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STRATEGIES TO REDUCE PESTICIDE RESIDUES IN FOOD: REMARKS ON PESTICIDE FOOD POISONING SCENARIOS IN NIGERIA (1958–2018)

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Abstract: Food insecurity across the globe, particularly in developing countries such as Nigeria, portends deleterious health challenges owing to various environmental contaminants, including the incursion of pesticides into the biosphere and the consequential retention of pesticide residues in food composition. This study provides a depth of insights into a comprehensive analysis on how the indiscriminate applications of pesticides have distorted both the terrestrial and aquatic ecosystems, altered the food chain and occasioned the appalling incidence of food poisoning. Available data from 1958 to 2018 on pesticide food poisoning scenarios in Nigeria were collected, collated and critically evaluated. The findings of this analytical study, which is navigated through a thorough content and discourse analysis of reliable information, unveiled the various channels through which pesticide residues had penetrated into the food chain and its hazardous implications on the plants, animals and humans being at the receiving ebb of the food chain. In addressing this dilemma, the authors suggested a seam of inter-connected critical and strategic tactical approaches to reduce pesticide poisoning, which, if adopted by the Nigerian government and that of other developing countries, will effectively combat the pervasiveness of this threat to food security.

Key words: action plans, environmental sustainability, Nigeria, pesticide residues, urban foods.

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Introduction

The ubiquity of pesticides is not contentious as pesticides are used everywhere, in plant protection, food storage facilities, animal and public health (residential buildings, hospitals, schools, parks), etc. The use of pesticides dates back to the era of ancient Sumerians about 4500 years ago, when the use of elemental sulfur was relied upon to control insects and mites attacking fruits and vegetables (Unsworth, 2010). Since this era, scientists have been particularly concerned about the risks linked with the use and abuse of pesticides in the environment generally. Broadly speaking, pesticides are substances that are used to protect humans against the insect vectors of disease-causing pathogens, to protect crop plants from competition from abundant but unwanted plants (i.e., 'weeds'), and to protect crop plants and livestock from diseases and depredations by fungi, insects, mites, and rodents (Rosenfeld and Feng, 2011). Synthetic pesticides, formulated to control various types of organisms, include insecticides, acaricides, rodenticides, molluscicides, nematicides, fungicides, herbicides and others. They may exist as liquids, gels, pastes, chalks, powders, granules, pellets, gases etc., with the active ingredient present at varying concentrations.

In Nigeria, synthetic pesticides were introduced in the 1950s, and their usage has been on the increase ever since their application in cocoa production (Adeyeye and Osinbajo, 1999; Adesuyi et al., 2018). It is estimated that about 125,000–130,000 metric tons of preparations of over 135 synthetic pesticides are imported and applied every year in Nigeria (Asogwa and Dongo, 2009; Erhunmwunse et al., 2012). Also, Nigerian farmers and householders have been relying heavily on pesticides for the control of various weeds, insects, and pathogens, leading to the high importation of these products (Adesuyi et al., 2018). The classes of pesticides usually employed for agricultural uses are insecticides (dichlorvos), fungicides (e.g. azoxystrobin, chloranil, thiabendazole etc.), herbicides (glyphosate, atrazine, etc.). Insecticides generally act as respiratory inhibitors, and insect growth regulators. Popular insecticide families include organochlorines, organophosphates, and carbamates. These chemicals are highly toxic, used extensively in both domestic and industrial settings and valued because of their fast action. However, some locally adulterated, uncertified, and expired chemical pesticides such as *Otapiapia* are locally manufactured and hawked by low-income dwellers in the streets of cities of Nigeria (Maduako, 2009; Musa et al., 2010a).

However, it is very important to point out that the primary essence of pesticide application is to control weeds, insects, mites and other harmful animals and plant pathogens at the time of crop cultivation and food storage (USEPA, 2017). The problem of poor transportation network remains one of the major setbacks in Nigeria and many other developing countries, and as such, farmers continue to face the difficulty of transporting their agricultural produce from the farm to the markets

in urban settlements where they are sold. Hence, they resort to applying pesticides on the farm produces in order to preserve them while it takes them days to transport them to their desired destinations.

Therefore, the common practice of applying Gamalin 20 to cocoa beans to prevent molds and maggot development is a usual practice in cocoa-trading zones in Nigeria. Many low-income households in Nigeria still depend on 'Sniper' to get rid of mosquitoes, cockroaches and other insects, even reptiles at home. Similarly, the prevalence of weevils on stored beans in various parts of Nigeria has led many bean traders to use 'Sniper', a toxic pesticide containing 2,2-dichlorovinyl dimethyl phosphate (DDVP), to control weevil infestation (Karigidi, 2018). Nigeria being a populous country and a net-importer of food has a reasonable number of people in the food business, many of whom utilize pesticides in their daily operation even to the extent of mixing together different pesticide formulations to eradicate pests without putting into consideration their harmful effects on the environment. It is the fact that over 98% of insecticides and 95% of herbicides applied in the environment reach a destination other than their target species due to pollution pathways such as air, water and soil (Miller, 2004 as cited in Babarinsa et al., 2018). Organochlorines are the most common pesticides applied to food crops on the field and during storage (Ogah et al., 2011).

Despite efforts to address indiscriminate usage, several factors are responsible for the continual use of pesticides. The most prominent factor driving pesticide use, as articulated by Xu et al. (2008), is the need to control a wide range of harmful organisms, including insects, plant pathogens, weeds, rodents etc., as well as to guard against the loss of crops and minimize the threats to human and livestock health. According to Xu et al. (2008), two main factors motivating the continual and excessive use of pesticides in our society include pesticide resistance and the simplifications in the agro-ecosystem (monocropping), causing imbalances in the agricultural ecosystems with a reduction in the density of natural enemies' populations and more intensive pest infestation, even the emergence of new pests. Furthermore, the poor awareness among farmers about the benefits and hazards associated with agricultural pesticides was reported in a study conducted in Bayelsa State, the southern part of Nigeria (Kainga et al., 2016). Low education levels of the rural populace, poor pesticide education or lack of information and training on pesticide safety measures, poor application technology, and inadequate protections leading to extensive misuse have also been identified as a major problem (Hurtig et al., 2003; Atreya, 2008; Ojo, 2016).

There are numerous ways pesticides can enter the food chain, during the pesticide production, their application during cultivation, the storage and distribution of agricultural produce, unintended contamination during household usage, the accidental discharge and surface run-offs of pesticides. When these chemicals are used injudiciously or when there is an accidental discharge or

spillage, pesticides may contaminate the soil, nearby groundwater and surface water (Erhunmwunse et al., 2012), thus contaminating potable water and even possibly endangering the aquatic organisms (e.g. fishes, crabs, and periwinkles). Children and pregnant women are most vulnerable to the hazard of pesticide exposure, perhaps because of their high metabolic rates. Exposure scenarios of children to pesticides are prevalent. Most of the times, children exposure to pesticides is because of pesticide usage to prevent mosquitoes, cockroaches and rodents, or due to storage within reach of children. Considering the behavior of children (e.g. crawling and hand-to-mouth activities) and the use of pesticides in schools and playgrounds (playing fields and parks), the exposure of children to pesticides is possible and could be lethal.

According to PAN International (2007), there is a global surge in the incidence of pesticide poisoning, with an estimate of 1–41 million people suffering health problems from exposure to pesticides annually. It is estimated that a minimum of 300,000 deaths is due to pesticide poisoning annually, with most of them (99%) from low- and middle-income countries (WHO, 2009). Pesticide residues in food have been responsible for several food poisoning and death cases in Nigeria (Shaibu, 2008), which is due to the injudicious use of chemicals on food products. A plethora of literature has emphasized the toxicity and persistence characteristics of pesticides in the environment, with limited studies on the extent of pesticide contamination and the safety measures needed to reduce pesticide residues in food.

Hence, this study attempts to address the following research questions:

i. To what extent are Nigerian local foods contaminated with pesticide residues?

ii. What safety measures are needed to minimize pesticide residues in food?

Thus, the questions above are critically explored through the analysis of the methodology, results and discussions, and conclusion on findings.

Material and Methods

This study relied on a review of relevant information about the subject matter using a wide range of appropriate key words (such as monitoring + pesticide residues + food + Nigeria) to search or filter peer-reviewed journals, books on "pesticide residues in Nigerian food markets" on scholarly search engines such as EBSCO Discovery Service (EDS), Google Scholar, Google Books, Microsoft Academic, ResearchGate, WorldWideScience, personal blogs, online newspapers and internet reports and others. Content analysis–a method of inquiry for the subjective interpretation of the content of the data of a text by the systematic process was utilized to capture and observe trends of pesticide poisoning cases and the possible measures that can be taken to reduce pesticide residues. Discourse

analysis was also utilized for studying text linguistics, i.e., tone, phonology, syntax, style and organization in academic research papers. Through the deconstruction of texts, implicit or hidden contents in academic research papers were made more obvious through reasoning and analytical thinking, thereby helping to sieve out reliable information on research objectives. Searches related to the first objective were limited to studies conducted in (or for) Nigeria, but the second objective utilized a broad range of works of literature without any geographical restrictions. Fifty (50) references were obtained for this study, but after careful selection and sieving, twenty-five (25) references were considered suitable for this purpose. In order to measure the extent of pesticide contamination, we gathered data on pesticide residue levels in Nigerian foods covering most states as well as pesticide poisoning cases in the country from 1958 to 2018 and examined the type of pesticide residues found, especially if they are on the list of persistent pesticides or banned.

Results and Discussion

The extent of pesticide contamination in Nigerian local foods

a. Pesticide residue levels in Nigerian foods

Tables 1 and 2 illustrate information on levels of pesticide residues in Nigerian foods as well as pesticide poisoning incidents in the country from 1958 to 2018. The presence of pesticide residues in foods and the events of food poisoning by pesticides is an indication that farmers or food dealers at one point or the other have used pesticides to preserve food loss or damage by the pest.

b. Pesticide poisoning incidences in Nigeria (1958–2019)

Pesticides are among the leading causes of death by self-poisoning, particularly in low- and middle-income countries (WHO, 2018). The worldwide deaths and chronic diseases due to pesticide poisoning amount to about 1 million per year (Environews Forum, 1999). Table 2 shows that pesticide poisoning in Nigeria dates back to decades and will occur again if adequate and legislative measures are not taken.

Table 1. Data on pesticide residue levels monitored in foods consumed by Nigerians across the States.

Musa et al. (2010b)	Smoked fish species, *Clarias* sp., *Gymnarchus niloticus* and *Tilapia* sp. were contaminated with DDT, dichlorvos and lindane.	Smoked fish	Northeast Nigeria
Aikpokpodion et al. (2012).	Forty percent (40%) of cocoa beans obtained from Ondo State had diazinon (organophosphate) residues while there was no detectable diazinon in cocoa beans from Cross River and Ogun States.	Cocoa beans	Ondo, Cross River and Ogun
Akinloye et al. (2011)	Paraquat residues weredetected in some crops, fruits and vegetables though these residues were within the PQ tolerance or maximum pesticide limits.	Radish, maize, plaintain, yam, tomato, pawpaw, waterleaf and other vegetables	Ogun
Gwary et al. (2012)	Dichlorvos, endrin and DDT were detected in both field samples (pre-storage), and stored samples (post-harvest). Dichlorvos was applied to prevent stored beans from insect attack.	Beans	Borno
Ogah and Coker (2012)	Maize sampled in the study contained residues of one or more organophosphate or carbamate pesticides. There is a high incidence of pesticide residues in maize sold in Lagos markets.	Maize	Various markets in Lagos State
Ogah et al. (2012)	Residues of DDT, endrin, endosulfan (except dieldrin) were detected in beans exceeded WHO MRLs. The estimated total diet intakes for aldrin and dieldrin exceeded their maximum permissible intakes by 100% and 17%, respectively.	Beans	
Gushit et al. (2013)	Residues of atrazine and other priority pollutants were detected in food samples.	Groundnut, potato, cucumber, rice straw, yam tuber, cassava, acha straw	Northern part of Nigeria
Akan et al. (2013)	Residues of organophosphorus pesticide residues (dichlorvos, diazinon, chlorpyrifos, and fenitrothion) were present in vegetables at alarming rates (exceeded WHO MRLs).	Vegetables (spinach, lettuce, cabbage, tomato and onion)	Borno State
Okoya et al. (2013)	Residues of organochlorine pesticides (cis-chlordane, α- endosulfan DDE and dieldrin and dieldrin) were detected in rivers in Ondo state. OCP levels were significantly ($P < 0.05$) higher in the dry season than in the wet season among the rivers studied.	Rivers	Ondo
Anzene et al. (2014)	Organochlorine pesticide (OCPs) residues were detected in post-harvest grains. Lindane and aldrin residues were above the maximum residue limits (MRLs) set by WHO/FAO.	Millet, guinea corn and maize grains	Nasarawa
Olufade et al. (2014)	Higher residues of aldrin and heptachlor were detected in cowpea and dried yam chips, which were generally above the EU-MRLs.	Cowpea, dried yam chips	Osun (Ile-Ife markets)
Akan et al. (2014)	Fish samples contained residues of OCP but within allowable limits.	Fish	Borno
Ogbeide et al. (2015)	High concentrations of organochlorine pesticides, specifically benzenehexachloride (α-BHC, γ-BHC, and β-BHC) were detected in water, sediment, and fish with a potential cancer risk for the local residents with lifetime consumption of pesticide-contaminated fish.	Water, fish	Edo State

Continued Table 1. Data on pesticide residue levels monitored in foods consumed by Nigerians across the States.

Shinggu et al. (2015)	OCPs were detected in water, sediments and two species of fish in Lake Geriyo, but higher levels of OCPs were found in the fish samples, higher in most cases, than the recommended WHO maximum residue levels (MRLs) of 0.05 mg/kg in food items.	Fish	Adamawa
Ogbeide et al. (2016)	Residues of organochlorine (hexachlorocyclohexane) were detected in surface water, sediment, and fish (Tilapia zilli and Clarias gariepinus). The study shows that there is a potential risk to humans exposed to contaminated water, sediment, and fish through ingestion, inhalation, and dermal routes of exposures.	Surface water, sediment, and fish	Edo
Njoku et al. (2017)	Residues of alachlor, atrazine and many other pesticides were detected in common vegetables with values greater than WHO MRLs.	Vegetables (spinach, lettuce)	Lagos
Olutona and Livingstone (2018)	Eleven OCP residues were detected in some brands of malt drinks at varying concentrations with endosulfan II above EU-MRLs.	Malt drinks	Osun
Omoyajowo et al. (2018)	Residues of different kinds of pesticides were detected in common fruits consumed, but watermelon exceptionally had residues of atrazine, clothianidin, omethoate and oxamyloxime above WHO/FAO MRLs.	Apple, watermelon and sweet orange	Lagos

Possible measures and action plans for reducing pesticide residues in foods

Reducing pesticide residues in food commodities requires conscious and concerted efforts on the part of the public and the government, given the presence of these residues may affect human health. The following approaches will go a long way in stemming the incidences of pesticide poisoning and other health-related crises associated with the ingestion of food contaminated with pesticides:

a. Thorough washing or rinsing

A plethora of studies have demonstrated that thorough washing or rinsing of food with different solvents may help reduce pesticide residues (Bhagirathi et al., 2001; Yuan et al., 2009). Washing fruits and vegetables with a salt solution is an appropriate way to remove pesticide residues from food surfaces. For instance, washing Chinese cabbage with a 1% saline solution for 10 minutes is effective in removing chlorothalonil residues (Lee and Chou, 1995). Ong et al. (1996) reported that chlorinated and ozonated water could help remove residues of azinphos-methyl, captan and formethanate-HCl on apple. Another study reported that washing potatoes with tap water or aqueous solutions of acetic acid effectively removes organochlorine and organophosphate residues (Zohair, 2001). Similarly, a study suggested that washing with sodium base (1%) or sodium hydrogen carbonate solution (1.5%) could reduce the level of chlorpyrifos-methyl insecticide and fenarimol fungicide in cherries (Hadjikinova et al., 2006). The treatment of

food with ozone water is effective in removing organochlorine residues (Kim et al., 2000; Wu et al., 2007; Vijayasree et al., 2013). Another study reported that soaking cowpea fruit in water for 15 minutes and later soaking in 0.1% edible vinegar for 15 minutes, followed by treatment with running tap water for 2 minutes, will effectively remove residues of chlorantranilipole on cowpea. Several studies have also suggested that a combination of washing, peeling, blanching, frying and cooking can remove pesticide residues effectively (Krol et al., 2000; Kang and Lee, 2005; Duhan et al., 2010; Huan et al., 2015; Keikotlhaile et al., 2010; Aondona et al., 2019). The aforementioned studies suggest that thorough washing or rinsing of food commodities will remove pesticide residues to a great extent and will reduce associated risks.

Table 2. Human poisoning incidences in Nigeria from 1958 to 2018.

State/location and year	Description of catastrophe	Citation(s)
Okebode, Southwest Nigeria:1958	All members of the family of a local chief, a prominent cocoa farmer at Okebode in southwestern Nigeria, were hospitalized after eating leaf vegetable undergrowth from a cocoa farm that had been earlier sprayed with lindane.	Ikpesu and Ariyo (2013)
Oyo State:1960	Many cases of human poisoning by Gammalin 20 (lindane) were reported at University College Hospital, Ibadan.	Igbedioh (1991)
Ondo State:1982	Twenty public health field workers were poisoned by malathion.	Igbedioh (1991)
Southwestern Nigeria:1986	Local people complained of nausea when they used water suspected to be contaminated with organophosphorous insecticide for making their local food (cassava meal).	Sridhar et al. cited in Sridhar and Ogbalu (1986)
Imo State:1988	A family of five (5) died after they had eaten the meals contaminated with pesticides.	Igbedioh (1991)
State not-specified: 2004	Carbofuran pesticide residues found on several batches of noodles manufactured in Nigeria may have resulted in 23 reported cases of vomiting and one death.	Olurominiyi and Emily (2011)
Cross River: 2008	112 people were hospitalized, and 2 people died after eating moin-moin and beans contaminated with an outrageously high level of organophosphate carbamates, fenitrothion and chlorpyrifos.	Shaibu (2008)
Gombe: 2008	Over 120 students at a public school were hospitalized after eating the meal contaminated with a high dose of lindane.	Shaibu (2008)
Gombe, Adamawa: 2011	Six family members died after eating moin-moin prepared from suspected poisoned beans.	Ikpesu and Ariyo (2013)
Cross River: 2011	Many citizens of Bekwarra Local Government Area of Cross River State were hospitalized after eating moin-moin and beans.	Ikpesu and Ariyo (2013)
Adamawa: 2018	A family of 4 died, shortly after they had eaten meals contaminated with pesticides during Ramadan fast.	Okon (2018)
Ilorin: 2018	A family of 4 died, 4 others were hospitalized after they had eaten meals contaminated with pesticides.	Okon (2018)
Bayelsa: 2018	Three family members, including a pregnant woman, died after eating a dinner contaminated with pesticides.	Okon (2018)
Yassharu village, Kafur LGA, Katsina	Some people suffered from pesticide poisoning after eating maize preserved with pesticides.	Ikpesu and Ariyo (2013)
Isua-Akoko Area of Ondo State	Four out of a 9-member family died after eating yam flour treated with pesticides.	Olulakin et al. (2015)

b. The regulation, adoption and implementation of harmonized guidelines for the registration of pesticides

As earlier reported by Ojo (2016), poor legislation and lack of enforcement of available legislation on pesticide use and distribution are a major cause of pesticide poisoning in Nigeria and even transcend beyond Nigeria. Hazards arising during the application of pesticides are mainly due to lack of information, knowledge and awareness, poor legislation or enforcement of legislation, and sales in the open market of pesticides (WHO, 1990). Governments of developing countries are beleaguered with pesticide mismanagement and handling by unlicensed retailers and smuggling across porous land borders (Keri, 2009). The regulations governing the use of chemicals are encapsulated in the ECOWAS Regulations on Pesticides which exhaustively entail monitoring, authorization, marketing, distribution and use of pesticides in the West Africa sub-region, while NAFDAC (National Food and Drug Administration Agency) is also saddled with the responsibility of monitoring compliance with safety and authorization of the use and distribution of these pesticides at the national level.

For instance, NAFDAC issues permits for specific brands of pesticide formulations used in Nigeria. Hence, the government or agency should regulate the marketing, storage, use, and disposal of pesticides and ensure that only pesticides that have been registered enter the market while those already in the country should be removed and disposed of correctly. The evaluation of the data submitted in the registration dossier should follow internationally accepted and agreed evaluation standards and procedures in as much as these are available. The harmonized protocol for bio-efficacy studies and the harmonized residue study protocol will help to inform appropriate policy and guidelines for the registration of pesticides and licensed trade of pesticides (Keri, 2009; WHO, 2010).

c. Proper pesticide labeling

Each pesticide allowed in the market should clearly show directions for use in the local language, the use for which it is intended, how and when it should be used, and the quantity should be specified. The label should contain conspicuous warnings regarding the consequences or dangers of its misuse to humans, plants, and the environment and should include statements about the route of entry and specific actions that must be taken to avoid exposure (Adesuyi et al., 2018). For instance, a pesticide label might read: “Poisonous if swallowed, inhaled, or absorbed through the skin” or “Avoid contact with eyes, quickly contact your doctor in case of any eventualities”. Thus, farmers and pesticide applicators should be encouraged to purchase protective clothing or kits at subsidized rates. The commission should be empowered to provide directives on the advertisement of all registered pesticides (Ojo, 2016; Adesuyi et al., 2018).

d. Education

Public sensitization and capacity building programs for farmers, agricultural extension workers, food wholesalers/retailers on good agricultural practices (GAPs) are critical for agricultural resilience and a safe environment. As echoed by Sridhar and Ogbalu (1986), there is an urgent need for the government to collect systematically and disseminate all the information available on pesticide production, importation, handling and usage, residues, occupational hazards and poisoning and pest resistance. Moreover, all should encourage GAPs and the integration of integrated pest management (IPM). GAPs refer to the basic environmental and operational conditions necessary for the production of safe, wholesome fruits and vegetables (NCSE, 2018) or "the proper use of pesticides to obtain maximum protection from pests" (Alexander and Anderson, 1984). The guiding principle of IPM is that pesticides should be used judiciously in conjunction with nonchemical means of pest control. Examples of GAPs include pesticide dose adjustment for tree-fruit spraying, worker hygiene and health, manure use and water quality throughout the production and harvesting process, proper storage and disposal of pesticides, understanding the characteristics of the application site (soil texture, slope, organic matter) before applying the pesticide. The food regulatory agencies in the country should ensure that pesticide applicators, especially those in agricultural activities, are trained and educated on the proper use of pesticides and the concept of integrated pest management (Adesuyi et al., 2018). The Nigerian government should also promote aggressive enlightenment campaigns through the efforts of both public and private sectors to create a high level of awareness amongst the citizenry about the available alternatives to synthetic pesticides. Training and sensitization programs on the judicious use of pesticides must be done at the village or community level to ensure grass-root participation while the cost of training and demonstrations may be shouldered by the manufacturer of these pesticides. The public should be enlightened on how to carefully dispose of empty pesticide containers and how important good personal hygiene is, such as washing their hands with soap after the use of pesticides (Ojo, 2016; Adesuyi et al., 2018).

e. The inspection of registered pesticides and monitoring of pesticide residues in locally-consumed products:

The effective post-marketing surveillance of registered pesticides will help to monitor the safety of pesticides after they have been released to the market. Prior to the use of any pesticides, the marketing authorization holder must submit a risk assessment plan to the regulator, which does not excise plans for post-authorization studies. The post-marketing surveillance of registered pesticides is critical in ensuring that newly marketed pesticides achieve the highest safety standards. All industrialized countries have food monitoring programs that measure pesticide

residues. Levels exceeding the maximum established limits have been reported occasionally in monitored food (Njoku et al., 2017; Omoyajowo et al., 2018). It has been reported that many foods consumed locally do not pass through monitoring and evaluation, unlike the cash crops or foods meant for export which must be passed through safety-check in agencies such as NAFDAC. Unfortunately, many Nigerian crops (such as cowpea, tubers, etc.) are rejected on account of a high level of residual pesticides. Sadly, there is no way an individual consumer can determine whether or not the food he/she consumes is laced with pesticides or not (Auwal-Ahmad and Awoyale, 2008; Ojo, 2016). Therefore, the consistent and stringent monitoring and evaluation of registered pesticides and of pesticide residues in locally-consumed products should be done and sustained, while tested protocols for safe disposal of expired, obsolete or otherwise unwanted pesticides should be put in place and well-publicized (Njoku et al., 2017; Omoyajowo et al., 2018; Adesuyi et al., 2018).

f. Alternatives to the use of synthetic pesticides

There are various alternative ways to control pests, which include cultivation methods, biological methods, pheromones and hormones, genetic controls, quarantine and irradiation (Raven and Berg, 2004).

Some of these methods are non-toxic methods that can be used in controlling insects in the home and gardens.

✓ Cultivation method: One way to reduce damage by crop pests is by interplanting (having alternating rows of different plants). The proper timing of planting, fertilizing and irrigating promotes healthy, vigorous plants that are more able to resist pests because they are not stressed by other environmental factors. Also, the rotation of crops can also help to control pests. When maize is not planted in the same field for 2 years in a row, the maize rootworm is effectively controlled (Raven and Berg, 2004).

✓ Botanical (or green) pesticides: This involves using plant extracts to control pests. In recent times, attention has been paid towards the exploitation of higher plant products as novel chemotherapeutics in plant protection (Dubey et al., 2008). Higher plants contain a wide spectrum of secondary metabolites such as phenols, flavonoids, quinones, tannins, essential oils, alkaloids, saponins and sterols. They are usually eco-friendly, safer for users and very effective when used correctly (Isman, 2000). For instance, essential oils possess anti-microbial and anti-fungal properties, and there is an urgent need to bio-prospect the pesticidal property of different essential oils and more efforts should be made scientifically to document the pesticidal plants and to investigate their bio-control efficacy.

The following are instances where plant products have been used to control pests: using distillates of a cocktail of herbs such as lemongrass, lime peels, and hyssop achieves 100% adult weevil and egg mortality within one hour of exposure

to infested beans, using neem oil and rotenone (Karigidi, 2019). Neem oil is extracted from the nut of the neem tree (*Azadirachta indica*), which contains the chemical *azadirachtin*. Rotenone is a natural insecticide extracted from the roots of certain tropical plants (Raven and Berg, 2004; DeAngelis, 2017).

✓ Pheromones: Pheromones are natural substances produced by animals to stimulate a response in other members of the same species. They are species-specific signals which enable communication between life-forms of the same species, i.e. intraspecific communication (Abd El-Ghany, 2019). Pheromones trigger a reaction in the recipient that causes changes in its behavior (Cork 2004). Since each insect species produces its own specific pheromones, once the chemical structure is known, it is possible to make use of pheromones to control individual pest species. Pheromones have been successfully used to lure insects, such as Japanese beetles, to traps, where they are killed.

According to Abd El-Ghany (2019), pheromones have been classified into eight types: aggregation pheromones (attract individuals of both sexes at food sites and reproductive habitats), alarm pheromones (alert members of the same species to the presence of a menace), oviposition-deterrent pheromones (discourage females from laying eggs in the same resource of another female), home recognition pheromones, sex pheromones (mediate the interaction between sexes of the same species and are mainly produced by females to attract males), trail pheromones (guide social insects to distant food sources), recruitment pheromones (induce nestmates to leave the nest and migrate to a work site or vice versa), royal pheromones (enables workers to recognize patronage like kings and queens, thereby maintaining the strain reproductive division).

✓ Genetic controls: Genetic engineering offers great promise in breeding pest-resistant plants more quickly, e.g. a gene from *Bacillus thuringiensis* has been introduced into several plants such as cotton. This has been effective against caterpillars that eat cotton leaves. Further work has been done to greatly increase the potential of Bt toxin as a natural pesticide by modifying the gene coding for the toxin, so that it affects a wider range of insect pests while studying resistance management strategies to curb pest resistance to GM crops.

✓ Quarantine: Governments attempt to prevent the importation of foreign pests and diseases by practicing quarantine or to restrict the importation of exotic plant and animal materials that might harbor pests. If a foreign pest is accidentally introduced, the quarantine of the area where it is detected helps prevent its spread. If a foreign pest is detected on a farm, the farmer may be required to destroy the entire crop.

