mineralogy, etc. [34].

Geochemists take advantage of cave rocks to investigate and enlighten in the geochemical structure of the regions. As previously seen, caves are formed by the combination of geological processes and other abiotic and biotic factors. Studying a cave can answer many questions, for example, the probable future geologic process in a region. In addition, the chemical aspects of a cave rock define the mineral sources founding in that cave. For the history of a region, paleontologists and archeologists can use caves where they found ancient used materials or human/animal skeletons [35]. On the other side, microbiologists and biologists are interesting in the adaptation of organisms in such extreme environments, explore to understand the different metabolisms undergoing these habitats. Therefore, the redox reactions that take place in caves, by microorganisms, are thought to contribute to the development of substances that could be used in different fields including clinical, pharmaceutical, bioremediation, food bioprocessing, and water sanitization [20, 36]. For this context, microbiological investigations in caves are multiplied over the world.

For economic uses, some caves are also opened for tourism. Cave tourism constitutes the main geological tourism in the world [37]. In many countries, the show caves are among their main economic revenues. We can enumerate the Rouffignac Caves in France opened in 1959 and visited by up to 500 visitors per day [38]. Further, more than 250,000 of visitors were reported to enter the Gongo Cave, located in the Pacitan regency in Indonesia, per year which contributes to more than 200,000 US$ per year in this regency economy [39]. Most of

the cave tourists are attracted by the natural aspect of caves. They are curious to discover new natural areas, especially geological formations. However, for further tourism attractions and facility of cave accession, humans make some improvements such as the addition of artificial light of different waves and construction of artificial walking paths. Additionally, the tourism feature of the cave can be for health rather than for recreation. It is reported that 8.5% of the Ballıca Cave (Turkey) tourists are of health tasks including asthma treatments [37].

Cave sports activities are of different levels depending on the experience of the cavers. They are generally considered extreme sports because of the dangerous aspects of some caves [49]. Cave diving is generally practiced in active caves that are filled with water. Some show caves consist of diving practices like the Dordogne valley in France [50]. However, cave diving expeditions could be for research goals: an exploration of new underground caves as well as studying these environments in different scientific fields. Divers sampled visible colonies of *Thiothrix spp*, identified in six of eight underwater caves in Florida for microbiological studies of these caves which revealed that this sulfide oxidizing mixotrophic bacteria play a role in cave formation [40].

It is reported that industrial uses in caves even if they are occasionally [41]. However, this field, which is not developed, consists of specialized forms of agriculture including mushroom farming, fish breeding, and cheese production.

## Are Cave Activities Impact Cave Microbial Diversity?

Caves and Earth's surface lands are quite different in their physicochemical characteristics. The impacts of caving on the cave ecosystem depend on the type of cave. As previously seen, speleogenesis is from different processes therefore some caves are constituted with strong rocks and typically dried. Some others are fragile, and present rivers or stream waters can drip on there. Further, the diversity of microorganisms depends on the abiotic factor of their environments [4, 42]. Here, we adopt the hypothesis which stands that each cave is unique, and speleology should be specific for a cave. Although the impacts of caving cannot be generalized, the probable cave damages especially cave microbial diversity threatening are illustrated by taking all the perspective of human activities in caves and cave types.

Limit of organic matter, stable temperature, high humidity, and low or total darkness are the best environmental conditions for the biodiversity of these ecosystems. However, diverse external factors can affect these sensitive conditions thereby impacts on the life of the biogenic part of caves as it demonstrated by [38]. Microorganisms are among the main cave biodiversity and they can appear as visible mats as well as invisible biofilms on cave walls, sediments, and pools [4]. Humans during their cave visits can bring to the cave nonnative microorganisms that can compete for the indigenous ones in such oligotrophic environments. They can introduce these microorganisms through their dresses, shoes, or by touching cave structures with their body or septic materials. Draws and human finger marks were reported respectively on Azorean lava tubes and Yarımburgaz Cave [30, 43]. The authors stand that a limited microbial development was observed in these areas in two decades [43]. This might be caused to the mechanical movement which leads to the removal of the native colonized microbes while the imported microorganisms did not survive under the extreme conditions of the cave.

It is suggested that the *ex-situ* microorganisms could not persist in such an environment. It is also shown that the endemic species can mitigate the introduced organic matters through bioremediation [44]. However, cavers can leave organic matters like hair, nails, the rest of their foods, or their excreta inside caves. These organic inputs can only be favorable to exotic microorganisms. The study of human contamination in Lechuguilla Cave was run, and authors found that *Staphylococcus aureus,* which is a human skin flora, *E. coli* which is a normal human intestinal flora, and high temperature *Bacillus* which are found in soils heated by sunlight, are increased during highly visit levels and reduced when the same areas are at rest from visit [45].

Besides that, lamps used by cavers during cave explorations or other activities are unfavorable for the cave living organisms, since caves are divided into four zones where the light is limited or completely absent. At the cave entrance, still there is light since the surface and underground environments meet on there. The light progressively diminishes to zero at the twilight zones and it is absent on the transition zones. The last zone consists of a completely dark zone where the relative humidity is high, and the temperature is nearly stable continuously [41]. Light providing by cavers together with the high humidity of the cave

environment will promote the development of phototrophs like algae which are generally grow at the limit of light penetration [41]. The development of such organisms affects not only the cave microorganisms but also the cave and speleothems that are forming by the interaction of native microbes and cave minerals.

The uncontrolled visit of humans in a cave or a long camp period inside caves can induce an increment of carbon dioxide level inside these environments. It is suggested that the high level of $CO_2$ detected in show caves, where the bat population is limited, is due to the huge number of visitors that receive those caves [39]. It is stated that the high concentration of $CO_2$ can inhibit mineral deposition in caves [46]. In this fact, possible lowering of cave chemoautotrophs can be noted. The disappearance of such primary producers can directly impact the other organisms of the same ecosystem. Furthermore, human explorations of caves can lead a temperature variability inside these underground surfaces and most changes are observed in show caves [39, 47]. In such a known thermostable environment, the direct impacts of a thermal change on the cave species are not yet determined. However, an adaptation of exogenous thermotolerant soil bacteria is probable if the air temperature of these environments did not return stable after few hours [45].

