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Vegetational History, Floral Preference of Honeybees (Apis mellifera var. adansonii) and Biodiversity Conservation Inferred from Recent Honey Pollen Analysis in Hawul Local Government Area of Borno State, Nigeria

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Abstract:
Pollen analytical examination was conducted using honey samples from four selected localities within Hawul Local Government Area of Borno State, Nigeria with the aim of ascertaining the species of plants that were utilized by honeybees in the course of honey production; vegetational history and biogeographical origin of honey and the taxa most preferred by honeybees. The samples were treated using standard palynological techniques and results showed that a total of 27,852 pollen grains count of 4665, 9513, 5669 and 8005 was recorded for Ngwa, Timpil, Peta and Bantali respectively. Study revealed that eighty- eight (88) pollen types belonging to forty-one (41) plant families were encountered. One (1) was identified to family level, seventy-four (74) to generic level, twelve (12) to species level and one (1) unidentified. The predominant pollen types in the four samples were those of Syzygium guineense, Psidium gaujava, Mangifera indica, Parkia biglobosa, Combretum spp., Vitellaria paradoxa, Elaeis guineensis, and Trichillia prieureana. The identified species originated from different taxa of trees, grass and shrubs. Findings revealed that the period of major honey production were between dry season to early raining season (October-April). The pollen assemblage reflects the vegetation of Hawul in Borno State to be Guinean Savanna type despite high level of human impact (anthropogenic activities) on the environment. Pollen weight was between 0.40 to 0.45 grams indicating that the honey samples were unadulterated. Adequate conservation of these indicator species is recommended for safety health and environmental sustainability using appropriate biotechnological interventions.

Keywords: Biodiversity, Floral preference, Honey, Pollen analysis, Vegetation history,

INTRODUCTION

The use of pollen grains and spores in environmental studies is primarily in its application to the study of vegetational history (Traverse, 1988). The relevance of pollen content to the vegetation of a region is related to the palynomorphs produced *in situ* and those supplied from the surrounding ecological zones (Ige & Essien, 2019). Conclusion about climate and human disturbances could be deduced from such analysis and they are termed secondary deductions (Erdtman, 1969). Fact gathered from such analysis could be useful to climatologists and oil explorationists among others (Moore & Webb, 1978). Basically, pollen analysis is a technique for reconstruction of former vegetation by means of the pollen grains recovered from sediments. Since the pollen grain exine is resistant, it may have a long geological life once it is incorporated into sediment, but only if the grains avoid mechanical attrition and chemical changes such as oxidation (Hopping, 1967).

The vegetation of an area is an integral and basic component of the ecosystem and is sensitive to changes in the ecosystem. Consequently, vegetation changes are themselves a response to and a reflection of variation in one or more of the factors of the environment, particularly climate (Essien, 2019). A close relationship exists between vegetation and the rest of the environment, particularly climate and soil. Thus, the flora of an area provides a good reflection of the major climatic regime of the area. The influence of climate on other components of the environment is so great that every other climatic zone has its own characteristic vegetation type (Ige, 2017). Plants are therefore among the best indicators of the environment especially of the climate, soil and fauna. Certain individual or assemblages of plants are known to be characteristic of specific ecological zone and the occurrence of the fossil pollen of such ecological indicator species in sediments is considered a reflection of contemporary ecological conditions. For example, the tropical rainforest is characterized by broad-leaved species, the savanna characterized by grasses, the desert by ephemeral and sclerophyllous plants and the cold regions of the world characterized by evergreen conifers (Essien, 2019).

The study of vegetation and the way in which it has been altered and developed in the course of time indicates past changes that have occurred in our terrestrial environment. Variations in climate and in the intensity of human activities in historic and prehistoric times have made their mark upon the vegetation, and the plants themselves have left a record of these changes in the form of vast quantities of pollen grains which have survived in contemporary sediments (Roberts, 1989). In Quaternary, however, the pollen grains can be directly referred to extant vegetation due to the proximity of Quaternary period with the present, proving "Present is key to the past". Pollen analysis, therefore, is an extremely powerful tool for the investigation of floristic and climatic changes that took place in the recent past (Ige, 2017).

Co-evolution and mutualism have been cited as examples of relationships between honeybees and flowering plants. Honeybees and flowering plants are mutually dependent; honeybees need flowering plants for food in the form of pollen and nectar, whereas plants need honeybees for pollination. Honey contains pollen grains which are collected by honeybees while foraging the flowers for nectar (Essien, 2020). The bee is the most valuable insect on planet earth. This is not because of the value of its direct products as they represent only 0.5% of the total agricultural production, but because of the enormous benefits accruing from the cross pollination of plants. This cross pollination ensures the improved quality and quantity of produce, fruits and seeds, improved species of self-germinating plants and also maintain the eco-balance on earth (Sivaram, 1995).

The honey bees (*Apis mellifera var. adansonii*), the pollinators of plants the world over; play a crucial role for wild and cultivated plants, especially in the tropics where insect pollination is vital (Winfree, 2010; Ollerton *et al*., 2011). Honeybees are one of the world's most essential pollinators capable of sustaining biodiversity and food security. Honey, which is produced by honeybees, is one of the most consumed foods with very high nutritional, pharmaceutical and medicinal value. It is made from nectar and pollen primarily through the action of digestive enzymes by a careful mixture, compression, dehydration and maturation processes in the beehive (Shubharani *et al*., 2013). For quality assurance purpose, the color, pollen diversity and abundance are a determinant of the nutritional richness and originality of honey. The pollen grains also reflect the honeybee preferred plants for pollen and/or nectar, geographical and organoleptic properties (Bogdanov & Martin, 2002; Anidiobu, 2016). As a matter of urgency, plants foraged by honeybees must be conserved for continuity if honey production is to be sustained and one of the ways to determine

these plants is through pollen analytical studies (Kayode & Oyeyemi, 2014; Byrant, 2018; Adekanmbi & Ogundipe, 2009). A combination of the insect and wind pollinated taxa found in a honey gives a unique understanding of the particular geographical location where the honey was produced and the plant communities in that region. This could shed more light on the important plants foraged by honeybees (Essien *et al*., 2022a).

Recently, there are evident cultural, agricultural, unscientific and uncontrolled practices threatening the flora of several part of Hawul Local Government Area, Borno State. The indiscriminate destruction of plants may lead to the loss of important honey plants. Information on pollen analysis in Hawul Local Government Area, Borno State is almost non-existent, limited or somewhat scarce. This expanding destruction of flora could lead to loss of biodiversity and important bee plants. Honeybees (*Apis mellifera* var. *adansonii*) forage on plants for nectar and pollen for the production of honey. Due to the loss of flora and long duration of production, adulteration of honey has also become rampant in Nigeria. Adulteration of honey simply means glucose, dextrose, molasses, corn syrup and invert sugar have been added into an original honey to probably increase quantity or add to the taste. The component (physical and chemical properties) confers the uniqueness of each honey and to be sure of its authenticity, it is vital to perform extensive honey compositional analysis like pollen analysis. Seeking to reveal the relative weight of pollen could be used in differentiating pure from adulterated honey.

This study, therefore, intends to ascertain among other things; the originality of honey produced and sold in Hawul, Borno State as well as the important bee plants that need to be conserved. Knowing the bee plants could be used as the basis of legalized protection and propagation of bee plants and farms. Pollen analytical studies have been found useful in deciphering such plants. The objectives, therefore, are to physico-chemically quantify the honey samples, carry out qualitative and quantitative analysis of pollen grains to determine the vegetational history and biogeographical origin of the honey samples; major season of honey production; floral preference of honeybees (*Apis mellifera* var. *adansonii*), and originality status of the honey samples.

