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Research article

Study of Spent Coffee Grounds Using Cytological Technique on Root Tip of *Allium cepa*

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Abstract: Coffee is a widely consumed beverage that has a major impact on economies, markets, and industries; however, the postproduction of coffee drinks results in wasted or spent coffee grounds (SCG). SCG is used as a plant growth medium; however, its suitable level of use is uncertain. This research determined the optimal amount of SCG for root development and its effect on the cells of *Allium cepa*. The SCG was mixed with sand (S) in different ratios (SCG:S = 40:60, 50:50, 60:40, 70:30, 80:20, 90:10) to evaluate their effects on root growth and cytotoxicity on cells. Root number and length were measured to assess growth, while the mitotic index (MI) and chromosomal abnormalities were analyzed to determine cytotoxic effects. The 40:60 SCG:S mixture produced the highest root growth compared to controls, whereas higher SCG ratios (70:30, 80:20, 90:10) reduced the MI. Various chromosomal abnormalities were observed, such as micronuclei, c-mitosis, fragmented chromosomes, chromosome bridges, sticky chromosomes, and laggard chromosomes. Therefore, the chemical compounds in SCG affected cytotoxicity on *A. cepa* roots. The most beneficial use of SCG for addition to plant growth media resulted from a precisely defined mixture to balance growth enhancement with potential cytotoxic risk.

Keywords: Cell aberration; root tip; cell division; spent coffee grounds; growth media

1. Introduction

Coffee belongs to the family Rubiaceae and the tribe Coffeeae [1]. More than 100 species of the genus *Coffea* are known, with the two varieties *C. arabica* and *C. canephora* being the most economically exploited species [2]. Coffee is one of the most popular beverages globally and is the second most-traded commodity after petroleum. Due to high demand, the coffee industry generates a variety of by-products. The process of producing coffee begins with harvesting coffee cherries, which are either dry- or wet-processed to produce green coffee, the standardized trading form [3]. However, the industrial production of coffee yields large quantities of by-products, including cherry husks, cherry pulps, silver skin, and ultimately, spent coffee grounds (SCG) [4]. SCG refers to the solid waste generated in serving individual coffee consumers or during the industrial production of instant coffee and the roasting of coffee beans [5,6]. During instant coffee production, an estimated 6 million tonnes of SCG are generated worldwide annually [6]. Furthermore, the accumulation of coffee waste is increasing in line with the increase in coffee consumption [7]. Disposing of SCG in large quantities in landfills could lead to pollution of water resources and the release of CO_2, which contributes to climate change [5]. Therefore, the

large volume of SCG generated during coffee production highlights the increasing importance of effective recycling.

SCG is a valuable organic waste that can be repurposed for economic, commercial, and domestic agricultural applications. SCG contains polysaccharides, sugars, proteins, minerals, lipids, caffeine, acids, alkaloids, and polyphenols [8,9]. Ramalakshmi et al. [10] found that the primary antioxidant in SCG is 5-caffeoylquinic acid (~6%). Bravo et al. [11] also noted substantial levels of total caffeoylquinic acids (6–13 mg/g) and dicaffeoylquinic acids (3–6 mg/g). Due to its chemical composition, antioxidant, emulsifying, and emulsion properties, as well as its high lipid content (mostly fatty acids), SCG has the potential for incorporation into topical products such as creams and sun protectors [9]. Additionally, SCG can supply nutrients and promote plant growth, particularly in dry agricultural areas that require improved soil fertility [12]. SCG directly enhances the soil's physical, chemical, and biological structure by leaching various nutrients, including nitrogen, phosphorus, and potassium, into the soil through water [13]. Consistently, the application rate and plant species, as well as the application of raw SCG, have shown positive effects on physical and chemical soil properties such as moisture content, soil porosity, and bulk density [13]. However, the high moisture content of SCG can produce odors and harbor insects [5], while different types of SCG have varying effects on the toxicity of the plant [14]. The compounds in SCG may interfere with seed germination and root elongation, thus raising concerns about its safe use in crop production [15,16].