Even it is difficult to study the microbial structure of the cave ecosystems, its disturbance appeared to be easier because of their minuscule and invisible sizes. While walking, climbing, or other mechanical force can compress the stones and further reduce the oxygen for the organisms living in the sediments or the cave surfaces. The decrease of humidity in Altamira Cave opened to tourists was reported to be from 90% to 75% [47]. Such decrease contributes to the drying of the cave sediments and surfaces: that is one other factor that induces the threat of cave microbial diversity.

## Perspectives for Cave Microbial Diversity Conservation

Although without human investigations the positive and negative impacts of Nature remain in the dark, the conservation of these natural resources should be studied before any activity. For the life of caves as well as the continuity of industrial and biotechnological natural resources, we should elaborate a protocol of cave using. Some countries through their caver

associations have already established laws and conditions necessary for the conservation of such environments. for the protection of the microbial diversity of the cave, we suggest that:

- Show caves should be limited to a few numbers of visitors per day and per hour.
- Cave managers should avoid artificial improvement inside the caves.
- Tourism in the cave should be for the nature of caves since every cave is unique, every cave has the own characteristics, so no need for artificial improvement of caves.
- Education about the importance of caves and cave microbial diversity should be exposed to people through conferences or other education systems.
- Collaboration between cave managers, cavers, and scientists, especially microbiologists: if any new formation in a cave is observed or a new cave part is expedited, new microbial diversity should probably be discovered.
- Cavers or anyone enter the cave environment should be conscient to never leave any waste inside the cave. Wastes should be put in bags and bring out of the cave.
- Show caves as well as those that are opened for only scientific research should have a rest period in which cave microbial diversity can process into the bioremediation of the cave.
- Every cave should be used as a specific fortune and avoiding bad habits, like film capture especially historical and cultural films, which contribute to the further destruction of caves since further constructions inside the caves are processed (e.g. the 'YOR' in the 1980s inside the Yarımburgaz Caves) [30].
- Pools, rivers, and other water sources founding inside the caves should not be accessible for cave visitors, despite sampling for scientific studies. For further sampling protocols, you can visit and read more in [48]
- Camps installed by scientific groups should take small places as much as possible and new technics used for the research should be improved in a manner to spent as much as possible a few times inside the caves.
- During caving, cavers should as much as possible change or wash their caving clothes and boots frequently, especially when they move from a cave chamber to another one.
- We should be careful and conscient that every touch or walk step is susceptible to disturb the invisible microbial cave structure.

- Uses of fire for warm-up or using cigarettes inside the cave should be banned since they are a source of the $CO_2$ increase, organic matter input as well as toxic matter input.
- Encouraging the microbiology studies of caves since few of them are explored until now. This can be by the multiplication of caver associations in countries which should collaborate and dividing the research through the different caves present in their countries.

## Conclusion

Caves are extreme environments where the life of microorganisms is important for the formation of cave structures and the continuity of life in a cave. These microorganisms are found to be sources of the most important industrial and biotechnological products. Caving is essential for the expedition of such natural environments. However, cave activities have impacts on the living organisms present in such ecosystems. For protecting and conservation of the natural feature of caves, visits to caves should be limited to a specific number of visitors per year and artificial improvement of caves should be avoided. We can say that where the human being has not been yet, remain obscure but unfortunately the disturbance is more likely to happen where he is.

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**Derleme makale / Review article**

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# Plastik Biyodegradasyonu: Genel Sorunlar ve Biyoteknolojik Çözümler

**Dilara Özden**[1]

**ÖZET**

Plastik kirliliği tüm dünyayı etkilediği gibi Türkiye'de de giderek artmakta olan çevresel bir sorundur. Plastik atıklarının uzaklaştırılması için kullanılan yöntemler yetersiz olup farklı çevresel sorunları beraberinde getirmektedir. Bu nedenle çevre dostu ve etkili bir yöntem geliştirilmelidir. Mikroorganizmaların plastik yüzeylerinde üreyebilmelerinin ve enzim aktiviteleriyle plastik biyodegradasyonu gerçekleştirebilmelerinin görülmesiyle, plastik kirliliği için mikroorganizmaların kullanılması potansiyel bir çözüm olarak görülmüştür. Birçok mikroorganizmanın plastik biyodegradasyonu gerçekleştirebildiği ve birçok enzimin biyodegradasyon süreciyle ilişkili olduğu çalışmalarla gösterilmiş olsa da plastik kirliliğine karşı biyodegradasyon etkili bir yöntem olarak kullanılamamaktadır. Biyoteknolojik yaklaşımlar ile biyodegradasyon iyileştirilerek sürecin hızlandırılması ve plastik kirliliğine karşı kullanımının yaygınlaşması amaçlanmaktadır. Bu derlemede mikroorganizmaların genel plastik biyodegradasyon mekanizmalarının yanı sıra, plastik biyodegradasyonunda görülen sorunlar ve sorunların iyileştirilmesi için biyoteknolojik yaklaşımların kullanımı ele alınmıştır. Biyodegradasyon için mikroorganizmaların bir arada kullanılması, biyosürfaktanların etkisi, genetik mühendisliği yaklaşımları, metagenomiks ve biyoinformatik yaklaşımları üzerinde durulmuştur. Önemli gelişmeler ve çalışmalara yer verilmiştir. Türkiye'de gerçekleştirilen çalışmalar ve plastik kirliliğine karşı yapılan araştırmalar da değerlendirilmiştir.