MATERIALS AND METHOD

Study Area

Ngwa, Timpil, Peta and Bantali are localities within Hawul Local Government Area of Borno State. Borno State lies in North Eastern Nigeria. Hawul geographical coordinates- Latitude: 10° 25′ 59″ North, and Longitude: 12° 14′ 49″ East. It has an area of 2,098km² and Altitude 328 m (1,076 ft). Hawul Climate has a Tropical savanna climate and a population of about 120,000 as at the 2006 census. The land of Hawul Local Government Area is covered with volcanic soil and have a rainfall concentration between May to November. The harmattan season between December and January is basically influenced by the North-East Trade winds. It has mean annual temperature of between 25 and 38°C.

Sample Collection

Four honey samples were collected from vendors who sources from the wild at the study area between the months of September and December, 2022. The honeys were extracted by pressing and squeezing the combs, filtered into a bottle through fine mesh-copper gauze to avoid introduction of debris. Once collected the samples were labelled and transported to the Laboratory, Department of Biology, Nigerian Army University Biu, for pollen analysis.

Determination of pH
Honey (10 g) was dissolved in 75 ml of distilled water in a beaker and vigorously mixed using a glass rod, pH electric meter was immersed in the honey and values were taken.

Honey Colour
The Munsell Soil Color Chart was used.

Pollen Analysis
Three basic procedures were followed; honey quantification/dilution, pollen acetolysis and microscopy. All procedures followed the recommendation and techniques reported in Louveaux *et al*. (1978), Agwu *et al*. (2013) and Erdtman (1969). Mounting and microscopic examination was carried out using two drops of pollen suspension in microscope slide sealed off with 18 x 18 mm glass cover slip. Counting was done using Olympus microscope at x400 magnification while detailed pollen morphological studies to aid identification was done using Leica microscope at x 1000 magnification. Reference slides, pollen atlas and photomicrographs (Sowunmi, 1978; 1995; Agwu & Akanbi, 1985; Agwu *et al*., 2013; Shubharani *et al*., 2013; Essien *et al*., 2022b) was used for identification.

Weight of Pollen Grains
Honey (50 ml) and beaker (71.65 g) was weighed using the weighing balance. The honey was diluted with 1000 ml of distilled water and the formular below was applied
Weight of pollen x factor of 20 = weight of beaker/liter of honey samples.

Data Analysis
The data was subjected to descriptive statistics of frequency counts and percentages only. The classification for representation of pollen types followed was the one recommended by Louveaux *et al.* (1978) for expressing pollen grain frequencies: Very frequent (over 45%), frequent (16-45%), rare (3-15%) and sporadic (> 3%).

RESULTS AND DISCUSSIONS

Table 1 showed the physicochemical properties of the four honey samples while Table 2 showed the pollen types recovered from the four honey samples. Absolute pollen counts are shown in Figure 1. Pollen counts of 4665, 9513, 5669, and 8005 were recorded for Ngwa, Timpil, Peta and Bantali respectively. The classification recorded by Louveaux *et al.* (1970) for expressing pollen grains frequencies was adopted: very frequent (over 45%), frequent (16-45%), rare (3-15%) and sporadic (less than 3%). Results showed that Bantali honey sample had forty-five (45) pollen types which was the highest while Peta had the lowest number of pollen types; that is twenty-seven (27), clearly an indication of the high diversity of pollen in Hawul, Borno State. Table 3. showed the floral source of the honey samples and findings revealed that all the honey samples were multifloral in nature and they belonged to category I. There were no very frequent pollen types in any of the samples, and there was only frequent pollen type in the sample from Ngwa. In Table 4; a detailed analysis of the vegetation types inferred from the pollen types and count showed that Hawul is a Guinean savanna type that is anthropogenically disturbed. According to the physicochemical properties of the four honey samples, the color ranged from light brown to dark brown. For the pollen weight; results showed that Timpil honey sample had the highest (0.45 g) while that of Ngwa was lowest (0.40 g). The pH values showed that Bantali honey had the highest pH value of 4.06 while Ngwa was lowest (3.44).

Table 1: Physicochemical properties of the four honey samples

Localities	Colour	Honey Weight collected (g)	Weight of pollen (g)	pH Value	Weight of honey (gram/litre)
Ngwa	Light-brown	10	0.40	3.44	1376
Timpil	Dark-brown	10	0.45	3.47	1301
Peta	Light brown	10	0.41	3.71	1290
Bantali	Dark-yellow	10	0.44	4.06	1355

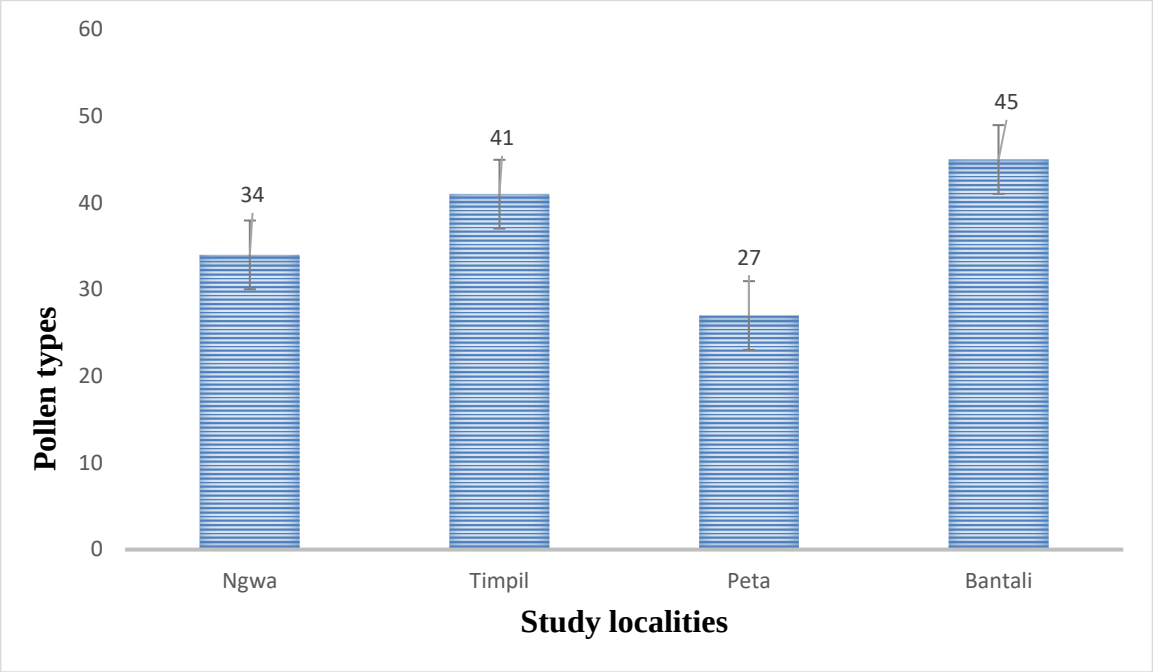

Fig. 1: Bar chart showing the number of identified pollen types in the four honey samples

The percentage of pollen representation based on plant families are shown in Fig 2. Results showed that the plant family Sapotaceae had (7.86 %), Bombacaceae (7.34 %), Solanaceae (4.84 %), Rubiaceae (3.22 %), Myrtaceae (5.56 %), Euphorbiaceae (5.15 %), Combretaceae/ Melastomataceae (4.03 %), Asteraceae (3.60 %), Aracaceae (5.50 %), Anacardiaceae (4.20 %). While the least abundant were Capparidaceae (0.15 %), Cyperaceae (0.19 %), Magnoliaceae (0.27 %), Rosaceae (0.25 %) and Aizoaeceae (0.28 %).