✓ Integrated pest management (IPM): IPM coordinates practices that reduce the quantity of pesticide use and exposure to chemicals. Since it is impractical to ban a large number of pesticides right now, implementing programs to reduce pesticide use is critical and IPM represents the most effective way to control and

manage pests because it uses cultural, mechanical, and biological pest controls where possible (Raven and Berg, 2004). The general goals of IPM are to: reduce pesticide levels in the environment; lessen hazards to people and the environment; increase the use of natural pest controls; develop a greater understanding of effective pest control methods and establish programs that will encourage voluntary participation. IPM is an important part of sustainable agriculture that optimizes natural controls by using agricultural techniques that discourage pests. In order to be effective, IPM requires a thorough knowledge of the life cycles, feeding habits, migration and nesting habits of the pests as well as all of their interactions with their hosts and other organisms. The complex, sophisticated knowledge needed to use IPM as stated above accounts for why it is not widespread.

The legal framework for the reduction of pesticide residues in local foods in Nigeria

The steady increase in the incursion of pesticide residues in the food chain right from 1958 to 2018 in Nigeria vividly unveils a symmetrical dilemma in the regulatory efficiency of administrative institutions empowered by the law to monitor the influx of pesticides into the market, and indeed the efficiency of legal enforcement agencies to prosecute erring individuals and companies that consistently endanger the lives of food consumers.

Section 135(1) of the FCCPA compels manufacturers and distributors of goods to notify the public of goods that can endanger their health and then withdraw such goods completely from the market. Sub-section (2) of the above section imposes strict penal consequences for the sale of contaminated foodstuffs on the marketer and sellers. However, it is saddening that despite the existence of these penal and punitive legal sanctions against food contamination that can result from the indiscriminate application of pesticides on foods, no individual or body corporate has been brought to book. Thus, this prosecutorial dereliction on the part of enforcement agencies continues to portend a setback on the effectiveness of the law in reducing the indiscriminate use of pesticides.

Hence, the role of pesticide producers in economic growth cannot be undermined, especially in countries such as Nigeria, where there are poor transport and storage facilities for agricultural produce, and as such, the law cannot stop the production of pesticides as a measure to curb the indiscriminate pesticide application on food. However, the law put in place regulatory measures on the importation, production, registration and even advertisement of all kinds of pesticides in Nigeria by establishing statutory corporations such as the National Agency for Food and Drugs Administration and Control (NAFDAC), the Federal Competition and Consumer Protection Commission (FCCPC) and the National

Environmental Standard and Regulation Enforcement Agency (NESREA), to regulate which kinds of pesticides can be imported or produced.

The NAFDAC Act and its subsidiary legislations empower NAFDAC to control the production, importation, registration of drugs such as pesticides and even prescribe standard specifications and guidelines for their products so that the kinds of pesticides that are imported or produced are not injurious to consumers' health. Hence, no pesticide can be put to sale in the market except they are registered, and they cannot be registered until NAFDAC has certified that they pose no injurious consequences on consumers' health.

It is further worthy to note that the National Environmental Standard and Regulation Enforcement Agency has the responsibility, under section 7(f) of the NESREA Act, to enforce the safe use of pesticides, especially at the stage of its application on farm crops and during food storage. Therefore, it is safe to aver that Nigeria has sufficient laws and agencies on pesticide regulations, but the enforcement of those laws and efficiency of those agencies are lacking (Ojo, 2016; Njoku et al., 2017). Thus, there is an urgent need for effective collaboration among NAFDAC, NESREA, and FCCPC, to prosecute erring persons and regulate the sale and usage of pesticides.

Therefore, it is submitted that there is a need for a state of emergency to be declared on food security through the inauguration of a task force on pesticide residues in foods. The task force will consist of governmental agencies of strategic importance and traders' associations for surveillance. The customs and immigration services must strictly stiffen and tighten up the Nigerian borders in order to curb the illegal smuggling of pesticides in the country. It is nevertheless admitted that this clean-up plan can only thrive in a corrupt-free environment. Hence, no meaningful result will be made if the border guards are the smugglers to be waded off. However, there is no doubt that an honest, committed and painstaking scrutiny at the borders will substantially reduce the presence of sub-standardized pesticide products in the market.

It is further submitted that the federal, state and local governments can build massive pest and disease resistant silos in every local government area, where agricultural produce can be stored in large quantities and also sold to traders. If this initiative is well implemented, it will save the traders the need to fumigate their stored food with pesticides, and by implication, there will be a significant reduction in pesticide residues in foods.

Conclusion

The pervasiveness of the indiscriminate use of pesticides for agricultural, preservative and domestic purposes remains unabated. Its grave consequences on human health continue to raise serious concerns bearing the urgency of effective

strategies for reducing pesticide residues in food. While the eradication of pesticides seems not to be feasible due to socio-economic reasons, the panaceas for pesticide food poisoning scenarios in Nigeria lie in the regulation and monitoring of pesticide production, importation, marketing and usage as well as the adoption of alternatives to synthetic pesticides.

In the light of the foregoing, this study concludes with the following recommendations:

i. Future studies should not only examine the potential risks of pesticide exposure but also establish a wide range of pesticide exposure patterns that appear to exist within Nigeria's populations.

ii. The Government and private sectors should sponsor public sensitization programs on the best handling of pesticides and the danger associated with the abuse and misuse of pesticides in Nigeria.

iii. The Government should prioritize and incentivize a zero-pesticide food production and distribution from the farmers down to the food wholesalers to consumer chain in order to support food security, economic goals and community benefits.

iv. The Government should encourage the public to patronize safe alternatives to chemical pesticides and to have an inclusive national pesticide policy that will guide against misappropriation, mishandling, and smuggling of uncertified pesticides.

v. NAFDAC should develop a comprehensive regulatory system that seeks to make possible the beneficial use of pesticides while minimizing their hazards to public health and the environment.

vi. NAFDAC and other regulatory agencies should be empowered to conduct periodic monitoring of food samples for pesticide residues; they should incorporate standard reporting procedures and establish a computerized database to collect data on pesticide residues of food samples in the market, and the residue testing program should include all toxic forms of pesticides, e.g. their metabolites and degradation products.

vii. Customs and immigration services should be tasked to monitor the smuggling of contraband pesticides through the porous borders.

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STRATEGIJE ZA SMANJENJE OSTATAKA PESTICIDA U HRANI: NAPOMENE O SCENARIJU TROVANJA PESTICIDIMA U HRANI U NIGERIJI (1958–2018)

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R e z i m e

Prehrambena nesigurnost širom sveta, posebno u zemljama u razvoju kao što je Nigerija, predstavlja zdravstvene izazove zbog različitih zagađivača životne sredine, uključujući prodor pesticida u biosferu i posledično zadržavanje ostataka pesticida u sastavu hrane. Ovo istraživanje pruža uvid u sveobuhvatnu analizu o tome kako je neselektivna primena pesticida ugrozila i kopneni i vodeni ekosistem, promenila lanac ishrane i izazvala užasnu učestalost trovanja hranom. Dostupni podaci od 1958. do 2018. o scenarijima trovanja pesticidima prisutnih u hrani u Nigeriji su prikupljeni, upoređeni i kritički procenjeni. Nalazi ove analitičke studije, koja se kreće kroz detaljnu analizu sadržaja i diskursa pouzdanih informacija, razotkrili su različite kanale kroz koje su ostaci pesticida prodrli u lanac ishrane i njihove opasne implikacije na biljke, životinje i ljude koji su pri kraju lanca ishrane. U rešavanju ove dileme, autori su predložili niz međusobno povezanih kritičnih, strateških i taktičkih pristupa za smanjenje trovanja pesticidima, koji će se, ako ih usvoje nigerijska vlada i vlade drugih zemalja u razvoju, efikasno boriti protiv rasprostranjenosti ove pretnje prehrambenoj sigurnosti.

Ključne reči: akcioni planovi, ekološka održivost, Nigerija, ostaci pesticida, urbana hrana.

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THE INFLUENCE OF THE APPLICATION OF DIFFERENT PLANT AQUEOUS EXTRACTS ON GRAIN AND PROTEIN YIELD IN SOYBEAN PRODUCTION

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Abstract: The aim of this study was to investigate the influence of aqueous extracts of different plant species on the grain and protein yield of soybean. The testing was conducted at the Institute of Field and Vegetable Crops in Novi Sad on the seeds of the NS Apolo variety. The aqueous extracts of the above-ground part of nettle, the above-ground part of nettle and comfrey, whole banana fruit, banana peel, onion bulbs leaves, the top parts of willow twigs and the top parts of soybean plants were foliarly applied. In addition to the untreated control variant, the experiment also included a distilled water control. Control with distilled water was to show whether the effect of aqueous plant extracts was due to plant material or just water. The results of the experiment showed that the use of aqueous extracts contributed to the increase in grain and protein yield. The increase in grain yield ranged from 9.48% to 15.34%, and the increase in protein yield from 9.31% to 16.16%. The best effect was achieved by applying the aqueous extract of the whole banana fruit and the aqueous extract of the mix of nettle and comfrey. By applying them each year, a significantly higher yield was achieved in relation to the control with distilled water.

Key words: aqueous extract, soybean, banana, nettle, comfrey.

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Introduction

Serbia is among the largest soybean producers in Europe. In the last ten years, soybean areas in Serbia have ranged from 144,000 to 202,000 hectares (Miladinov et al., 2020). In the last ten years, the average soybean yield has been 2.7 t/ha, with a minimum of 1.7 t/ha in 2012 and a maximum of 3.5 t/ha in 2014, which makes Serbia rank among the countries with the highest average yield in Europe (http://www.stat.gov.rs). Within soybean production, in addition to achieving high and stable yields, grain quality, i.e., the content of protein and oil in soybean grain, is also very important (Đukić et al., 2020). Most of the world's population uses meat, milk and eggs as sources of protein. However, with the increase in the number of inhabitants on Earth and the increase in the price of animal proteins, the need for a new source of plant proteins was created, so special attention was paid to the use of protein-oil crops, among which soybeans occupy the most significant place (Žilić et al., 2006). The increased acceptance of soybean proteins is due to manifold soybean qualities, good functional properties in food applications, high nutritional value, availability and low cost (Barać et al., 2004). Soybeans are used in the form of various products for human consumption. Therefore, it is necessary that part of soybean production be without the use of mineral fertilizers and pesticides (Dozet et al., 2019). Organic agriculture is a system of ecological production management that promotes biodiversity, organic matter, nutrient circulation and biological activity of land (Kovačević and Oljača, 2005). Organic production is based on a modern scientific understanding of ecology and agriculture and fully supports and monitors technological development and mechanization (Cvijanović et al., 2013). An increasing number of researches are aimed at finding the application of alternative measures in plant production in order to avoid unwanted consequences (Mamlić et al., 2021). In order to increase the yield and quality of products, foliar treatments with various plant extracts are increasingly applied (Dozet et al., 2017). Herbal extracts can be a significant source of various nutrients. The amount of nutrients depends on the plant species from which the aqueous extract is made, and the land on which the plant species grew. Also, herbal extracts are partially insecticides and fungicides due to bioactive chemicals found in the prepared treatment solution (Kim et al., 2005).

The aim of this study was to determine the influence of foliar fertilization with various plant extracts on the grain yield and soy protein yield per unit area, depending on the year, i.e., the amount of precipitation and temperature during the growing season.

Material and Methods

The object of research was the soybean cultivar 'NS Apolo' developed at the Institute of Field and Vegetable Crops in Novi Sad, Serbia. The field trials were conducted during three vegetative seasons (2019, 2020, and 2021) at the Rimski Šancevi experimental field (45°20′ N 19°51′ E, 80 m a.s.l.) near Novi Sad, Serbia. The trial was set up on chernozem soil type as a randomized complete block design (RCBD) with four replications under the conditions of dry farming. The plot size was 10 m^2. The inter-row spacing of 50 cm and the intra-row spacing of 4.5 cm were applied. Each year the plots were rotated with maize. Prior to sowing, soybean seeds were inoculated with the NS Nitragin microbiological fertilizer as a standard agrotechnical measure in soybean production.

During the three years of the experiment, neither significant disease occurrences nor insect occurrences were observed. Soybean seeds of the NS Apolo variety were sown in the first decade of April. Plants were treated twice. The first treatment was done in the V6 vegetative stage, and the second treatment was done in the R1 reproductive stage.

Treatments with the foliar application of aqueous extracts were as follows:

1. control – without any foliar spray;
2. distilled water (H_2O) only;
3. an aqueous extract of nettle (*Urtica dioica*) – prepared from above-ground parts, i.e., nettle tree and leaves;
4. an aqueous extract of nettle (*Urtica dioica*) and comfrey (*Symphytum officinale*) – prepared from the above-ground parts of nettle and comfrey in a ratio of 3:1;
5. an aqueous extract of the banana fruit (*Musa x paradisiaca*) – prepared from the ripe fruits of the banana, along with the peel;
6. an aqueous extract of banana peel (*Musa x paradisiaca*);
7. an aqueous extract of onion bulb leaves (*Allium cepa*);
8. an aqueous willow extract (*Salix matsudana*) – prepared from the tops of willow twigs;
9. an aqueous extract of soybean (*Glycine max*. (L.) Merr.) – prepared from the top parts of soybean plants.

For the preparation of plant extracts, one kilogram of plant material was used, which was cut into small pieces, the size of 1–4 cm. After cutting, the plant material was submerged in 10 l of rainwater and left to ferment with daily stirring. After fermentation, the obtained plant extract was filtered through gauze and left to stand at a temperature of 18–22 °C in glass bottles, hermetically sealed. Prior to the foliar treatment of soybean plants, the aqueous extract was diluted with distilled water in a ratio of 1:15. An application rate of 300 l per hectare was used.

Mechanical harvesting was conducted with a small harvester for plot trials (Wintersteiger elite). After basic plot harvesting, grain weight and moisture content were measured, and yield was calculated in kg ha^{-1}, with a moisture level of 14%. The protein content in the grain was measured by a spectrophotometer following the principle of the NIR technique. Based on the soybean grain yield and protein content, the protein yield per unit area was calculated.

Weather conditions. In 2019, the average daily air temperature was 19.5 °C, and the amount of precipitation during the growing season was 418.6 mm, with a deficit of 124 mm (Figures 1 and 2). From the date of sowing to the emergence of plants, 30.7 mm of precipitation was recorded, which was sufficient for uniform soybean emergence with the existing soil moisture. In the period from emergence to maturation, 310.7 mm of precipitation was recorded. The period of drought lasted from July 21 until the end of the vegetation period and amounted to 52 days. In 2019, during vegetation, there were 36 days with temperatures above 25 °C.

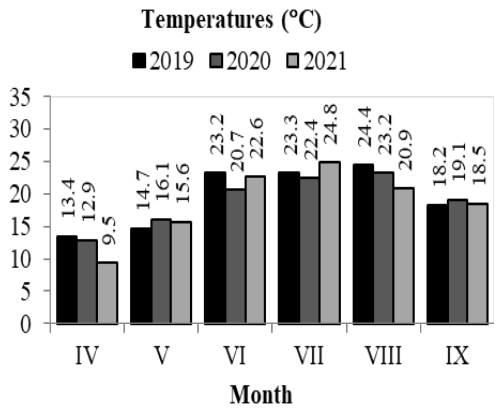

Figure 1. Average monthly temperatures during the vegetation season.

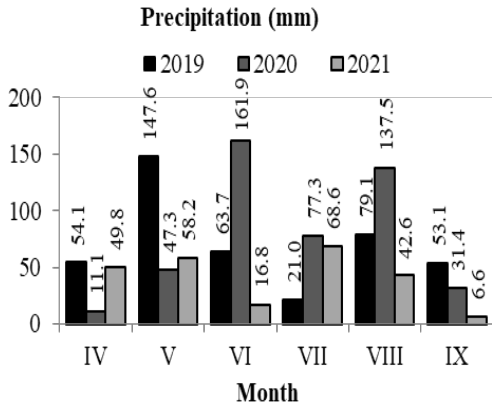

Figure 2. Average monthly precipitation during the vegetation season.

In 2020, the average daily air temperature was 19.1 °C, while the amount of precipitation was 466.5 mm during the growing season. In 2020, the most favourable conditions for soybean production were observed in the precipitation and air temperature distribution. There was no deficit of precipitation and drought that year, i.e., there was an excess of precipitation of 9.1 mm. During the soybean growing season, 17 days with temperatures above 25 °C were recorded.

In 2021, the average daily air temperature was 18.7 °C, while the amount of precipitation was 256 mm during the vegetation. The length of the vegetation period was significantly extended during 2021 due to the cold period. The vegetation period lasted for 142 days (Table 1). The precipitation deficit was 243.8

mm (Table 2). During the vegetation period of soybeans in 2021, the dry period began on June 19 and lasted until the plants matured, i.e. until September 19. The length of the dry period was 93 days, and 30 days with temperatures above 25 °C were recorded.

Table 1. Main dates for soybean vegetation from 2019 to 2021.

Year	Date of				Length of vegetation, days
	sowing	germination	maturation	harvest	
2019	15 April	02 May	10 September	12 September	132
2020	08 April	04 May	14 September	17 September	133
2021	24 April	01 May	19 September	20 September	142

Table 2. Potential and actual evapotranspiration during soybean vegetation from 2019 to 2021.

Year	SM	PV	PE	ET	PD	DS
2019	34.80	310.7	481.4	357.3	-124.1	21.07.
2020	42.31	415.4	462.1	471.2	+9.1	-
2021	46.01	190.2	480.1	236.2	-243.8	19.06.

Note. SM – soil moisture reserves during sowing time (mm), PV – precipitation during vegetation period (mm), ETP – potential evapotranspiration (mm), ETR – real (actual) evapotranspiration (mm), PD – precipitation deficit (mm), DS – drought start (day).

The results of the research were processed by analyzing the variance of the two-factorial experiment (Hadzivukovic, 1991), and the significance of the differences in treatment means was tested by the LSD test at 0.01 and 0.05 significance levels (statistical software “Statistica v.10.0”).

Results and Discussion

Soybean grain yield. Most aqueous extracts used as fertilizers or pesticides are largely the result of traditional knowledge, which is passed down from generation to generation. Plant material for the preparation of aqueous extracts is easily available, and their use does not economically burden plant production like foliar commercial fertilizers. There is scientific evidence that the use of certain aqueous extracts has a beneficial effect on plants (Lashin et al., 2013). However, for most aqueous extracts, scientific evidence does not exist even though they are traditionally used in agriculture. Some of the most commonly recommended preparations are aqueous plant extracts made from stinging nettle (Godlewska et al., 2020). The results of the research showed a significant influence of the year and weather conditions on the soybean grain yield. The highest yield was achieved

in 2020 (3783.03 kg ha^{-1}), which was by 39.32% higher compared to 2021. A significantly higher yield can be explained by the fact that, in 2020, there was no deficit of precipitation, i.e. even a surplus of 9.1 mm was recorded, while in 2021, the deficit was 256 mm with a dry period of 93 days (Tables 1 and 2). Also, in 2020, during the vegetation, 17 days with temperatures above the optimal ones for normal growth and development of plants were recorded (Figure 1). In 2021, this period lasted significantly longer, as many as 30 days. Observing the influence of aqueous extracts on soybean grain yield during three years (Table 3), it can be concluded that, on average, the best effect was achieved by applying an aqueous extract of the whole banana fruit. The 3-year averaged yield was 3329.94 kg ha^{-1}, which is by 15.34% higher than in the control. The application of an aqueous extract of nettle and comfrey achieved a yield of 3300.20 kg ha^{-1}, which is by 14.58% higher than the control. Aqueous extracts of banana peel and nettle had a similar effect on soybean yield. The increase amounted to 13.07% and 12.99%, respectively. Aqueous extracts of soybean and onion bulb leaves had a similar effect. Their effect increased the yield by 11.87% and 11.46% (Table 3). The positive effect of the aqueous extract of the banana fruit, as well as the peel, can be attributed to the chemical composition of this fruit. Banana fruit is rich in potassium, phosphorus, calcium, manganese, magnesium, selenium, and it also contains vitamins C, B and A. Banana peel contains phosphorus which affects the flowering of the plant, then potassium which promotes plant development and growth, while calcium makes plants stronger (Sidhu and Zafar, 2018).

Dozet et al. (2017) also found a beneficial effect of the aqueous extract of nettle and comfrey on soybean grain yield. By applying the aqueous extract of these two plant species, the soybean yield was increased by 8.31%. The same authors also determined the beneficial effect of the aqueous nettle extract on soybean grain yield. Similar results of the positive and significant impact of the application of plant extracts, especially the nettle extract, are emphasized in the research of Ljubović (2015). The best results using the nettle extract were obtained with the average weight of fresh above-ground lettuce. Zavišić et al. (2015), in their research, found that the use of nettle extracts aims to protect crops from diseases and pests. Aqueous nettle extracts fermented for 14 and 21 days showed an inhibitory effect on the mycelium growth of the *Botrytis cinerea*. The aqueous nettle extract is rich in nitrogen, phosphorus, calcium, magnesium and iron and promotes plant growth (Rivera et al., 2012). If the effects of aqueous extracts are observed in relation to the second control (water only), it can be concluded that a significant increase in yield was achieved by using the aqueous extract of whole banana fruit (8.71%) and the aqueous extract of nettle and comfrey (7.95%). Observing the impact of aqueous extracts per year, it can be seen that the best effect was achieved by the foliar application of the aqueous extract of the whole banana fruit in 2019 (Table 3).

Table 3. The influence of aqueous extract application on the yield of soybean (kg ha^{-1}).

Treatment (B)	Years (A)			Average B
	2019	2020	2021	
Control	3032.50	3462.27	1962.49	2819.09
Water	3246.10^{ns}	3580.75^{ns}	2230.89^{ns}	3019.25^{ns}
Nettle	3477.65*	3872.40*	2369.96*	3240.00*
Nettle+comfrey	3524.73**	3963.50**	2412.37*	3300.20**
Banana fruit	3586.03**	3927.18**	2476.62**	3329.94**
Banana peel	3482.14*	3911.14*	2335.41*	3242.90*
Onion bulb leaves	3447.81*	3790.86^{ns}	2312.85*	3183.84*
Willow twigs	3365.42*	3715.11^{ns}	2262.11^{ns}	3114.21^{ns}
Soybean plant	3474.96*	3824.06*	2296.82*	3198.61*
Average A	3404.15**	3783.03**	2295.50	3172.00

*, ** Significant at 0.05 and 0.01 probability levels, respectively (LSD test); ns = not significant.

Factor	$LSD_{0.05}$	$LSD_{0.01}$
A	542.25	695.61
B	301.72	426.26
AxB	330.53	456.49
BxA	289.11	413.45

The yield was 3586.03 kg ha^{-1}, which is by 15.44% higher than in the control. Aqueous extracts of banana peel and nettle had a similar effect on soybean yield. The increase amounted to 12.91% and 12.80%, respectively. Aqueous extracts of soybean plants and onion bulb leaves also had a similar effect. Their effect increased the yield by 12.73% and 12.05%. If the effects of aqueous extracts in relation to water are observed, it can be concluded that a significant increase in yield was achieved by using an aqueous extract of the whole banana fruit (9.48%). In 2020, the best result was achieved by applying the aqueous extract of nettle and comfrey. The yield was 3963.50 kg ha^{-1}, which is by 12.65% higher than in the control. Using aqueous extracts of whole banana fruit and banana peel, the increase in yield was 11.84% and 11.48%, respectively, while using the nettle extract, the increase amounted to 11.59%. A similar effect was achieved by applying the aqueous soy extract. If the effects of aqueous extracts in relation to water are observed, it can be concluded that a better effect was achieved in 2020 than in 2019. A significant increase was achieved by applying an aqueous extract of nettle and comfrey (9.66%), whole banana fruit (8.82%) and banana peel (8.45%). A significant increase, but lower, was achieved by using aqueous extracts of nettle and soybean plants (7.53% and 6.36%). The effect of aqueous extracts in 2021 differs significantly from 2019 and 2020. Compared to the control, the increase in yield ranged from 13.24% to 20.76%. The best result was achieved by applying an aqueous extract of banana fruit. The yield was 2476.62 kg ha^{-1}, which is by 20.76%

higher than in the control. By applying aqueous extracts of nettle and comfrey and nettle, soybean grain yield was increased by 18.65% and 17.19%, respectively. An aqueous extract of banana peel increased the yield by 15.97%, an aqueous extract of onion peel by 15.15%, and an aqueous soy extract increased the yield by 14.56%. Unlike the previous two years, the application of water had a better effect than the aqueous extract of willow twigs. If the effects of aqueous extracts in relation to water are observed, it can be concluded that a significant increase in yield achieved only by using an aqueous extract of the whole banana fruit was 6.20%.

Protein yield. The results of the research showed a significant influence of the year and weather conditions on soybean protein yield. The highest yield was achieved in 2020 – 1461.56 kg ha^{-1}, which is by 38.59% higher compared to 2021 (Table 4).

Table 4. The influence of the aqueous extract application on the protein yield (kg ha^{-1}).

Treatments (B)	Years (A)			Average B
	2019	2020	2021	
Control	1178,68	1333,51	763,56	1091,92
Water	1257,65^{ns}	1367,99^{ns}	859,03^{ns}	1161,56^{ns}
Nettle	1353,41^{ns}	1502,99^{ns}	923,14^{ns}	1259,85^{ns}
Nettle+comfrey	1378,86*	1543,28*	954,64^{ns}	1292,26*
Banana fruit	1402,98*	1524,51^{ns}	979,45*	1302,31*
Banana peel	1358,82^{ns}	1506,74	908,73^{ns}	1258,10^{ns}
Onion bulb leaves	1346,54^{ns}	1469,12^{ns}	908,75^{ns}	1241,47^{ns}
Willow twigs	1301,54^{ns}	1429,57^{ns}	880,99^{ns}	1204,03^{ns}
Soybean plant	1357,92^{ns}	1476,30^{ns}	898,93^{ns}	1244,38^{ns}
Average A	1326,27	1461,56	897,47	

Factor	$LSD_{0.05}$	$LSD_{0.01}$
A	193,45	328,49
B	147,32	256,17
AxB	156,42	266,24
BxA	138,24	145,36

The best effect was achieved by applying an aqueous extract of the whole banana fruit. The achieved protein yield was 1302.31 kg ha^{-1}, which is by 16.16% higher than in the control. By applying the aqueous extract of nettle and comfrey, a protein yield of 1292.26 kg ha^{-1} was achieved, which is by 15.50% higher than in the control. The use of other extracts did not significantly affect protein yield. If the influence of aqueous extracts in relation to water treatment is observed, it can be concluded that no aqueous extract has led to a significant increase in protein yield. Observing the impact of aqueous extracts per year, it can be seen that, in 2019, the

best effect was achieved by foliar application of an aqueous extract of the whole banana fruit. In this treatment, protein yield was 1402.98 kg ha^{-1}, which was by 15.99% higher than the control. When using the aqueous extract of nettle and comfrey, the increase was 14.52. In 2020, the use of the aqueous extract of nettle and comfrey significantly increased the protein yield by 13.59%. In 2021, a significant result was achieved by applying an aqueous extract of the whole banana fruit. Protein yield was increased by 22.04% compared to control. Weather conditions in some years have a huge impact on the variation of yield, protein and oil content in soybean grain (Dozet et al., 2019). The application of aqueous nettle extracts increased both the protein content and the oil content in the grain, i.e., there was an increase in the capacity for the accumulation of nutrients in the grain (Dozet et al., 2018).

Conclusion

The results show that foliar application of the different plant aqueous extracts can increase grain yield and protein yield. The increase in grain yield ranged from 9.48% to 15.34%, and the increase in protein yield from 9.31% to 16.16%. The best effect was achieved by applying the aqueous extract of the whole banana fruit and the aqueous extract of the mix of nettle and comfrey. By applying them each year, a significantly higher yield was achieved in relation to the control with distilled water. The best effect was achieved in the year with the largest precipitation deficit.

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UTICAJ PRIMENE VODENIH EKSTRAKATA RAZLIČITIH BILJAKA NA PRINOS ZRNA I PROTEINA U PROIZVODNJI SOJE

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R e z i m e

Cilj ovog rada je bio da se ispita uticaj vodenih biljnih ekstrakata na prinos zrna i proteina kod soje. Ispitivanje je obavljeno u Institutu za ratarstvo i povrtarstvo u Novom Sadu na sorti NS Apolo. Za folijarnu primenu korišćeni su vodeni ekstrakti: nadzemnog dela koprive, nadzemnog dela koprive i gaveza, ceo plod banane, kora banane, listovi lukovice crnog luka, vršni delovi grančica vrbe i vršni delovi biljaka soje. Pored kontrolne varijante, eksperiment je uključivao i kontrolu – destilovanu vodu. Kontrola destilovanom vodom će pokazati da li je efekat vodenih biljnih ekstrakata posledica biljnog materijala ili vode. Rezultati analize su pokazali da je upotreba vodenih ekstrakata doprinela povećanju prinosa zrna i prinosa proteina. Povećanje prinosa kretalo se od 9,48% do 15,34%, a povećanje prinosa proteina od 9,31% do 16,16%. Najbolji efekat je postignut primenom vodenog ekstrakta celog ploda banane i vodenog ekstrakta koprive i gaveza. Njihovom primenom svake godine postignut je znatno veći prinos u odnosu na kontrolu vodom.