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# Plastic Biodegradation: General Problems and Biotechnological Solutions

**ABSTRACT**

Plastic pollution is a significant environmental problem. The damage caused by plastic pollution to the environment is increasing all over the world, as in Turkey. Plastic waste management methods are insufficient, and they cause different environmental pollution problems. Therefore, there is a need for an effective and environmentally friendly method. With the discovery of the microorganisms that can grow on plastics and degrading plastics enzymatically, plastic biodegradation became a potential solution for plastic pollution. Although there are lots of microorganisms and enzymes which are responsible for plastic biodegradation, the biodegradation process is not efficient to use against plastic pollution problem. Biotechnological approaches are used to improve efficiency of plastic biodegradation process and increase the usage. The general mechanism of plastic biodegradation, disadvantages and biotechnological approaches based on those problems were discussed in this review. Plastic biodegradation by microbial communities, effects of biosurfactants, genetic engineering approaches, metagenomics and bioinformatics approaches are emphasized. In addition, researches performed against plastic pollution in Turkey were evaluated.

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## Giriş

Plastik kirliliği bütün dünyayı ilgilendiren önemli çevresel sorunlardan biridir. Türkiye'de 2010 yılında günlük üretilen plastik atığı kişi başına 0,21kg; üretilen yıllık plastik atık ise 5,6 milyon tondur. Akdeniz'de 2013 yılında deniz yüzeyinde bulunan plastik atığı 23,150 tondur [1]. Plastik kirliliğinin etkileri Türkiye'de artan tüketim ve üretimle beraber giderek artmakta ve görünür hale gelmektedir. 2018 yılında dünya genelinde açığa çıkan plastik atığı 359 milyon tondur [1,2]. 2019 yılında üretilen plastik, Avrupa'da 57,9 milyon ton, dünya genelinde ise 368 milyon tondur [3]. COVID-19 salgını dünyada maske, eldiven gibi tek kullanımlık plastiklerin kullanımının artmasına ve böylelikle plastik atık sayısının 2020 yılında 2019'a göre %30 artmasına sebep olmuştur [4]. Plastik kirliliği dünya genelinde tüketimin artması, plastik ürünlerinin kullanımının ve üretiminin artması, nüfusun artması ile beraber giderek daha da ciddi bir sorun haline gelmektedir.

Plastik atıklarının uzaklaştırılması için en çok kullanılan yöntemler sırasıyla plastik atıklarının arazilerde depolanması ve yakılmasıdır. Plastik atıklarının geri dönüştürülerek tekrar kullanılması maliyetli bir yöntem olduğu için tercih edilmemektedir [5]. Arazilerde depolama yöntemi, atıkların biriktirilmesine dayanan bir yöntemdir. Atıkların doğadan kaybolmasını sağlamamakla beraber benzen, dioksin gibi çevreye zararlı kimyasalların açığa çıkmasına neden olur ve uzun vadede toprak verimliliği olumsuz yönde etkilenir. Bir diğer uzaklaştırma yöntemi olan plastik atıklarının yakılması ise karbondioksit gibi zararlı gazların açığa çıkmasına neden olmaktadır [6]. Kullanılan yöntemler çevreye zararlı olması, plastik atıklarının tam olarak uzaklaştırılamaması ve uygun maliyetli olmaması sebebiyle yeterli değildir. Bu nedenle uygun maliyetli, çevre dostu ve etkili bir yöntem geliştirilmesi gerekmektedir.

Plastik atıklarının biriktirildiği alanlarda, okyanuslar ve denizlerdeki mikroorganizmaların incelenmesi birçok mikroorganizmanın plastik biyodegradasyonuna (biyo-bozunma) sebep olduğunu göstermiştir. Plastik biyodegradasyonu yapabildiği görülen iki balmumu kurdu *Galeria mellonella* ve *Plodia interpunctella*'nın bağırsağında yaşayan mikroorganizmaların incelenmesi çalışmalarıyla plastik biyodegradasyonu gerçekleştirebilen farklı mikroorganizmalar da bulunmuştur [7,8]. Böylelikle plastik atıklarının uzaklaştırılması için

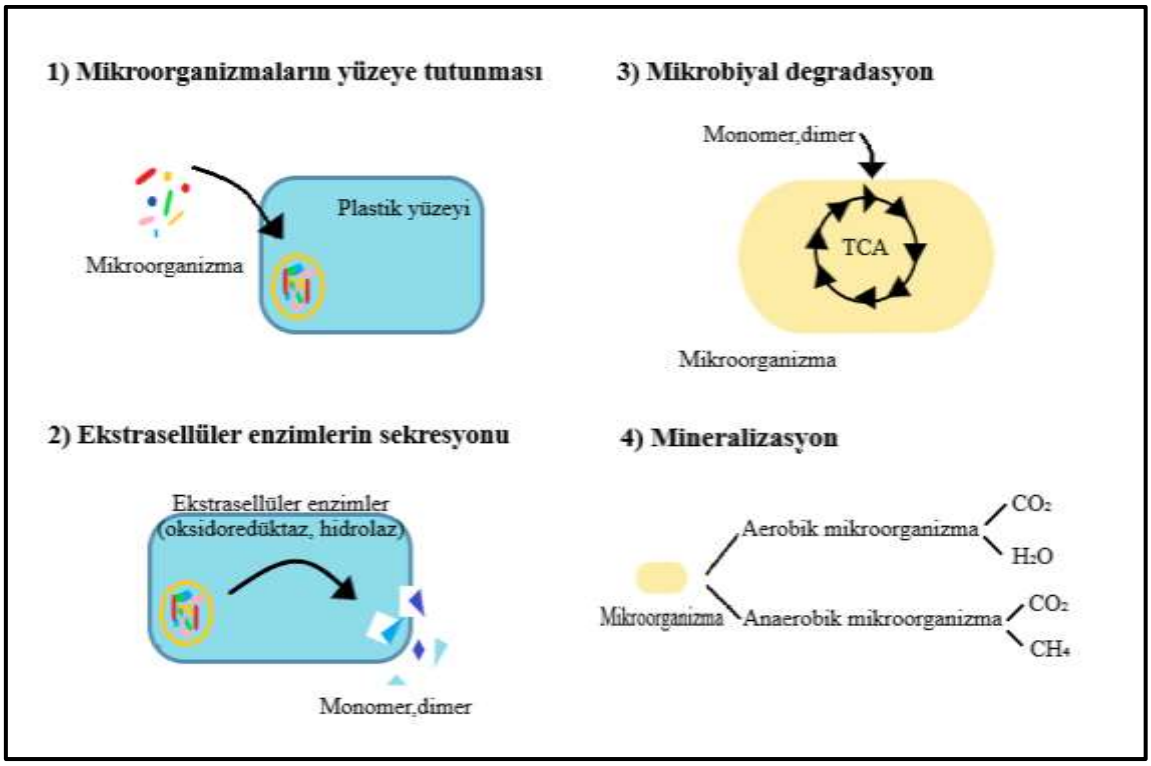
mikroorganizmaların kullanılması, yani biyodegradasyon potansiyel bir çözüm olarak ortaya çıkmış ve zamanla popüler bir konu haline gelmiştir.