Study revealed that eighty- eight (88) pollen types belonging to forty-one (41) plant families were encountered. One (1) was identified to family level, seventy-four (74) to generic level, twelve (12) to species level and one (1) unidentified (Tabel 2). The predominant pollen types in the four samples were those of *Syzygium guineense, Psidium gaujava, Mangifera indica, Parkia biglobosa, Combretum* spp., *Vitellaria paradoxa, Elaeis guineensis,* and *Trichillia prieureana*

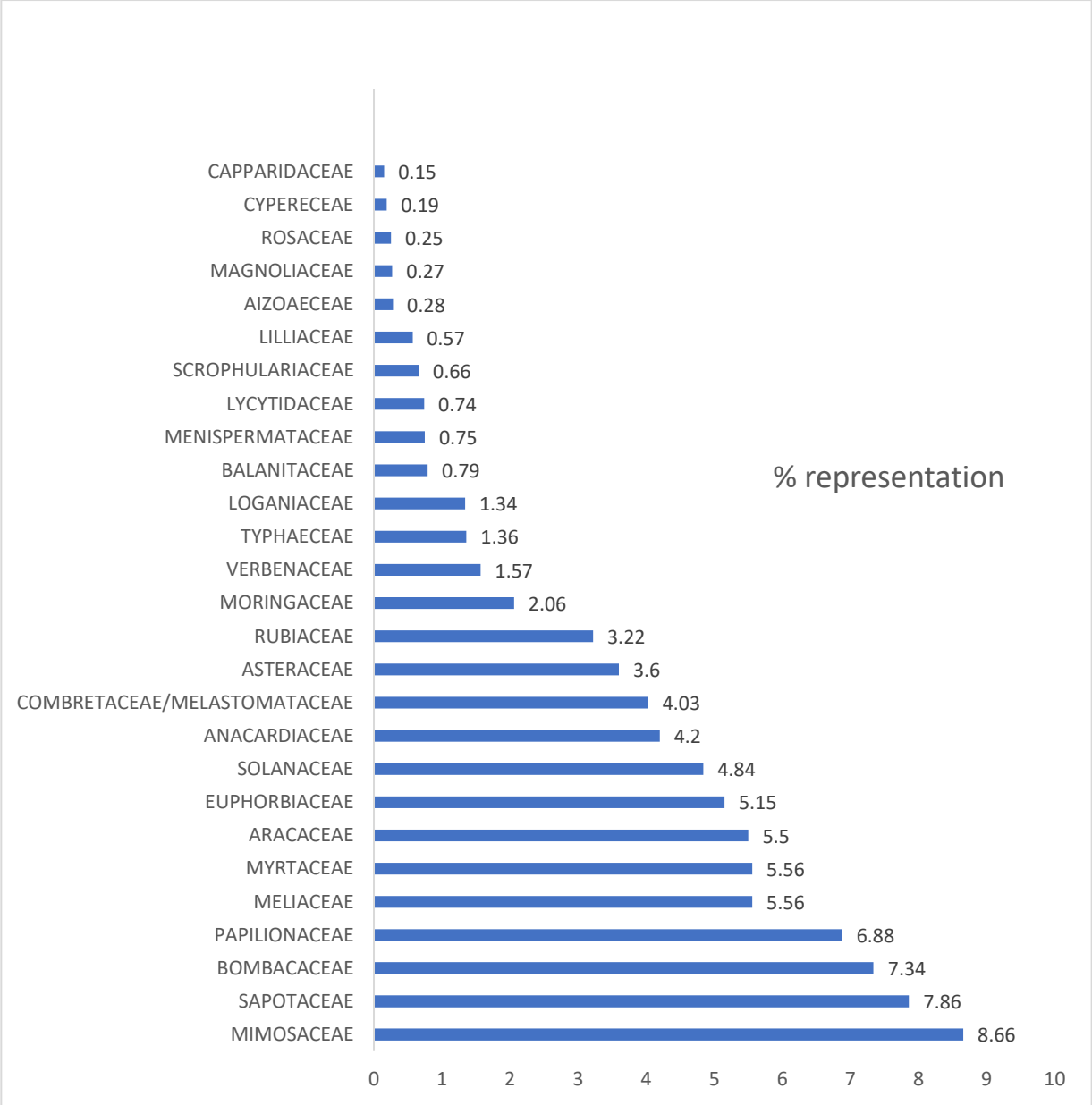

Fig 2: Percentage representation of plant families in the four pollen samples from the study area

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Table 2: Absolute pollen counts from the four honey samples studied

	Pollen types/families	Localities in Hawul									
		Ngwa	%	Timpil	%	Peta	%	Bantali	%	Total	%
1	**ACANTHACEAE**										
	Crossandra nilotica.							200	2.5	200	0.72
	Hypoestes elliottii			431	4.53					431	1.55
	Justicia spp.			209	2.20					209	0.75
2	**AIZOAECEAE**										
	Corbichonia decumbens	106	2.27							106	0.38
3	**ANACARDIACEAE**										
	Curronia volubilis							60	0.75	60	0.22
	Herria reticulata							65	0.81	65	0.23
	Mangifera indica					589	10.39	405	5.06	994	3.75
4	**ARECACEAE**										
	Elaeis guineensis			430	4.52	555	9.79	546	6.82	1531	5.50
5	**ASCLEPIADACEAE**										
	Curroria volubilis					57	1.01	113	1.41	170	0.61
6	**ASTERACEAE**										
	Aspilia africana	305	6.54			127	2.24			423	1.55
	Bidens pilosa	168	3.60							168	0.60
	Synedrella nodiflora	27	0.58	229	2.41					256	0.92
	Tridax procumbens	147	3.15							147	0.53
7	**BALANITACEAE**										
	Balinite orbicularis							221	2.76	221	0.79
8	**BOMBACACEAE**										
	Bombax buonopozense					417	7.36	105	1.31	522	1.87
	Bombax malabarieum					183	3.23			183	0.66
	Bombax spp.			57	0.60					57	0.20
	Ceiba pentandra			450	4.73	508	8.96	325	4.06	1283	4.16
9	**BORAGINACEAE**										
	Cordia africana	200	2.29							200	0.72
	Cordia sinensis	206	4.42							206	0.74
	Cordia vignei					48	0.85			48	0.17
	Heliotropium spp.							80	1.00	80	0.29
10	**CAESALPINACEAE**										
	Acacia rubida			50	0.53					50	0.18
	Brachystegia eurycoma			53	0.56					53	0.19
	Daniella oliveri							182	2.27	182	0.65
	Delonix elata							10	0.12	10	0.04
	Delonix regia			200	2.10					200	0.72
	Paramacrolobium coeruleum			36	0.38					36	0.13
	Senegalia mellifera	4	0.10							4	0.01
	Tephrosia purpurea							32	0.4	32	0.11
	Vauchellia reficiens	12	0.26	22	0.22					34	0.12
11	**CAPPARIDACEAE**										
	Capparis tementosa	29	0.62							29	0.10
	Cleome angustifolia	13	0.28							13	0.05
12	**COMBRETACEAE/ MELASTOMATACEAE**										
	Combretum spp.	309	6.62			474	8.36	340	4.25	1123	4.03
13	**CONVOLVULACEAE**										
	Ipomoea cordifolia							307	3.84	307	1.10
	Ipomoea spp.	156	3.34							156	0.56
14	**CUCURBITACEAE**										
	Coccinia grandis	37	0.79	43	0.45			24	0.3	104	0.37
	Luffa echinata			328	3.45			7	0.09	335	1.20