Ključne reči: vodeni ekstrakt, soja, banana, kopriva, gavez.

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CORRELATIONS OF MORPHO-AGRONOMIC TRAITS AND FORAGE QUALITY PROPERTIES IN DIVERSE RED CLOVER (*TRIFOLIUM PRATENSE* L.) COLLECTIONS

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Abstract: Red clover is an important perennial forage legume and a rich source of highly nutritional voluminous forage for livestock feed, which is continuously improved by plant breeding efforts that rely significantly on trait correlation studies. A two-year field trial was conducted at Rimski Šančevi, Novi Sad, Serbia. The aim of this research was to assess correlations of important morpho-agronomic traits and forage quality properties of 46 red clover accessions of diverse origins. The highest Spearman's correlation coefficient (0.97, $p<0.01$) was found between the branch number and internode number per stem in the first experimental year. The green mass yield and the dry matter yield in both years had very high (0.95, $p<0.01$) and high (0.86, $p<0.01$) correlations, respectively. The stem height and internode number per stem had intermediate correlations in both years (0.68, $p<0.01$, and 0.50, $p<0.01$, respectively), and stem height exhibited an intermediate correlation with green mass yield (0.57, $p<0.01$, and 0.62, $p<0.01$, respectively) and dry matter yield (0.60, $p<0.01$, and 0.56, $p<0.01$, respectively) in both years. Thus, the indirect selection for higher plants with higher numbers of internodes per stem may contribute to accomplishing higher yield performance per plant. Although the branch number showed the highest positive correlation ($p<0.01$) with internode number per stem and an intermediate positive correlation ($p<0.01$) with stem height, this trait had a low correlation with green mass yield and dry matter yield and cannot be used as a criterion for indirect selection.

Key words: *Trifolium pratense* L., morphological traits, agronomic traits, indirect selection, forage quality.

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Introduction

A primary agricultural benefit of red clover (*Trifolium pratense* L.) is its ability to fix atmospheric nitrogen (N) via symbiosis with root nodule bacteria, Rhizobium, and to contribute available N to grasslands, improving soil fertility and increasing yield, both of subsequent crops with leys and companion grasses (Ciaran and Ratnieks, 2021). As animal feed, red clover has a higher N content than grass and can improve livestock performance via the superior nutritive value of grass plus clover forages versus grass alone (Fraser et al., 2004). It is widely sown for forage in Europe because of its high yield of digestible organic matter, metabolizable energy and protein content (Staniak, 2019). Usual tests for defining forage quality are dry matter (DM), pH, crude protein (CP), available protein, ammonia nitrogen (as % NH3/TN), acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, and ash (Fulgueira et al., 2007).

Wild populations, as well as landraces and conventional cultivars of red clover, are naturally diploid (2n = 2x = 14), with gametophytic self-incompatibility, i.e. they need to be cross-fertilized before seed set. The modern tetraploid red clover cultivars (2n = 4x = 28) have been developed using autopolyploidy from diploid genotypes through chromosome doubling (Taylor and Quesenberry, 1996). The cultivated types of red clover had a similar level of genetic diversity, implying that modern red clover breeding programs did not negatively affect either genetic diversity or population structure (Osterman et al., 2021).

Genetic diversity is crucial to the breeders when improving agricultural plant species. The complexity of the red clover genome with high heterozygosity and heterogeneity has been an obstacle in genomic analyses (Li et al., 2019). Phenotypic distinctness in morpho-agronomic traits and forage quality properties within a population relies on genetic variation, but it is also influenced by environmental conditions (precipitation, temperatures) and by cutting frequency, plant maturity stage at cutting time, and cutting height (Swarup et al., 2021).

The favorable tendency of plant breeding encompasses the improvement of the multiple traits at the same time, however, it is difficult to achieve because of genetic correlations among different traits (Breseghello and Coelho, 2013). The correct assessment of genetic correlations requests large sample sizes and the existence of the genetic generic data, which may not always be available. Thus, phenotypic correlations are often supposed to reflect genotypic correlations (Sodini et al., 2018). The correlations are caused by pleiotropic genes, physical linkage of genes on the chromosome, or by the genetic structure of a population (Breseghello and Coelho, 2013). If two traits are favorably correlated, selection can simultaneously improve both. The indirect selection of chosen traits can lead to the improvement of the strongly positively correlated trait in the same direction

(Neyhart et al., 2019). On the other hand, unfavorable correlations are common and often cause problems for breeding.

The aim of this research was to assess and to analyze correlations of morpho-agronomic traits and forage quality properties of 46 red clover accessions tested in a two-year field trial and to show the performance of examined traits via frequency distributions. The obtained results can be used to consider the relationships of examined traits, especially the possibility for the indirect selection of traits that are strongly correlated with the main breeding goals, and for a simultaneous selection of multiple traits within the context of further red clover breeding.

Material and Methods

Plant material, field trial and experimental design

Plant material was selected from the red clover collection that belongs to the Institute of Field and Vegetable Crops, Novi Sad, Serbia. It was represented by 46 diploid and tetraploid red clover accessions, cultivars or populations originating from 17 different countries (Table 1).

The field trial was conducted at Rimski Šančevi (altitude 84 m, 45°20' N, 19°51' E), Novi Sad, Serbia, during the 2011 and 2012 growing seasons, and the sowing was performed at the beginning of April 2011. A field trial was arranged as a randomized complete block design with three replications (10 plants per repetition), with a row spacing of 80 × 80 cm (hill cropping), and a sowing depth of 2.5 cm.

The soil at Rimski Šančevi is a chernozem type, with extremely favorable pedological characteristics. The agro-chemical properties of the soil were analyzed in the Laboratory for Soil and Agroecology of the Institute of Field and Vegetable Crops, Novi Sad. Soil samples were slightly alkaline, with a medium content of $CaCO_3$, poor in humus, rich in total nitrogen, and with a high content of easily accessible phosphorus and potassium. No pesticides, chemical fertilizers, and irrigation were applied during the trial.

The location of Rimski Šančevi is characterized by a temperate-continental climate, with an average annual temperature of 11.3°C and an average precipitation of 631 mm. There was no significant difference in meteorological conditions between the two experimental years. During the vegetation period (from April to October), average monthly temperatures were higher than the long-term average. Maximum and minimum air temperatures were higher, while the total amount of precipitation was lower than the long-term average. The pronounced precipitation deficit was observed between June and September in both years. Relative humidity was generally lower than the long-term average during both years.

Table 1. The name, origin, type, and ploidy level of accessions from the red clover collection.

Genotype	Origin	Type	Ploidy level
89 E-0	Bulgaria	population	2n
91 E-44	Bulgaria	population	2n
91 E-63	Bulgaria	population	2n
Lutea	Germany	cultivar	2n
Amos	Denmark	cultivar	4n
Avala	Serbia	cultivar	2n
BGR1	Romania	population	2n
BGR2	Romania	population	2n
BGR3	Romania	population	2n
Bjorn	Sweden	cultivar	2n
Bolognino	Italy	population	2n
Bradlo	Slovakia	population	2n
Britta	Sweden	cultivar	2n
Čortanovci	Serbia	population	2n
Diana	Hungary	cultivar	2n
Dicar	France	cultivar	4n
Fertody	Hungary	cultivar	2n
Italia centrale	Italy	population	2n
Kora	Sweden	cultivar	2n
Krano	Denmark	cultivar	2n
Lemmon	Belgium	cultivar	2n
Lucrum	Germany	cultivar	2n
Marina	Serbia	cultivar	2n
Marino	Germany	cultivar	2n
Mercury	Belgium	cultivar	2n
NCPGRU2	Ukraine	population	2n
NCPGRU3	Ukraine	population	2n
NCPGRU4	Ukraine	population	2n
NCPGRU5	Ukraine	population	2n
Nemaro	Germany	cultivar	4n
Nessonas	Greece	cultivar	2n
Noe	France	cultivar	2n
NS-Mlava	Serbia	cultivar	2n
Quinekel	Chile	cultivar	2n
Renova	Switzerland	cultivar	2n
Rotra	Belgium	cultivar	4n
SA1	Australia	population	2n
SA3	Australia	population	2n
SA4	Australia	population	2n
Sofia52	Bulgaria	population	2n
Titus	Germany	cultivar	4n
Triton	Germany	cultivar	4n
Una	Serbia	cultivar	2n
Violeta	Bolivia	cultivar	2n
Violetta	Belgium	cultivar	2n
Vivi	Sweden	cultivar	4n

The evaluation of morpho-agronomic traits and forage quality properties

A detailed evaluation of the studied traits was carried out from the first cutting in July during both years. Each morpho-agronomic trait was determined by measuring 30 individual plants (10 plants per elementary plot) for each accession. Dry matter yield was determined after drying the plant samples at 105 °C to a constant weight.

In the first growing season, the following morphological traits were analyzed: internode number per stem (IN), branch number (BN), stem height (SH) (cm), stem thickness (ST) (cm), middle leaflet length (MLL) (mm), middle leaflet width (MLW) (mm), green mass yield (GMY) (g plant^{-1}) and dry matter yield (DMY) (g plant^{-1}).

In the second growing season, the following morpho-agronomic traits and forage quality properties were determined: internode number per stem, stem height, green mass yield, dry matter yield, crude protein content (CP) (AOAC, 1990), content of acid detergent fiber (ADF) (Van Soest, 1963), and content of neutral detergent fiber (NDF) (Van Soest and Wine, 1967). For the analysis of forage quality, plant samples were taken from the second cut of the second growing season, when about 25% of the flowers appeared (7 days after three heads of a plant had begun to flower). For each accession, three average bulk green mass samples were taken and dried at 60°C for 48h. Each bulk sample contained ten single plants.

Statistical analysis

The frequency distributions were determined for all investigated traits. The obtained mean values of agro-morphological traits and forage quality properties of red clover accessions were used to calculate the Spearman's correlation coefficient. The Spearman's coefficient was calculated according to the formula Eq. (1) (Zar, 1984):

$$r_s = 1 - 6\sum d^2/(n^3 - n) \quad (1)$$

where d is the difference between ranks for each x_i, y_i is a data pair, and n is the number of these pairs. This type of correlations represents a nonparametric criterion of the statistical strength for the relationship between two variables, and it is independent of the type of their distribution. In terms of intensity and strength, the values of the correlation coefficients (both Pearson's and Spearman's) can be classified into the following categories: very high (0.90–1.00), high (0.75–0.90), intermediate (0.5–0.75), low (0.30–0.50), and very low (0.00–0.30). The R software (R Core Team, 2017) was used to calculate Spearman's rank correlation coefficients.

Results and Discussion

The performance of studied red clover accessions

Frequency distributions of values of red clover morpho-agronomic traits in the first experimental year are shown in Figure 1.

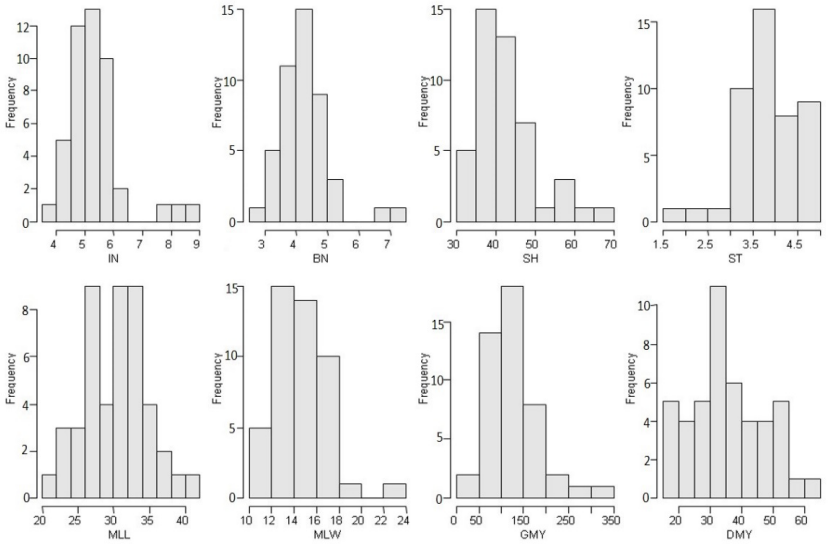

Figure 1. Frequency distributions of red clover traits in the first year. IN – internode number per stem, BN – branch number, SH – stem height (cm), ST – stem thickness (cm), MLL – middle leaflet length (mm), MLW – middle leaflet width (mm), GMY – green mass yield (g plant^{-1}), DMY – dry matter yield (g plant^{-1}).

The internode number per stem ranged from 3.87 to 8.53. The smallest branch number was 2.83, and the highest was 7.47. The variation for stem height was from 32.67 to 65.27 cm. The frequency distributions on the histogram for IN, BN, and SH were moved toward lower (below-average) values, and the most frequent classes were 5.0–5.5, 4.0–4.5, and 35–40 cm, respectively. The stem thickness ranged from 1.93 to 4.95 cm. The frequency distribution on the histogram for ST was moved toward higher (above-average) values, and the most frequent class was between 3.5 and 4.0 cm. The middle leaflet length varied from 21.97 to 40.10 mm, with the frequency distribution most resembling normal distribution with the three equal most frequent classes. The middle leaflet width showed variation from 10.60 to 23.40 mm. The green mass yield ranged from 40.33 to 319 g plant^{-1}. The frequency distributions on the histogram for MLW and GMY were moved toward lower (below-average) values, and the most frequent classes were 12–14 mm and

100–150 g plant^{-1}, respectively. The smallest value of dry matter yield was 16.77 g plant^{-1}, and the highest was 60.43 g plant^{-1}. The frequency distributions for DMY were uniform, with the exception of the most frequent class (30–35 g plant^{-1}) and the least frequent class (55–60 g plant^{-1}).

Frequency distributions of morpho-agronomic traits and forage quality parameters determined in the second year are shown in Figure 2.

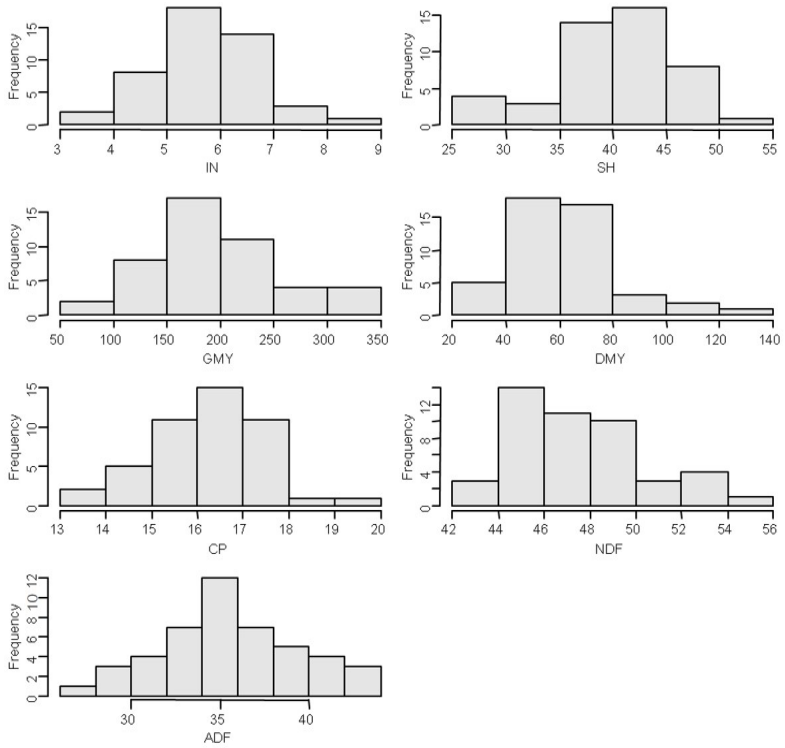

Figure 2. Frequency distributions of red clover traits in the second year. IN – internode number per stem, SH – stem height (cm), GMY – green mass yield (g plant^{-1}), DMY – dry matter yield (g plant^{-1}), CP – crude protein content (%), NDF – neutral detergent fiber content (%), ADF – acid detergent fiber content (%).

The internode number per stem was in the range of 3.33 to 8.73. The stem height of red clover accessions ranged from 26.77 to 50.23 cm. The lowest green mass yield and dry matter yield were 75.33 and 23.27 g plant^{-1}, respectively, and the highest values for GMY and DMY were 348.00 and 130.80 g plant^{-1}, respectively. The lowest value of the crude protein content was 13.73%, and the highest was 19.23%. The content of acid detergent fiber ranged from 27.78% to 42.60%. The content of neutral detergent fiber was in the interval from 43.08% to 55.54%. The frequency distributions for IN, GMY, CP, and ADF most resembled

normal distribution, and the most frequent classes were 5–6, 150–200 g plant^{-1}, 16–17%, and 34–36%, respectively. The frequency distributions for DMY and NDF were moved toward lower (below-average) values, and the most frequent classes were 40–60 g plant^{-1}and 44–46%, respectively. The frequency distribution for SH was moved toward higher (above-average) values, and the most frequent class was between 40 and 45 cm.

Correlation analysis

The assessment of correlation coefficients is important for plant breeders from the aspect of the indirect selection of traits correlated with the main breeding objectives and for a simultaneous selection of multiple traits because a correlation between them can reduce the response to selection and expected genetic advance. The values of the Spearman's correlation coefficient of eight morpho-agronomic traits of red clover in the first year are shown in Table 2.

The highest positive significant correlation coefficient was found between the internode number per stem and the branch number (0.97, $p<0.01$) (Table 2). Also, a very high, positive correlation (0.95, $p<0.01$) was observed between the green mass yield and the dry matter yield. Middle leaflet length and middle leaflet width had a high correlation (0.77, $p<0.01$). The correlation coefficients between internode number per stem and stem height (0.68, $p<0.01$), branch number and stem height (0.64, $p<0.01$), stem height and dry matter yield (0.60, $p<0.01$), stem height and green mass yield (0.57, $p<0.01$), were intermediate and positive.

Table 2. The Spearman's rank correlation coefficient for red clover morpho-agronomic traits in the first year.

	IN	BN	SH	ST	MLL	MLW	GMY	DMY
IN		0.97**	0.68**	0.37*	−0.05^{ns}	0.00^{ns}	0.41**	0.48**
BN			0.64**	0.38**	−0.06^{ns}	−0.04^{ns}	0.39**	0.47**
SH				0.36*	0.35*	0.42**	0.57**	0.60**
ST					0.41**	0.44**	0.32*	0.35*
MLL						0.77**	0.37*	0.43**
MLW							0.31*	0.34*
GMY								0.95**

ns – not significant; * – significant at the 0.05 probability level; ** – significant at the 0.01 probability level; IN – internode number per stem; BN – branch number; SH – stem height; ST – stem thickness; MLL – middle leaflet length; MLW – middle leaflet width; GMY – green mass yield; DMY – dry matter yield.

Low correlations were observed between the following morphological traits: internode number per stem and green mass yield (0.41, $p<0.01$), internode number per stem and dry matter yield (0.48, $p<0.01$), branch number and dry matter yield

(0.47, p<0.01), middle leaflet length and dry matter yield (0.43, p<0.01), stem thickness and middle leaflet length (0.41, p<0.01), stem thickness and middle leaflet width (0.44, p<0.01), stem height and middle leaflet width (0.42, p<0.01).

Also, lower than previously, positive and highly statistically significant correlations were found between branch number and green mass yield (0.39, p<0.01), and between branch number and stem thickness (0.38, p<0.01). In the first growing season, a low correlation was observed between the following pairs of agro-morphological traits: stem thickness and green mass yield (0.32, p<0.05), middle leaflet length and green mass yield (0.37, p<0.05), middle leaflet width and green mass yield (0.31, p<0.05), stem thickness and dry matter yield (0.35, p<0.05), middle leaflet width and dry matter yield (0.34, p<0.05), stem height and stem thickness (0.36, p<0.05), stem height and middle leaflet length (0.35, p<0.05), and internode number per stem and stem thickness (0.37, p<0.05).

The values of Spearman's correlation coefficients among four morpho-agronomic traits and forage quality properties of red clover in the second experimental year are shown in Table 3.

Table 3. Spearman's correlation coefficients for red clover morpho-agronomic traits and forage quality properties in the second year.

	IN	SH	GMY	DMY	CP	NDF	ADF
IN		0.50**	0.27^{ns}	0.44**	−0.21^{ns}	−0.30*	−0.27^{ns}
SH			0.62**	0.56**	−0.26^{ns}	−0.12^{ns}	0.07^{ns}
GMY				0.86**	−0.08	−0.06^{ns}	−0.04^{ns}
DMY					−0.13	−0.11^{ns}	−0.12^{ns}
CP						−0.21^{ns}	−0.03^{ns}
NDF							0.43**

ns – not significant; * – significant at the 0.05 probability level; ** – significant at the 0.01 probability level; IN – internode number per stem; SH – stem height; GMY – green mass yield; DMY – dry matter yield; CP – crude protein content; ADF – acid detergent fiber content; NDF – neutral detergent fiber content.

The highest value of the correlation coefficient for the analyzed traits of red clover accessions in the second year was observed between green mass yield and dry matter yield (0.86, p<0.01). The correlation coefficients between stem height and green mass yield (0.62, p<0.01), stem height and dry matter yield (0.56, p<0.01), and between internode number per stem and stem height (0.50, p<0.01), were intermediate.

A low correlation was obtained for internode number per stem and dry matter yield (0.44, p<0.01). The only significant correlation among forage quality properties, although low, was between the content of neutral detergent fiber and the content of acid detergent fiber (0.43, p<0.01). The crude protein content was not significantly correlated either with morpho-agronomic traits or with other forage

quality properties. The only significant correlation between morpho-agronomic traits and forage quality properties, although very low, was shown between internode number per stem and the content of neutral detergent fiber (−0.30, $p<0.01$).

Many researchers (Vasiljević et al., 2006; Tucak et al., 2013; Hoekstra et al., 2018) identified significant relationships between green mass yield and dry matter yield and red clover stem height, suggesting that selection for a longer stem could lead to an increase of the biomass yield. Asci (2011) found the existence of a higher significant correlation between plant height and dry matter yield (0.87, $p<0.01$) than in our study and a smaller significant correlation between plant height and internode number per stem (0.30, $p<0.01$) than in our study. Tucak et al. (2013) found significant correlations for the following morpho-agronomic traits: GMY-DMY (0.93, $p<0.01$), GMY-PH (0.85, $p<0.01$), DMY-PH (0.81, $p<0.01$), GMY-NI (0.74, $p<0.01$), DMY-IN (0.65, $p<0.01$), PH-IN (0.79, $p<0.01$), GMY-MLW (0.72, $p<0.01$), GMY-MLL (0.79, $p<0.01$), DMY-MLW (0.54, $p<0.05$), DMY-MLL (0.62, $p<0.01$), PH-MLW (0.60, $p<0.01$), PH-MLL (0.84, $p<0.01$), MLL-MLW (0.72, $p<0.01$), which were similar to the results of our research, but with higher values, for all pairs of traits except for GMY-DMY and MLL-MLW, which had higher values in our research. These authors also reported a positive significant intermediate and high correlation of the internode number per stem and the middle leaflet width and the middle leaflet length, respectively, in contrast to our research, where insignificant correlations between these traits were observed. Unlike our research, which showed insignificant correlations between CP and ADF, and between CP and NDF, Tucak et al. (2013) reported significant negative correlations between CP-NDF (−0.73, $p<0.01$) and CP-ADF (−0.64, $p<0.01$), and positive between NDF-ADF (0.76, $p<0.01$). The most important forage quality parameters in red clover include protein content, the content of crude fiber, neutral detergent fiber content, acid detergent fiber content, the content of lignin, digestibility of the forage, and leaf to stem ratio (Tucak et al., 2021). Reiné et al. (2020) examined the nutritional quality of plant species in Pyrenean hay meadows of high diversity and, for red clover, determined negative associations between CP and ADF, CP and NDF, and positive between NDF and ADF.

Conclusion

Considering frequency distributions, the studied red clover collection had a considerable range of variability for the observed morpho-agronomic traits as well as for the forage quality properties. The stem height and internode number per stem had an intermediate, positive, and significant correlation, and stem height exhibited an intermediate significant correlation with green mass yield and dry matter yield. Thus, the indirect selection for higher plants with higher numbers of internodes per

stem may contribute to the selection toward accomplishing higher yield performance per plant. Although the branch number showed the highest, positive, significant correlation with internode number per stem and an intermediate, positive, and significant correlation with stem height, this trait had a low correlation with green mass yield and dry matter yield and cannot be used as a criterion for indirect selection. The only significant correlation between morpho-agronomic traits and forage quality properties, although very low and negative, was observed between internode number per stem and the content of neutral detergent fiber.

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KORELACIJE MORFOLOŠKO-AGRONOMSKIH OSOBINA I POKAZATELJA KVALITETA KRME RAZNOLIKE KOLEKCIJE CRVENE DETELINE (*TRIFOLIUM PRATENSE* L.)

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R e z i m e

Crvena detelina je važna višegodišnja krmna leguminoza koja predstavlja bogat izvor visoko nutritivne voluminozne krme za stočnu hranu, koja se kontinuirano poboljšava oplemenjivanjem. Dvogodišnji poljski ogled je bio postavljen na lokalitetu Rimski Šančevi u Novom Sadu, u Srbiji. Cij ovog istraživanja je bio da se utvrde korelacije unutar i između važnih morfološko-agronomskih osobina i pokazatelja kvaliteta krme za 46 varijeteta crvene deteline. Najviša visoko značajna vrednost za Spirmanov koeficijent korelacije (0,97, $p<0,01$) utvrđena je između broja grana i broja internodija po stabljici u prvoj eksperimentalnoj godini. Prinos zelene mase i prinos suve materije su bili u značajnoj veoma jakoj (0,95, $p<0,01$) korelaciji u prvoj godini, a jakoj (0,86, $p<0,01$) korelaciji u drugoj godini istraživanja. Visina stabljike i broj internodija po stabljici su bili u umerenoj, pozitivnoj, značajnoj korelaciji u obe godine (0,68, $p<0,05$, odnosno 0,50, $p<0,05$). Visina stabljike je ostvarila umerenu, pozitivnu, značajnu korelaciju sa prinosom zelene mase (0,57, $p<0,05$, odnosno 0,62, $p<0,05$) i prinosom suve materije (0,60, $p<0,05$, odnosno 0,56, $p<0,05$) u obe godine. Indirektna selekcija na nivou biljke sa većim brojem internodija po stabljici može doprineti postizanju većeg prinosa po biljci. Iako je broj grana pokazao najveću, pozitivnu, značajnu korelaciju sa brojem internodija po stabljici i umerenu, pozitivnu značajnu korelaciju sa visinom biljke, broj grana je imao nisku korelaciju sa prinosom zelene mase i prinosom suve materije, i ne može služiti za indirektnu selekciju.

Ključne reči: *Trifolium pratense* L., morfološke osobine, agronomske osobine, indirektna selekcija, kvalitet krme.

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THE ASSESSMENT OF CADMIUM AND LEAD IN ORGANIC AND CONVENTIONAL ROOT AND TUBER VEGETABLES FROM THE SERBIAN MARKET

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Abstract: Global organic agriculture and consumption of organic food has continuously increased over the past decades. The aim of the research was to determine and compare cadmium (Cd) and lead (Pb) concentrations in organic and conventional root and tuber vegetables from the Serbian market. Samples of three root and tuber vegetables commonly consumed in Serbia, including potatoes, carrots and beetroots, were collected at two green markets and four supermarkets in the territory of the city of Belgrade, Serbia. Concentrations of Cd and Pb in fresh weight were determined by atomic absorption spectroscopy (AAS). Mean concentrations of Cd and Pb in two types of vegetables were compared by the t-test. Cd and Pb concentrations in both types of vegetables were below allowable limits. Potato mean Cd concentration was significantly lower in the organic than in the conventional type (0.021 mg kg^{-1} and 0.037 mg kg^{-1}, respectively). In carrots, it was the opposite, Cd concentration was higher in the organic type, but the difference was not significant either between the two types or for beetroots. Results indicated lower Pb levels in organic potatoes and beetroots, and higher Pb levels in organic carrots, but differences between means were not significant in all tested vegetables. Obtained results are not conclusive, but they indicate lower or similar concentrations of both metals in organic vegetables in comparison to conventional types.

Key words: vegetables, cadmium, lead.