Bu derlemede biyodegradasyon süreciyle beraber, mikroorganizmaların plastik atıklarının uzaklaştırılmasında kullanımının arttırılması için geliştirilen biyoteknolojik yaklaşımlara yer verilmiştir. Plastik biyodegradasyonunun biyoteknolojik çözümler ile gerçekleştirilmesiyle elde edilen yararlı sonuçlara ve Türkiye'de bu yaklaşımlar doğrultusunda gerçekleştirilen araştırmalara değinilmiştir.

## Biyoremediasyon ve Biyodegradasyon

Biyoremediasyon mikroorganizmaların metabolik aktiviteleri sonucu kimyasal atıkların ve çevreye zararlı materyallerin etkisinin azaltılmasını ya da çevreden temizlenmesini sağlamasıdır [9]. Kimyasal atıklar, ağır metaller, pestisitler, zenobiyotikler, hidrokarbonlar, plastikler gibi sağlık ve ekoloji açısından zararlı birçok kirletici için biyoremediasyon çalışmaları gerçekleştirilmektedir [10,11]. Mikroorganizmaların ve enzimlerin çevre kirliliğinin giderilmesi için optimize edilmesi ve biyodegradasyon mekanizmalarının etkinleştirilmesi ile etkili biyoremediasyon gerçekleştirilebilir [12].

Biyodegradasyon, mikroorganizmaların doğal olarak polimerleri ve organik maddeleri daha küçük maddelere parçalayabilmesidir [13]. Plastik biyodegradasyonu, mikroorganizmaların plastiğin yüzeyine tutunarak plastiği çoğalmak için karbon kaynağı olarak kullanabilmesi olarak tanımlanabilir [5].




**Şekil 1** Biyodegradasyonun 4 temel aşaması

Plastik biyodegradasyonu mikroorganizmaların enerji mekanizmalarına göre aerobik ve anaerobik olmak üzere ikiye ayrılır. Mikroorganizmalar arasında süreçte farklılıklar bulunsa da plastik biyodegradasyonu genel olarak Şekil 1'de gösterildiği gibi 4 temel adımdan oluşmaktadır. Mikroorganizmaların, bakterilerin ve mantarların plastik yüzeyine tutunarak koloni oluşturması ilk adımdır. Plastikler yüksek moleküler ağırlıkları nedeniyle hücre içine alınamazlar. Mikroorganizmalar ekstrasellüler enzimlerin, hidrolaz oksidoredüktaz gibi depolimerazların sekresyonu ile plastiği daha küçük parçalara ayırarak monomer ve dimer plastik parçalarını oluştururlar. Monomer ve dimer parçalar kolaylıkla çözünerek hücre içine girebilir ve hücre içi enzimlerle enerji kaynağı olarak kullanılabilir. Aerobik mikroorganizmalarda son ürün olarak karbondioksit ve su ürünleri oluşurken, anaerobik mikroorganizmalarda karbondioksit ve metan gibi ürünler oluşur. Bu ürünlerin oluşmasına genel olarak mineralizasyon adı verilir [14,15].

Plastik biyodegradasyon veri tabanı olan PBMD'in (Plastics Microbial Biodegradation Database) verilerine göre bu zamana kadar 950 mikroorganizmanın plastik biyodegradasyonu gerçekleştirebildiği veya plastik biyodegradasyonu süreci ile ilgili olduğu belirtilmiştir [16].

**Tablo 1** Plastik biyodegradasyonu gerçekleştirebilen bazı bakteri ve mantarlar ile plastiklerde gözlenen ağırlık kayıpları

| Mikroorganizma | Plastik | Süre (gün) | Ağırlık Kaybı (%) | Referans |
|---|---|---|---|---|
| Bakteri | | | | |
| *Bacillus subtilis* | PE | 60 | 23,15 | [17] |
| *Streptomyces* sp. | PE | 6 ay | 46,16 | [18] |
| *Pseudomonas citronellolis* | PVC | 45 | 10,00 | [19] |
| *Bacillus cereus* | PP | 40 | 12,00 | [20] |
| *Sporosarcina globispora* | PP | 40 | 11,00 | [20] |
| *Bacillus wiedmannii* | PE | 90 | 5,39 | [21] |
| Mantar | | | | |
| *Aspergillus flavus* | PE | 100 | 5,50 | [22] |
| *Coriolus versicolor* | N.E.B.P* | 93 | 20,00 | [23] |
| *Spicaria* spp. | PUR | 21 | 22,90 | [24] |
| *Aspergillus fumigatus* | PUR | 21 | 39,50 | [24] |
| *Alternaria solani* | PUR | 21 | 63,60 | [24] |
| *Cephalosporium* sp. | PS | 56 | 2,17 | [25] |

*: Nişasta esaslı biyoplastik

Polietilen (PE), polivinil klorür (PVC), polipropilen (PP), polietilen tereflatat (PET), poliüretan (PUR), polistiren (PS) gibi birçok plastiğin biyodegradasyona uğrayabildiği çalışmalarla gösterilmiştir. Plastik biyodegradasyonundan sorumlu olan bazı mikroorganizmalar ve etkili oldukları plastikler, ağırlık kayıpları ile birlikte **Tablo 1**'de özetlenmiştir. Plastik biyodegradasyonunda kütinaz, proteaz, esteraz, lipaz, lakkaz, PETase enzimlerinin önemli olduğu ve 79 genin plastik biyodegradasyon süreciyle ilgili olduğu belirtilmiştir [16].