15	**CYPERACEAE**										
	Cyperus crassipes							54	0.67	54	0.19
16	**EUPHORBIACEAE**										
	Alchornea cordifolia	109	2.35			43	0.79			154	0.55
	Euphorbia grandicornis	189	4.05					361	4.51	550	1.97
	Euphorbia hirta							164	2.05	164	0.59
	Euphorbia hypericifolia							5	0.06	5	0.02
	Euphorbia spp.	58	1.24					205	2.56	263	0.94
17	*Ricinus communis*							302	3.77	302	1.08
	GENTIANACEAE										
18	*Crawfurdia lanceolata*	160	1.68							160	0.57
	LILIACEAE										
19	*Aleo turkenensis*							160	2.00	160	0.57
	LOGANIACEAE										
	Strychnos spinosa			240	2.52			132	1.65	372	1.34
20	**LYCYTIDACEAE**										
	Crateranthus letesturi	207	4.44							207	0.74
21	**MAGNOLIACEAE**										
	Magnolia coco			56	0.59	20	0.35			76	0.27
22	**MELIACEAE**										
	Trichillia prieureana	906	19.42	582	6.12	22	0.49	39	0.49	1549	5.56
23	**MENISPERMACEAE**										
	Cuculus hirsutus							209	2.61	209	0.75
24	**MIMOSOIDEAE**										
	Acacia spp.			224	2.35			130	1.62	354	1.27
	Adenanthera pavonina			255	2.68	97	1.71			352	1.26
	Mimosa pigra					28	0.49			28	0.10
	Mimosa spp.	47	1.01			18	0.32	304	3.80	369	1.32
	Parkia biglobosa			809	8.50	520	9.17			1329	4.77
	Pentaclethra macrophylla	12	0.26							12	0.04
25	*Xylia* spp.					5	0.10			5	0.02
	MORINGACEAE										
26	*Moringa oleifera*			507	5.33			67	0.84	574	2.06
	MYRTACEAE										
	Eucalyptus globus			182	1.91					182	0.65
	Euginea nodiflora	20	0.43	147	1.55	30	0.53			197	0.71
	Psidium guajava	234	5.02	504	5.30			7	0.01	745	2.67
	Syzygium guineense			571	6.00	191	3.37	772	9.64	1534	5.51
27	**PAPILIONACEAE**										
	Bauhinia championionii	102	2.17							102	0.37
	Flemingia strobilifera			270	2.84			9	0.11	279	1.00
	Macrotyloma africanum	15	0.32							15	0.05
	Milletia pinnata							5	0.06	5	0.02
28	**PHYLLANTHACEAE**										
	Phyllanthus spp.			520	5.47					520	1.87
29	**POACEAE**	22	0.47	7	0.07			41	0.51	70	0.25
30	**PROTEACEAE**										
	Protea elliottii			285	3.00					285	1.02
31	**ROSACEAE**										
	Rosa pricei	30	0.64	75	0.79	51	0.90			156	0.56
	Rubus pinnatisepaus			6	0.06					6	0.02
32	**RUBIACEAE**										
	Cephallanthus occidentalis			532	5.60					532	1.91
	Morellia senegalensis					161	2.86	137	1.71	298	1.04
	Sacrocephalus latifolius			52	0.55	6	0.11	18	0.22	76	0.27
33	**RUTACEAE**										
	Citrus spp.	72	1.5	122	1.28	139	2.45			333	1.20
34	**SAPINDACEAE**										
	Cordospermum halicacabum	104	2.23					116	1.45	220	0.79
	Paullinia pinnata	90	1.93	134	1.41					224	0.80

35	**SAPOTACEAE**										
	Mimusops warneckii	137	2.94							137	0.49
	Northia spp.							124	1.55	124	0.45
	Vitellaria paradoxa	584	12.52	421	4.43	430	7.59	492	6.15	1927	6.92
36	**SCROPHULARIACEAE**										
	Stemodia serrata							184	2.30	184	0.66
37	**SOLANACEAE**										
	Solanum melongena			136	1.43	689	12.15	522	6.52	1347	4.84
38	**TYPHACEAE**										
	Typha latifolia	6	0.13	10	0.11	256	4.52	107	1.34	379	1.36
39	**VERBENACEAE**										
	Phylla nodiflora			121	1.27			315	3.94	436	1.57
40	**VITACEAE**										
	Cissus quadrangularis			45	0.47					45	0.16
41	**INDETERMINATA**	2	0.04	2	0.02	3	0.05	2	0.02	9	0.03
	Total pollen count	**4665**	**100**	**9513**	**100**	**5669**	**100**	**8005**	**100**	**27852**	**100**

Table 3: Floral sources of the honey samples from Hawul

Samples	**Pollen type**				**Remark on floral origin**	**Pollen count/ Category**
	Very Frequent (> 45%)	**Frequent (16 – 45%)**	**Rare (3 – 15.9%)**	**Sporadic (< 3%)**		
Ngwa	-	*Trichillia prieureana* (19.42).	*Psidium guajava* (5.02), *Cordia sinensis* (4.42), *Aspilia africana* (6.54), *Tridax procumbens* (3.15), *Cordia sinensis* (4.42), *Ipomoea* spp. (3.34), *Euphorbia grandicornis* (4.05), *Vitellaria paradoxa* (12.52).	*Synedrella nodiflora* (0.58), *Vauchellia reficiens* (0.26), *Capparis tementosa* (0.62), *Alchornea cordifolia* (2.35), *Mimosa* spp. (1.01), *Eugenia nodiflora* (0.43), *Rosa pricei* (1.50), *Typha latifolia* (0.13), Poaceae (0.47). *Vauchellia reficiens* (0.22), *Coccinia grandis* (0.45), *Crawfurdia lanceolate* (1.68), *Eugenia nodiflora* (1.55), *Rosa pricei* (0.79).	Multifloral	4,665/ **l**
Timpil	-	-	*Elaeis guineensis* (4.52), *Psidium guajava* (5.30), *Syzygium guineense* (6.00), *Vitellaria paradoxa* (4.43). *Parkia biglobosa* (8.50), *Phyllantus* spp (5.47), *Ceiba pentandra* (4.75), *Trichillia prieureana* (6.22).	*Rubus pinnatisepalus* (0.06), *Sarcocephalus latifolius* (0.55), *Citrus* spp. (1.28), *Solanum melongena* (1.42), *Typha latifolia* (0.11), Poaceae (0.07).	Multifloral	9, 513 /**l**
Peta	-	-	*Elaeis guineensis* (9.79), *Mangifera indica* (10.39), *Ceiba pentandra* (8.96), *Syzygium guineense* (3.37), *Parkia biglobosa* (9.17), *Vitellaria paradoxa* (7.59), *Solanum melongena* (12.15), *Typha latifolia* (4.52).	*Alchornea cordifolia* (0.79), *Trichillia prieureana* (0.49), *Mimosa* spp. (0.32), *Mimosa pigra* (0.49), *Eugenia nodiflora* (0.53), *Rosa pricei* (0.90), *Sarcocephalus latifolius* (0.11), *Citrus* spp. (2.45).	Multifloral	5,669 / **l**

Bantali	-	-	*Mangifera indica* (5.06), *Elaeis guineensis* (6.82), *Ceiba pentandra* (4.06), *Combretum* spp. (4.25), *Ipomoea cordifolia* (3.84), *Mimosa* spp. (3.80), *Syzygium guineense* (9.64), *Vitellaria paradoxa* (6.15), *Solanum melongena* (6.52), *Phyla nodiflora* (3.94).	*Heliotropium* spp. (1.00), *Delonix elata* (2.27), *Coccinia grandis* (0.30), *Luffa echinata* (0.09), *Euphorbia hirta* (2.05), *Trichillia prieureana* (0.49), *Moringa oleifera* (0.84), *Psidium guajava* (0.01), Poaceae (0.51), *Morellia senegalensis* (1.71), *Sarcocephalus latifolius* (0.22), *Typha latifolia* (1.34).	Multiflor al	8,005/ **I**

***Floral origin:** selected based on most represented (frequently and rare occurring) plant species
Categories: I (<20,000), II (20,000 – 100,000), III (100,000 – 500,000), IV (500,000 – 1,000,000) and V (>1,000,000)

Table 4: Vegetation inference from pollen types recovered from the four honey samples from Hawul