Although the agronomic potential of SCG as a soil amendment has been thoroughly investigated for various plant species, there has been no published information on the cytotoxicity of SCG in plant cells, particularly using the *Allium cepa* test. Cytological studies can provide insights into the mechanisms of SCG toxicity and better evaluate its suitability for sustainable agricultural applications. Currently, a cytological technique is used to examine chromosome abnormalities. The onion plant is an ideal model for studying chromosomal changes due to its moderate number of chromosomes (2n = 16), suitable size, low cost, high sensitivity, and reproducibility [17]. Therefore, analyzing *A. cepa* root tip cells is a convenient method for determining the cytotoxic impact of SCG on the growth of onion roots and chromosome aberrations under a microscope.

2. Materials and Methods

2.1 Preparation of aceto-orcein dye

A sample (1 g) of orcein dye powder was dissolved in 45 mL of acetic acid and 55 mL of distilled water. Then, the orcein solution was boiled until completely dissolved. After that, the solution was passed through Whatman filter paper and stored in dark bottles, where it was used as a 1% w/v aceto-orcein dye solution for chromosome staining.

2.2 Onion cultivation

Spent coffee grounds (SCG) were derived from roasted coffee powder that had been used to prepare coffee beverages, dried at 40°C, and then sieved to obtain a uniform particle size. A sample of 10 onion bulbs was placed in trays containing different treatments of sand combined with SCG for approximately 7 days, until the emergence of new roots at 25±2°C. For the root cytotoxicity assay, six treatments of each growth medium were varied in the following order: SCG:S, 40:60, 50:50, 60:40, 70:30, 80:20, and 90:10, and then compared to a positive control (100% SCG) and a negative control (100% S). The experiment was performed in triplicate for each SCG-to-sand ratio.

2.3 Measurements of the number and length of onion roots

After the cultivation period, the roots of the onions from each treatment were cleaned. The lengths and number of root tips were measured for three onion bulbs from each treatment. Subsequently, the remaining seven onion bulbs were transferred to a fixative solution containing ethanol and glacial acetic acid in a 3:1 ratio for 24 hours at 4°C. Then, the roots were transferred to 70% v/v alcohol at the same temperature. These treated roots were used in subsequent steps for preparing onion root cell slides.

2.4 Cytotoxicity assay on root cells

Once cut, the rootlets were soaked in a fixative solution for 24 hours at 4°C. The root tips (1–3 mm in length) were hydrolyzed in 1 N hydrochloric acid at room temperature for 5 minutes and then stained with aceto-orcein for 5 minutes. Following staining, they were squashed onto a glass slide to determine the mitotic phase for the percentage mitotic index (MI) and to assess for the percentage of chromosomal cell aberrations, using equations (1) and (2), respectively. This was done by viewing the slides under a light microscope (Olympus CX 23) with a 400× objective lens; 200 cells were considered per slide for three slides in each treatment.

$$\% \text{ MI} = \frac{\text{Number of cells in mitotic phases}}{\text{Total number of cells counted}} \times 100 \quad (1)$$

$$\% \text{ Cell aberration} = \frac{\text{Number of aberration cells}}{\text{Total number of cells counted}} \times 100 \quad (2)$$

2.5 Statistical analysis

The SPSS software program, version 29, was used to analyze the number and length of roots, as well as the number of cells. One-way analysis of variance with a 5% significance level ($p < 0.05$) was applied to compare the averages. All treatments were conducted in triplicate, with the data presented as the mean ± standard deviation (SD).