Introduction

Organic agriculture is currently practiced in 187 countries, and the organic share of total agricultural land is 1.5% globally (Willer et al., 2021). Over the past decade, global organic food and drink sales increased from 54.9 to 106.4 billion US dollars (Sahota, 2011; Sahota, 2021). A remarkable increase in the organic market is associated with consumers' demand for organic food. Numerous studies have

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been carried out to evaluate consumers' practices and food choice motives. Organic food consumers are highly motivated by the environment, ethics and health aspects (Kesse-Guyot et al., 2013). In addition, they are relatively highly educated and more physically active than other food consumer groups (Kesse-Guyot et al., 2013). Organic product consumption is also associated with specific diet characteristics, including more fruits and vegetables (Eisinger-Watzl et al., 2015). Studies conducted in Serbia indicated that organic food consumers are motivated to buy organic food mostly by health aspects and the support to organic producers (Vlahović and Šojić, 2016; Vehapi, 2015).

Demand for organically grown vegetables has also increased due to consumers' interest in food safety. Although the consumption of vegetables provides essential nutrients for humans, heavy metal contamination of vegetables causes a great concern regarding human health. An increased level of heavy metals such as cadmium (Cd) and lead (Pb) in vegetables has been reported in China (Huang et al., 2014), India (Gupta et al., 2008; Sharma et al., 2008), and other countries.

Cd and Pb concentrations in crops vary, with leafy and root vegetables generally having higher concentrations than fruits or seeds. The source of contamination of vegetables with Cd and Pb can be soil, water or air (Elgallal et al., 2016; El-Kady and Abdel-Wahhab, 2018). Chen et al. (2013) showed that the application of large amounts of low-grade fertilizers could be a source of heavy metals in soil. Also, a long-term application of Cd-containing phosphate fertilizers can increase Cd concentrations in vegetative and also reproductive plant parts (Grant et al., 2010). However, post-harvest vegetable contamination with heavy metals during transport, storage and marketing has also been shown (Kassouf et al., 2013). High exposure to these metals has negative effects on human health. Thus the excess Cd can damage kidneys, while the excess Pb can affect the neurodevelopment in children (Satarug, 2018; Gundacker et al., 2021).

Available data on Cd and Pb in organic and conventional vegetables are often controversial. While synthetic agrochemicals are much lower in organic than in conventional fruits and vegetables, usually, there is no difference between them in environmental pollutants such as heavy metals. It is generally accepted that they cannot be avoided through organic farming practices (see review by Magkos et al., 2006). Based on an assessment of Cd and Pb in a variety of organically produced foodstuffs available in the Greek market, Karavoltsos et al. (2008) suggested that the majority of certified organic products may have a lower level of Cd and Pb.

According to the author's best knowledge, there is no available data on Cd and Pb in organic vegetables in Serbia. This study aimed to compare concentrations of Cd and Pb in commonly consumed root and tuber vegetables in Serbia, including potato (*Solanum tuberosum* L.), carrot (*Daucus corota* L.) and beetroot (*Beta vulgaris* L. ssp. *vulgaris*) from the Serbian market.

Material and Methods

The collection and preparation of samples

Samples of edible parts of commonly consumed root and tuber vegetables, including organic and conventional potatoes, carrots and beetroots, were collected in March 2016 from two green markets and four supermarkets located in the territory of the city of Belgrade, Serbia. All available organic products originated from different producers, and the same number of samples of conventional products was collected. Samples of both organic and conventional vegetables were collected as follows: five potato samples, five carrot samples, and four beetroot samples. All collected organic vegetables were labeled with a national symbol for organic products, or an organic certificate was available.

All samples were washed with tap water to remove soil particles and then rinsed with deionized water. Afterwards, samples were placed on the cellulose paper to remove excess water. Each sample consisted of three roots or tubers. Samples were peeled, chopped into small pieces, and ground in a high-speed blender. In total, 14 samples of each organic and conventional vegetable were prepared.

The digestion of samples and analyses

Samples of 1 g of fresh weight were digested with 7 ml of HNO_3 + 2 ml of H_2O_2 in a microwave oven (Ethos EZ, Milestone). After digestion, samples were transferred to 50-ml flasks, and deionized water was added. The concentration of cadmium and lead was determined by AAS (GBC SensAA).

Results and Discussion

In the present study, vegetables were selected based on the edible part, root or tuber. The number of samples was determined based on the availability of organic vegetables at that moment. According to the Serbian Regulation and Commission Regulation (EU) 2021/1323, the maximum level of Cd in potatoes and carrots is 0.10 mg kg^{-1} fresh weight, and for beetroots, it is 0.06 mg kg^{-1} fresh weight. According to the Serbian Regulation and Commission Regulation (EU) 2021/1317, the maximum level of Pb in root and tuber vegetables is 0.10 mg kg^{-1} fresh weight.

Although Pajević et al. (2018) recently reported both Cd and Pb concentrations above the maximum level by Serbian and EU legislations in potatoes, carrots and beetroots, in the present study, this was not the case (Tables 1, 2 and 3). Based on mean values, Cd and Pb concentrations varied considerably between examined vegetables. While potatoes had the highest level of both metals, the means for carrots and beetroots were similar.

In organic potatoes, the Cd level ranged from 0.014 to 0.030 mg kg^{-1}, whilst in conventional potatoes, the span was from 0.019 to 0.057 mg kg^{-1}. The t-test showed that the mean Cd concentration was significantly lower in organic than in conventional potatoes (0.021 mg kg^{-1} and 0.037 mg kg^{-1}, respectively) (p<0.05) (Table 1). This is in accordance with results by Hadayat et al. (2018), who evaluated metals in organic and conventional potatoes collected from supermarkets in Florida, USA. On the other hand, the mean Cd concentration in carrots was 2-fold higher in the organic than in the conventional type (0.006 and 0.003 mg kg^{-1}, respectively), but the difference was not significant (Table 2), whilst in both types of beetroots, it was 0.004 mg kg^{-1} (Table 3).

Table 1. Cd and Pb concentrations in commercially available organic and conventional potatoes.

Sample #	Cd (mg kg^{-1} FW)		Pb (mg kg^{-1} FW)	
	Organic	Conventional	Organic	Conventional
1	0.014	0.019	0.063	0.100
2	0.019	0.032	0.100	0.097
3	0.021	0.047	0.027	0.066
4	0.030	0.057	0.064	0.069
5	0.024	0.030	0.024	0.049
Mean	0.021	0.037	0.056	0.076
Median	0.021	0.032	0.063	0.069
t-test	*		ns	

*indicates a significant difference (p<0.05); ns – not significant; # – number.

Table 2. Cd and Pb concentrations in commercially available organic and conventional carrots.

Sample #	Cd (mg kg^{-1} FW)		Pb (mg kg^{-1} FW)	
	Organic	Conventional	Organic	Conventional
1	0.002	0.003	0.028	0.009
2	0.003	0.005	0.010	0.010
3	0.021	0.002	0.028	0.013
4	0.002	0.002	0.012	0.012
5	0.002	0.003	0.013	0.009
Mean	0.006	0.003	0.018	0.010
Median	0.002	0.002	0.013	0.009
t-test	ns		ns	

*indicates a significant difference (p<0.05); ns – not significant; # – number.

A meta-analysis performed by Hoefkens et al. (2009) also indicated that the Cd level was significantly lower in organic than in conventional potatoes, whilst the opposite was for carrots. However, Hadayat et al. (2018) reported much lower Cd concentrations in organic than in conventional carrots, whilst no differences between the two were observed in the Czech Republic by Krejčová et al. (2016).

Table 3. Cd and Pb concentrations in commercially available organic and conventional beetroots.

Sample #	Cd (mg kg^{-1} FW)		Pb (mg kg^{-1} FW)	
	Organic	Conventional	Organic	Conventional
1	0.003	0.005	0.016	0.017
2	0.007	0.006	0.018	0.022
3	0.004	0.004	0.013	0.014
4	0.002	0.003	0.010	0.018
Mean	0.004	0.004	0.014	0.017
Median	0.003	0.004	0.014	0.017
	ns		ns	

* indicates a significant difference (p<0.05); ns – not significant; # – number.

In the present study, Pb concentrations in organic and conventional potatoes ranged from 0.024 to 1.00 mg kg^{-1}, and from 0.049 to 1.00 mg kg^{-1}, respectively (Table 1). Although the mean Pb concentration in organic potatoes was lower than in the conventional type (0.056 and 0.076 mg kg^{-1}, respectively), the difference between them was not significant (p<0.05). In organic carrots, Pb had a wider span than in the conventional type, ranging from 0.010 to 0.028 mg kg^{-1}, and from 0.009 to 0.013 mg kg^{-1}, respectively, and the mean Pb concentration was higher in the organic than in the conventional type (0.018 and 0.010 mg kg^{-1}, respectively) (Table 2). These results are supported by Hoefkens et al. (2009), who reported much lower Pb concentrations in organic potatoes, but those were higher in organic carrots in comparison to the conventional type. Results by Malmauret et al. (2002) also indicated the Pb level in organic carrots that exceeded the maximum level in France. On the other hand, in the present study, the mean Pb concentration in beetroots was slightly higher in the conventional than in the organic type (0.017 and 0.014 mg kg^{-1}, respectively). Differences between organic and conventional carrots and beetroots in the mean Pb concentration were not significant (p<0.05) (Tables 2 and 3). Similar results for carrots were reported by Krejčová et al. (2016). In addition, a recent study by Cámara-Martos et al. (2021) indicated no differences in heavy metals between organic and conventional Brassicaceae vegetables.

Conclusion

Concentrations of Cd and Pb in organic and conventional potatoes, carrots and beetroots were below the maximum level determined by the Serbian and the EU Regulations. Obtained results are not conclusive, but they indicate a lower or similar concentration of both metals in organic vegetables in comparison to conventional types. Further research with a large number of samples is needed to evaluate the quality of organic vegetables in terms of toxic metals.

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KADMIJUM I OLOVO U ORGANSKOM I KONVENCIONALNOM KORENASTOM I KRTOLASTOM POVRĆU NA TRŽIŠTU U SRBIJI

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R e z i m e

Organska poljoprivreda i konzumiranje organske hrane u svetu su u stalnom porastu prethodnih decenija. Cilj istraživanja je bio da se odrede i uporede koncentracije kadmijuma (Cd) i olova (Pb) u organskom i konvencionalnom povrću, dostupnom na tržištu u Srbiji. Uzorci korenastog i krtolastog povrća – krompira, mrkve i cvekle sakupljeni su na dve zelene pijace i četiri supermarketa na teritoriji grada Beograda. Koncentracije Cd i Pb u svežoj masi merene su metodom atomske apsorpcione spektrofotometrije (AAS). Srednje vrednosti koncentracija Cd i Pb dva tipa povrća su poređene t-testom. Koncentracije Cd i Pb u oba tipa povrća bile su niže od maksimalno dozvoljenih. Srednja vrednost Cd bila je značajno niža u organskom nego u konvencionalnom krompiru (0,021 mg kg^{-1} odnosno 0,037 mg kg^{-1}). Suprotni rezultati su dobijeni za mrkvu, koncentracija Cd je bila viša u organskoj mrkvi, ali razlika između dva tipa nije bila značajna, kao ni za cveklu. Rezultati su ukazali na niži nivo Pb u organskom krompiru i cvekli, i njegov viši nivo u organskoj mrkvi, ali razlike između dva tipa nisu bile značajne. Na osnovu dobijenih rezultata ne može se izvesti nedvosmislen zaključak, ali rezultati ukazuju na niži ili sličan nivo oba metala u organskom u odnosu na konvencionalno povrće.

Ključne reči: povrće, kadmijum, olovo.

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EFFECTS OF THE FOLIAR SPRAY OF GROWTH REGULATORS ON THE FATTY ACID COMPOSITION OF SAFFLOWER UNDER ORGANIC AND CHEMICAL SOIL FERTILIZATION

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Abstract: In the current study, we evaluated the impacts of organic and chemical fertilizers as well as the foliar application of growth regulators on quantitative traits and seed oil content of safflower grown in northwestern Iran. The experiment was done as a split-plot (3 × 5), and the main plot was assigned to different fertilizers, including organic fertilizer (FYM: farmyard manure 20 t ha^{-1}), full chemical fertilizer (FCF) and no fertilization "control" (NF). The sub-plots were allocated to foliar spray treatments, which included control (no-spray application; S_0), choline (S_1), chitosan (S_2) and salicylic acid (S_3). The results showed that the application of fertilizers significantly affected the oil and protein content of the seed. However, the effect of FYM was more prominent than FCF on oil qualitative characteristics. A significant positive correlation was observed between oil content, protein content, and some fatty acid composition such as oleic acid, arachidonic acid, stearic acid, and palmitic acid. The highest values of the previously mentioned traits were obtained using $FYMS_1$, $FCFS_1$ and $FYMS_3$. This trend was also clearly obvious in the content of linoleic acid as the main fatty acid in safflower oil. Among the foliar spraying treatments, the most improving effect was obtained with the use of choline. In conclusion, improving soil conditions through the application of FYM and appropriate amounts of chemical fertilizers is one of the most important agronomic management measures to improve the oil quality of safflower seeds.

Key words: choline, fatty acid composition, linoleic acid, iodine value, quantitative analysis.

Introduction

Safflower (*Carthamus tinctorius* L.) is an annual, broadleaf oilseed crop and a member of the family Compositae or Asteraceae, but also it can be considered a

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multi-purpose plant. The safflower plant provides a useful vegetable and cosmetic oil and attractive dyes for textiles. It is also used for extracting oil from seeds that are used as edible oil or in the paint industry. Today, this crop supplies oil, meal, birdseed, and some by-products (residue from oil processing) for the food and industrial product markets, although this crop is now primarily grown for the oil (Zemour et al., 2021). It originates from the Asian Fertile Crescent, and previous evaluations clearly show that safflower has a good ability to adapt to drought and high temperatures (Zemour et al., 2019; Yeloojeh and Saeidi, 2020). Therefore, its introduction in semi-arid regions would constitute an alternative to the development of oilseed crops.

However, under semi-arid conditions, soil fertility in systems with low and asymmetric annual precipitation, hot summer and cold winter, is constrained by environmental extremes. In most areas with a semi-arid climate, these soils have inherently low fertility, low availability of nitrogen and phosphorus, low water-holding capacity, high pH, low soil organic matter (ranging from 0.1 to 3%), shallowness, stoniness, and other specific problems (Garcia-Franco et al., 2018). However, climate change during the last decades has intensified these restrictions. Semi-arid areas in the West Asia and North Africa (WANA) are vast and significant. Hence, these lands are of great global significance even if their agricultural production potential is relatively low. However, proper soil fertility management is of prime importance in increasing crop production. In this moor, it seems that the improvement of soil conditions and nutritional statuses of the plant and increasing vegetative growth also affect the qualitative components of the extracted oil.

In the above conditions, it appears that incorporating organic matter can have several benefits for agricultural soils. Organic matter causes soil to clump and form soil aggregates, improving soil structure. With better soil structure, permeability (infiltration of water through the soil) improves, in turn improving the soil's ability to take up and hold water (Barton et al., 2016). Farmyard manure is a key fertilizer in organic and sustainable soil management. The successive application of farmyard manure can also increase crop production and has the potential to reduce chemical fertilizer requirements. Because of the development of the livestock industry, farmyard manure is an available, relatively cheap and potential source of multiple nutrients for improving soil chemical, physical and biological properties (Nouraein et al., 2019).

In addition, it appears that some foliar spraying treatments can provide better plant growth in semi-arid conditions by stimulating defense mechanisms and growth encouragement (Gajc-Wolska et al., 2018). Chitosan (Ch) is a carbohydrate derivative from chitin, and it seems that its foliar application enhances the protection of crops against fungal attacks and reduces disease severity (El Amerany et al., 2020). Choline is a water-soluble vitamin recognized as a growth regulator under multiple stress conditions by improving growth, oxidative defense, and

secondary metabolism (Riaz et al., 2021). Also, salicylic acid (SA) is a plant hormone that has been described to play an essential role in the activation and regulation of multiple responses to biotic and abiotic stresses, and it has been revealed that spraying plants with SA solution is favorable for plant growth and helps to protect them against abiotic stresses (Maruri-López et al., 2019).

Although some studies have previously examined the effects of foliar application, a number of questions regarding differences in their impact under different nutritional conditions remain to be addressed. The major objective of this study was to investigate the effect of the foliar application of growth regulators under chemical and organic fertilizer management on safflower oil quality.

Material and Methods

Site description

The response of safflower fertilizer management and foliar spray of growth regulators were studied at the Research Farm in the Kharajou region, Northwestern Iran (longitude: 46°53′E; latitude: 37°31′N; altitude: 1780 m) during the growing season of 2016–2017 under irrigated conditions. The study area is a part of regional semi-arid highlands; in terms of climate, the area has been classified (according to the Köppen and Geiger classification system) as a cold semi-arid area (Peel et al., 2007). The annual average precipitation is 310 mm, with a maximum in April, and one-third of the precipitation includes snow, and about 78% of the precipitation occurs during the growing seasons. The previously fallowed field was prepared by plowing three times with a tractor-mounted cultivator and planking with the last plowing. The site was covered by a fine mixed, mesic, typical cacixerepts soil, exhibiting a xeric moisture regime. The soil was clay loam texture and contained 2.34 $g.kg^{-1}$ organic matter, 0.54 $g.kg^{-1}$ total N, 9.38 $mg.kg^{-1}$ available phosphorus (P), and 171.28 $mg.kg^{-1}$ available potassium (K) in the 0–20-cm soil layer. The organic matter content of the soil was low (<0.5%) at the initiation of the experiment. Decomposed farmyard manure was applied annually, and tillage operations were performed in early March.

Experimental design

Seeds of the hardy facultative safflower (*Carthamus tinctorius* L.) CV. ‘Esfahan’ were purchased from the Pakan Bazr Company. The experiment was laid out according to a split-plot (3×4), with main plots arranged as an RCBD with three replications and a net plot size of 5 × 4 m. The main plot was assigned to different fertilizers, including organic fertilizer (application of 20 t ha^{-1}farmyard manure), full chemical fertilizer and unfertilized condition “control” (C). The sub-plots were allocated to foliar spray treatments, which included control (no-spray application), choline, chitosan and salicylic acid (100 ppm). Full chemical fertilizer included 200 kg ha^{-1} N-P-K (20:10:5) fertilizer and 10 kg ha^{-1} complete

micronutrient fertilizer (Fe = 7%, Zn = 3.7%, Mn = 3%, Cu = 0.25%, B = 0.25%, Mo = 0.35%) which were used as two split applications, i.e. a half as a pre-plant (starter fertilizer) and a half as a post-emergence side-dress application during stem elongation. Each plot included twenty rows, 5 m long and 75 cm apart. Seeds were sown 20 cm apart at 5-cm depth. In the interspaces, the small terraces of 1.5 m were considered to prevent contamination by surface run-off containing fertilizers. Irrigation was applied every week to replace soil water lost due to evapotranspiration. Growth regulators were sprayed over the foliage to the point of run-off during the stem elongation (BBCH=30), flowering (BBCH=30) and head growth (BBCH=71). Low weed, disease, insect and pest pressures were maintained by using cultivation and recommended amounts of pesticides and herbicides.

The evaluation of fatty acids

The harvest occurred 140 days after the emergence of the plants. The plants were collected from 1 m^2 of each plot before threshing, and manual cleaning of the seeds was performed. Total protein was estimated by the Kjeldahl method according to Nosheen et al. (2016). The moisture content was determined using the gravimetric method by drying a sub-sample for 24 h at 105°C. To evaluate the oil content, the extraction was performed using a Soxhlet extractor under laboratory conditions using petroleum ether solvent. The extraction was performed with 2 g of ground seeds. The iodine value of oil was determined according to the standard method (Cd 1-25) introduced by AOCS (1993). The saponification value was measured according to the standard method (Cd 3-25) suggested by AOCS (1993). The fatty acid compositions were analyzed according to Pasandi et al. (2018) by GC–MS and GC-FID instruments. The determination of the fatty acid profile was carried out by GC equipped with a flame ionization detector (Agilent 6890N, USA) and a TC-FFAP capillary column (60 m×0.25 mm internal diameter, 0.25 μm). The temperature program was as follows: starting at 150 °C and then heating to 190 °C at 5 °C/min, after 2 min followed by heating from 190 °C to 250 °C at 5 °C/min. The final temperature (250 °C) was held for 8 min. The fatty acids used as standards for the GC analyses (palmitic, stearic, and oleic acids) were from Sigma. The injector and detector temperatures were both set at 250 °C. Injections of the methylated sample (1 μL) were made in the splitless mode. To convert the fatty acid compositions to fatty acid methyl esters, 0.2 mL of 2 N KOH and 1.5 mL of hexane were added to 0.1g of safflower oil. The mixture was then centrifuged at 2500 rpm for 1 min. Then, the FAME was separated and analyzed.

Statistical analysis

Data analysis was conducted using SAS version 9.4 (SAS Institute Inc). Mean values were compared according to Steel and Torrie (1980) by least significant difference (LSD) at $P < 0.05$.

Results and Discussion

The highest seed protein contents were recorded for plants grown under FYM applied conditions, which were followed by the FCF. The foliar application of choline had the greatest effect on increasing the seed protein content, followed by the foliar application of chitosan and salicylic acid. Interestingly, choline foliar application was able to increase the protein content even under NF conditions (Figure 1).

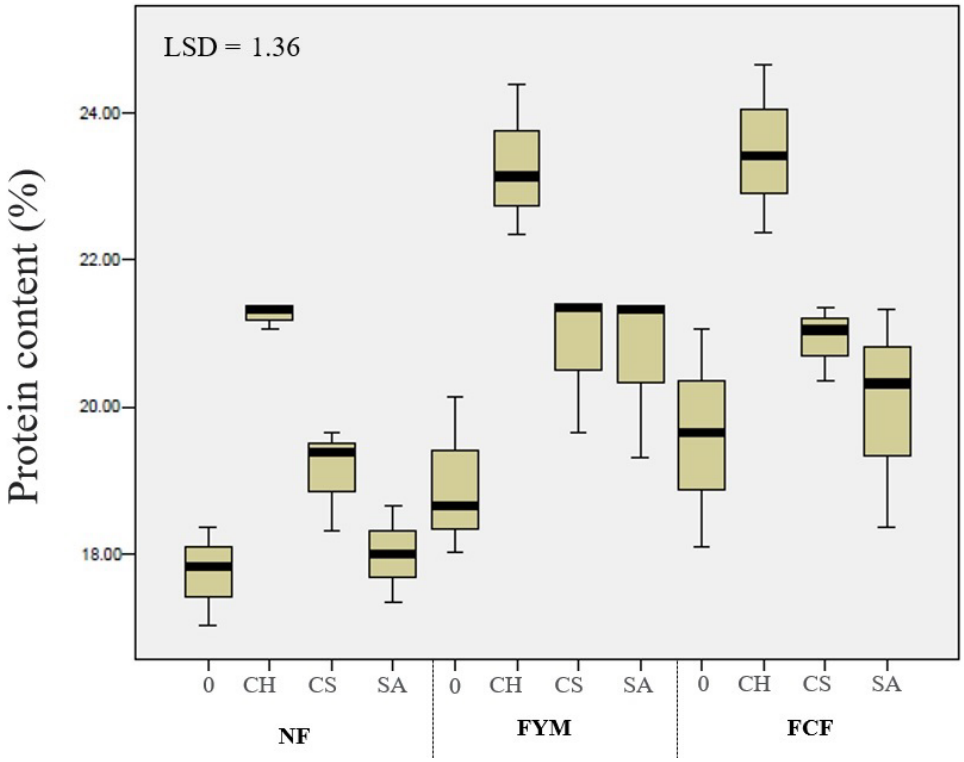

Figure 1. Effects of fertilizer application and foliar spray treatments on the seed protein content of safflower plants grown in a semi-arid region in northwestern Iran. NF: no fertilizer application, FYM: farmyard manure, FCF: full chemical fertilizer, S_0: control (no-spray application), S_1: a foliar spray of choline, S_2: a foliar spray of chitosan, S_3: a foliar spray of salicylic acid. The cap bar refers to the standard error. The dark horizontal line in each box indicates the average combined treatment. The columns with a difference higher than LSD (least significant difference) are statistically different at the $P < 0.05$ level.

The results of the analysis of variance (ANOVA) showed that seed oil and protein content were significantly affected by fertilizers and foliar sprays ($p <$

0.01). The highest oil content was recorded in plants grown under FYM or FCF applied conditions and sprayed with choline. Although all foliar treatments improved the oil content when compared with control (no-spayed plants), the salicylic acid had a slightly greater improving effect than chitosan. The positive effect of the choline spray on seed oil content was quite evident regardless of fertilizer conditions (Figure 2).

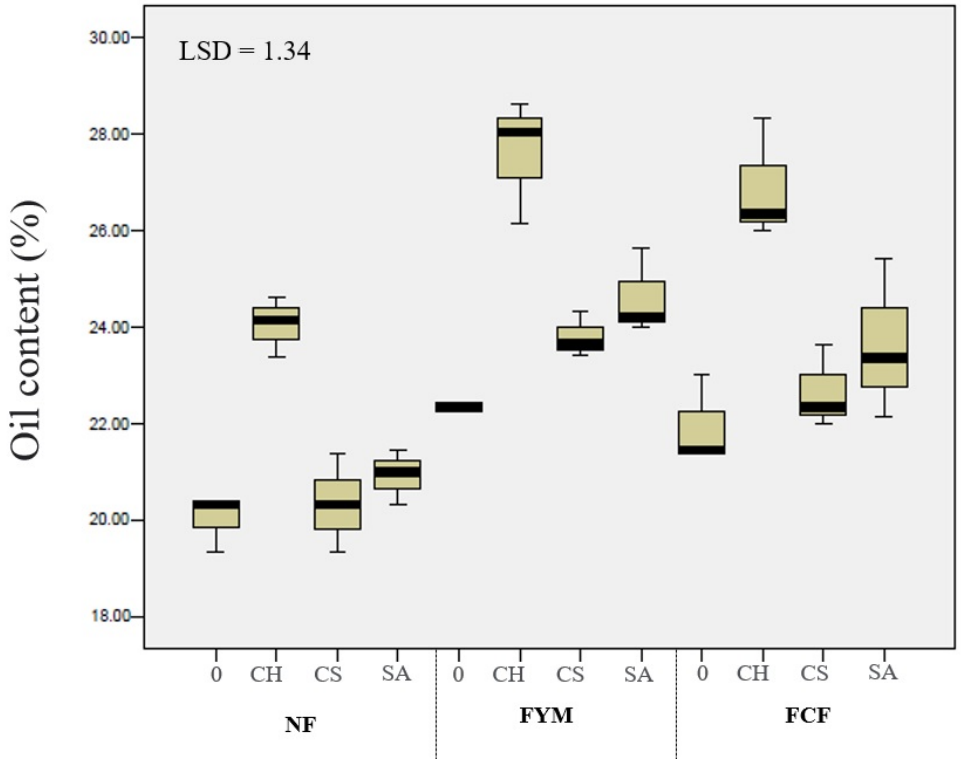

Figure 2. Effects of fertilizer application and foliar spray treatments on extracted oil content from safflower plants grown in a semi-arid region in northwestern Iran. NF: no fertilizer application, FYM: farmyard manure, FCF: full chemical fertilizer, S_0: control (no-spray application), S_1: a foliar spray of choline, S_2: a foliar spray of chitosan, S_3: a foliar spray of salicylic acid.

Table 1 lists the most abundant fatty acids found in the analyzed oil as affected by soil fertilization and foliar sprays. The oil contents of safflower seeds ranged from 20.08% to 29.51%. Oil analysis revealed that the main fatty acid seed samples were linoleic acid (67.51% ± 1.26%), oleic acid (6.30% ± 0.52%), palmitic acid (5.83% ± 0.7%), stearic acid (2.98% ± 0.35%), arachidonic acid (1.01% ±

0.04%), linolenic acid (2.31% ± 0.2%), lauric acid (1.63% ± 0.17%) and myristic acid (1.48% ± 0.14%). The evaluation of seed oil content showed that the main effect of fertilizers and foliar sprays was statistically significant regarding this seed quality parameter ($p < 0.05$). ANOVA showed that fertilizers and foliar sprays significantly affected the linoleic acid content. FYM and FCF increased linoleic acid content by 10% and 8% compared to control. Also, the choline spray on plants increased linoleic acid content by 9.3%.

Table 1. Effects of foliar sprays of growth regulators under organic and chemical soil fertilization on the percentage of fatty acids in safflower seeds.