Vegetation type represented from absolute pollen counts						
		Lowland rainforest	**Open forest**	**Savanna**	**Human impacted**	**Suggestive inference on biogeographical origin of honey**
Selected pollen types		*Brachystegia eurycoma, Ceiba petandra, Vitellaria paradoxa, Pentaclethra macrophylla*	*Acacia* spp., *Combretum* spp., *Elaeis guineensis, Alchornea cordifolia*	*Bauhinia championionii, Daniella oliveri Phyllanthus* spp., *Parkia biglobosa, Trichillia priuereana,* Poaceae, *Morellia senegalensis, Adenanthera pavonina, Parkia biglobosa, Syzygium guineense.*	*Solanum melongena, Mangifera indica*, *Protea elliottii, Justicia* spp., *Delonix Regia, Euphorbia* spp., *Euphorbia hirta, Moringa oleifera,*	
Total pollen count		**2645**	**7,305**	**9,500**	**9,102**	
Localities	Ngwa (%)	18. 56	21.46	37.38	22.50	- Guinea savanna
	Timpil (%)	30.12	20.50	37.15	12.23	- Guinea savanna
	Peta (%)	20.77	13.52	28.98	36.73	- Human impacted
	Bantali (%)	26.11	28.60	32.72	12.57	- Guinean savanna
Total pollen indicator of the vegetation (%) of Hawul		**10.57**	**26.22**	**34.11**	**29.10**	**Hawul in Borno State is largely Guinean Savanna**

Total pollen count = 27,852

Vegetation History and Biogeographical Origin of Honey

The determination of a biogeographical origin of honey is based on the entire spectrum being consistent within the flora of that particular region (Louveaux *et al.*, 1978). The abundance of *Ceiba pentandra*, *Combretum* spp., *Elaeis guineensis*, *Parkia biglobosa*, *Solanum melongena*, *Syzygium guineense*, *Trichilia prieureana,* and *Vitellaria paradoxa* indicates farmland and homesteads and it also reflects the vegetation of lowland rainforest and guinea savanna. The occurrence of the pollen of the above listed plants in the pollen spectrum of the studied samples confirms their biogeographical origin reflecting guinean savanna ecovegetation type that is anthropogenically disturbed. Findings agree favourably with the report of Agwu & Okeke (1997); Essien *et al*. (2022c) as well as Essien & Olaniyi (2023).

According to pollen analysis of these honey samples, derived and guinea savanna taxa were the highest pollen contributor (34.11%) followed by human impact taxa (29.10%), open forest taxa (26.22% and lowland rainforest taxa (10.57%). The suggestive vegetational inference inferred from this honey pollen analysis revealed that Hawul in Borno State is Guinean savanna vegetation type that is human impacted (anthropogenically disturbed). The pollen analysis shows a fairly similar floral composition for the entire honey samples studied which is in line with the work of Sowunmi (1976) in Southeastern Nigeria and the high floral diversity of the forested-savanna ecozone by Agwu *et al*. (2013) in Northcentral Nigeria. The percentage of human impact indicator species could be attributed to anthropogenic activities in this region such as the activities of herdsmen (livestock grazing, annual bush burning, etc.), deforestation, urbanization, and agricultural activities in line with Essien *et al*. (2022a) reports. From Table 4, there were clear indications that the region of Hawul is largely guinean savanna which is been impacted by humans although with little variation with respect to the different study localities. The vegetation type seems to be same as that of Northcentral Nigeria (Aina *et al*., 2014; Agwu *et al*., 2013; Essien *et al*., 2022). This further validates the fact that Southern Borno State is likely a mosaic of lowland rainforest/Guinea savanna. It is reported that most Nigerian honey comes from the savanna regions or mosaic of lowland rainforest and secondary grassland (Sowunmi, 1976).

Season of Honey Production

Most plants flower during the dry seasons, allowing honeybees graze during those times. To produce honey in the study area efficiently, this study examined the numerous pollen types and their distinct flowering seasons. According to Dalziel (1937) and Keay (1959) studies, flowering seasons differ for different plants. For example, *Mangifera indica* (February-May), *Morellia senegalensis* (November to January; March to April), *Mimusops warneckei* (April to June), *Alchornea cordifolia* (October to November; June - August), *Bombax buonopozense* (January to March), *Brachystegia eurycoma* (April to May), *Daniella oliveri* (November to January; March to April), *Delonix regia* (April to August), *Elaeis guineensis* (October-April), *Parkia biglobosa* (December to April), *Paullinia pinnata* December to January), *Trichilia prieureana* (January to March), *Tridax procumbens* (June to September), *Vitellaria paradoxa* (April to June). According to Sowunmi (1976) and Agwu & Akanbi (1985), *Parkia biglobosa*, and *Phyllanthus* spp. all have flowering periods between January and October. These flowering seasons can be used by beekeepers to maximize the production of honey in the study area.

Floral Preference of Honeybees (*Apis mellifera* var. *adansonii*)

Pollen analysis of honey samples examined indicates the presence of pollen types of different plants species, most likely a reflection of more species diversity characteristics of Human impacted Guinean Savanna vegetation type. The determination of the floral origin of honey is

based on the relative frequencies of pollen types of various nectar producing plants species in the honey samples. Generally, entomophilous plants were observed to be more abundant in the pollen spectrum of each honey sample studied and the honey from the source localities were rich in pollen types.

In terms of floral sources, this study revealed that all the honey samples were multifloral (Table 3); suggesting that honeybees (*Apis mellifera* var. *adansonii*) collected honey by gathering a variety of pollen and nectar that they found to be most appealing. According to Agwu *et al*. (2013), Kayode & Oyeyemi (2014), Adeonipekun *et al*. (2016), Adekanmbi & Ogundipe (2009), and Essien *et al*. (2022c), the majority of Nigerian honeys fall into the type I description of Parades and Bryant (2019). The pollen types from the least abundant families may not have been fully domesticated, or their pollen does not rank among the top choices for honeybees.

The study showed that all honey samples were multifloral (Table 3), implying that honeybees (*Apis mellifera* var. *adansonii*) foraged for several preferred pollen and nectar sources to produce the honey. Agwu and Njokuocha (2004) reported that the differences which were observed in the number of contributing plant species in the honey samples may be attributed to the variation in edaphic factors, microclimate, lack of uniformity in the establishments of plants (including flowering period) and selective behaviors of bees during their foraging activities.

Originality of Honey

Deciphering the botanical or ecological origin and the authenticity of honey samples from Hawul, Borno State, Nigeria was the focus of this study. Complimentarily, knowing the best times for apiculture by understanding the flowering seasons of the plant was another objective. Having seen evident impact of humans in the study location, pollen analytical study shed more light on the important bee plants that may require preservation for continuous supply of quality honey in Borno State. The study found that all honey samples were acidic in nature (Table 1) and pollen weight revealed that the honey sample were not adulterated. Cases of honey adulteration have been reported in many cities in Nigeria. For example, Agwu *et al*. (2013) from Dekina; Aina *et al*. (2014) from Kogi East; Anidiobu (2016) from Kabba; Essien *et al*. (2022a) from Ijumu has been reported to be good. This study confirms those from Hawul, Borno State; that were randomly sampled are also of good quality. Honey quality can be measured by its pollen diversity and count (Ige & Modupe, 2010; Oyeyemi, 2017; Essien *et al*., 2022c). The high diversity of pollen types (Figure 1) further supports the originality of the honey samples (Bogdanov & Martin, 2002).

CONCLUSION

Pollen analysis is still an indispensable method for the determination of vegetational history and biogeographical origin of honey; major season of honey production; floral preference of honey bees, and purity status of honey based on its floral and geographical origin. It can to some extent, reflect the floristic characteristics of the area the honey was collected from. This study has revealed some important indicator species of vegetation types in Hawul as well as honey bees (*Apis mellifera* var. *adansonii*) preferred pollen and nectar sources. These plants include those of *Syzygium guineense, Psidium gaujava, Mangifera indica, Parkia biglobosa, Combretum* spp., *Vitellaria paradoxa, Elaeis guineensis,* and *Trichillia prieureana* worthy of conservation and their sustainable exploitation managed in the apiculture to enhance large scale production of honey in Hawul Local Government Area of Borno State, Nigeria. The study further revealed that the region of Hawul in Borno State is largely Guinean Savanna and is currently being impacted by human activities of subsistence.