3. Results and Discussion

3.1 Effect of SCG on the growth of onion roots

SCG provides sufficient nutrients and supports plant growth, with numerous studies investigating its use as an alternative to chemical fertilizers for promoting plant growth. For example, Hechmi et al. [12] reported that raw SCG contained significant concentrations of organic matter (62.6%), nitrogen (2.4%), phosphorus (0.47%), and potassium (0.94%). SCG residues from instant coffee preparation could be distinguished by their fine particle size and relatively high humidity levels, ranging from 69.7% [18] to 80% [6]. Additionally, raw SCG had a high water-holding capacity (70–73 g per 100 g of SCG), which could supply water to the plant [19]. Furthermore, the pH of raw SCG was moderately acidic, ranging from 3.9 [14] to 5.52 [20], indicating its potential use as a bio-fertilizer for many plant species. These findings from other studies support the current results on onion root growth, based on different combinations of SCG and sand as the onion plant medium. The growth medium mixed with SCG resulted in greater root length and number of roots (2.07 ± 0.48 cm and 17.33 ± 2.31 roots, respectively) than the 100% sand-negative control group. The best effects on root length were observed when using the SCG:S ratios of 40:60 and 50:50 (4.61 ± 0.50 and 3.16 ± 0.44 cm, respectively). The number of roots for 40:60 was 31.00 ± 4.58 (Table 1). When the SCG-to-sand ratio exceeded 50, the length of the roots decreased. The mixtures with ratios of 60:40 and 70:30 were not significantly different from the negative control group (Table 1). Therefore, these ratios did not lead to improved growth of the onion roots. The ratios of 80:20 and 90:10 significantly reduced root length compared to the negative control group (0.90 ± 0.18 and 0.75 ± 0.19 cm, respectively). However, these ratios were consistent with the positive control group, where the growth medium was 100% SCG (1.11 ± 0.10 cm), suggesting that the presence of certain substances in the SCG may excessively inhibit root growth in such growth media (Figure 1 F–H). For the different mixtures of SCG and sand, ranging from 50:50 to 90:10, there was no significant increase in the number of roots compared to the control groups (Table 1), indicating that the specified ratios of SCG to sand did not affect the number of roots (Figure 1). Therefore, the optimal ratio of SCG-to-sand for promoting the growth of onion roots (both the length and number of roots) was 40:60 (Figure 1 B). Caliskan et al. [21] demonstrated that the application of 20% SCG + 80% sand media as the growth medium for stone pine (*Pinus pinea*) produced the greatest root collar diameter and number of lateral branches. However, higher SCG rates (30%) decreased the seedling height, maximum root length, total needle weight, stem weight, main root weight, and lateral root weight [21]. The current results for root growth of *A. cepa* showed that the 60% SCG rate resulted in decreased root length, suggesting that such higher SCG rates affected root development. Ribeiro et al. [16] anticipated that the presence of other chemicals in raw SCG, such

as caffeine (CFN) and phenolic compounds, inhibited seed germination. Raw (fresh and air/oven-dried) SCG generally contains significant concentrations of phenolic compounds [8] and tannin and CFN [16]. CFN can have positive and negative effects on small plants, as the caffeine found in coffee can affect them depending on its concentration. Using caffeine at an optimal concentration can accelerate root growth, increase the frequency of root formation, and increase the number of roots following root pruning in small plants [22]. CFN extracted using supercritical CO_2 from SCG corresponded to 18–48% of the extracted compounds from coffee beans and 8–31% from roasted coffee [21]. CFN concentrations exceeding 0.1% can harm plant tissue by slowing or stopping root formation and shoot growth, and causing tissue necrosis [23]. SCG generally contains high levels of polyphenols, which are induced mainly by phytotoxic chemicals in the raw material [8,24]. In addition, phytotoxicity may be due to the high tannin concentrations in SCG [25], which can be toxic to root formation. The roots play a crucial role in absorbing and transporting water and minerals. Additionally, SCG derivatives, such as biochar, contain carboxylic acids that exhibit phytotoxic effects by inhibiting seed germination in lettuce (*Lactuca sativa*) [26]. The primary mechanism by which SCG may inhibit plant growth is by reducing nitrogen release, which is related to the amount of chlorophyll [13]. Furthermore, the carbon-nitrogen ratio (C/N) of raw SCG ranges from 20 to 32, which is higher than the C/N ratio in most horticultural soils and far higher than that of soil microbial populations [13].

Table 1. Length and number of onion roots after 7 days of growth in different spent coffee grounds (SCG) and sand (S) mixtures

Growth medium (SCG:S)	Root length (cm)	Number of roots
0:100	2.07 ± 0.48^{c}	17.33 ± 2.31^{c}
40:60	4.61 ± 0.50^{a}	31.00 ± 4.58^{a}
50:50	3.16 ± 0.44^{b}	24.33 ± 3.31^{b}
60:40	2.00 ± 0.65^{c}	18.00 ± 1.73^{c}
70:30	1.91 ± 0.31^{c}	16.67 ± 2.08^{c}
80:20	0.90 ± 0.18^{d}	17.00 ± 2.65^{c}
90:10	0.75 ± 0.19^{d}	16.67 ± 5.51^{c}
100:0	1.11 ± 0.10^{d}	19.00 ± 1.00^{bc}

Note: Values (mean ± SD) with different lowercase superscripts in column indicate significant ($p<0.05$) differences.