Birçok mikroorganizmanın plastik biyodegradasyonu gerçekleştirebildiği, plastik biyodegradasyonunda hangi enzimlerin önemli olduğu ve hangi enzimlerin degradasyondan sorumlu olduğu yapılan araştırmalarda görülse de mikrobiyal degradasyon ticari olarak plastik atıklarının uzaklaştırılması için kullanılamamaktadır. Biyodegradasyon sürecinin yavaş olması ticari olarak kullanımını engellemektedir. Plastik yüzeylerinde fonksiyonel grupların eksik olması ve plastiğin yüzey hacim oranı mikroorganizmaların yüzeye tutunmasını yavaşlatan ya da engelleyen faktörlerdir. Polipropilen, polistiren gibi plastiklerin hidrofobik doğası ve plastiklerde kristalize

yapının fazla olması da mikroorganizmaların yüzeye tutunmasını engelleyerek biyodegradasyonu etkilemektedir. Mikroorganizmalar açısından ise biyodegradasyondan sorumlu enzimlerin az miktarda bulunması ve aktivitesinin düşük olması sürecin yavaşlamasına sebep olmaktadır [14,26].

## Biyodegradasyonun Arttırılması ve Biyoteknolojik Yaklaşımlar

Mikroorganizmalar tarafından gerçekleştirilen plastik biyodegradasyonunun veriminin arttırılması ve ticari kullanımının yaygınlaştırılması için biyoteknolojik yaklaşımlar kullanılmaktadır. Tek bir mikroorganizmanın kullanılmasının yerine mikrobiyal komüniteler oluşturulması, biyosürfaktanların eklenmesi ya da mikroorganizmalar tarafından ürettirilmesi, genetik mühendisliği yaklaşımlarıyla önemli enzimlerin düzenlenmesi, metagenomik ve biyoinformatik analizlerin uygulanması sürecin iyileştirilmesi için uygulanmakta olan yaklaşımlardır [14,27,28].

**Mikrobiyal komüniteler**

Doğal sistemlerin örnek alınmasıyla farklı türler bir arada kullanılarak mikrobiyal komüniteler geliştirilmektedir. Plastik yüzeylerinde mikroorganizmaların biyofilm oluşturması ile tek tür mikroorganizmanın aktivitisine göre metabolik aktivitenin arttığı bilinmektedir [29]. Mikrobiyal komünitelerde biyodegradasyon, mikroorganizmaların metabolitik aktivitelerinin birleşmesi ile gerçekleşir. Bu sebeple mikroorganizmaların plastik biyodegradasyonu için bir arada kullanımı ve kültürü ile biyodegradasyon süreci hızlandırılmaktadır [28].

Farklı mikroorganizmaların bir arada kullanılması ile türler arasında mikroorganizmalardan birinin faaliyetinin, diğer mikroorganizmanın faaliyetini etkileyerek plastik biyodegradasyonunu sağlamasından dolayı biyodegradasyon artar. *Noyosphingobium sp*. sadece metiyonin eklendiğinde PVA (Polivinil alkol) biyodegradasyonu gerçekleştirebilen bir mikroorganizmadır. *Xanthobacter flavus* ile beraber kültürlendiğinde ise metiyonine gerek kalmadan PVA biyodegradasyonun etkili şekilde gerçekleşebildiği görülmüştür. Biyodegradasyon için gerekli olan metiyoninin *Xanthobacter flavus*'un metabolik aktivitesinden elde edildiği düşünülmektedir [30].

Seneviratne ve arkadaşlarının (2006) gerçekleştirdikleri çalışmada DPE (Bozunabilir polietilen) üzerinde koloni oluşturabilen mikroorganizmaların incelenmesi ile etkili büyüme gerçekleştirebilen iki mikroorganizma belirlenmiştir. *Penicillium frequentans* ve *Bacillus mycoides* DPE üzerinde 21 gün ayrı ve birlikte kültüre edilmiştir. Sadece

*Penicillium frequentans* ile kültüre edilen DPE'ye göre mikroorganizmaların birlikte kullanımı ve biyofilm oluşumu sayesinde ağırlık kaybının 14 kat arttığı görülmüştür [31].

Çetin ve Çıtak (2013), izole edilen mikroorganizmaların tekli olarak kültüründe PVA (Polivinil alkol) biyodegradasyonu gözlenmezken, mikroorganizmaların çoklu kültüründe biyodegradasyon gözlendiğini belirtmiştir. Böylelikle simbiyotik olarak PVA biyodegradasyonu sağlanabildiği ve 16-25 günde başlangıç değerine göre %90 PVA miktarında azalmaya sebep olduğu belirtilmiştir [32].

Dwicania ve arkadaşlarının (2019) gerçekleştirdiği çalışmada, *Pseudomonas aeruginosa* ve *Brevilbacterium sp*. bakterilerinin birlikte kültürü ile 30 günde PE (Polietilen)'de %7 ye kadar ağırlık kaybı sağlanmıştır [33]. Bir diğer çalışmada *Tenebrio molitor* larvasından izole edilen *Acinetobacter sp.* ve *Bacillus sp*. PE biyodegradasyonu için birlikte kültüre edilmiştir [34]. Çalışmada PE degradasyonu için iki farklı bakteri türünün bir arada kullanımı ile 30 günde plastik miktarında %18 azalma sağlanabildiği ve plastik biyodegradasyonunun farklı mikroorganizmaların bir arada kullanımı ile arttırılabileceği gösterilmiştir [34].