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A Review on Principles of FISH and GISH and its Role in Cytogenetic Study

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Abstract:
This review article was reviewed at Bahir Dar University College of Agriculture and Environmental science in 2018. A genome can be considered as all the DNA sequences of an organism together. However, we also can see it as all its chromosomes and all its genes. Conventional cytogenetic technique always studies at a particular stage of cell division. Classical cytological studies using chromatin staining still contribute much to the present appreciation of chromosomal diversity in wild species. This approach allows the detection of gross karyotypic alterations such as changes in chromosome number and morphology and gives an overview of chromosome behavior in mitosis and meiosis. However, many recent cytogenetic studies have used FISH to map single cloned or Polymorphic Chain Reaction-amplified sequences to chromosomes or GISH with total genomic DNA probes to identify the parental origin of chromatin in hybrids and allopolyploids. Fluorescence In situ hybridization and Genome In situ hybridization's role in improving our understanding of interspecific hybridization, and polyploidy, genetic mapping, and plant breeding. These methods also have a role in clarifying patterns of chromatin folding, interphase nuclear organization, and chromatin distribution in different cells of the cell cycle and development. Thus, this review was reviewed to create an understanding of the principles of GISH and FISH and their applications in Cytogenetic study.

Keywords: Cytogenetic, DNA, FISH, GISH, Hybridization

INTRODUCTION

The age of classical cytogenetic has, however, been largely superseded by the implementation of DNA techniques during the past few decades. In situ hybridization (FISH) is now recognized as an important technique in many areas of molecular biological research. The technique is used to locate the physical position of a known DNA sequence on a chromosome (Devi et al., 2005). Cytogenetic techniques have become necessary components of studies of the organization of the genome and its association with chromatin.

Cytogenetic research and chromosome analysis are the main aspects of genomics and genetic sciences. Molecular cytogenetic techniques, such as in situ hybridization methods, are admirable tools to analyze the genomic structure and function, chromosome constituents, recombination patterns, alien gene introgression, genome evolution, aneuploidy, and polyploidy (Andres et al., 2013 Younis et al., 2015).

After in situ hybridization technique development by John et al (John et al., 1969) and Gall and Pardue (John et al., 1969), various approaches were achieved such as radioactively labeled probes improved into nonradioactive probes labeled with biotin (Rayburn and Gill, 1985) and detection by indirect (antibody-fluoro chrome conjugate) and direct (fluoro chrome detection) staining.

Genomic in situ hybridization (GISH) was first used to discriminate the genomes of the intergeneric hybrid between parental genomes, that is, Hordeum chilense and Scale africanum (Schwarzacher et al., 1989). Genomic in situ hybridization (GISH) is an efficacious technique that used for genome differentiation of one parent from the other by utilizing special chromosome labeling techniques. GISH has a tip role in cytogenetics for the investigation of the evolutionary relationship of crops and identification of an inserted region in the parent from the alien species. GISH technique follows the same protocol as the fluorescent in situ hybridization (FISH) technique. However, genomic and blocking DNA utilization in GISH differentiate it from FISH analysis. In plant species, genome organization and homology study are carried out by the use of genomic in situ hybridization (GISH). In addition, karyotype analysis of many plant species has been performed by the GISH technique (Ksiazczyk et al., 2010).

Objective

To Review the Principles of GISH and FISH and its Application for Cytogenetics Study

LITERATURE REVIEW

Applications of FISH and GISH

Classical cytological studies using chromatin staining still contribute much to the present appreciation of chromosomal diversity in wild species. This approach allows the detection of gross karyotypic alterations such as changes in chromosome number and morphology and gives an overview of chromosome behavior in mitosis and meiosis. However, many recent cytogenetic studies have used FISH to map single cloned or PCR-amplified sequences to chromosomes or GISH with total genomic DNA probes to identify the parental origin of chromatin in hybrids and allopolyploids. FISH and GISH together have shed much light on many biological phenomena. These methods also have a role in clarifying patterns of chromatin folding, interphase nuclear organization, and chromatin distribution in different cells of the cell cycle and development (Leitch, 2000).

Some very useful studies have been conducted utilizing these techniques in both animals and plants. Initially, studies made involved repeated DNA including sat-DNA from Drosophila and mouse. The application of in situ hybridization techniques in plants has lagged compared to its use in mammalian cytogenetics.

Chromosome Mapping:

The utilization of in situ hybridization technology is of particular interest to those engaged in chromosome walking or genome mapping projects. FISH has been utilized in many plants to identify chromosomes accurately, using species-specific repetitive sequences, ribosomal genes, and even unique sequences. Because of their universal occurrence and redundancy, ribosomal genes are of great value for karyotype analysis and comparative studies of genome organizations. FISH techniques using fluorochrome allow the visualization of multigenic families, such as 5s and 18s-5.8-26s ribosomal RNA genes for their location chromosomes Physical localization of multicopy gene families, such as 5S and 18S-26 rRNA genes have been reported in wheat2, tomato3, barley4, garlic5 and in Aegilops umbellulata6. In cotton, multi-copy genes were mapped on specific chromosomes in meiosis7. Recently, FISH has been used for the physical mapping of ribosomal genes, microsatellites, and transposable DNA sequences on the sugar beet chromosome (Schmidt et al., 1994; Schmidt et al., 1996).

Genome Analysis:

GISH permits the characterization of the genome and chromosome of hybrid plants, allopolyploid species, and recombinant breeding lines. Thus, the ancestry of hybrid and polyploid species can be elucidated by genomic southern and in situ hybridization. In essence, the analysis involves the hybridization of labeled genomic DNA from suggested ancestors or relatives to chromosome spreads or Southern blots of DNA from the species under investigation. Hybridization strength, uniformity, and presence of positive or negative bands are then assessed to indicate relationships. Traditionally, genome relationship was analyzed by the study of chromosome painting but there may be several limitations of chromosome pairing. The amount of pairing not only depends on the degree of homology between the pairing chromosomes but also on genetic and environmental factors. A multicolor FISH (MFish) using total genomic DNA probe is a promising approach for simultaneously discriminating each genome in natural or artificial amphidiploids. It uses various fluorescence dyes to represent different painting probes at the same time. Moreover, this technique is a powerful tool for investigating genome homology between polyploid species and their diploid progenitors. Using fluorescent probes produced by shearing the total genomic DNA of a particular progenitor species, it may be possible to identify all chromosomes belonging to a particular genome of the amphidiploids. Multicolor in situ hybridization has been used to distinguish three genomes in hexaploid wheat20. Biotinylated total genomic DNA of the diploid A-genome progenitor Triticum Urartu, digoxigenin- a belled total genomic progenitor Aegilops squares, and non-labeled total genomic DNA of one of the possible B genome progenitors Ae. speltoides were hybridized in situ to metaphase chromosome spreads of Triticum aestivum cv. Chinese Spring. For detection, only two fluorochromes, fluorescein, and rhodamine, were used. The A, B, and D genomes were simultaneously detected by their yellow, blue, and red fluorescence, respectively. (Bennett et., 1999) by using genomic in situ hybridization, demonstrated the allopolyploids origin of Milium montianum (2n=22) and the homology between eight large chromosomes of this species and M. vernal (2n=8).