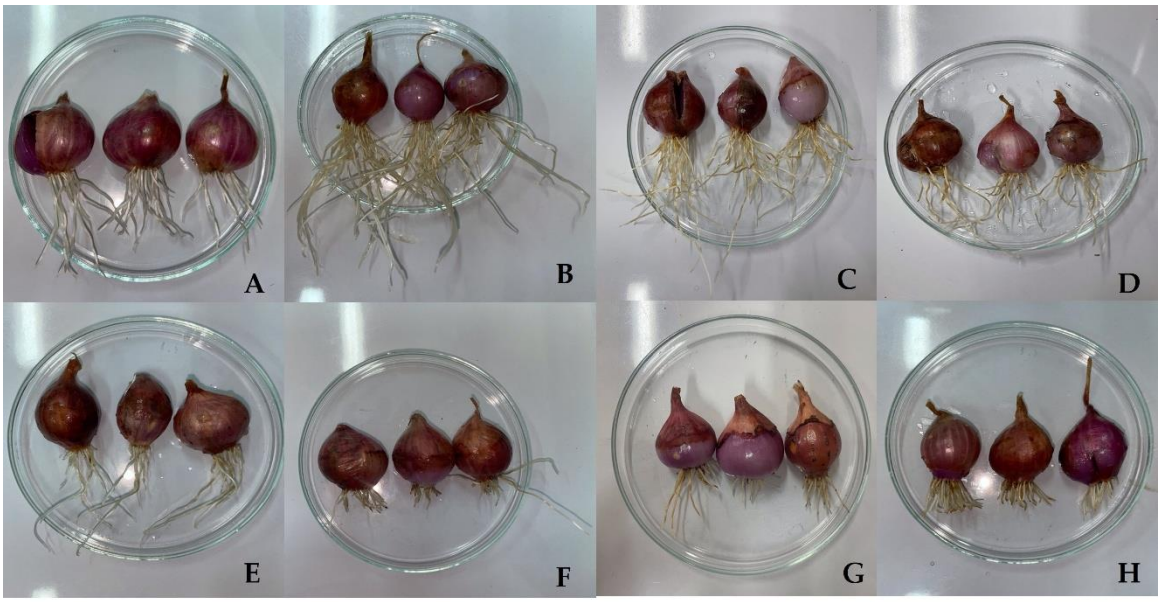

Figure 1. Onion bulb roots planted in various ratios of SCG-to-S for 7 days: (A) 0:100, (B) 40:60, (C) 50:50, (D) 60:40, (E) 70:30, (F) 80:20, (G) 90:10, (H) 100:0