Treatment		PA	SA	OA	LA	LL	AC	LU	MA
NF	Control	4.41±0.45	2.45±0.20	4.40±0.46	62.98±1.32	1.04±0.01	0.68±0.02	1.43±0.21	1.62±0.09
	Choline	5.74± 0.47	2.49±0.43	6.63±1.41	66.80±1.27	1.27±0.11	0.80±0.09	2.06±0.05	2.00±0.30
	Chitosan	4.45±0.53	2.64±0.09	5.15±0.70	63.57±0.87	1.06±0.15	0.83±0.10	2.62±0.04	1.83±0.10
	Salicylic acid	3.66±0.33	2.22±0.07	4.46±0.31	62.76±1.44	1.22±0.09	0.69±0.03	2.01±0.19	1.91±0.09
FYM	Control	5.21±0.50	3.02±0.08	6.49±0.64	68.32±1.65	3.01±0.46	1.08±0.00	1.20±0.09	1.78±0.31
	Choline	8.40±0.13	3.96±0.62	9.25±0.04	73.68±0.67	4.77±0.29	1.40±0.02	1.40±0.12	2.04±0.37
	Chitosan	6.01±0.88	2.96±0.42	7.27±0.19	68.67±1.28	3.31±0.57	1.12±0.04	1.39±0.33	1.94±0.15
	Salicylic acid	6.85±0.89	3.43±0.40	6.65±0.63	68.26±1.29	3.54±0.25	1.29±0.01	1.57±0.18	1.94±0.16
FCF	Control	6.25±1.16	3.42±0.74	5.23±0.49	66.04±1.26	1.98±0.11	0.91±0.04	1.16±0.09	0.66±0.11
	Choline	7.81±1.30	3.46±0.40	7.32±1.00	72.97±1.22	2.36±0.12	1.12±0.00	2.11±0.23	0.65±0.00
	Chitosan	5.76±1.10	3.06±0.56	6.17±0.06	68.00±1.24	2.15±0.10	1.08±0.11	1.55±0.47	0.70±0.02
	Salicylic acid	5.42±0.68	2.69±0.24	6.64±0.40	68.11±1.71	2.04±0.20	1.02±0.07	1.18±0.07	0.69±0.03
LSD		1.80	1.02	1.44	3.22	0.60	0.13	0.52	0.46

NF: no fertilizer application, FYM: farmyard manure, FCF: full chemical fertilizer, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid, LL: linolenic acid, AC: arachidonic acid, LU: lauric acid, MA: myristic acid. In each column, if the difference between the means is greater than the LSD (Least Significant Difference) value, then the means are significantly different ($P \leq 0.05$).

The mean comparison of palmitic acid content among different foliar spraying treatments also showed the beneficial effects of choline foliar application on improving the amount of this fatty acid. However, the effect of other foliar spraying treatments was different under organic and chemical fertilizer conditions, so the greatest effect of foliar spraying treatments was observed under FYM applied conditions. Furthermore, the foliar application of salicylic acid under NF or CFC conditions caused a significant reduction in the content of this fatty acid. The examination of oleic acid content also showed the effectiveness of choline foliar application so that oleic acid content in plants sprayed with choline was 71%

higher than in no-sprayed plants, while the highest amount of oleic acid was observed in plants grown under FYM applied conditions (Table 1). The evaluation of linolenic acid and arachidonic acid content revealed that the effect of foliar spraying treatments, especially choline, was only visible and significant under FYM and FCF applied conditions.

Responses of lauric acid content to fertilizer treatments were somewhat different from other fatty acids, so the highest amount of this fatty acid was observed under no fertilization conditions. The highest amount of lauric acid (2.06%) was obtained by using chitosan under NF conditions. The mean comparison of myristic acid amount between different treatments surprisingly showed that the application of chemical fertilizers reduced the amount of this fatty acid by 173% compared to the NF conditions (Table 1). Our results showed that fertilizer application significantly affected oil qualitative characteristics. It is important to highlight the fact that soils of semi-arid regions with the Mediterranean climate are faced with serious limitations in terms of nutrient supply for the plant and physical and chemical properties due to low precipitation and lack of proper crop management. Since the experiment was performed under full irrigation conditions, it appears that the application of organic and chemical fertilizers by improving the soil physicochemical properties and providing the essential elements has provided better plant growth and a sufficient supply of photoassimilates. Under such conditions, improving the source-sink relationship in the plant leads to a greater supply of amino acids needed for protein biosynthesis in the filling seeds, as well as the precursors needed for fatty acid biosynthesis and increases the seed oil content (Poisson et al., 2019). The protein and oil contents of seeds are crucial factors for farmers and industry. However, up to the present time, the impact of soil fertilization and foliar sprays on the oil quality of the seed has rarely been taken into account in safflower, which could be re-introduced as a forgotten and drought-tolerant oil crop in semi-arid regions.

The quality of vegetable oil is highly related to fatty acid composition and the ratio of saturated to unsaturated fatty acids. The increasing amount of unsaturated fatty acids increases the quality, however, it will result in a decrease in the shelf life of the produced oil. Although the application of fertilizers and foliar sprays increased almost all saturated and unsaturated fatty acids, the highest numerical increase was related to unsaturated fatty acids (oleic acid; 18:1 and linoleic acid; 18:2). The increasing unsaturated fatty acid content with soil fertilization could be attributed to the improvement of nutrient availability and supply for the oil metabolism (Pasandi et al., 2018). This result ties well with previous studies wherein the inoculation of arbuscular mycorrhizal fungus on the soybean seed improved the oil quality and unsaturated fatty acid content (Amani Machiani et al., 2021).

The results of correlation analysis were corroborated by principal component analysis (PCA) (Figure 3). The correlation coefficient between any two traits is approximated by the cosine of the angle between their vectors. In Figure 3, the most prominent relation was the strong positive association between protein content, palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid content and iodine value. Furthermore, PCA analysis showed that the highest values of mentioned traits were obtained by the foliar spray of choline under FYM and FCF conditions ($FYMS_1$ and $FCFS_1$). The increasing worldwide demand for vegetable protein for human nutrition (vegetarian or vegan diets) has led to a wider search for sources of vegetable protein, thus making safflower seed proteins interesting alternatives due to their acceptable quality. In our study, a strong linear positive correlation between protein content and oil content was determined. This might be due to increased source strength for photoassimilates supply and increased photoassimilate utilization (sink strength). These findings are directly in line with previous findings (Janmohammadi et al., 2014; Nouraein et al., 2019).

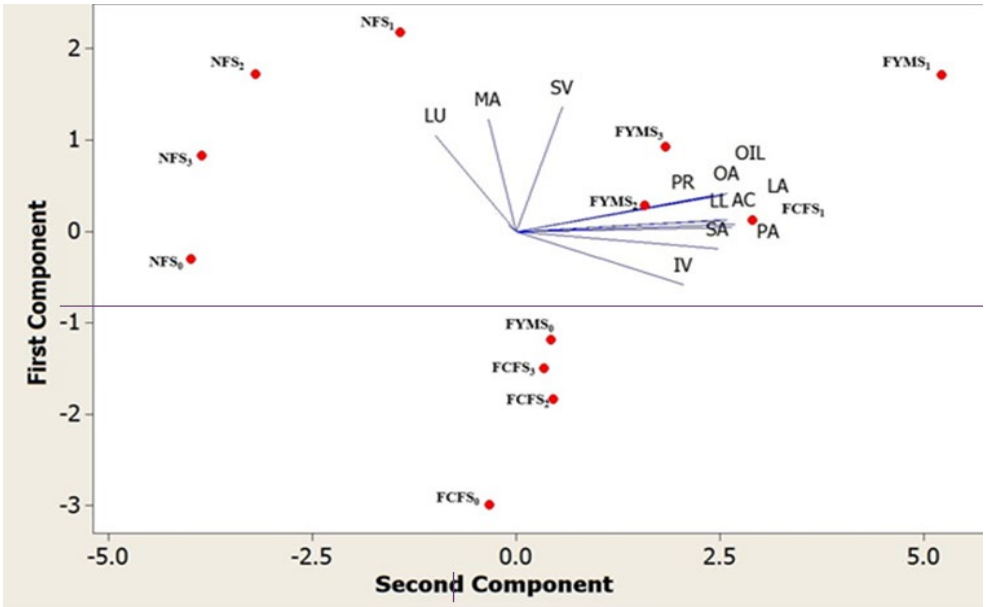

Figure 3. The bi-plot of the first two principal components (PC1 and PC2) for the spatial distribution of seed oil qualitative traits and combined treatment (fertilizer × foliar spry). NF: no fertilizer application, FYM: farmyard manure, FCF: full chemical fertilizer, S_0: control (no-spray application), S_1: a foliar spray of choline, S_2: a foliar spray of chitosan, S_3: a foliar spray of salicylic acid, PA: palmitic acid, SA: stearic acid, OA: oleic acid, LA: linoleic acid, LL: linolenic acid, AC: arachidonic acid, LU: lauric acid, MA: myristic acid, IV: iodine value, SV: saponification value.

The assessment of the iodine value of oil showed that fertilizer application significantly affected this parameter. The mean comparison of the iodine value between combined treatments showed that the application of organic and chemical fertilizers increased the iodine value (Figure 4).

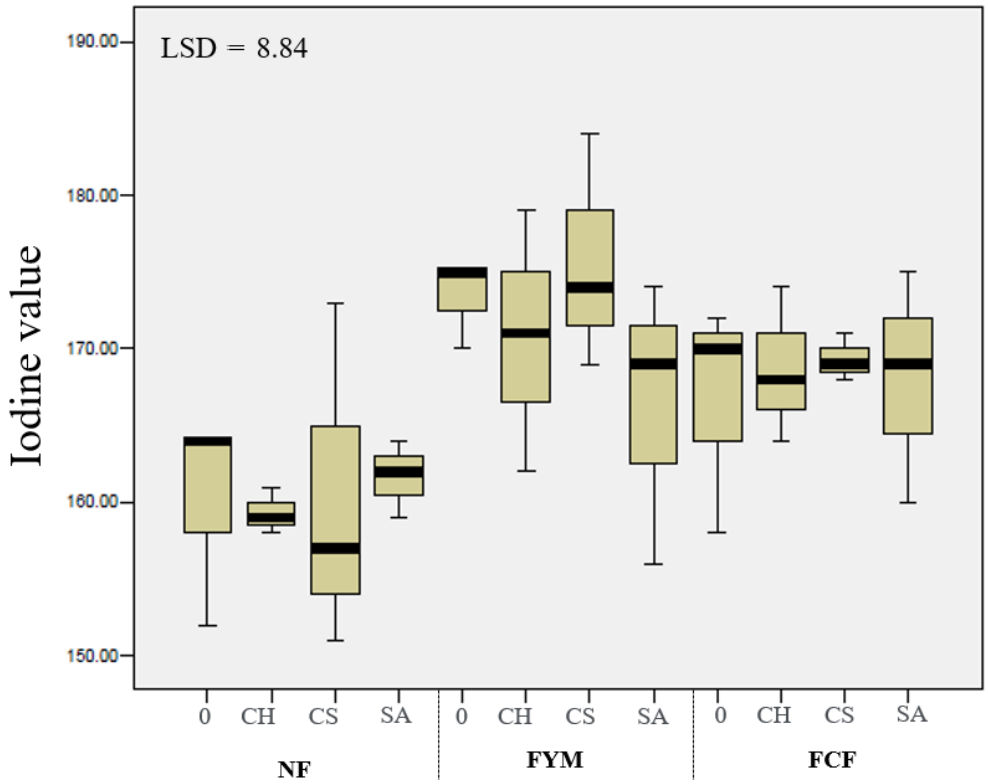

Figure 4. Effects of fertilizer application and foliar spray treatments on the iodine value of the safflower seed oil extracted from plants grown in a semi-arid region in northwestern Iran. NF: no fertilizer application, FYM: farmyard manure, FCF: full chemical fertilizer, S_0: control (no-spray application), S_1: a foliar spray of choline, S_2: a foliar spray of chitosan, S_3: a foliar spray of salicylic acid.

The evaluation of the iodine index indicates that with improving the plant growth conditions, the number of unsaturated fatty acids in the oil increased. In agreement with our hypothesis, a noticeable change in oil characteristics was recorded in response to the soil fertilization management and foliar sprays. Superior results are seen for distinguished effects of choline spray compared to other foliar treatments. Choline is an organic nitrogen compound, and it is the precursor of the osmoprotectant glycine betaine, and also it protects cells against

oxidative damage by reactive oxygen species (Salinas et al., 2013). Under certain assumptions, its distinctive effects can be attributed to its properly utilized concentration, its chemical compatibility with the nature of cell membranes and its greater penetration, stimulation of defense processes and improvement of plant growth. Our results highlighted synergetic effects on soil fertilization (especially with FYM) and the foliar use of growth regulator or protector efficiencies to improve the oil quality of safflower.

Our results showed that the efficiency and effectiveness of foliar spraying treatments were only evident and significant when the plant was grown under adequate nutritional status and suitable soil conditions. Based on these results, it is clear that soil management is a higher priority in the studied area. Our results cast a new light on the application of FYM as an inexpensive, accessible and eco-friendly source to improve seed oil quality. A difference between the effects of FYM and FCF can only be attributable to the positive and significant effects of FYM on improving soil physical properties. It is worth discussing these interesting facts revealed by the results of Nouraein et al. (2019). Overall, the promising finding was that improving the physical and chemical conditions of the soil and providing an essential situation for basic plant growth can significantly increase the effectiveness of foliar treatments and improve seed oil quality.

Conclusion

In conclusion, this study showed that the different fertilization or foliar spray treatments resulted in different responses of qualitative oil composition. Although the application of organic and chemical fertilizers increased the oil and protein contents compared to the control (no fertilization), the improving effect of FYM application on the quality aspects of oil was greater than that of chemical fertilization. Among the foliar spraying treatments, the greatest effect was related to choline, so even under no fertilization conditions, some of its positive effects were observed. Our results showed that with the improvement of plant nutritional conditions through the application of fertilizers, the effect of foliar application treatments on oil properties became more noticeable. In summary, the application of the organic fertilizer with the foliar spray of choline had the greatest impact on the composition of safflower seed oil and improved oil quality. Future studies could fruitfully explore this issue further by focusing on biochemical pathways of oil biosynthesis in seeds during filling stages under different nutritional statuses of the plant.

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UTICAJ FOLIJARNOG TRETMANA REGULATORIMA RASTA I ORGANSKIH I MINERALNIH ĐUBRIVA NA SASTAV MASNIH KISELINA ŠAFRANIKE

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R e z i m e

U ovoj studiji procenjivali smo uticaje organskih i mineralnih đubriva, kao i folijarnu primenu regulatora rasta na kvantitativne osobine i sadržaj ulja u semenu šafranike koja se uzgaja u severozapadnom Iranu. Eksperiment je postavljen kao podeljena parcela (3 × 5), a glavna parcela je imala tretmane različitim đubrivima, uključujući organsko đubrivo (ST: stajnjak 20 t ha^{-1}), mineralno đubrivo (MĐ) i bez đubrenja – 'kontrola' (BĐ). Na potparcelama su bili folijarni tretmani regulatorima rasta, koji su uključivali kontrolu (bez tretmana; S_0), holin (S_1), hitozan (S_2) i salicilnu kiselinu (S_3). Rezultati su pokazali da primena đubriva značajno utiče na sadržaj ulja i proteina u semenu. Međutim, uticaj stajnjaka je bio izraženiji od uticaja mineralnog đubriva na kvalitativne karakteristike ulja. Uočena je značajna pozitivna korelacija između sadržaja ulja, sadržaja proteina i sastava nekih masnih kiselina kao što su oleinska kiselina, arahidonska kiselina, stearinska kiselina i palmitinska kiselina. Najveće vrednosti prethodno navedenih osobina dobijene su korišćenjem STS_1, $MĐS_1$ i STS_3. Ovaj trend je takođe bio očigledan u sadržaju linolne kiseline kao glavne masne kiseline u ulju šafranike. Među folijarnim tretmanima, najviše poboljšanja postignuto je upotrebom holina. Poboljšanje osobina zemljišta primenom stajnjaka i odgovarajućih količina mineralnih đubriva je jedna od najvažnijih agrotehničkih mera za poboljšanje kvaliteta ulja semena šafranike.

Ključne reči: holin, sastav masnih kiselina, linolna kiselina, količina joda, kvantitativna analiza.

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THE GROWTH AND NUTRIENT UPTAKES OF YELLOW PASSION FRUIT (*PASSIFLORA EDULIS* VAR. FLAVICARPA) SEEDLINGS IN RESPONSE TO ORGANIC FERTILISER APPLICATION UNDER TROPICAL CONDITIONS

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Abstract: Passion fruit is valued for its intense flavoured juice used in juice mixes. Though cultivation is prominent in many tropical regions, it is a new crop in southwest Nigeria regardless of the favourable ecology. New crop establishment depends on meeting the nutrient requirements, among other growth resources. Two experiments were conducted at Abeokuta to evaluate the optimum rate(s) of Sunshine Organic Fertilizer® (SOF) required for the growth of the seedlings. The SOF was applied at 0, 0.5, 1.0, 1.5, 2.0, and 2.5 t/ha in experiments arranged in Completely Randomised Design (CRD) replicated thrice. The application of SOF improved the growth of seedlings, but a rate above 2.0 t/ha resulted in depressed growth. In experiment 1, the application of 0.5 t/ha SOF gave the highest values of 152.7 cm, 19 mm and 19.0 for vine length, vine diameter and the number of leaves. In experiment 2, the number of leaves was the highest at 1.0 t/ha though similar to 1.5 and 2.0 t/ha. The application of SOF had a significant influence on the seedling leaf area (LA) in the experiments. The largest LA occurred in seedlings treated with SOF at 2.0 and 2.5 t/ha. Dry weight and foliar nutrient uptakes in both experiments were significantly influenced by SOF application. The application of SOF at 0.5 t/ha in experiment 1 resulted in higher N uptake, but the reverse was the trend in experiment 2, with higher nutrient uptakes at 1.5–2.5 t/ha SOF. Conclusively, an optimal rate of 2.0 t/ha had positive effects on seedling response.

Key words: fertiliser rate, growth, new crop, nutrient toxicity, nutrient uptakes.

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Introduction

Passion fruit (*Passiflora edulis* var. flavicarpa) is cultivated for fresh consumption and, more importantly, for the juice used as a flavouring for fruit juice mixes and wine. It is the most cultivated of the *Passifloraceae* family and native to Brazil though cultivation has spread to most tropical and subtropical regions of Africa, Asia, Australia and New Zealand (Morton, 1987; Knight and Sauls, 1994). Passion fruit has immense potentials as an industrial crop in southwest Nigeria, but there is little or no awareness of these potentials that can motivate commercial production (Joseph-Adekunle and Olubode, 2020). Passion fruit was introduced from Europe into Nigeria in the 1980s and is currently grown in Kaduna, Plateau and some western states of Nigeria in home gardens for juice making (Alegbejo, 2004; Aiyelaagbe et al., 2004; Joseph-Adekunle, 2006). These few localized production attempts did not follow standard production packages or fertiliser recommendations for optimum growth. Research results from previous works indicated that passion fruit responded to fertiliser application at the juvenile growth phase (Fagbayide and Joseph-Adekunle, 2002; Aiyelaagbe et al., 2005) though with conflicting rates. However, other researchers reported that the application of organic fertilisers, whether fortified or not, enhanced the development, production and fruit quality of sweet passion fruit (*Passiflora alata Dryland*) and yellow passion fruit as they developed adequate attributes for consumption (Damato et al., 2005; Britto et al., 2005; Joseph-Adekunle and Fagbayide, 2008) Meeting nutrient requirements is one of the essential factors for new crop establishment. Omotoso and Akinrinade (2013) reported positive responses on the growth and fruit quality of pineapple using applied fertilisers. The notion that organic fertiliser can be applied *ad libitum* needs to be examined in the face of growing environmental concern, especially from an organic point of view (Allen and Mangan, 2015). Tagliavini and Scandellari (2013) are of the opinion that nutrient uptake by crops is the backbone of proper fertiliser application in sustainable agriculture. The amounts of nutrients taken up under field conditions can be approximated to the amount of nutrients in the new biomass produced by the tree crop in one year. The experiments should determine the optimum rates of Sunshine Organic Fertilizer® (SOF) and industrially processed municipal wastes required for the growth and nutrient uptake of juvenile yellow passion fruit seedlings. These experiments were, therefore, conducted to assess the growth responses of yellow passion fruit (YPF) seedlings, dry matter accumulation and nutrient uptakes as influenced by rates of Sunshine Organic Fertilizer® (SOF).

Materials and Methods

Two experiments were conducted at the Federal University of Agriculture, Abeokuta, Nigeria (3° 25′ E 7° 15′ N, 100 m above sea level). The SOF rates were 0, 0.5, 1.0, 1.5, 2.0 and 2.5 t/ha based on the N requirement for passion fruit growth in the first experiment and repeated in experiment 2 as the validation of experiment 1. The soil and fertiliser were analysed for physical and chemical properties. Two seedlings were assigned to each treatment, and treatments were laid into Completely Randomised Design (CRD) with three replicates. Four-month-old seedlings of yellow passion fruit (YPF) were transplanted into 16-litre pots perforated at the bottom, previously filled with 20 kg of topsoil; the SOF was premixed with the soil two weeks before transplanting the seedlings. The seedlings were transplanted at the rate of one seedling per pot. Measurements were taken 15 weeks after transplanting on vine length in cm (VL) measured 3 cm above soil level to the tip of the longest vine, number of lateral vines (NLt), number of leaves (NL), vine diameter (VD in mm), and leaf area (LA cm^2). Leaf area was determined non-destructively at 16 weeks after transplanting (a week after measurement of growth parameter) by using the formula $Y = 11x - 49.2r^2$, where x = leaf length, $r^2 = 0.94$ (correction factor), Y = LA (Aiyelaagbe et al., 2005). Dry matter was determined 16 weeks after transplanting (WAT) by partitioning parts of sampled seedlings and oven drying at 65°C to constant weight. Nutrient contents of the dried leaf samples were analysed for nutrient concentration using standard procedures described by AOAC (1990), and nutrient uptakes calculated using the seedling dry foliar weight (g). Data were subjected to analysis of variance (ANOVA) using Genstat 12th edition and significant means separated using Duncan's Multiple Range Test (DMRT at $p \leq 0.05$).

Results and Discussion

The soil was sandy loam, slightly acidic, with low % N; moderate available P and Zn; with extremely low K in line with critical levels of soil nutrients (Chude et al., 2012). The Sunshine Organic Fertilizer® was low in N and P contents, but K content and total carbon concentrations were very high (Table 1).

Vegetative growth response of yellow passion fruit to Sunshine Organic Fertilizer® (SOF) application

The application of SOF had significant effects on the vegetative parameters of yellow passion fruit seedlings in both experiments. The longest vine was obtained in experiment 1, with the application of 0.5 t/ha, which was significantly different from other SOF rates except for 1.0 t/ha. The application of other rates of SOF and control on yellow passion fruit seedlings, however, resulted in lower vine length

values. In experiment 2, a higher SOF rate of 1.5 t/ha had the longest vine but was not significantly different from other rates of SOF compared to 0.5 t/ha in experiment 1. The thickest vine diameter was obtained in seedlings treated with SOF at 0.5 t/ha in the experiment. This was followed by seedlings that received SOF of 1.5 t/ha, while SOF applied at 2.0 and 2.5 t/ha produced seedlings with significantly lower vine diameter values. In experiment 2, application rates above 0.5 t/ha resulted in vine diameter in the range of 14–17 mm, which was lower compared to experiment 1.

Table 1. Pre-transplanting soil and Sunshine Organic Fertilizer® physical and chemical properties.

Variables	Soil values	Sunshine Organic Fertilizer® values	
pH in water (1:1)	6.50	NA	NA
Macronutrients			
N (g/kg)	0.78	Total N (g/kg)	
Available P (mg/kg)	12.25	Total P (g/kg)	10.00
Total carbon (g/(g/kg)	2.16	Total carbon (g/kg)	9.87
Exchangeable bases (cmol/kg)		Exchangeable bases (g/kg)	22.62
Ca	3.87	Ca	63.75
Mg	0.96	Mg	4.31
K	0.07	K	6.86
Na	0.23	Na	3.97
Micronutrients (mg/kg)		Micronutrients (mg/kg)	
Mn	43.80	Mn	230.60
Zn	13.42	Zn	169.00
Cu	0.27	Cu	43.70
Fe	3.65	Fe	15.88
Particle size distribution (g/kg)		NA	NA
Sand	764		
Clay	60	NA	NA
Silt	176		
Textural class	Sandy loam		

The seedlings treated with SOF in experiment 1 produced the number of leaves in the range of 10 and 19 and were not significantly different. However, in experiment 2, the number of leaves almost doubled per SOF rate. The application of 1.0 t/ha recorded the highest number of leaves, which was similar to rates of 1.5 and 2.0 t/ha. The application of SOF had a significant influence on the seedling LA in the two experiments. The seedlings treated with SOF produced a smaller leaf area in the first experiment compared to the second. The application of SOF at higher rates resulted in the largest leaf area as seen in seedlings treated with SOF at 2.5 t/ha in experiment 1, and at 2.0 t/ha in experiment 2. The applied SOF had a

significant effect on the number of laterals vines of the seedlings with control, 0.5 and 1.5 t/ha rates recording similar higher numbers and those seedlings treated with SOF at 1.0 and 2.0 t/ha produced the lowest mean number of lateral vines. Seedlings in experiment 2 treated with SOF at 2.5 t/ha had the significant highest value, followed by 0.5, 1.0 and 1.5t/ha, respectively. The observed apparent higher values in control for most of the vegetative parameters could be due to time lag taken for the mineralization of the SOF treated seedlings, hence, the need to augment nutrient status early to enhance a vigorous start. The depression of the growth parameters like vine length, number of leaves and number of lateral vines except for leaf area with the application of SOF at higher rates may be due to the excessive availability of some nutrients that could cause nutrient imbalance or antagonism or toxicity. Fageria (2001) has stated that, in crop plants, the nutrient interactions are generally measured in terms of growth response and nutrient concentrations. However, the observed trend of depressed growth at higher SOF rates is in line with the stance of Mimolana and Or (2000) that fertilisers should be applied in synchrony with crop demand in smaller quantities during the growing season. Improved growth responses have been affirmed by many researchers who reported that young plants developed large amounts of vegetative and root growth in response to adequate soil nutrient qualities occasioned by soil amendment (Aruleba and Fasina, 2004; Berberich et al., 2006). However, the depressed growth in seedlings treated with high rates of organic fertiliser beyond 1.5t/ha in the pots negated these earlier findings. The mind-set that excessive application of organic fertiliser or compost to the soil is not detrimental is misleading. It is against this backdrop that Mangan (2016) stated that excessive fertiliser application can create many soil and crop problems (Table 2).

Table 2. Effects of Sunshine Organic Fertilizer® application on the growth of yellow passion fruit seedlings 15 weeks after transplanting.

SOF (t/ha)	Vine length (cm)		Vine diameter (mm)		Number of Leaves		Number of lateral vines		Leaf area (cm^2)	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
0	194.3	202.2	17.0bc	13.0b	27.3	30.0b	2.9a	1.3ab	522.0	695.0
0.5	152.7	187.1	19.7a	7.0b	19.3	27.7bc	2.4ab	0.7b	497.0	730.0
1.0	127.3	188.2	19.0ab	16.0a	10.4	34.0b	1.0c	1.6ab	497.0	690.o
1.5	83.0	212.3	18.7ab	14.0a	13.3	33.0ab	1.8ab	1.4ab	440.0	698.0
2.0	83.0	200.1	14.0d	16.0a	12.0	33.4ab	0.7c	1.6ab	531.0	807.0
2.5	101.7	195.3	15.0cd	17.0a	11.0	29.0b	1.3bc	1.7a	543.9	624.0
SE	4.2	43.50	1.39	1.90	9.25	1.45	0.64	0.19	10.40	61.00

Means in the columns without letters are not significantly different at $p \leq 0.05$; SE = The standard error of the mean, SOF = Sunshine Organic Fertilizer®; Expt 1 = The first experiment; Expt 2 = The second experiment.