**Biyosürfektanların eklenmesi ve ürettirilmesi**

Biyosürfektanlar; mikroorganizmalar tarafından üretilen, yüzey aktif ve amfifilik bileşenlerdir. Mikroorganizmaların yüzeylerinde ve hidrofobik maddelerin yüzeylerinde değişikliklere sebep olurlar. Yüzey gerilimini azaltarak etkileşimi kolaylaştırır ve çözünürlüğü arttırarak hidrofobik organik bileşiklerin biyo-erişilebilirliğini arttırırlar. Biyodegradasyon sürecini iki şekilde etkileyerek plastik biyodegradasyonunun artmasını sağlayabilirler. İlk olarak biyosürfektanlar mikroorganizmaların substratlara erişimini arttırabilir ya da bakteri hücrelerinin plastik yüzeyleriyle daha kolay etkileşmesini sağlayabilirler [35,36].

Mukherjee ve arkadaşlarının (2016) gerçekleştirdikleri çalışmada *Bacillus licheniformis* tarafından üretilen biyosürfektanın yüzey gerilimini azaltarak *Lysinibacillus fusiformis*'in PE yüzeyine tutunmasını arttırdığı ve PE oksidasyonunu arttırması ile PE biyodegradasyonunu arttırdığı belirtilmiştir [37]. Vimala ve Mathew'ın (2016) elde ettiği sonuçlara göre *Bacillus subtilis* tarafından sürfektan üretimi (Sürfaktin) gerçekleştirilmesi ile 30 günde PE'de %9,26'ya kadar ağırlık kaybı sağlandığı belirtilmiştir [38]. Biyosürfektanların amfifilik yapısıyla mikroorganizmaların

hidrofobik yüzeylere tutunmasını sağladığı veya arttırdığını böylelikle biyodegradasyon hızının arttırılabileceği görülmüştür. Furukawa ve arkadaşlarının (2019) gerçekleştirdikleri çalışmada ise *TfCut2 (Thermobifida fusca, kütinaz)* enzimiyle katyonik sürfektan dodesil trimetil amonyumun ($C_{12}$-N $(CH_3)_3^+$) birlikte eklenmesiyle PET ağırlık kaybının 2 kat arttığı ve 24 saatte %15 ağırlık kaybına ulaşıldığı görülmüştür [39]. Eklenilen katyonik sürfektan reaksiyon hızını 13 kat arttırmıştır [39].

Gerçekleştirilen çalışmalardan elde edilen sonuçlarda da görüldüğü gibi biyosürfektanların mikroorganizmalar tarafından üretilmesi ya da biyodegradasyon sürecinde eklenmesiyle mikroorganizmaların plastik yüzeylerine tutunması daha etkili duruma getirilebilir. Böylelikle biyodegradasyon sonucu plastiklerde gözlenen ağırlık kaybı artmaktadır. Aynı zamanda substratlara erişimin artmasıyla reaksiyon hızının artması ve daha hızlı biyodegradasyon sağlanabilir.

Türkiye'de *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Pseudomonas* spp gibi mikroorganizmalarda biyosürfektan üretiminin petrol kirliliği üzerindeki etkileri gibi farklı biyoremediasyon çalışmaları gerçekleştirilmektedir [40,41,42]. Fakat plastik biyodegradasyonuna yönelik biyosürfektanların etkilerinin incelendiği bir çalışma görülmemiştir.

**Genetik modifikasyonlar**

Moleküler klonlama, enzim modifikasyonları, metabolik yolakların düzenlenmesi gibi çeşitli moleküler modifikasyonlar ile plastik biyodegradasyonu iyileştirilebilmektedir. Genetik modifikasyonlar ile mikroorganizmaların plastik biyodegradasyon ile ilgili özellikleri düzenlenerek plastik biyodegradasyon süreci hızlandırılabilmektedir [43,44].

Enzim aktivitesinin arttırılması için protein mühendisliği yaklaşımları kullanılarak enzimin verimliliğinin düzenlenmesi sağlanmaktadır. Enzimlerin aktif bölgelerinde gerçekleştirilen mutasyonlar sonucunda enzim aktiviteleri incelenerek plastik biyodegradasyonunu arttırabilecek daha etkili enzimler tasarlanabilir. *Ideonella sakaiensis* PETase enziminin aktif bölgesinde gerçekleştirilen ikili mutasyonlar sonucunda PET (Polietilen tereftalat) degradasyonunun arttığı görülmüştür [45]. *Ideonella sakaiensis* PETase enziminde çoklu mutasyonların gerçekleştirilmesi ve plastik biyodegradasyonu açısından yararlı olanların seçilmesiyle enzim tekrardan düzenlenerek DuraPETase enzimi tasarlanmıştır [46]. PET biyodegradasyonunun 10

günde 400 kat arttığı ve 20 günde eklenen plastiklerin degradasyonunun tamamlanabildiği belirtilmiştir [46].

Plastik biyodegradasyonunda önemli olduğu görülen enzimlerin farklı organizmalarda yüksek verimde ve miktarda ekspresyonunun sağlanması ile biyodegradasyon arttırılabilmektedir. *Fusarium solani pisi* kütinaz enzimi ve *Cellulomonas fimi* selüloz bağlanma bölgesinin *Escherichia coli*'de ekspresyonu ile 48 saatte PET plastiklerinde %0,90 azalma belirtilmiştir [47]. *Pseudomonas* sp. alkan hidroksilaz geninin (*alkB*) *Escherichia coli* BL21'de ekspresse edilmesi ile 80 gün LMWPE (Düşük moleküler ağırlıklı polietilen) inkübasyonu sonucunda %19,3 karbon minerilizasyonu ve daha aktif biyodegradasyon sağlanabildiği görülmüştür [48]. *Aspergillus niger* lipaz enziminin *Pichia pastoris*'de ekspresyonu ile PLA (polilaktik asit) ve PCL (polikaprolakton) plastiklerinde biyodegradasyon gerçekleşebildiği gözlenmiştir [49]. Kim, Park ve arkadaşlarının (2020) gerçekleştirdikleri çalışmada bir yeşil alg olan *Chylamydomonas reinhardtii*'de PETase enziminin ekspresyonu ile 4 haftada eklenen PET'in tamamen degrade olması sağlanmıştır [50]. Doğal süreçte mikroorganizmalarda enzim miktarının az olması ya da yavaş elde edilmesi ekspresyon kapasitesi daha yüksek bir organizmanın kullanılması ile aşılabilir.