3.2 Cytotoxicity of SCG on onion root cells

Examination of the amount of SCG that causes toxicity to cells and chromosomes in onion root cells identified that when SCG-exposed cells were examined, the MI decreased. Compared to the control group, the decrease in MI of dividing onion root tip cells leads to a corresponding increase in chromosomal abnormalities. This increase has been reported to be directly proportional to the chemical concentration and the duration of exposure to the onion roots [27]. After 7 days of growth, the growth medium consisting of large amounts of SCG mixed with sand (70:30 or higher) led to a significant decrease in the MI compared to the negative control group. However, the SCG:S mixture of 60:40 had lower toxicity than the 70:30 medium, although the MI values (37.67 ± 1.04 and 34.83 ± 1.15, respectively) were not significantly different. In contrast, the 60:40 mixture of SCG and sand had a significantly higher MI than the positive control group (Table 2), indicating that the 60:40 ratio began to affect the onion root cells, leading to an increase in abnormalities and inhibition of cell division at the root tip, resulting in a decrease in root length compared to the negative control group (Figure 1D). Furthermore, a correlation was observed between the number of abnormal cells and the decrease in MI (Table 2). In addition, the 40:60 and 50:50 growth media had MI values of 41.50 ± 2.65 and 40.67 ± 1.53, respectively. These ratios did not significantly impact the decrease in MI compared to the negative control group (Table 2). Hence, they were suitable for promoting cell division in onion roots, leading to increased root length (Figures 1B and C). However, the 100% SCG resulted in increased root length corresponding to the MI values for the 80:20 and 90:10 treatments. The cell abnormalities resulting from toxicity due to excessive mixing of SCG included micronuclei, c-mitosis, fragmented chromosomes, chromosome bridges, sticky chromosomes, and laggard chromosomes (Figure 2F–L). The number of these abnormalities varied with each set of growth media treatments. The mechanisms underlying these effects likely relate to the chemical composition of SCG, which contains caffeic acid, coumaric acid, and protocatechuic acid [28]. These compounds are known to produce reactive oxygen species (ROS), which leads to oxidative stress and the disruption of essential cellular enzymes [29]. Similar trends have been observed in studies where *A. cepa* was exposed to coffee extracts, resulting in dose-dependent declines in germination and mitotic activity [30]. The abnormalities observed in onion root cells can be attributed to the presence of CNF, as reported by Chandraker et al. [31]. They examined beverages containing CNF, such as coffee, tea, and Coca-Cola®, to observe their impact on the normality of *A. cepa* root cells. They observed that the concentration of the beverages and the duration of exposure to the onion roots led to the formation of abnormal cells with characteristics such as c-mitosis, stickiness, adherent chromosomes, and laggard chromosomes. Furthermore, *A. cepa* remains a reliable model for identifying cytogenetic disruptions caused by environmental pollutants due to its sensitivity and cost-effectiveness [17]. However, a limitation of the current study was the lack of chemical analysis of the SCG extracts to establish dose-response relationships and to determine the specific chemical or chemicals responsible for SCG toxicity. Nevertheless, this approach should support the development of safe and sustainable methods for using SCG in agriculture.

Table 2. Abnormal cells and MI values observed in onion root cells during mitotic cell division

Growth medium (SCG:S)	% MI	MN	CM	FC	CB	SC	LC	Cell aberration (number)	Cell aberration (%)
0:100	41.67 ± 0.76^{a}	10.33 ± 1.15^{ab}	4.00 ± 1.00^{cd}	7.00 ± 0.00^{a}	14.67 ± 1.53^{a}	22.67 ± 0.58^{d}	28.33 ± 2.08^{cd}	87.00 ± 1.00^{cd}	43.50 ± 0.50^{cd}
40:60	41.50 ± 2.65^{a}	12.00 ± 4.00^{a}	2.67 ± 2.08^{cd}	6.67 ± 3.21^{a}	12.67 ± 1.53^{a}	24.00 ± 2.65^{d}	26.67 ± 4.16^{d}	84.67 ± 6.11^{d}	42.33 ± 3.06^{d}
50:50	40.67 ± 1.53^{a}	6.33 ± 1.15^{bc}	5.00 ± 1.00^{bc}	10.33 ± 1.53^{a}	13.67 ± 2.08^{a}	26.33 ± 1.53^{cd}	27.67 ± 1.53^{d}	89.33 ± 3.15^{cd}	44.67 ± 1.76^{cd}
60:40	37.67 ± 1.04^{ab}	4.33 ± 2.08^{c}	3.33 ± 1.53^{cd}	6.33 ± 1.15^{a}	13.33 ± 1.53^{a}	31.00 ± 3.46^{bc}	35.00 ± 3.46^{abc}	93.33 ± 1.53^{bcd}	46.67 ± 0.76^{bcd}
70:30	34.83 ± 1.15^{bc}	10.33 ± 3.06^{ab}	4.33 ± 2.08^{cd}	8.67 ± 4.16^{a}	14.67 ± 2.08^{a}	27.00 ± 2.00^{cd}	29.67 ± 6.51^{bcd}	94.67 ± 1.53^{bc}	47.33 ± 0.76^{bc}
80:20	33.67 ± 1.89^{bc}	5.67 ± 1.53^{bc}	12.67 ± 1.15^{a}	9.00 ± 2.00^{a}	13.33 ± 1.53^{a}	30.00 ± 2.00^{bc}	32.00 ± 1.00^{abcd}	102.67 ± 3.79^{ab}	51.33 ± 1.89^{ab}
90:10	31.00 ± 4.77^{c}	6.00 ± 4.58^{bc}	7.67 ± 2.08^{b}	8.67 ± 2.52^{a}	15.00 ± 3.00^{a}	32.33 ± 4.73^{b}	38.33 ± 5.03^{a}	108.00 ± 11.27^{a}	54.00 ± 5.63^{a}
100:0	33.15 ± 0.87^{c}	5.00 ± 2.00^{c}	1.67 ± 0.58^{d}	6.67 ± 1.15^{a}	14.33 ± 2.08^{a}	37.33 ± 2.08^{a}	35.33 ± 1.15^{ab}	100.33 ± 3.51^{ab}	50.17 ± 1.76^{ab}