Effects of Sunshine Organic Fertilizer® application on the dry matter production of yellow passion fruit seedlings

The application of SOF at 0.5 and 2.5 t/ha resulted in the highest leaf dry weight, while SOF at 1.0 and 2.0 t/ha produced lower leaf dry weight. Yellow passion fruit seedlings treated with SOF at 1.5 and 2.5 t/ha produced the highest vine dry weight with tendrils attached and were similar though not statistically different from the control, SOF at 0.5 and 2.0 t/ha. The seedlings with SOF applied at 2.0 t/ha had the highest root dry weight and were significantly different from control and other SOF rates. The application of SOF at 2.5 t/ha resulted in the lowest seedling root dry weight, while other SOF rates had similar values. The highest total dry matter was obtained in YPF seedlings with SOF applied at 1.5 t/ha. The control with SOF applications at 2.0, 2.5 and 0.5 t/ha had lower values. In experiment 2, the application of the SOF at the different rates had significant effects on the biomass of seedlings with the highest leaf dry weight, vine and tendril dry weight and total dry weight occurring at 15 WAT. The application of SOF at 1.0 t/ha, however, resulted in the highest root dry weights. Conversely, the application of SOF at 0.5 t/ha and 2.5 t/ha resulted in consistently lowest leaf, vine and tendril dry weights. The observed trends supported the findings of Cavalcante et al. (2013) regarding the use of humic acid. They reported that humic substances sprayed positively affected the aerial part, root system and improved the quality of yellow passion fruit seedlings (Table 3).

Table 3. Effects of the Sunshine Organic Fertilizer® application on vegetative dry matter (g/plant) production in yellow passion fruit seedlings 16 weeks after transplanting.

SOF	Leaf dry Weight		Vine + Tendril dry weight		Root dry weight		Total dry weight	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
0	6.0ab	11.0bc	26.0ab	51.0b	4.0bc	9.0bc	36.0a	71.0b
0.5	7.0a	9.0c	23.0bc	40.0d	4.0bc	12.0ab	34.0ab	61.0c
1.0	5.0b	12.0b	20.0c	53.0a	6.0ab	8.0c	31.0b	73.0a
1.5	6.0ab	13.0a	27.0a	47.0c	4.0bc	11.0b	37.0a	71.0b
2.0	5.0b	13.0a	23.0bc	50.0b	8.0a	10.0b	36.0a	73.0a
2.5	7.0a	11.0bc	27.0a	40.0d	2.0c	13.0a	36.0a	64.0bc
SE	0.41	0.38	1.26	0.96	0.94	0.45	1.02	3.92

Means in a column with the same letters are not significantly different at $p \leq 0.05$, SOF = Sunshine Organic Fertilizer® in t/ha; SE = The standard error of the mean; Expt 1 = Experiment 1 and Expt 2 = Experiment 2.

Effects of the Sunshine Organic Fertilizer® application on yellow passion fruit seedling nutrient uptakes

The application of SOF in experiment 1 had a significant influence on foliar N, P, K, Mg, Fe, Mn and Cu uptakes in the yellow passion fruit seedlings in experiment 1. Plants that were treated with 0.5 t/ha SOF had maximum N uptake. This was followed by seedlings that did not receive SOF and those that received 2.5 t/ha SOF rate though insignificantly different. Higher rates of SOF resulted in significantly lower values of N uptake.

The P uptake was the highest in seedlings treated with SOF at 2.0 t/ha. Other rates had similar values except for SOF supplied at 1.5 t/ha without P uptake. The application of the SOF at 0.5 and 2.5 t/ha resulted in the significantly highest foliar K uptake while other rates had similar lower values. Foliar Ca and Zn uptakes of YPF seedlings were not significantly influenced by the SOF rates. This negated the findings of Tagliavini and Scandellari (2013), who found that Ca is often the nutrient absorbed at the highest amount, followed by N or K in the case of highly productive trees. Several factors affect nutrient uptake, including tree age, yields, tree vigour, but these effects also depend on the type of nutrient and specific crop characteristics. Some crops, such as grapes and apple, absorb relatively less N than peach, kiwifruit and orange. An increase in nutrient uptake is attributed to the increased availability of nutrients (Tagliavini and Scandellari, 2013). In experiment 2, yellow passion fruit seedlings without SOF and those treated with 0.5 t/ha had the significantly highest value compared to other SOF rates. The foliar Fe uptake was the highest in seedlings that received SOF at 2.5 t/ha. Yellow passion fruit seedlings without SOF, those with SOF applied at 2.0 and 2.5 t/ha had significantly higher foliar Mn uptakes. The highest foliar Cu uptake was observed in seedlings that received 0.5 t/ha of SOF, followed by seedlings that received SOF at 2.0 and 2.5 t/ha (Tables 4 and 5).

Table 4. The foliar nutrient uptake (g/plant) of yellow passion fruit seedling as influenced by the Sunshine Organic Fertilizer® application 16 weeks after transplanting (Experiment 1).

SOF (t/ha)	N	P	K	Ca	Mg	Zn	Fe	Mn	Cu
0	0.016ab	0.001a-c	0.031ab	0.011	0.009a	0.265	1.93bc	0.455a	8.25b
0.5	0.024a	0.001a-c	0.060a	0.002	0.009a	0.349	1.90bc	0.347ab	15.5a
1.0	0.011b	0.001a-c	0.050ab	0.00	0.00c	0.388	2.1ab	0.204bc	8.50b
1.5	0.11b	0.00c	0.031b	0.005	0.001c	0.244	3.82ab	0.228ab	8.75ab
2.0	0.012b	0.001a-c	0.023b	0.012	0.008ab	0.264	1.39c	0.379a	10.25ab
2.5	0.013b	0.002a	0.062a	0.001	0.004a-c	0.570	4.86a	0.429a	10.25ab
SE($p \leq 0.05$)	0.004	0.000	0.014	0.004	0.003	0.145	1.177	0.089	1.753

Means in a column with the same letters are not significantly different ($p \leq 0.05$) SE = The standard error of the mean, SOF = Sunshine Organic Fertilizer®.

Table 5. The foliar nutrient uptake (g/plant) of yellow passion fruit seedlings as influenced by the Sunshine Organic Fertilizer® application (Experiment 2).

SOF t/ha	N	P	K	Ca	Mg	Zn	Fe	Mn	Cu
0	0.025b	0.001b	0.024b	0.022bcd	0.011b	0.558bc	3.85b	0.968b	0.058b
0.5	0.016c	0.002a	0.021b	0.024bcd	0.014a	0.554bc	5.25b	1.417a	0.024b
1.0	0.020bc	0.002a	0.019b	0.026a-d	0.011bc	0.406bc	4.20b	0.875bc	0.200a
1.5	0.029a	0.002a	0.025a	0.026a-d	0.013ab	0.675ab	4.97b	1.365ab	0.288a
2.0	0.029a	0.002a	0.026a	0.019cde	0.013ab	0.667ab	8.11a	0.948b	0.000b
2.5	0.024b	0.001b	0.025a	0.017de	0.012ab	0.735a	3.53b	1.380ab	0.016b
SE	0.010	0.0004	0.004	0.005	0.002	0.150	3.000	0.303	0.1404

Means in a column with the same letters are not significantly different at $p \leq 0.05$ SE = The standard error of the mean, SOF = Sunshine Organic Fertilizer®.

Higher rates of SOF resulted in significantly lower values of N uptake. This is in line with the assertion of Mimolana and Or (2000) that nutrient uptake is reported to be influenced by soil properties, crop characteristics and growing conditions.

Regression models for the total dry weight (TDW) to total nutrient uptakes in yellow passion fruit seedlings as affected by the Sunshine Organic Fertilizer® application

Under experiment 1, yellow passion fruit seedlings treated with SOF showed positive significant data adjustments to linear regression models as functions of SOF rates with the total uptakes of N, Mn and Fe. These were characterised by a total dry weight (TDW) increase up to 65 g/plant for total N uptake with SOF added at 2.0 t/ha, 63 g/plant for total Mn uptake with an SOF rate of 2.5 t/ha and 68 g/plant for total Fe uptake at an SOF rate of 2.5 g/ha (Figures 1, 2 and 3).

The TDW showed a quadratic function in relation to Cu and K total uptakes. The maximum TDW of about 55 g/plant was obtained for total Cu uptake at above 2.0 t/ha SOF application rate, beyond which there was a decrease in TDW. For total K uptake, the TDW of about 56 g/plant was obtained at an SOF rate of 2.0 t/ha, beyond which there was a decrease in total K uptake (Figures 4 and 5).

Yellow passion fruit seedlings treated with SOF in the first experiment showed positive significant data adjustments to linear regression models as functions of SOF rates with the total uptakes of N, P and K. The regression models for the total dry weight (TDW) showed a quadratic function in relation to N, P and K total uptakes. The maximum TDW of about 55 g/plant was obtained for K uptake.

In experiment 2, TDW of about 56 g/plant was obtained at SOF rate of 2.0 t/ha, beyond which, there was a decrease in total K uptake (Figures 6–8).

A balanced supply of essential nutrients is one of the most important factors in increasing growth and crop yields. In crop plants, the nutrient interactions are generally measured in terms of growth response and change in the concentration or uptake of nutrients. Positive (synergistic) interaction is noted when the presence of two nutrients results in an increase in crop yield that is more than adding only one. Similarly, if adding the two nutrients together produces less yield as compared to individual ones, the interaction is negative (antagonistic). When there is no change, there is no interaction, and this is true for organic fertilizers.

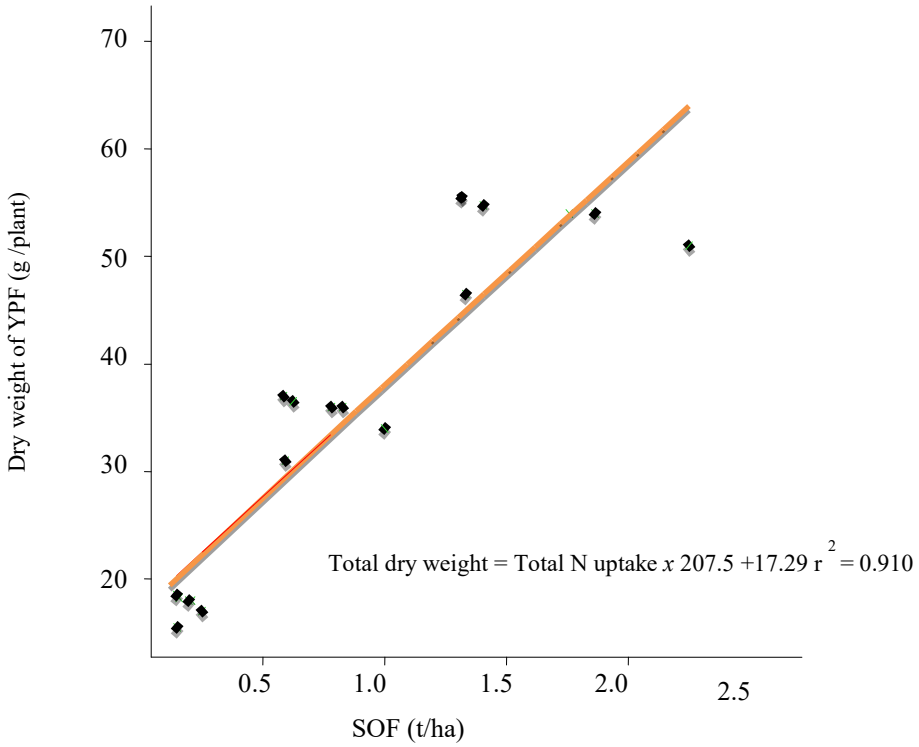

Figure 1. Total dry weight and total N uptake in YPF seedlings as influenced by the SOF rates in experiment 1.

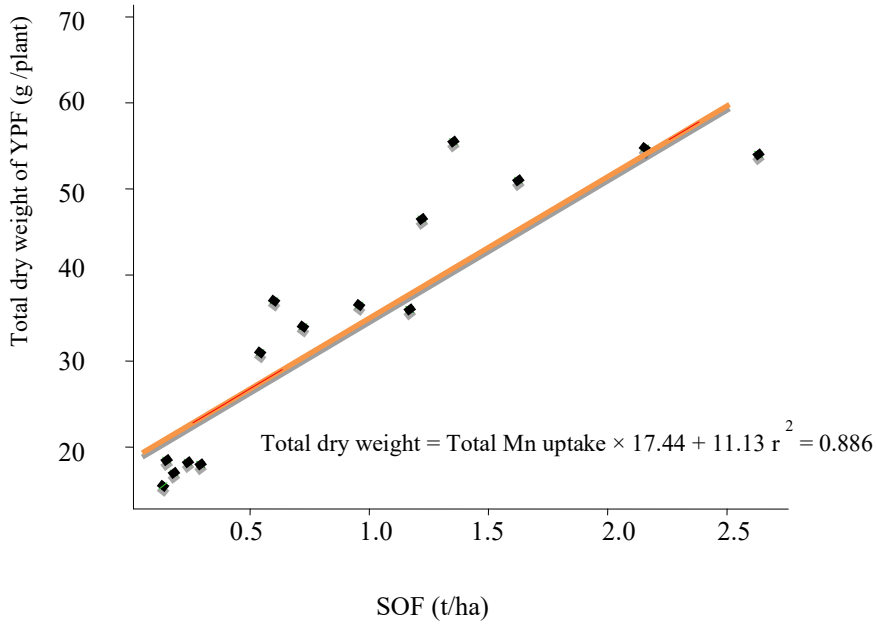

Figure 2. Total dry weight and total Mn uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment.

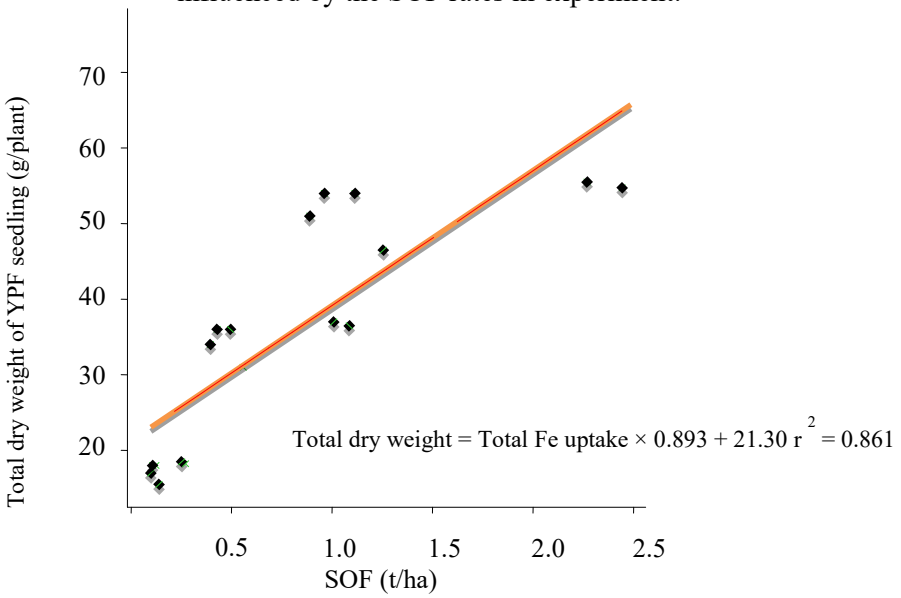

Figure 3. Total dry weight and total Fe uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment 1.

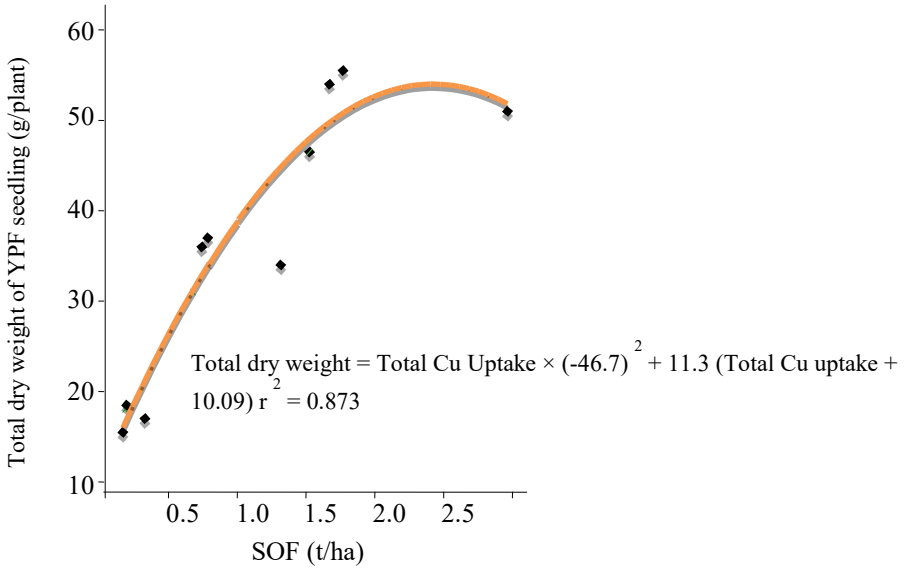

Figure 4. Total dry weight and total Cu uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment 1.

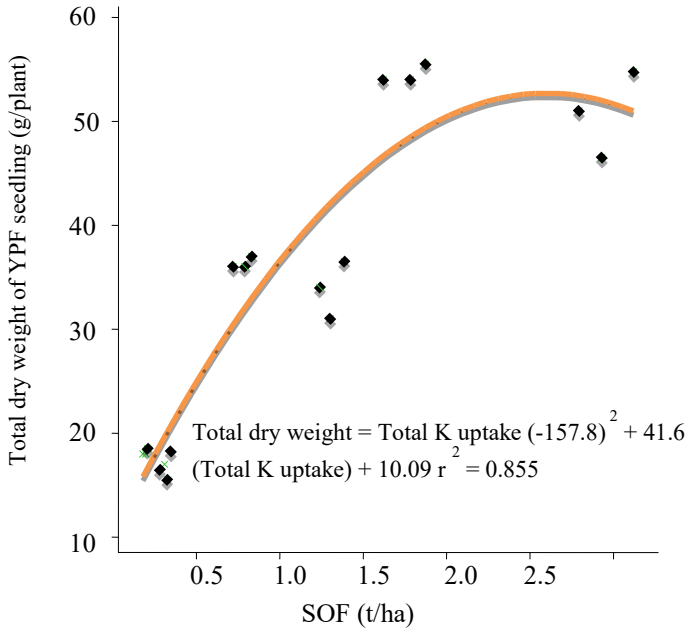

Figure 5. Total dry weight and total K uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment 1.

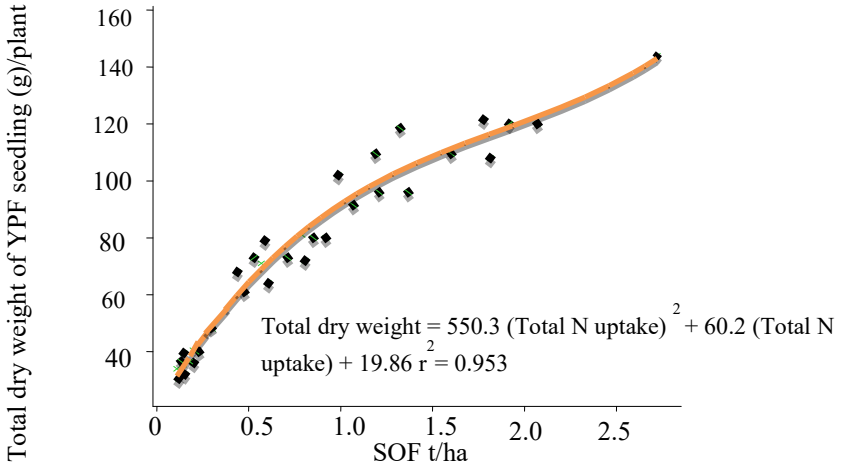

Figure 6. The relationship between total dry weight and total N uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment 2.

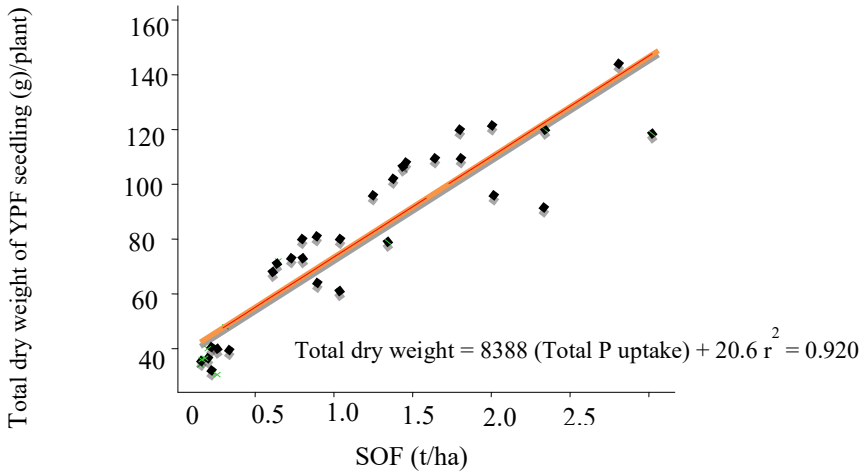

Figure 7. The relationship between total dry weight and total P uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment 2.

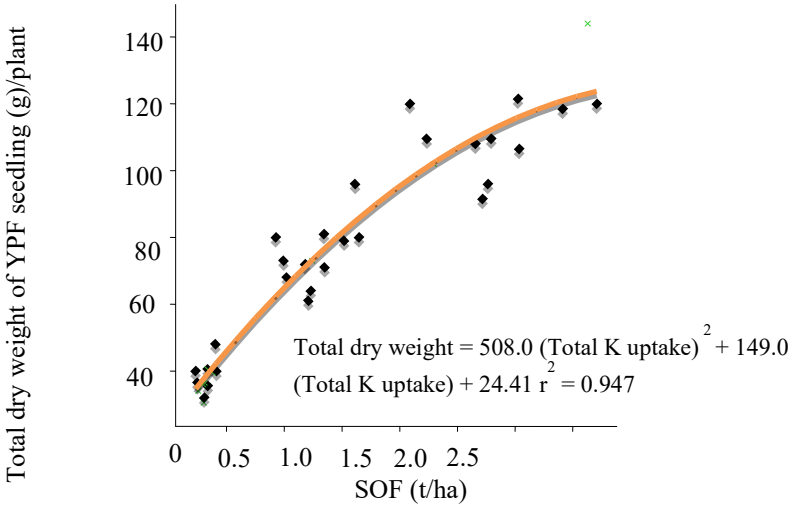

Figure 8. The relationship between total dry matter and total K uptake in yellow passion fruit seedlings as influenced by the SOF rates in experiment 2.

Conclusion

Applications of Sunshine Organic Fertilizer® at low rates enhanced the vegetative growth of yellow passion fruit at an early stage of growth, while a deleterious effect at higher rates was observed.

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RAST I USVAJANJE HRANLJIVIH MATERIJA SADNICA MARAKUJE (*PASSIFLORA EDULIS* VAR. FLAVICARPA) KAO ODGOVOR NA PRIMENU ORGANSKOG ĐUBRIVA U TROPSKIM USLOVIMA

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R e z i m e

Marakuja je cenjena zbog soka intenzivnog ukusa koji se koristi u mešavinama sokova. Iako je uzgajanje zastupljeno u mnogim tropskim regionima, to je nova kultura u jugozapadnoj Nigeriji bez obzira na povoljnu ekologiju. Zasnivanje novih useva zavisi od zadovoljavanja potreba za hranljivim materijama među ostalim resursima rasta. Dva eksperimenta su sprovedena u Abeokuti da bi se procenila/e optimalna količina organskog đubriva *Sunshine Organic Fertilizer*® (SOF) potrebnog za rast sadnica. SOF je primenjen u količini od 0, 0,5, 1,0, 1,5, 2,0 i 2,5 t/ha u eksperimentima raspoređenim u potpuno randomizovanom dizajnu u tri ponovljanja. Primena organskog đubriva SOF poboljšala je rast sadnica, ali je količina iznad 2,0 t/ha dovela do smanjenja rasta. U eksperimentu 1, primena od 0,5 t/ha organskog đubriva SOF dala je najveće vrednosti od 152,7 cm, 19 mm i 19,0 za dužinu loze, prečnik loze i broj listova. U eksperimentu 2, broj listova je bio najveći pri količini od 1,0 t/ha, ali slične vrednosti su dobijene kod količine od 1,5 i 2,0 t/ha. Primena organskog đubriva SOF u eksperimentima je imala značajan uticaj na lisnu površinu sadnica. Najveća lisna površina se javila kod sadnica tretiranih organskim đubrivom SOF u količini od 2,0 i 2,5 t/ha. Suva masa i usvajanje hranljivih materija preko lista u oba eksperimenta bili su značajno uslovljeni primenom organskog đubriva SOF. Primena doze od 0,5 t/ha organskog đubriva SOF u eksperimentu 1 dovela je do većeg usvajanja N, ali je obrnut trend bio prisutan u eksperimentu 2 sa većim usvajanjem hranljivih materija od 1,5 do 2,5 t/ha organskog đubriva SOF. Konačno, optimalna količina od 2,0 t/ha imala je pozitivan efekat na odgovor sadnica.

Ključne reči: količina đubriva, rast, novi usev, toksičnost hranljivih materija, usvajanje hranljivih materija.

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IN VITRO SENSITIVITY OF *COLLETOTRICHUM ACUTATUM* ISOLATES FROM STRAWBERRY TO TEBUCONAZOLE, PROCHLORAZ, FLUDIOXONIL AND THIOPHANATE-METHYL

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Abstract: The objective of this study was to determine the *Colletotrichum acutatum* isolates sensitivity to several chemical compounds *in vitro* and to find their possibilities as a potential control agents for anthracnose disease on strawberry. *C. acutatum* J.H. Simmonds, a causing agent of anthracnose, is a very important pathogen of strawberry, which leads to devastating losses in its production. The pathogen is effectively controlled by the fungicides. Thus their application is necessary for achieving high yield and fruit quality. The sensitivity of 14 isolates of *C. acutatum*, collected from commercial strawberry plantations in Serbia, to four fungicides, was examined by an *in vitro* sensitivity assay. Based on the results of morphological, pathogenic and molecular characterization (up to the complex level), all 14 isolates were determined as *C. acutatum*. The commercial formulation of tebuconazole, fludioxonil, prochloraz and thiophanate-methyl were used for the sensitivity test. The mycelial growth assay method was used to investigate isolates sensitivity to fungicides. The tested isolates were very sensitive to prochloraz and fludioxonil, with mean EC_{50} values of 0.067±0.062 mg L^{-1} and 0.093±0.043 mg L^{-1}, respectively. Significantly higher mean EC_{50} values were observed for tebuconazole (1.473±0.878 mg L^{-1}) and thiophanate-methyl (1.718±1.592 mg L^{-1}). The toxicity of tested fungicides in the mycelial growth assay of *C. acutatum* isolates indicates the potential implementation of these fungicides in the protection programs against strawberry anthracnose disease.

Key words: fungicide sensitivity, anthracnose, tebuconazole, prochloraz, fludioxonil, thiophanate-methyl.

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Introduction

The cultivated garden strawberry (*Fragaria ananassa* Duch.) is a very important berry crop with a major economic value worldwide. Turkey, Spain and Poland are the leading strawberry producers in Europe, with a total production of 415.150, 366.161 and 196.972 tons, respectively (Milivojević, 2018). According to the data obtained from the Statistical Office of the Republic of Serbia, strawberry was grown on over 6700 ha with a total production of 30.483 tons in Serbia in 2020.

There are many limiting factors responsible for the major losses in strawberry production. Strawberry anthracnose, caused by species from the *Colletotrichum* genus, is a very destructive and widespread disease of cultivated strawberries. *Colletotrichum* spp. contains an extremely large number of pathogens, and early studies reported *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *C. fragariae* A. N. Brooks and *C. acutatum* as the most common strawberry anthracnose pathogens (Freeman and Katan, 1997). Recent molecular methods for the identification of *Colletotrichum* species have demonstrated that these species are, in fact, "species complexes" composed of numerous diverse species (Damm et al., 2012). One of the most frequent species complex on strawberry, *C. acutatum* (teleomorph *Glomerella acutata* Guerber & J. C. Correll), infects other fruits (stone, pome and berry fruits), grapes and vegetables (tomatoes, peppers), and many other crops (Howard et al., 1992; Bernstein et al., 1995; Smith, 2002). The pathogen is widespread in almost all areas of strawberry growing but primarily has tropical and subtropical distribution. All parts of the plant are affected, and the typical symptoms are flower blight, leaf spots, petiole lesions, crown rot, root rot and fruit rot (Haack et al., 2018). According to the findings of Ivanovic et al. (2007), fruit rot can cause over 80% strawberry yield losses in Serbia if conditions for disease development are favorable.