Transgene Detection:

Chromosomal position and local chromatin structure are thought to have a profound effect on the level of gene expression. Variable transgene expression and silencing occur frequently in transgenic plants. In the past few years, there has been much interest in the correlation between transgene expression and cytogenetic position (Kohli et al., 2003). In plants that have been genetically engineered by particle bombardment or Agrobacterium-mediated transformation, the integration mechanism does not appear to be sequence-dependent and presumably occurs via illegitimate recombination at double-strand breaks in the genomic DNA (Svitashev et al., 2002).

Analysis of Soma Clonal Variation:

Soma-clonal variations arising in tissue culture have been looked upon as a novel source of genetic variation for crop improvement. Tissue culture phases may impose stress, and induce instability (chromosome breakage and DNA transposition) leading to karyotyping changes. Genetic instability may be associated with the fraction of repeated sequences of DNA present in the plant genome (Bebeli et al., 1990). Analysis of genetic variation in regenerated plants is necessary for the identification and utilization of the proper soma clonal variation for crop improvement. Examination of the chromosomal distribution of 5S and 18S-26S rRNA is useful in identifying the types of genomic changes that might occur during in vitro culture (Maluszynska et al., 1993).

Karyotyping Using FISH:

Rather than distinguishing chromosomes from different genomes in hybrids or allopolyploids, karyotyping techniques are used to differentiate the non-homologous chromosomes within a genome. Such procedures are important for detecting chromosomal aberrations, defining which chromosomes are involved in cases of aneuploidy, for studies of chromosomal behavior, and the genomic localization of repetitive DNA sequence arrays, individual loci, or transgene insertion sites. The most commonly used probes for fluorescence in situ hybridization (FISH) karyotyping in plant species are the 5S and 25S rRNA genes, tandemly repeated sequences near telomeres, and centromere-specific repeats. A limited number of tandemly repeated sequences, which provide a large target for hybridization by a single probe, make chromosome identification possible by FISH procedures in Arabidopsis thaliana (Koorneef et al., 2003). Pinus species (Hizume et al., 2002) and Norway spruce (Vischi et al., 2003); however, in the latter two cases there are some ambiguities in the ability to distinguish different members of the karyotype. By combining these FISH landmarks with other chromosomal characters such as arm ratio and heterochromatic regions, precise karyotyping has been reported for the cytologically well-characterized inbred line of maize (Sadder et al.,2001; Chen et al., 200) and selected lily species (Lim et al., 2000).

Phylogenetic Applications of GISH:

A new area of GISH experiments is its utilization as a tool in phylogenetic studies, a new application of GISH that we propose to term GISH-phylogenetic. Molecular systematics based on DNA sequences is sometimes limited by the lack of sequence data. Moreover, different algorithms have been used to construct phylogenetic trees that provide for different assumptions and treat data differently. As a result, various phylogenetic approaches applied to the same dataset may generate different phylogenetic trees (reviewed in Whelan et al., 2001). In this case, an independent control system could help to decide, which tree represents the true species relationships. Lim et al. (2007) applied a combination of GISH and FISH with tandem repeat probes to study the evolution of Nicotiana polyploid species. Allopolyploid divergence in Nicotiana is associated with several phenomena, e.g., parental genome DNA losses, retro element activity, and intergenomic homogenization (Leitch et al., 2008). A consequence of these homogenization processes is that GISH works on natural Nicotiana allopolyploids only if allopolyploidy occurred less than 5 million years ago. In natural polyploid species, of this age and older, distinguishing parental genomes using GISH was not possible. The authors argued that GISH failure represented a near-complete genome turnover associated with long-term genome diploidization. By comparing GISH patterns in allopolyploids of different ages, including synthetic allopolyploids that mimic natural polyploid species, Lim et al. (2007) attempted to reconstruct the evolutionary time scales for "genome turn-over".

Genomic Constitution:

Horticultural crop genomic compositions and parental chromosomes can be investigated by utilizing GISH methods such as in strawberries (Nathewet and Yanagi, 2014; Choi et al., 2007). GISH analysis can significantly distinguish the different genomes, foremost the evaluation based on chromosome size in each genome. Discrimination of the different genomes proved the incidence of rearrangements after interspecific hybridization (Reis et al., 2014). Karyotype and genomic study of sour cherry proved that GISH could differentiate chromosomes between parental species chromosomes. Based on size and centromere position, karyotype results of Prunus a valium and Prunus cerasus clearly distinguish chromosomes from one another. The utilization of genomic DNA as a probe helps in hybridization with species-specific repetitive sequences which are dispersed over the genome. Hybridization distribution in 32 chromosomes

of sour cherry showed that 16 chromosomes came from P. avium, while the rest of the 16 chromosomes from P. fruticose. These findings ensured that P. cerasus genome constituents are composed of P. fruticose and P. avium (Schuster and Schreiber, 2000).

Karyotyping by GISH:

Karyotypes of many plant species are characterized by chromosomes of rather a uniform size, shape, and banding patterns (Schubert et al., 2001). Considering classical banding methods only N banding (in only a limited number of species) and C banding are feasible in plants. Besides FISH, the whole gDNA can be used in some cases to karyotype chromosomes. Genome-specific repeats have frequently a non-random distribution, forming clusters within heterochromatin blocks. As the hybridization signals often coincide with C-bands, this approach is referred to as GISH-banding. Based on GISH-banding, a universal reference karyotype for rye has been created (Zhou et al., 2008). The GISH banding pattern coincided not only with Giemsa C-banding but also with DAPI patterns (DAPI C-banding (Markova et al., 2006), whereby the fluorescence was brighter at the GISH bands, suggesting the presence of AT-rich DNA sequences at these loci. In some species with relatively small genomes, GISH probes label preferentially (peri) centromeric regions, leaving chromosome arms mostly unlabelled (Fig. 2a). A critical 1C genome size of 0.6 pg has been proposed, below which GISH labeling of entire chromosomes usually fails (Raina and Rani, 2001). In some species, GISH labeling seems to be almost exclusively based on centromeric satellite repeats (centromeric GISH, English). On the other hand, the hybridization pattern of some repetitive sequences can mimic GISH. One particular repetitive sequence could be dispersed along the whole genome of only one parent; hence it can recognize parental genomes in hybrid (e.g., Cermak et al., 2008). For example, only pericentromeric regions were revealed in hybridogenous Boechera genotypes (Brassicaceae) upon GISH using gDNA of B. holboellii and B. stricta. This pattern has been caused by genome-specific centromeric repeats (Kantama et al., 2007). GISH-based labeling of entire chromosomes could be potentially achieved by the use of exceptionally high concentrations of hybridizing gDNA and longer hybridization times, as shown for the allopolyploid species Arabidopsis suecica (Ali et al., 2004), having a comparable genome size as Boechera species.

Difference Between FISH and GISH

Fluorescence In-situ Hybridization:

A laboratory technique for detecting and locating a DNA sequence or a gene on a chromosome within the genome. It is the process of painting/staining/ the whole or part of a chromosome with fluorescence molecule to identify chromosomal abnormality and the presence of a region of DNA or RNA within the chromosome. Chromosomes can be seen if they are stained and magnified under the microscope. The technique relies on exposing chromosomes to a small DNA sequence (probe) that has a fluorescent molecule attached to it. So that scientists can visualize the location of a particular gene to check for a variety of chromosomal abnormalities. (Source: https://www.majordifferences.com/2015/10/difference-between-fish-andgish.html#. wx04btqvziv).

On the other hand, a molecular cryptogenic technique uses fluorescent probes that bind to only those parts of the chromosome with a high degree of sequence complementarity. It was developed by biomedical researchers in the early 1980s (Langer et al., 1982) and is used to detect and localize the presence or absence of specific DNA sequences on a chromosome. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. FISH is often used for finding specific features in DNA for use in genetic counseling, medicine, and

species identification. FISH can also be used to detect and localize specific RNA targets (mRNA and miRNA) in cells, circulating tumor cells, and tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues (Amann et al., 2008).