Note: Values (mean ± SD) with different lowercase superscripts in column indicate significant ($p<0.05$) differences. Abbreviations: MI = mitotic index, MN = micronucleus, CM = C-mitosis, FC = fragmented chromosome, CB = chromosome bridges, SC = sticky chromosomes, and LC = laggard chromosome.

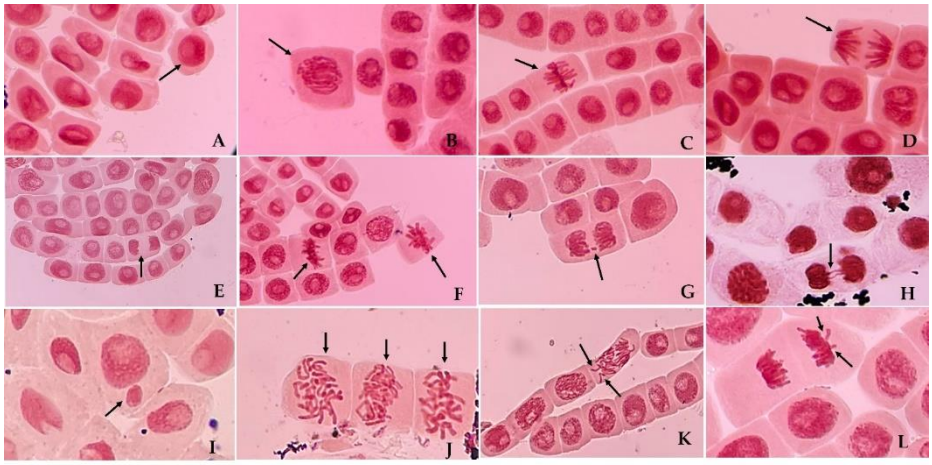

Figure 2. Characteristics of normal and abnormal cell division in onion root tip cells at 400× magnification: (A) Interphase, (B) Prophase, (C) Metaphase, (D) Anaphase, (E) Telophase, (F) Sticky chromosome, (G) Fragmented chromosome, (H) Chromosome bridges, (I) Micronucleus, (J) C-mitosis, (K and L) Laggard chromosome

4. Conclusions

Spent coffee grounds (SCG) can be utilized as a growth medium to promote plant growth. The experiments on onions demonstrated that the optimal SCG-to-sand ratio for influencing root growth was 40:60. During the onion root cell toxicity testing of SCG, it was observed that using a growth medium with a 60:40 ratio resulted in increased abnormalities in the onion root cells. This mixture inhibited cell division at the root tips, which was correlated with abnormal cells and a decrease in the MI. Using SCG in optimal amounts can promote root growth in plants, but excessive use may lead to cell toxicity and hinder growth. Consequently, SCG might be an alternative to organic amendments for plants. However, further research is needed to investigate the chemical profiling of SCG in relation to various plant species, as well as to conduct cytotoxic and physiological assessments. This would help clarify the dose-response relationships and toxicity mechanisms and facilitate the development of processes or tests for evaluating SCG in field conditions. Such a comprehensive approach could ultimately lead to the safe and sustainable incorporation of SCG in agricultural systems.

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