Effective control of *Colletotrichum* spp. can be achieved using a combination of chemical and non-chemical measures. The use of fungicides has major importance among other control methods, and successful protection highly depends on timely applications of multi- and single-site fungicides (Mertely et al., 2017a, b). Currently, there are no fungicides registered in the Serbian pesticide market for the strawberry anthracnose control, so chemical management of anthracnose is a challenge since farmers usually opt for the application of fungicides registered for other strawberry pathogens. Fungicides which are commonly used and highly effective for fruit rot disease control are azoxystrobin, pyraclostrobin and the combinations of fludioxonil+cyprodinil and boscalid+pyraclostrobin. Multi-site preventive fungicides, for example, captan, also provide sufficient control of *C. acutatum*, however, the weekly applications during the season are required since there is no curative effect (Mertely et al., 2017b). Generally, recent researchers

suggested that *Colletotrichum* infection can be controlled by numerous fungicides: copper compounds, dithiocarbamates, phtalimides, triazoles and other chemicals like imazalil, prochloraz and fludioxonil (Cao et al., 2017; Gao et al., 2018; Baggio et al., 2018; He et al., 2019).

Differences in fungicide sensitivity among species from a single geographical location might be a result of fungicide selection pressure and variations, which are reflected in significant species heterogeneity within the complex itself. Therefore, knowing the fungicide sensitivity profile of species is important and necessary to create adequate protection programs and help farmers to apply only effective fungicides to overcome potential production problems (Dowling et al., 2020).

The objective of this study was to determine the *C. acutatum* isolates sensitivity to several chemical compounds *in vitro* and to find their possibilities as a potential control agents for anthracnose disease on strawberry.

Material and Methods

Fungal isolates

Colletotrichum acutatum researched in this study was isolated from anthracnose-symptomatic strawberry fruits, using a method given by Dhingra and Sinclair (1995). The samples were collected in 2019 from the strawberry production areas in the Rasina district in Serbia. Fruit fragments (1cm long) cut from the place between healthy and necrotic tissues were washed under running tap water for 10 minutes, surfaced sterilized with 5% sodium hypochlorite solution for 5 min, and then rinsed three times with sterile distilled water. The disinfested fragments were put on a blotting sheet for 20 minutes to dry. Thereafter, the fragments were moved to a Petri plate containing a combination of PDA and antibiotic media and incubated at 25ºC for 10–14 days. Pathogen colonies with the typical morphological characteristics of *C. acutatum* were transferred to new dishes with a PDA medium. Based on the results of morphological, pathogenic and molecular characterization (up to the complex level), all 14 isolates were determined as *C. acutatum*.

Pathogenicity tests

Two representative isolates (C.a. 10 and C.a. 2) of *C. acutatum* were chosen for pathogenicity tests. Each isolate was tested on five fruits. The fruits were surface sterilized in 96% ethanol for 1 min, then rinsed in sterile distilled water and dried at 20ºC. After that, the fruits were wounded with a sterile dissecting needle and inoculated with each isolate by applying fragments of the colony in the middle of the injured fruits. Wounded but non-inoculated fruits were used as a negative control. The inoculated fruits were placed in a glass jar with a lid containing water-soaked paper wool and incubated at 25°C and a 12h photoperiod. Symptoms were

evaluated 2–3 days after inoculation by a visual examination of necrotic surfaces. The experiment was repeated two times.

In vitro sensitivity to fungicides

Isolates' sensitivity to four selected fungicides was evaluated based on the mycelial growth assay method described by Zhang et al. (2012).

The commercial formulations of tebuconazole (Folicur 250 EW, 250 g a.i./L, Bayer, Germany), fludioxonil (Flux, 225 g a.i./L, Galenika-Fitofarmacija, Serbia), prochloraz (Mirage 45 EC, 450 g a.i./L, Adama Makhteshim Ltd., Israel) and thiophanate-methyl (Galofungin T, 450 g a.i./L, Galenika-Fitofarmacija, Serbia) were dispersed in sterile, distilled water to prepare stock solution series. Final concentrations of fungicides for the sensitivity test in PDA medium were: 0.1, 0.2, 0.5, 1, 5 and 10 mg a.i./l for tebuconazole, 0.02, 0.05, 0.1, 0.25 and 0.5 mg a.i./l for fludixonil, 0.01, 0.02, 0.1, 0.5, 1 and 5 mg a.i./l for prochloraz and 0.1, 1, 5, 50 and 500 mg a.i./l for thiophanate-methyl.

Mycelial plugs (10 mm in diameter) were cut from colony margins of 5-day-old cultures with a cork borer and transferred to Petri dishes with fungicide-amended PDA in three replications for each concentration. No fungicide was added to the control plates. The fungal colonies were incubated at 25ºC for 10 days. When colonies reached 75% of the area in the control plate, colony radial growth (in mm) in all plates was measured. Measurements were performed in three directions in each Petri plate, and then the percent inhibition (PI) per each fungicide was calculated using the following formula:

$$PI = \frac{a-b}{a} x100 \quad (1)$$

where a was the colony diameter of control plates and b was the colony diameter of fungicide-amended plates.

The fungicide concentration that effectively inhibited radial fungal growth by 50% (EC_{50}) was determined for each isolate and fungicide. The probit analysis was used for the calculation of EC_{50} values. The resistance factor (RF) of each isolate was calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate in the study, where isolates were grouped as follows: sensitive (RF < 3), moderately resistant (RF = 4–20), resistant (RF = 20–100) and highly resistant (RF > 100) (Gouot, 1994; Dekker, 1995).

Results and Discussion

Pathogenicity

Both tested isolates (C.a. 2 and C.a. 10) were able to infect their host. Inoculated strawberry fruits showed typical anthracnose symptoms and developed brown and necrotic lesions around the fruit wounds (Figure 1). *C. acutatum* was reisolated from the symptomatic fruit parts, according to Koch's postulates. No symptoms were observed on the negative control.

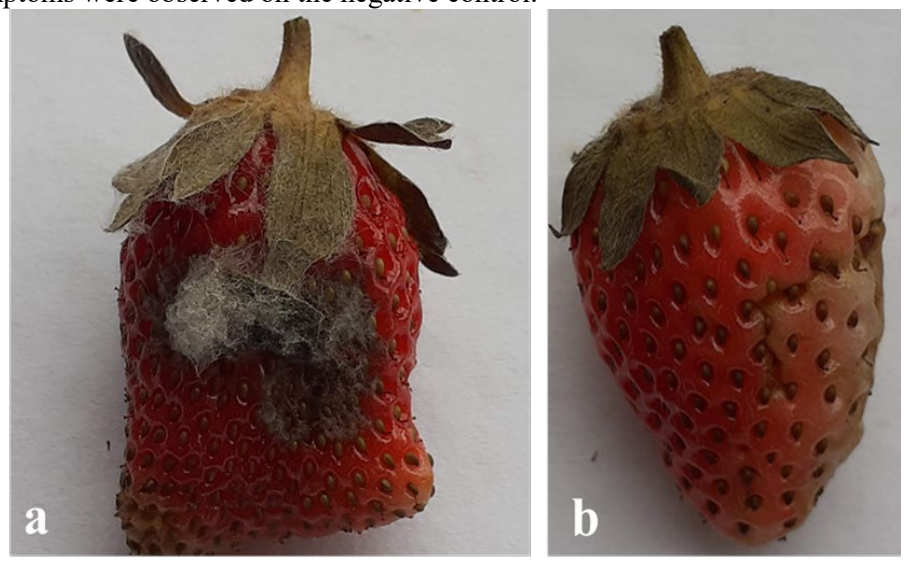

Figure 1. The pathogenicity test of the selected C.a. 10 isolate; a strawberry fruit with an anthracnose symptom (a) and a negative control (b).

In vitro sensitivity of the studied *C. acutatum* isolates

The highest toxicity of tebuconazole was observed for isolate C.a. 10 (EC_{50}=0.791 mg L^{-1}) and the lowest for isolate C.a. 2 (EC_{50}=4.483 mg L^{-1}). The EC_{50} values for the remaining isolates were from 0.842 to 1.701 mg L^{-1}. RFs for tebuconazole for thirteen isolates were below 3, whereas C.a. 2 had RF of 5.7. The regression coefficients (*b*) for all tested isolates were below 1.64.

The studied isolates were very sensitive to prochloraz. Among 14 isolates of *C. acutatum*, the highest sensitivity was observed for isolate C.a. 16 (EC_{50}=0.020 mg L^{-1}), while C.a. 2 had the lowest sensitivity with the value of EC_{50}=0.272 mg L^{-1}. RFs for ten isolates were below 3, for one (C.a. 8), it was 3.1, while for three isolates (C.a. 2, C.a. 5, C.a. 7), RFs were between 4.2 and 13.6. The regression coefficients (*b*) were below 0.93.

All tested isolates expressed a relatively uniform response to fludioxonil (Figure 2), where EC_{50} values ranged from 0.030 to 0.184 mg L^{-1}. The highest toxicity of fludioxonil was observed for isolate C.a. 7 (EC_{50}=0.030 mg L^{-1}), while the lowest toxicity was determined for isolate C.a. 18 (EC_{50}=0.184 mg L^{-1}). RFs were below 3 for eight isolates, while the remaining six isolates had RFs between 3.8 and 6.1. The regression coefficient (*b*) ranged from 1.04 to 2.21.

Differences in the sensitivity of *C. acutatum* isolates to the examined fungicides are presented in Tables 1–2.

Table 1. *In vitro* sensitivity of *C. acutatum* isolates to tebuconazole and prochloraz.

	Tebuconazole			Prochloraz		
Isolate code	EC_{50} mg L^{-1}	*b*	RF	EC_{50} mg L^{-1}	*b*	RF
C.a. 15	0.842	1.14	1.1	0.031	0.71	1.6
C.a. 27	1.701	1.33	2.1	0.037	0.78	1.9
C.a. 16	1.023	1.30	1.3	0.020	0.74	1
C.a. 13	1.611	1.64	2	0.027	0.75	1.4
C.a. 18	0.990	1.24	1.2	0.058	0.61	2.9
C.a. 14	1.462	1.29	1.8	0.036	0.73	1.8
C.a. 2	4.483	1.01	5.7	0.272	0.75	13.6
C.a. 1	1.154	1.28	1.5	0.055	0.91	2.8
C.a. 8	1.501	1.26	1.9	0.061	0.82	3.1
Ca. 26	1.010	1.32	1.3	0.048	0.82	2.4
C.a. 4	1.310	1.42	1.6	0.053	0.93	2.7
C.a. 5	1.322	1.35	1.7	0.084	0.69	4.2
C.a. 10	0.791	1.15	1	0.035	0.85	1.8
C.a. 7	1.424	1.46	1.8	0.114	0.81	5.7

(b) – expressing the relative sensitivity of the isolates; (RF) – the resistance factor; it is calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate in the experiment.

The calculated EC_{50} for thiophanate-methyl ranged from 0.209 mg L^{-1} (C.a. 1) to 4.953 mg L^{-1} (C.a. 10). The EC_{50} for the other investigated isolates ranged from 0.367 mg L^{-1} to 3.878 mg L^{-1}. RFs for seven tested isolates were below 3, whereas for the other six, they were between 3.7 and 18.5, and for the remaining one (C.a. 10), it was 23.7. The regression coefficients (*b*) were below 0.90.

Numerous reports testify that *C. acutatum* affects a wide range of hosts (Damm et al., 2012). For farmers, the best management strategy in plant disease control depends on timed applications of preventive and systemic fungicides. This research examined *in vitro* sensitivity of *C. acutatum* isolates to four fungicides with different modes of action. Tebuconazole and prochloraz act by inhibiting C14-demethylation in the ergosterol biosynthesis (FRAC 3; G1), fludioxonil inhibits MAP/Histidine kinase in osmotic signal transduction (FRAC 12; E2), while thiophanate-methyl affects the inhibition of ß-tubulin assembly in mitosis (FRAC 1; B1).

Table 2. *In vitro* sensitivity of *C. acutatum* isolates to fludioxonil and thiophanate-methyl.

Fludioxonil				Thiophanate-methyl		
Isolate code	EC_{50} mg L^{-1}	*B*	RF	EC_{50} mg L^{-1}	*b*	RF
C.a. 15	0.130	1.80	4.3	0.367	0.57	1.7
C.a. 27	0.085	1.16	2.8	0.387	0.58	1.8
C.a. 16	0.129	1.83	4.3	0.462	0.59	2.2
C.a. 13	0.123	1.98	4.1	0.374	0.57	1.8
C.a. 18	0.184	1.59	6.1	2.460	0.73	11.8
C.a. 14	0.079	1.04	2.6	0.328	0.57	1.6
C.a. 2	0.037	1.82	1.2	0.772	0.52	3.7
C.a. 1	0.084	1.88	2.8	0.209	0.54	1
C.a. 8	0.057	1.60	1.9	0.474	0.48	2.3
Ca. 26	0.134	1.94	4.5	3.066	0.59	14.7
C.a. 4	0.035	1.78	1.2	3.478	0.90	16.6
C.a. 5	0.076	1.90	2.5	3.878	0.79	18.5
C.a. 10	0.115	2.21	3.8	4.953	0.73	23.7
C.a. 7	0.030	1.67	1	2.840	0.81	13.6

(b) – expressing the relative sensitivity of the isolates; (RF) – the resistance factor; it is calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate in the experiment.

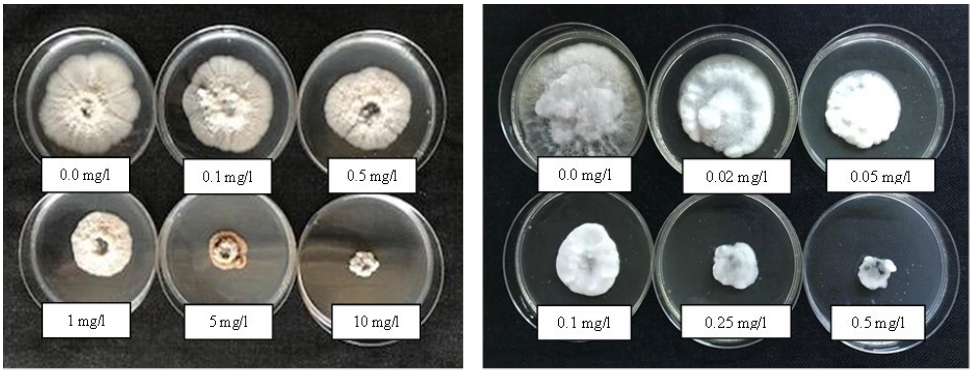

Figure 2. Mycelial growth at concentrations of 0.0, 0.1, 0.5, 1, 5 and 10 mg L^{-1} for tebuconazole (left) and of 0.0, 0.02, 0.05, 0.1, 0.25 and 0.5 mg L^{-1} for fludioxonil (right).

Significant differences in the sensitivity of *Colletotrichum* to various fungicides are represented in several studies. Greer et al. (2011) state that *C. gloeosporioides* isolates are highly sensitive to benzimidazole fungicides, whereas isolates of *C. acutatum* are resistant to them. In this study, most of the isolates of *C. acutatum* were sensitive to the selected fungicides. The EC_{50} values were in the range from 0.030 to 0.184 mg L^{-1}, 0.020 to 0.272 mg L^{-1}, 0.791 to 4.483 mg L^{-1} and 0.209 to 4.953 mg L^{-1} for fludioxonil, prochloraz, tebuconazole and thiophanate-

methyl, respectively. Therefore, prochloraz was the most toxic fungicide, with average EC_{50} values of 0.067±0.062 mg L^{-1}. Besides prochloraz, fludioxonil was the fungicide which showed a strong inhibitory effect on mycelial growth, with a mean EC_{50} value of 0.093±0.043 mg L^{-1}. Comparing with other fungicides tested in this study, *C. acutatum* isolates showed less sensitivity to tebuconazole and thiophanate-methyl. Mean EC_{50} values were 1.473±0.878 mg L^{-1} for tebuconazole and 1.718±1.592 mg L^{-1} for thiophanate-methyl.

The results of this study for tested fungicides (except for thiophanate-methyl) are relatively similar to those reported by many other researchers for *in vitro* studies. Cao et al. (2017) determined the sensitivity of *Colletotrichum* species complexes to DMI fungicides, and the results showed that the mean EC_{50} value for prochloraz was 0.040 μg ml^{-1}. In the study conducted by Gao et al. (2018), where they investigated the sensitivity of 205 isolates of *C. acutatum,* EC_{50} values for fludioxonil were in the range from 0.011 to 0.080 μg ml^{-1} with a mean EC_{50} value of 0.031±0.057 μg ml^{-1}. According to the findings by He et al. (2019), the mycelial growth of different *Colletotrichum* species, including *C. acutatum,* was highly inhibited by tebuconazole, and EC_{50} values ranged from 0.36 to 0.48 mg L^{-1}. Baggio et al. (2018) reported that tested isolates of *C. acutatum* were insensitive to thiophanate-methyl, and EC_{50} values could not be determined. In their study, thiophanate-methyl was not able to inhibit the mycelial growth of *C. acutatum*, and the potential explanation they gave relates to the fact that the tested isolates were simply classified as belonging to the complex *C. acutatum* and were not determined further. In our study, isolates were also determined as a *C. acutatum* complex, but thiophanate-methyl had an inhibitory effect on tested isolates, which potentially indicates that these are different species within the complex compared to the previous research by Baggio et al. (2018). However, in our study, one isolate (C. a. 10) with RF value of 23.4 could potentially be characterized as resistant. However, since we have done molecular identification up to the complex of *C. acutatum* (not further), we cannot claim with certainty that the resistance is present in the population.

The recent data show that *C. acutatum* species complex currently contains 41 species in total, which indicates the great variability in the sensitivity of *Colletotrichum* isolates to different fungicides. A potential solution for further investigations may be to use more precise molecular studies to distinguish the isolates, which may help explain the variations.

Conclusion

According to the obtained results, it can be concluded that tebuconazole, prochloraz, fludioxonil and thiophanate-methyl are effective against the mycelial growth of *C. acutatum*. The mycelium was highly inhibited by prochloraz and

fludioxonil, while tebuconazole and thiophanate-methyl showed less inhibition in this study. Considering that the lack of registered plant protection products for controlling *C. acutatum* on strawberries in Serbia is an additional problem, these results indicate the potential integration of tested fungicides in the protection programs against strawberry anthracnose disease. Since this is preliminary research on fungicide sensitivity, further trials are needed to clarify the differences in the effects of these fungicides in the field conditions.

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OSETLJIVOST IZOLATA *COLLETOTRICHUM ACUTATUM* SA JAGODE NA TEBUKONAZOL, PROHLORAZ, FLUDIOKSONIL I TIOFANAT METIL *IN VITRO*

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R e z i m e

Cilj ovog istraživanja bio je da se ispita osetljivost izolata *Colletotrichum acutatum* na nekoliko različitih fungicida u uslovima *in vitro* i da se pronađe njihova potencijalna uloga u strategiji zaštite jagode od prouzrokovača antraknoze. *C. acutatum* J. H. Simmonds, prouzrokovač antraknoze, veoma je važan patogen jagode koji dovodi do ogromnih gubitaka u njenoj proizvodnji. Ovaj patogen se efikasno suzbija fungicidima, pa je njihova primena neophodna za postizanje visokog prinosa i kvaliteta plodova. U ovom istraživanju ispitivana je osetljivost 14 izolata *C. acutatum in vitro*, prikupljenih iz komercijalnih zasada jagode u Srbiji, na četiri fungicida. Svih 14 izolata determinisano je do nivoa kompleksa *C. acutatum* na osnovu morfoloških, patogenih i molekularnih karakteristika. Za test osetljivosti korišćene su komercijalne formulacije tebukonzola, fludioksonila, prohloraza i tiofanat-metila. Metoda praćenja porasta micelije na hranljivoj podlozi korišćena je za određivanje osetljivosti izolata na fungicide. Ispitivani izolati su bili veoma osetljivi na prohloraz i fludioksonil, sa srednjim EC_{50} vrednostima od 0,067±0,062 mg L^{-1} odnosno 0,093±0,043 mg L^{-1}. Značajno veće srednje EC_{50} vrednosti dobijene su kod tebukonazola (1,473±0.878 mg L^{-1}) i tiofanat-metila (1,718±1,592 mg L^{-1}). Toksičnost testiranih fungicida na porast micelije izolata *C. acutatum* ukazuje na mogućnost implementacije ovih fungicida u programe zaštite od prouzrokovača antraknoze na jagodi.

Ključne reči: osetljivost na fungicide, antraknoza, tebukonazol, prohloraz, fludioksonil, tiofanat-metil.

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A COMPARISON OF METHODS FOR THE DETECTION OF *PHYTOPHTHORA INFESTANS* ON POTATOES IN MAURITIUS

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Abstract: Late blight, a disease caused by oomycota, *Phytophthora infestans*, is a greater threat to the potato crop than any other disease in Mauritius. This disease remains the most challenging to manage once symptoms have appeared, thus requiring rapid detection for effective disease management. The aim of this study was to compare different methods for early detection of the causal agent of potato late blight. Conventional culture-based methods involved the direct isolation of *P. infestans* from infected leaves on Carrot Piece Agar (CPA), Carrot Sucrose Agar (CSA), Commercial Potato Dextrose Agar (CPDA), Fresh Potato Dextrose Agar (FPDA-1 and FPDA-2), Oatmeal Agar (OMA), Pea Sucrose Agar (PSA) and Water Agar (WA) without antibiotic supplementation. Mycelial growth on agar was subsequently identified using molecular techniques. A culture-independent method was also attempted whereby total genomic DNA was directly extracted from symptomatic leaves with mycelial growth followed by PCR amplification with ITS5/ITS4 primers and sequencing. The different media ranked in the following decreasing order of performance: PSA >>> CSA ~ FPDA-1 > CPA ~ CPDA ~ OMA, with growth appearing on PSA within 7 days without contamination. DNA sequencing confirmed the identity of the agent recovered from PSA and from diseased leaves to be *P. infestans*. Findings of this study point to an optimum nutritive medium for recovering and culturing *P. infestans* from leaves with foliar blight without the use of antibiotics. Alternatively, a culture-independent method can be used for rapid detection and identification during routine disease surveillance.

Key words: late blight, morphology, pea sucrose agar, sequencing.

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Introduction

Potato, *Solanum tuberosum* L. is the world's fourth most significant crop after rice, wheat, and maize and the first among the non-grain crops (Campos and Ortiz, 2020). Potatoes are widely considered as a "one-stop-shop" for human nutrition (Stewart and Mcdougall, 2012). Moreover, the potato crop is known for its relative ease of cultivation compared to cereals (Gebru et al., 2017; Campos and Ortiz, 2020). Potato is an important strategic commodity for Mauritius (Ministry of Agro-Industry and Food Security, 2016). In 2018, the production and per capita consumption of potatoes were 17,033 tonnes (FAOSTAT, 2019) and 16.41 kg/year (Statistics Mauritius, 2018), respectively in Mauritius. The main cultivars of potatoes planted are 'Spunta', 'Delaware', 'Belle Isle' (FAREI, 2021) and a newly released cultivar 'Vigora' (Ramdhin, personal communication). However, similar to other *Solanaceous* crops, potatoes are susceptible to a variety of diseases (Amsel and Bishop, 2008), especially late blight, which can have devastating consequences for its cultivation (Sparks et al., 2014).

Potato late blight is caused by oomycota, *Phytophthora infestans* (Mont.) de Bary. It has been reported that *Phytophthora* spp. can affect both cultivated and wild plants, thus causing major losses in agriculture and disrupting the natural forest ecosystem (Ho, 2018). Environmental factors such as temperature, relative humidity and rainfall strongly influence *P. infestans* infection and disease development on potato plants (Arora et al., 2014). According to Whisson et al. (2016), sporangia of *P. infestans* on leaves can spread rapidly in growing areas by water and/or wind. In general, ideal conditions for infection and late blight development include night temperatures of 10–16^{o}C accompanied by light showers, followed by several days of temperatures of 13–16^{o}C with a relative humidity of above 90% (Platt, 2008; Kirk, 2009; Kirk et al., 2013). Relative humidity of >90% and temperatures of 16–21^{o}C can be classified as favourable for spore production and sporangial germination (Platt, 2008). According to the Mauritius Meteorological Services (MMS), average night minimum temperatures can drop to 16.4^{o}C during the coolest months of the island (July and August) (MMS, 2021). During the potato production season, local climatic conditions significantly contribute to the spore's dissemination and germination.

Late blight is by far the most important potato disease worldwide (Platt, 2008; Kirk et al., 2013) as well as for Mauritius (Ibrahim and Taleb-Hossenkhan, 2017). Potato late blight occurs annually in many potato production areas worldwide and can result in 100% crop losses (Platt, 2008). In 2004, an outbreak of late blight occurred in Mauritius, and significant yield losses were recorded for the cultivar 'Spunta', previously reported as relatively tolerant (FARC, 2004; Neeliah et al., 2006). Similarly, in June 2020, another potato late blight outbreak was reported in Mauritius (Ponnappa-Naiken, 2020). Given that late blight constitutes a great threat

to potato production in Mauritius, there is a need for early detection of the pathogen for effective management of the disease.

Conventional culture-based diagnoses are well-known, although they are time-consuming, labour-intensive and technically challenging despite being very sensitive and accurate (Drenth et al., 2006; Khan et al., 2017). *P. infestans* attacks mainly living or freshly injured plant material making isolation of the pathogen from necrotic tissue difficult due to the presence of saprobes, which quickly overgrow the pathogen (Drenth and Sendall, 2001). Considering the very high reproductive potential and the rapid rate of spread of *P. infestans*, (Khan et al., 2017), the management of late blight is inherently more difficult than other diseases. Management strategies depend primarily on the early diagnosis and detection of the causal agent in the potato fields. Compared to traditional methods, PCR is rapid and accurate for specific identification of *P. infestans* in plant material (Hussain et al., 2013; Khan et al., 2017). The objective of this study was to compare the effectiveness and turnaround time of culture-dependent and culture-independent detection and identification of the causal agent of late blight affecting potato plants.

Material and Methods

Site visit and sample collection

In August 2020, an open potato field of the Spunta cultivar, located in Vacoas (20°19'38.9"S 57°29'38.3"E) in the super-humid agro-climatic zone of Mauritius, was surveyed for late blight symptoms. Disease incidence was estimated by counting the number of plants with disease symptoms from 100 random plants and repeated 4 times. In addition, a total of 25 samples of symptomatic leaves were collected, labelled and transported to the Microbiology laboratory of the University of Mauritius.

Microscopic examination of infected leaves

The infected leaves were surface-sterilised by rubbing with cotton wool, moistened with 70% ethyl alcohol, and incubated in humid chambers at 15±3°C for 24 h. After incubation, some mycelia were taken at the sporulating lesion using a sterile needle and placed on a drop of cotton blue lactophenol dye on a clean microscope slide. A cover slip was placed over the dye drop and examined under 400x magnification using a bright-field compound microscope (Euromex, The Netherlands).

Preparation of culture media

A total of eight different culture media were compared in terms of their ability to recover and isolate the pathogen. Carrot Piece Agar (CPA) (Werres et al., 2001),

Carrot Sucrose Agar (CSA) (Hussain and Hussain, 2016; Kumbar, 2017), Fresh Potato Dextrose Agar (FPDA), Oatmeal Agar (OMA) (Hussain and Hussain, 2016) and Pea Sucrose Agar (PSA) (Stammler, 2006), were all prepared from freshly available ingredients. FPDA was prepared by boiling fresh potatoes in distilled water until completely softened (Fresh Potato Dextrose Agar Version 1 [FPDA-1]) (Hussain and Hussain, 2016) or still firm (FPDA-2) (Kiraly et al., 1970). The ready-to-use commercially available media used included Potato Dextrose Agar (CPDA, HiMedia, India) and Water Agar (WA, HiMedia, India) (*Appendix*).

Comparison of techniques for recovery of *Phytophthora infestans* from diseased tissue

The direct transfer of mycelial growth to media

After overnight incubation of symptomatic potato leaves in humid chambers, any visible growth of the pathogen was aseptically transferred onto the differently prepared media (CPA, CPDA, CSA, FPDA-1, FPDA-2, OMA, PSA and WA) using a method adapted from Tumwine et al. (2000). Briefly, mycelia were carefully picked up using a sterile needle and placed on each medium without touching the leaf tissue. The plates were then incubated at 15±3^{o}C in the dark for 3–7 days, depending on the growth rate of *P. infestans* on the medium. Three independent replicates were carried out for each medium.

Transfer of infected leaves onto healthy potato tubers

Within this method, two approaches were attempted. Firstly, healthy tubers were surface-sterilised by wiping with 70% ethanol and aseptically cut into 5-mm thick slices. These slices were then placed in 90-mm sterile Petri dishes onto which small pieces of infected leaves, cut at the sporulating border of the lesion, were placed and covered with lids. The dishes were then incubated at 15±3^{o}C for 14 days, until sporulation around the slices was observed. The sporangia and hyphae were carefully picked and transferred onto the different media prepared as described above (Tumwine et al., 2000; Gamboa et al., 2019).