Gerçekleştirilen mutasyonların diğer yaklaşımlarla birleştirilmesiyle beraber daha hızlı ve yüksek verimde biyodegradasyon gerçekleştirilebilmektedir. *Thermobifida fusca* kütinaz (*TfCut2*) enziminin aktif bölgesinde gerçekleştirilen ikili mutasyonlar ve katyonik sürfektanın bir arada kullanılmasıyla PET degradasyonunun 13 kat artması, 24 saatte %90 plastik ağırlığının azalması sağlanmıştır [39]. Gerçekleştirilen çalışmada bu zamana kadar belirtilen en yüksek PET hidrolaz değeri elde edilmiştir. *Thermobifida cellulosilytica* kütinaz 1 (*Thc_Cut1*) enzimi yüksek konsantrasyonlarda enzim elde edilebilen ve biyoteknolojide ekspresyon sistemleri açısından artan ilgi gören *P.pastoris* kullanılarak ekspresse edilmiştir [51]. Glikozilasyon bölgesinde gerçekleştirilen mutasyon ile PBS (Polibütilen süccinat) hidrolizi 96 saatte %92'ye ulaşmıştır [51].

Enzimlerde gerçekleştirilen mutasyonların etkilerinin ve enzimin aktivitesinin gözlenmesi ile plastik biyodegradasyonu için önemli olan enzimlerin yapısal özellikleri anlaşılmaktadır. Mutasyon çalışmaları plastik biyodegradasyonu sırasında açığa çıkan monomer-dimer substratlara enzimin bağlanabilme özelliklerinin incelenmesine de olanak sağlamaktadır. Mikrobiyal enzimlerin ve genlerin modifikasyonu ile plastik

biyodegradasyonu sürecinde açığa çıkan substratlara karşı mikroorganizmaların spesifik aktivitesi arttırılıp biyodegradasyon süreci iyileştirilebilir [52]. Palm ve arkadaşlarının (2019) gerçekleştirdikleri çalışmada, PET biyodegradasyonu sırasında önemli olan MHETase enziminin yapısının incelenmesi ile enzimin aktif merkezinin substrata bağlanma özellikleri belirlenmiştir. Katalitik özelliklerin arttırılması için MHETase enzim varyantı geliştirilerek BHET (bis- (2-hidroksietil) teraftalat) substratına enzimin özgüllüğü arttırılmıştır [53] .

Türkiye'de gerçekleştirilen çalışmalarda enzimlerin aktivitelerinin düzenlenmesine yönelik yaklaşımlara rastlanmamıştır.

**Metagenomiks ve biyoinformatik**

Metagenomiks genetik materyalin direk olarak çalışılması, sekans ya da fonksiyonlarının izlenmesi ile mikrobiyal komüniteler ve kültüre edilemeyen organizmalar ile ilgili bilgi vermektedir [54]. Mikroorganizmaların kültür ortamında çoğaltılarak incelenmelerinin yanı sıra nükleik asitlerin, proteinlerin ve lipitlerin direk olarak çalışılması birçok biyoremediasyon çalışmasıyla beraber plastik biyodegradasyonu için de önem kazanmaktadır [55]. Metaproteomiks, metatranskriptomiks ve metabolomiks çalışmalarının biyodegradasyon çalışmalarına dahil edilmesi mikroorganizmalar arasında fonksiyonel ve yapısal farklılıkların daha detaylı ve hızlı analizine olanak sağlamaktadır [55].

Metagenomiks çalışmaları farklı koşullarda mikroorganizmaların metabolizmalarında gerçekleşen değişimleri gösterir. Ekstrasellüler ve intrasellüler metabolitler, plastik biyodegradasyonu için önemli ve yeni yolakların aydınlatılması ve sorumlu enzimlerin bulunması için incelenmektedir. Genom analizleri ile mikroorganizmaların plastiği karbon kaynağı olarak kullanabilme potansiyelleri incelenebilmektedir. Genom analizleri örneklerdeki mikroorganizmaların türlerinin tayin edilmesi için kullanılarak plastik biyodegradasyonu gerçekleştirebilen yeni mikroorganizmaların keşfedilmesini de sağlamaktadır [56].

Meyer-Cifuentes ve arkadaşlarının (2020) gerçekleştirdikleri çalışmada denizden elde edilen mikroorganizma örnekleri, mikroorganizma komünitelerinin polifenolik plastikleri (PF) biyodegradasyon süreçleri açısından, metagenomiks analizlerle incelenmiştir. *Marinobacter* ve *Gammaproteobacteria*'nın plastik yüzeyinde büyüyebildikleri ve PF depolimerizasyonunda görevli olan *Ples* genini kodladıkları

görülmüştür. Elde edilen mikrobiyal komünitenin proteomiks ve transkriptomiks değişimleri incelenerek 6 PETase benzeri enzimin alifatik-aromatik polimer degradasyonundan sorumlu olabileceği görülmüştür [29].

Pinnell ve Turner'ın (2019) benzer bir yaklaşımla gerçekleştirdikleri çalışmada, denizlerden elde edilen mikrobiyal komünite örneklerinin metagenomiks analizleri gerçekleştirilmiştir. PHA (Polihidroksialkanoat) degradasyonunda, sülfat indirgeyen bakterilerin (SRB) önemli olduğu gösterilmiştir. Esteraz, depolimeraz ve adenil sülfat redüktaz (*aprBA*) enzimlerinin biyodegradasyondaki rolü ile *Desulfovibrio*, *Desulfobacteraceae* ve *Desulfobulbaceae* gibi plastik biyodegradasyonu gerçekleştirebilen yeni türler rapor edilmiştir [57].