The second approach involved the sandwich method. A healthy potato tuber was washed with running water, followed by surface sterilisation by wiping with 70% alcohol. The tuber was cut aseptically into two halves between which the infected leaf was placed. The whole sandwich was secured with an elastic band and subsequently incubated at 15±3^{o}C for 4-9 days, followed by transferring of any visible mycelial growth from the tubers onto each medium as described above (Sobkowiak and Śliwka, 2017).

Isolation from sporangial suspension

A sporangial suspension of the causal agent was prepared by gently washing each infected leaf with ca. 1 ml of deionised water into a sterile glass Petri dish to dislodge the sporangia. This suspension was used in two different steps. Firstly, an aliquot of 100 µl of the suspension was spread-plated on the different media mentioned above and plates were incubated at 15±3°C for up to 7 days with the daily examination.

Secondly, some healthy leaves were artificially wounded using a sterile needle. They were then inoculated with 20 µl of the sporangial suspension and incubated in humid chambers at 15±3°C (Harrison et al., 1990; Lees et al., 2012). Any visible growth on inoculated leaves was then isolated and sub-cultured onto each medium as described in the first section.

Sub-culturing and microscopic examination of cultures

After 7–21 days, intermittent sub-culturing was done by transferring actively growing hyphae at the tips of the cultures onto fresh media (Tumwine et al., 2000). Sub-cultured plates were incubated at 15±3°C in the dark for another 20 days. The 20 day-old cultures were then examined under the microscope by picking the mycelial growth using a sterile needle, and similar procedures were conducted as described in the previous section.

Molecular identification of *Phytophthora infestans*

After 27–41 days of incubation, putative isolates of *P. infestans* on PSA were selected for molecular identification since growth was more prolific on PSA compared to the other media. Briefly, mycelial growth was scraped from the plate, weighed, and ground with liquid nitrogen prior to extraction using the Cetyl Trimethyl Ammonium Bromide (CTAB) method. For the detection purposes, a culture-independent method was included, in which total genomic DNA (leaf and causal agent) was directly extracted from the ground infected leaf tissue with liquid nitrogen (Khan et al., 2017; Riit et al., 2016), followed by DNA extraction using the CTAB method (Ranghoo and Hyde, 2000).

The ITS regions of rDNA were amplified using the ITS primers, ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCGCTTATTGATATGC-3') (Ristaino et al., 1998). The thermal cycling parameters comprised initial denaturation at 95°C for 3 min followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s followed by a final extension at 72°C for 5 min. DNA sequencing reactions were done using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) following the protocol outlined by the manufacturers. Sequencing reaction products were purified by the ExoSAP method and were directly sequenced in both directions using an automated sequencer (ABI

3500 DNA sequencer (Applied Biosystems) at Inqaba Biotechnical Industries (Pty) Ltd, South Africa, using the same primers as for amplification. Forward and reverse sequences were assembled and edited using CLC Main Workbench Version 7.6 (https://www.qiagenbioinformatics.com/). Consensus sequences were computed using the ClustalW (Thompson et al., 1994), integrated in MEGA6 software (Tamura et al., 2013), and deposited in GenBank (http://www.ncbi.nlm.nih.gov). All generated sequences were compared with each other by calculating nucleotide (nt) similarities, as well as with previously deposited *Phytophthora* spp. isolates available in GenBank, using the similarity search tool BLAST.

Results and Discussion

In-field disease incidence and symptomatology

The estimated disease incidence of late blight in the potato field was ca. 40%, and the disease was found to spread over a moderate distance, as shown in Figure 1A. The foliage of the potato plants infected by late blight was observed to have numerous characteristic dark lesions near the tips and margins, progressing to a pale green colour at the sporulating border (circled yellow) (Figure 1B).

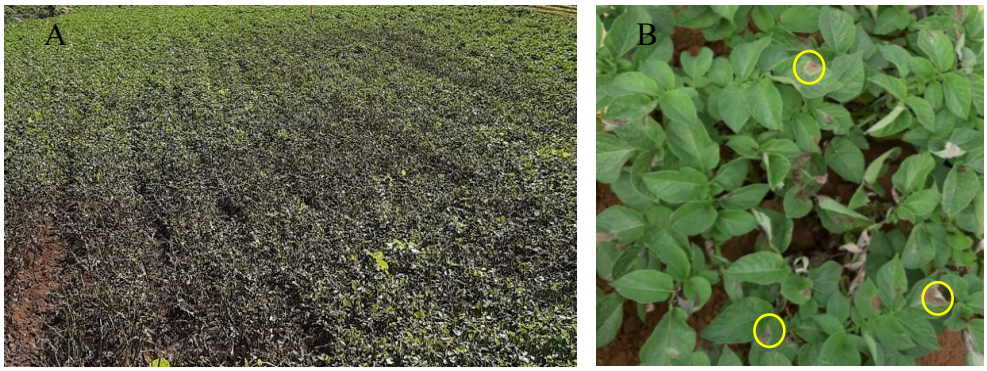

Figure 1. A: Late blight in a potato field at Vacoas, Mauritius, B: large dark lesions on leaves of potato plants with late blight.

Morphological identification

The microscopic examination of infected leaves (Figure 2A) revealed the presence of lemon-shaped ovoid and semi-papillate sporangia (Figure 2B), with an average length:width ratio of sporangia being 1.9. As for the sporangiophores observed, they were branched with swelling just below the sporangium (Figure 2B) and had an average width and length of 4.4 µm (n=50) and 622.8 µm (n=50), respectively. These morphological characteristics were similar to those described

by other researchers such as Drenth and Sendall (2001) and Sobkowiak and Sliwka (2017) and were tentatively identified as *P. infestans.*

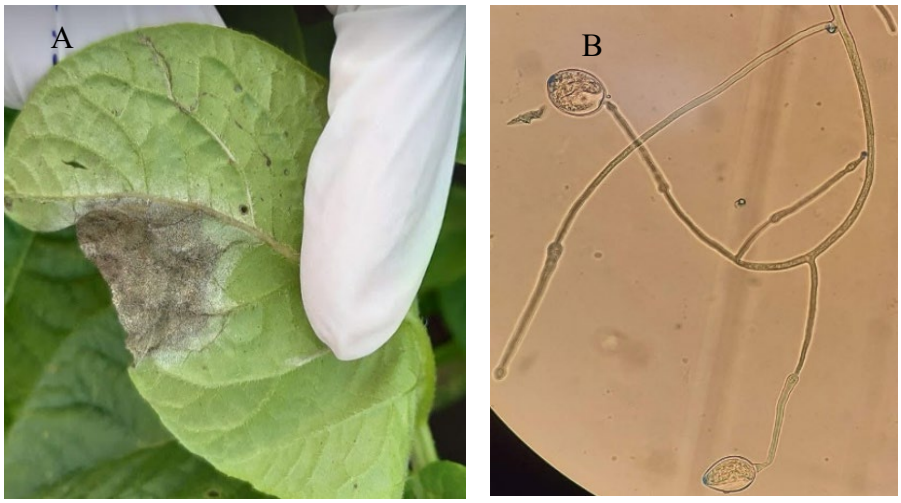

Figure 2. A: Visible mycelial mass of *Phytophthora infestans* on the abaxial surface of leaves, B: ovoid, semi-papillate sporangia on sympodial sporangiophores observed under a bright-field microscope (400x magnification).

Comparison of techniques for recovery of *Phytophthora infestans* from diseased tissue

P. infestans was isolated using different techniques and media to determine the optimum condition for the recovery of the agent. Indeed, optimisation techniques are important for extending the life of samples collected without risking the loss of the agent of interest (Tumwine et al., 2000). Our findings revealed that isolation of *P. infestans* was rendered difficult due to contamination by secondary pathogens present on the leaves or tubers, despite the use of aseptic techniques. In fact, no growth or occasional outgrowth by background contaminants was observed on incubated plates. The contamination could be attributed to the absence of antibiotics in the media, possibly favouring the growth of bacterial contaminants, as reported in the study done by Sarker et al. (2020). Equally, using "healthy" leaves or tubers for recovery of *P. infestans* could present further risks of contamination by other microorganisms. Indeed, the contamination of plates has been attributed to the growth of background microflora on the host tissue (Tumwine et al., 2000) or delayed sub-culturing of hyphal tips onto media (Sarker et al., 2020). On the other hand, the direct transfer of mycelial growth from infected leaves onto media was successful in this study, as demonstrated by the onset of growth after 7 days of incubation. In addition, the risk of contamination of the

culture was minimised by intermittent sub-culturing. Tumwine et al. (2000) also emphasised the importance of sterile techniques and regular sub-culturing.

Performance comparison of different culture media

The direct transfer of mycelial growth from infected leaves to media resulted in white, slightly fluffy, irregular colony growth on PSA after 7–20 days of incubation, while much slower growth was observed on CSA and FPDA-1. Observation of PSA plates under a microscope revealed lemon-shaped sporangia together with sporangiospores. Based on the colony morphology and characteristics of sporangia, the pathogen was tentatively identified as *Phytophthora infestans*. Hence, *P. infestans* could be successfully recovered on PSA from freshly infected potato leaves using a simple and cost-effective method, namely the direct transfer of mycelial growth from infected leaves to PSA without antibiotics.

In addition, it is worth mentioning that no antibiotics were used in the preparation of the various media, making it a very cost-effective method for analyses of samples collected during routine screening. Moreover, compared to PSA, CSA and FPDA-1, other media (CPA, CPDA, FPDA-2, OMA and WA) did not promote the growth of the pathogen since microscopic examination of the plates revealed the presence of sporangia, which were initially transferred from the infected leaves, without sporangiophores. The absence of sporangiophores could be attributed to the lack of one or more nutrients needed for the development of *P. infestans* (Kumbar, 2017; Sarker et al., 2020). For instance, Kumbar (2017) noted that the absence of sucrose in CPA inhibited the sporangial germination of *P. infestans*.

Molecular identification of *Phytophthora infestans* from culture and total genomic DNA

The sequence of the representative isolates obtained from the culture-dependent approach (Accession No. MW794194) was compared with those available in GenBank. It was found to share 99–100% nucleotide similarity with over 100 *P. infestans* isolates, 100% with an isolate from *Solanaceous* crops, Accession No. EU200296 (Vargas et al., 2009). Similarly, the sequence of the PCR product obtained from the total DNA from the culture-independent route (Accession No. MZ504994) also shared 99.50% nucleotide similarity with over 100 *P. infestans* isolates, with 95.50% *P. infestans* isolate (MK507866) from the tomato from the United Kingdom (Pettitt et al., 2019). The isolates included in this study are the first molecularly characterised *P. infestans* isolates from Mauritius.

However, the culture-dependent method took around 30 days (Figure 3) and proved to be very time-consuming, as also noted by other researchers (Hussain et al., 2005; Hussain et al., 2013). Since sporangia can germinate within a few hours under favourable conditions, early diagnosis and detection of the causal agent in

infected potato fields are thus necessary for successful disease control (Khan et al., 2017), and this has prompted the development of faster PCR-based methods (Judelson and Tooley, 2000; Hussain et al., 2005; Haverkort et al., 2009; Lees et al., 2012).

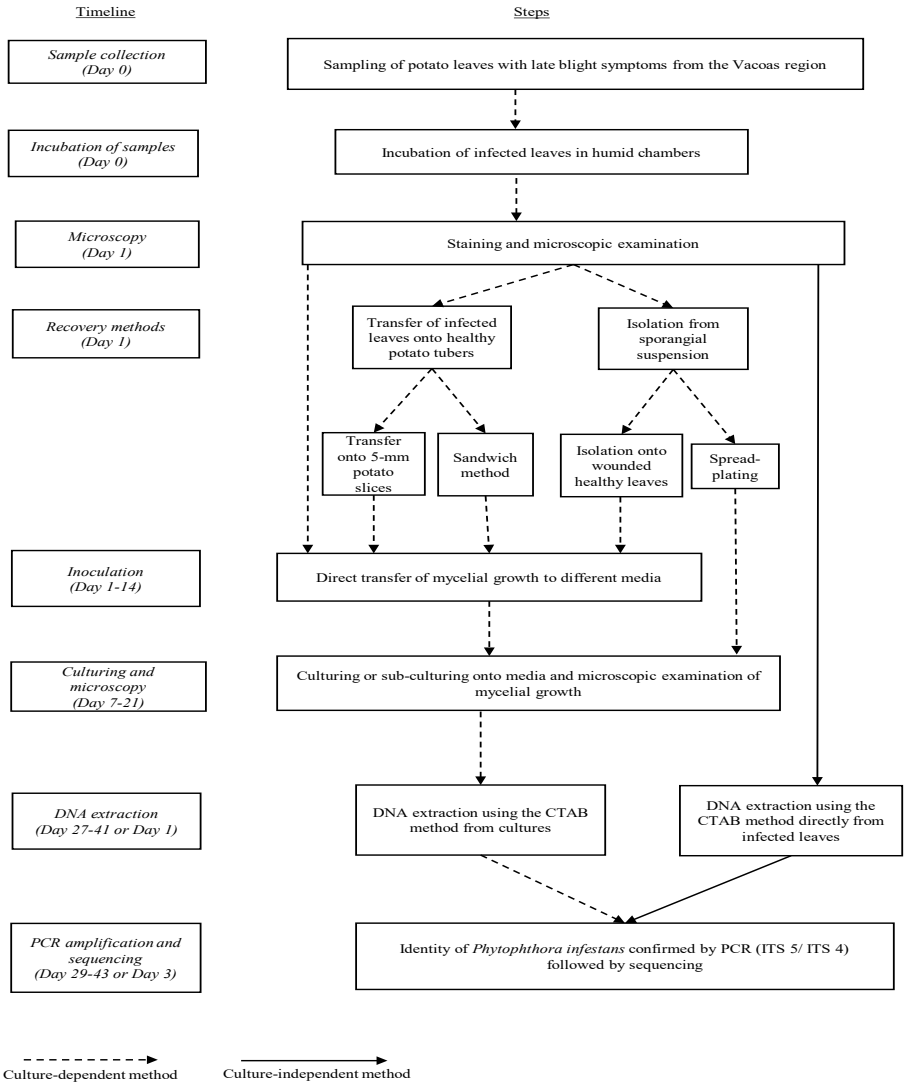

Figure 3. A flow diagram showing the different steps for the culture-dependent and culture-independent methods for the identification of the causative agent from diseased potato leaves.

Extraction of the total genomic DNA from infected leaves followed by PCR and sequencing enabled accurate confirmation of the identity of the etiological agent of foliar blight within 3 days of sample collection (Figure 3). Molecular techniques are being increasingly deployed for the diagnosis of foliar late blight (Judelson and Tooley, 2000; Drenth and Sendall, 2001; Hussain et al., 2005; Haverkort et al., 2009; Lees et al., 2012; Khan et al., 2017) since the symptoms are similar to other fungal diseases, abiotic disorders, or fungicide injuries (Pscheidt, 1991). The primers ITS5/ITS4 were found reliable for rapid taxonomic identification of *Phytophthora* species, as also reported by Ristaino et al. (1998). In our study, direct microscopic observation of diseased leaves for morphological characteristics typical of *P. infestans* coupled with molecular confirmation by the culture-independent approach enabled fast and accurate pathogen detection.

A flow diagram showing the different steps for the culture-dependent and culture-independent methods is shown in Figure 3.

Conclusion

The culture-based approach for isolation of *P. infestans* on PSA without the use of antibiotics followed by molecular identification proved to be both cost-effective and successful although it was more time-consuming compared to the culture-independent method. *In situ* microscopic examination of symptomatic leaf tissues coupled with total genomic DNA extraction, PCR using ITS5/ITS4 primers and sequencing, resulted in the quick and accurate identification of the pathogen. Besides being considerably faster and semi-specific, the culture-independent method is particularly useful in laboratories with limited financial resources and personnel.

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Appendix

Carrot Piece Agar (CPA) (Werres et al., 2001): carrots 50 g; agar 20 g; distilled water 1000 ml.

Grate the fresh carrots. Place the grated carrots directly in a conical flask and add the agar, followed by the distilled water. Autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Carrot Sucrose Agar (CSA) (Hussain and Hussain, 2016; Kumbar, 2017): carrots 220 g; distilled water 1000 ml; sucrose 20 g; agar 9 g.

Cut the fresh carrots into pieces. Boil the cut carrots in 500 mL distilled water until cooked. Comminute the warm carrots in a blender for 1 min at high speed. Filter through a 3–4 layered muslin cloth and squeeze out the juice from the residue. Make up the filtrate to 1 L and add the sucrose and agar. Autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Commercial Potato Dextrose Agar (CPDA): PDA (HiMedia) 39.1 g; distilled water 1000 ml.

Add PDA to water, autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Fresh Potato Dextrose Agar Version 1 (FPDA-1) (Hussain and Hussain, 2016): potatoes 225 g; 1000 ml distilled water; 20 g dextrose; 20 g agar.

Cut the peeled and washed potatoes into pieces. Boil the cut potatoes in distilled water until fully cooked. Strain the solution and keep the filtrate for use. Make up the filtrate to 1 L and add the dextrose and agar. Autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Fresh Potato Dextrose Agar Version 2 (FPDA-2) (Kiraly et al., 1970): potatoes 200 g; distilled water 1000 ml; dextrose 20 g; agar 20 g.

Cut the peeled and washed potatoes into pieces. Boil the cut potatoes in distilled water for 10–15 min and make sure that the boiled potatoes remain firm. Strain the solution and keep the filtrate for use. Make up the filtrate to 1 L and add the dextrose and agar. Autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Oatmeal Agar (OMA) (Hussain and Hussain, 2016): oats 20 g; distilled water 1000 ml; agar 20 g.

Mix oats with distilled water. Boil the mixture for 10 min. Filter the solution through a muslin cloth. Make up the filtrate to 1 L and add the agar. Autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Pea Sucrose Agar (PSA) (Stammler, 2006): frozen peas 150 g; distilled water 1000 ml; glucose 5 g; agar 20 g.

Boil the frozen peas in distilled water until fully cooked. Filter the homogenised mixture through 3–4 layers of a muslin cloth. Make up the filtrate to 1 L and add the glucose and agar. Autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

Water Agar (*WA)* (Hussain and Hussain, 2016): agar 20 g; distilled water 1000 ml.

Add agar to water, autoclave the mixture at 121°C for 20 min and then cool to 50°C in a water bath.

UPOREDNO OCENJIVANJE METODA ZA DETEKCIJU PATOGENA KROMPIRA *PHYTOPHTHORA INFESTANS* NA MAURICIJUSU

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R e z i m e

Plamenjača, bolest koju izaziva oomiceta *Phytophthora infestans* predstavlja veću opasnost za usev krompira nego bilo koja druga bolest na Mauricijusu. Nakon pojave simptoma, suzbijanje je vrlo teško, što ukazuje da je neophodno ustanoviti način brze detekcije u cilju obezbeđenja primene efikasnih mera suzbijanja. Osnovni cilj ovih istraživanja bio je da se uporede različite metode za ranu detekciju prouzrokovača plamenjače krompira. Metode zasnovane na konvencionalnim metodama uključile su direktnu izolaciju *P. infestans* iz zaraženih listova bez dodavanja antibiotika na podlogu od komadića mrkve (CPA), podlogu od mrkve i saharoze (CSA), komercijalni krompir dektrozni agar (CPDA), svež krompir dekstrozni agar (FPDA-1 and FPDA-2), podlogu od ovsa (OMA), podlogu od graška i saharoze (PSA) i vodeni agar (WA). Porast micelije na podlogama identifikovan je korišćenjem molekularnih metoda. Direktna detekcja bez prethodne izolacije takođe je primenjena. Ukupna genomna DNA direktno je ekstrahovana iz listova sa simptomima i sporulacijom, nakon čega je PCR amplifikacija i sekvenciranje obavljeno primenom prajmera ITS5/ITS4. Po opadajućoj pogodnosti, podloge se mogu poređati na sledeći način: PSA >>> CSA ~ FPDA-1 > CPA ~ CPDA ~ OMA, pri čemu je porast na PSA bio bez kontaminacija i vidljiv nakon sedam dana. Sekvenciranje je potvrdilo identitet *P. infestans* kako sa PSA tako i direktno iz zaraženih listova. Dobijeni rezultati ukazuju na značaj izbora optimalne hranljive podloge bez antibiotika za izolaciju i gajenje *P. infestans* direktno iz listova sa plamenjačom. S druge strane, metoda direktnog dokazivanja bez izolacije može da se koristi za brzu detekciju i identifikaciju tokom rutinskog nadzora nad pojavom bolesti.

Ključne reči: plamenjača, morfologija, podloga od graška i saharoze, sekvenciranje.

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Ballard, J. (1998). *Some significant apple breeding stations around the world*. Selah, Washington.

Web site
Platnick, N.I. (2010). The world spider catalog, version 10.5. *American Museum of Natural History*. Retrieved February 12, 2016, from http://research.amnh.org/entomology/spiders/catalog/index.html

Summary
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All graphs, diagrams and photographs should be titled "Figure" (1, 2, etc.). They should be placed in the text. Graphs and diagrams should be computer drawn, using 9 font size and a maximum width of 13 cm, so that they can be legible and distinct after the size reduction. The overuse of colours and hues should be avoided for aesthetic reasons. The detailed legend without abbreviations for each graph and

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Only standardised abbreviations should be used in the paper. Measure units should be expressed using International System of Units (SI). The abbreviations can be used for other expressions provided these expressions are stated in the full form when appear for the first time with the abbreviated form in the brackets. Values from 1 to 9 can be written in letters, but others numerically.

Nomenclature
The complete nomenclature (chemical and biochemical, taxonomical, genetic etc.) must be adjusted to international codes and commissions, such as *International Union of Pure and Applied Chemistry, IUPAC-IUB Combined Commission on Biochemical Nomenclature, Enzyme Nomenclature, International Code of Botanical Nomenclature, International Code of Nomenclature of Bacteria* etc.

Formulae
All formulae and equations in the paper should be worked out by means of the programme "WORD Equation". An ample space should be left around the formulae for the sake of visibility. Subscripts and superscripts should be clear. Greek letters and other non-Latin symbols should be explained when they are first used. The meaning of all symbols should be given immediately after the equation where these symbols are first used. Equations should be numbered by Arabic numerals, serially in brackets, at the right-hand side. Each equation must be mentioned in the text as Eq. (1), Eq. (2), etc.

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Ključne reči
Ključne reči su termini ili fraze koje najbolje opisuju sadržaj članka za potrebe indeksiranja i pretraživanja. Broj ključnih reči može biti od 3 do 10. Navode se ispod sažetka. Naslov „Ključne reči“ piše se boldovano i uvlači jednim

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Uvod

Uvod treba da sadrži informacije o dosadašnjim istraživanjima po navedenom pitanju i šta se datim istraživanjem želi postići. Prilikom osvrta na literaturu, navesti autora i godinu, a autora citirati u spisku literature. Naslov „Uvod“ piše se sa prvim velikim slovom, centrirano i boldovano, nakon čega sa jednim razmakom ispod naslova sledi tekst uvoda poravnat po levoj i desnoj margini. Svaki novi pasus uvlači se jednim tabulatorom. Ova pravila važe i za sva ostala poglavlja.

Materijal i metode

Materijal i metode treba izložiti jasno uz objašnjenje svih primenjenih postupaka u radu. Opšte poznate metode izložiti kratko, a detaljnije ih objasniti ukoliko se odstupa od ranije objavljenih postupaka. Za radove eksperimentalnog karaktera obavezno navesti način statističke obrade podataka. U ovom poglavlju, kao i u poglavlju „Rezultati i diskusija“, po potrebi se mogu dati i određena podpoglavlja.

Rezultati i diskusija

U poglavlju „Rezultati i diskusija“ interpretiraju se podaci dobijeni na osnovu zapažanja i izvršenih eksperimenata. U komentaru rezultata treba se pozivati na literaturu koja se navodi na kraju rada, čime se obezbeđuje poređenje dobijenih rezultata sa dosadašnjim saznanjima u toj oblasti.

Zaključak

U zaključku treba ukratko navesti najznačajnije rezultate dobijene u radu. Izbegavati nabrajanje svih rezultata istraživanja sa ponavljanjem brojčanih vrednosti koje su prethodno već navedene u poglavlju „Rezultati i diskusija“. Zaključak ne sme da sadrži reference.

Zahvalnica

Zahvalnica treba da sadrži naziv i broj projekta, odnosno naziv programa u okviru koga je rad nastao, kao i naziv institucije koja je finansirala projekat ili program.

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Primeri navođenja referenci su sledeći:

Periodičan časopis
Gvozdenović, S., Saftić Panković, D., Jocić, S., & Radić, V. (2009). Correlation between heterosis and genetic distance based on SSR markers in sunflower (Helianthus annuus L.). *Journal of Agricultural Sciences*, *54*, 1-10.

Knjiga
Steel, R.G.D., & Torrie, J.H. (1980). *Principles and procedures of statistics*. New York: McGraw-Hill Book Company.

Poglavlje u knjizi
Bell, R.L., Quamme, H.A., Layne, R.E.C., & Skirvin, R.M. (1996). Pears. In J. Janick & J.N. Moore (Eds.), *Fruit breeding, Volume I: Tree and tropical fruits.* (pp. 441-514). New York: John Wiley and Sons, Inc.

Zbornik
Behera, T.K., Staub, J.E., Behera, S., Rao, A.R., & Mason, S. (2008). One cycle of phenotypic selection combined with marker assisted selection for improving yield and quality in cucumber. In M. Pitrat (Ed.), *Proceedings of the IXth EUCARPIA meeting on genetics and breeding of Cucurbitaceae* (pp. 115-121). Avignon.

Teza
Singh, N.K. (1985). *The structure and genetic control of endosperm proteins in wheat and rye*. University of Adelaide.

Izveštaj
Ballard, J. (1998). *Some significant apple breeding stations around the world*. Selah, Washington.

Veb sajt
Platnick, N.I. (2010). The world spider catalog, version 10.5. *American Museum of Natural History*. Retrieved February 12, 2016, from http://research.amnh.org/entomology/spiders/catalog/index.html

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Tabele
Tabele obeležene arapskim brojevima (1, 2, itd.) praćene naslovom treba da se nalaze na odgovarajućem mestu u tekstu, u fontu 9. Maksimalna širina tabela treba da bude 13 cm. One treba da budu jasne, što jednostavnije i pregledne. Treba izbegavati vertikalne crte, a broj kolona ograničiti tako da tabela ne bi bila preširoka. Takođe, treba izbegavati nepotrebnu upotrebu horizontalnih crta. Naslov tabele, poravnat po levoj i desnoj margini, sa tačkom na kraju, navodi se sa jednim razmakom iznad tabele. Ispod tabele treba dati detaljno objašnjenje skraćenica, simbola i znakova korišćenih u samoj tabeli. Svaka tabela mora biti pomenuta u tekstu.

Ilustracije
Svi grafikoni, dijagrami i fotografije treba da se nazovu „Slika“ (1, 2, itd.). Prilažu se na odgovarajućem mestu u tekstu. Grafikone i dijagrame treba uraditi fontom 9, u crno-beloj tehnici i sa maksimalnom širinom od 13 cm. Voditi računa da oni budu čitki i jasni i nakon redukcije veličine. Za svaki grafikon i dijagram treba obezbediti detaljnu legendu bez skraćenica. Fotografije moraju biti visokog kvaliteta da bi se tehnički mogle dobro reprodukovati. Prilažu se u „TIF“ ili „JPG“ formatu, u crno-beloj tehnici. Naslov ilustracije, poravnat po levoj i desnoj margini, sa tačkom na kraju, navodi se sa jednim razmakom ispod ilustracije. Svaka ilustracija mora biti pomenuta u tekstu.

Skraćenice i jedinice
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Nomenklatura

Celokupna nomenklatura (hemijska i biohemijska, taksonomska, genetička itd.) mora biti usklađena sa međunarodnim kodeksima i komisijama, kao što su *International Union of Pure and Applied Chemistry, IUPAC-IUB Combined Commission on Biochemical Nomenclature, Enzyme Nomenclature, International Code of Botanical Nomenclature, International Code of Nomenclature of Bacteria* itd.

Formule

Sve formule i jednačine u radu moraju biti urađene pomoću programa „Word Equation". Pri pisanju formula, radi preglednosti, ostaviti dovoljno praznog prostora oko same formule. Subskripti i superskripti treba da budu jasni. Prilikom pisanja jednačina treba dati smisao svih simbola odmah posle jednačine u kojoj se simbol prvi put koristi. Jednačine treba da budu numerisane arapskim brojevima, serijski u zagradama, na desnoj strani linije. Svaka jednačina mora biti pomenuta u tekstu kao Eq. (1), Eq. (2), itd.

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