Sekanslama teknolojilerinin ve biyoinformatik analizlerin gelişmesiyle beraber metagenomiks analizleri ilerlemektedir. 16S rRNA sekanslama tekniği mikrobiyal komünitelerin çalışılması için kullanılan önemli bir yöntemdir [58]. Sekanslama teknolojilerinin gelişmesi, maliyetlerinin düşmesi ile metagenomiks çalışmalarının ilerlemesi ve kültürden bağımsız metotlar ile mikrobiyal komünitelerin incelenebilmesi sağlanmıştır [58]. Biyoinformatik ve metagenomiks, plastik biyodegradasyonu ile ilgili enzim ve gen bilgileri elde edilerek degradasyon yolaklarının daha iyi anlaşılması ve plastik biyodegradasyonunun iyileştirilmesi için kullanılmaktadır [59]. Elde edilen bilgiler genetik mühendisliği yaklaşımları ile birlikte kullanılarak enzimlerin aktivitesinin arttırılması sağlanabilir.

UM-BBD (University of Minnesota Biocatalysis/ Biodegradation database), OxDBase, MetaCyc gibi veri tabanları üzerinden biyodegradasyonla ilgili genlere, enzimlere ve biyodegradasyon yolaklarına ulaşılabilmektedir [60]. BioSurfDB metagenom, organizma bilgileri, biyodegradasyon yolakları, enzimler hakkında bilgi vermekle beraber biyosürfaktanların kullanımı ve sonuçları hakkında bilgilerin elde edinebilindiği bir veri tabanıdır [61]. PICRUSt bakteri protein fonksiyonlarının 16S rRNA metagenomiks verilerine göre belirlenmesini sağlayan ve biyoremediasyon potansiyallerine erişilmesini sağlayan bir veri tabanıdır [59]. Önceden belirtildiği gibi PBMD diğer veri tabanlarının yanı sıra plastik biyodegradasyonu için oluşturulmuş bir veri tabanıdır.

Oyewusi ve arkadaşlarının (2020) gerçekleştirdikleri çalışma da, Tuz Gölü'ndeki mikroorganizmaların biyoinformatik ve metagenomiks analizleri sonucunda

biyoremediasyon ve biyoteknoloji açısından önemli özelliklerinin olduğu ve ileri çalışmaların gerçekleştirilmesi gerektiği bildirilmiştir [59]. Benzer yaklaşımlarla Akdeniz gibi plastik kirliliğinin yoğun görüldüğü denizlerdeki mikrobiyal komünitelerin incelenmesi ile değerli bilgilerin elde edilebileceği düşünülmektedir. Bu veriler, bahsedilen biyoteknolojik yaklaşımlarla birleştirilerek önemli sonuçlar elde edilebilir.

## Sonuç

Plastik biyodegradasyonu, tüm dünyada artmakta olan plastik kirliliğinin azaltılmasında günümüzde kullanılan plastik atık uzaklaştırma yöntemlerinin aksine çevre dostu olması sebebiyle potansiyel çözüm olarak görülmektedir. Plastik atıklarının bulunduğu alanlardaki mikroorganizmaların incelenmesi, mikroorganizmaların çeşitli plastiklerde biyodegradasyon gerçekleştirebildiğini göstermiştir. Süreçte aktif olan birçok bakteri, mantar ve enzimler bildirilmiştir. Fakat biyodegradasyonun yavaş gerçekleşmesi ve etkisinin düşük olması nedeniyle mikroorganizmaların plastik atıklarının uzaklaştırılması için ticari kullanımı bulunmamaktadır. Mikroorganizmalarda enzim miktarının veya aktivitesinin düşük olması ve plastik yüzeyinin özelliklerinden dolayı mikroorganizmaların yüzeye tutunamaması sürecin yavaş olmasının nedenleridir.

Plastik biyodegradasyonunun kısıtlayıcı etmenleri biyoteknolojik yaklaşımlar kullanılarak iyileştirilmeye ve bertaraf edilmeye çalışılmaktadır. Doğada mikroorganizmaların etkileşimleri ve ortak metabolik süreçlerde görev almaları örnek alınarak farklı tür mikroorganizmaların bir arada kullanılması ve mikrobiyal komünitelerin oluşturulması yaklaşımı geliştirilmiştir. Mikroorganizmalar arası etkileşim ile biyodegradasyon süreci hızlanır mikroorganizmalar ek düzenleyicilere gerek kalmadan aktivite gösterebilir [30,34]. Mikroorganizmalarının plastik yüzeyine tutunmaları ve substrata erişimi ise biyosürfektanların eklenmesi veya üretimi sayesinde arttırılabilir.

Plastik biyodegradasyonunda enzim aktivitelerinin arttırılması için protein mühendisliği, mutasyon çalışmaları ve diğer genetik modifikasyonların kullanılması sürecin iyileşmesine yüksek miktarda katkı sağlamaktadır [50,51]. Metagenomiks analizleri; mikrobiyal komünitelerin, enzimlerin ve yolakların direk incelenebilmesi sayesinde önemli bilgiler verir.

Türkiye'de plastik biyodegradasyonu için gerçekleştirilen çalışmalar incelendiğinde birçok yaklaşımlar için çalışma eksikliklerinin bulunduğu görülmüştür. Bu alanda yapılan çalışmalar arttırılarak plastik kirliliğine karşı yeni çözümler üretilmelidir.

Tüm dünya açısından önemli bir sorun haline gelen plastik kirliliği; metagenomiks çalışmaların, biyoinformatik veri tabanlarının artması ve diğer biyoteknolojik yaklaşımların da bir arada kullanılması ile azaltılabilir ve engellenebilir. Plastik biyodegradasyonunun iyileştirilmesi ve var olan sorunların çözülmesi, biyodegradasyonun ticari boyutunu da arttıracaktır.

**Çıkar Çatışması**

Yazarın çıkar çatışması oluşturabilecek herhangi bir kurum ya da kuruluşla ilgisi bulunmamaktadır.

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**DECLARATION:** This work is part of Gülfidan KUYUMCU's MA thesis. Articles on 27-29 September 2017 held in Bayburt in Turkey was presented as a Oral Presentation I. International Organic Agriculture and Biodiversity Symposium

# Genetic Analysis Related To Organized Genetic Changes in Potato And Processed Potatoes

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