Basic Features of FISH:

The detection of chromosomal DNA in cytological preparations was initially based on the application of isotopic labeled RNAs and DNAs. A breakthrough came with the introduction of fluorescent labels, (in) directly linked to DNA probes and visualized under the fluorescence microscope. The technology of FISH appeared superior to previous in situ technologies in providing better spatial resolution along with the potential of simultaneously using different fluorescence systems for multi-probe analysis. The number of targets to be analyzed simultaneously depends on the number of fluorochromes with different excitation and emission wavelengths (Nederlof et al. 1990). In a large-scale study involving all major classes of repeats, patterns that are representative and unique for each chromosome can be produced. In a comprehensive study of the sugar beet (Beta vulgaris) genome, the positions of major classes of repeats and gene-rich regions were assessed concerning the heterochromatic pericentromeric regions and gene-rich distal euchromatin regions of the chromosomes (Schmidt et al., 1998). Thus, in summary, FISH adds a powerful new tool to the existing battery of cytological techniques, and will play an important role in future efforts to determine genome structure.

Genomic In-situ Hybridization:

The first procedure to use fluorescent labels to distinguish chromosomes in the plant kingdom involved the process of genomic in situ hybridization (GISH) (Schwarzacher et al., 1989). This widely applied cytogenetic technique provides a direct visual method for distinguishing parental genomes and analyzing genome organization in interspecific hybrids, allopolyploid species, and interspecific introgression lines (Jiang and Gill, 1994.) In GISH, labeled total genomic DNA is used as a probe with unlabeled genomic DNA from another species as a blocking agent. Because the chromosomal sequences that are common to the two species contributing to the analyzed specimen are hybridized with unlabeled DNA, the labeled probe, especially the portion containing species-specific dispersed repetitive sequences (such as transposable elements), hybridizes to only one of the two sets of chromosomes.

Procedures of FISH and GISH

FISH Procedure:

1. Cells cultured, harvested, prepared on microscopic slides and are denatured (now DNA is single stranded for probe attachment)
Cells on metaphase stage of division is selected (as maximum condensation on metaphase stage)
2. Fluorescently labeled hybridization probe is added
(The hybridization probe is a short fragment of DNA that has a fluorescent dye attached that enable scientist to visualize the site of probe attachment. A typical FISH probe would be 10 - 100 kb long)
3. If the DNA corresponding to the probe is present in the sample, then the fluorescently labeled probe will attach to the DNA and will be visible under a fluorescent microscope.
4. This allows deletions (no fluorescent spot at the expected position) and rearrangements (spot present, but in an unexpected chromosomal location) to be detected. Thus helps in diagnosis of genetic diseases.

Source: https://www.majordifferences.com/2015/10/difference-between-fish-and gish.html#. WxO4BTQvzIV.

DNA and RNA Probes:
RNA probes can be designed for any gene or any sequence within a gene for the visualization of mRNA (Louzada et al., 2012; Lee et al., 2011) lncRNA and miRNA in tissues and cells. FISH is used by examining the cellular reproduction cycle, specifically the interphase of the nuclei for any chromosomal abnormalities (Bernasconi et al., 2008). FISH allows the analysis of a large series of archival cases much easier to identify the pinpointed chromosome by creating a probe with an artificial chromosomal foundation that will attract similar chromosomes. The hybridization signals for each probe when a nucleic abnormality is detected. Each probe for the detection of mRNA and lncRNA is composed of 20 oligonucleotide pairs, each pair covering a space of 40–50 bp. For miRNA detection, the probes use proprietary chemistry for specific detection of miRNA and cover the entire miRNA sequence (Bernasconi et al., 2008).

Probes are often derived from fragments of DNA that were isolated, purified, and amplified for use in the Human Genome Project. The size of the human genome is so large, compared to the length that could be sequenced directly, that it was necessary to divide the genome into fragments. (In the eventual analysis, these fragments were put into order by digesting a copy of each fragment into still smaller fragments using sequence-specific endonucleases, measuring the size of each small fragment using size-exclusion chromatography, and using that information to determine where the large fragments overlapped one another.) To preserve the fragments with their DNA sequences, the fragments were added into a system of continually replicating bacteria populations. Clonal populations of bacteria, each population maintaining a single artificial chromosome, are stored in various laboratories around the world. The artificial chromosomes can be grown, extracted, and labeled, in any lab containing a library. Genomic libraries are often named after the institution in which they were developed. An example is an RPCI-11 library, which is named after the Roswell Park Cancer Institute in Buffalo New York. These fragments are about 100 thousand base pairs and are the basis for most FISH probes.

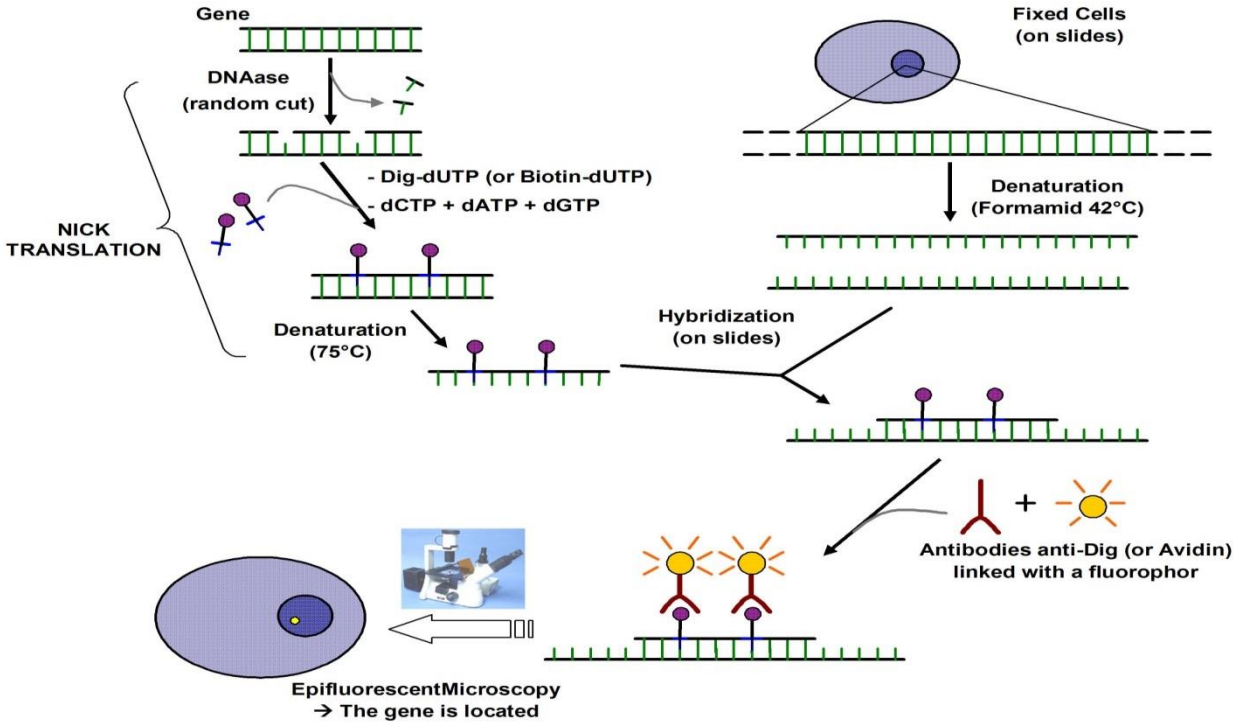

Figure 1: Scheme of the principle of the FISH Experiment to localize a gene in the nucleus.
(Source: https://en.wikipedia.org/wiki/Fluorescence_in_situ_hybridization#Probes_%E2%80%93_RNA_and_DNA).

GISH Procedure:

Extracting a total of genomic DNA from one of the species (to be used as a probe). Chromosome preparation of species two being studied repeated sequences in both species anneal quickly than the unique sequences of the genome. This helps in assessing genome relationship between species

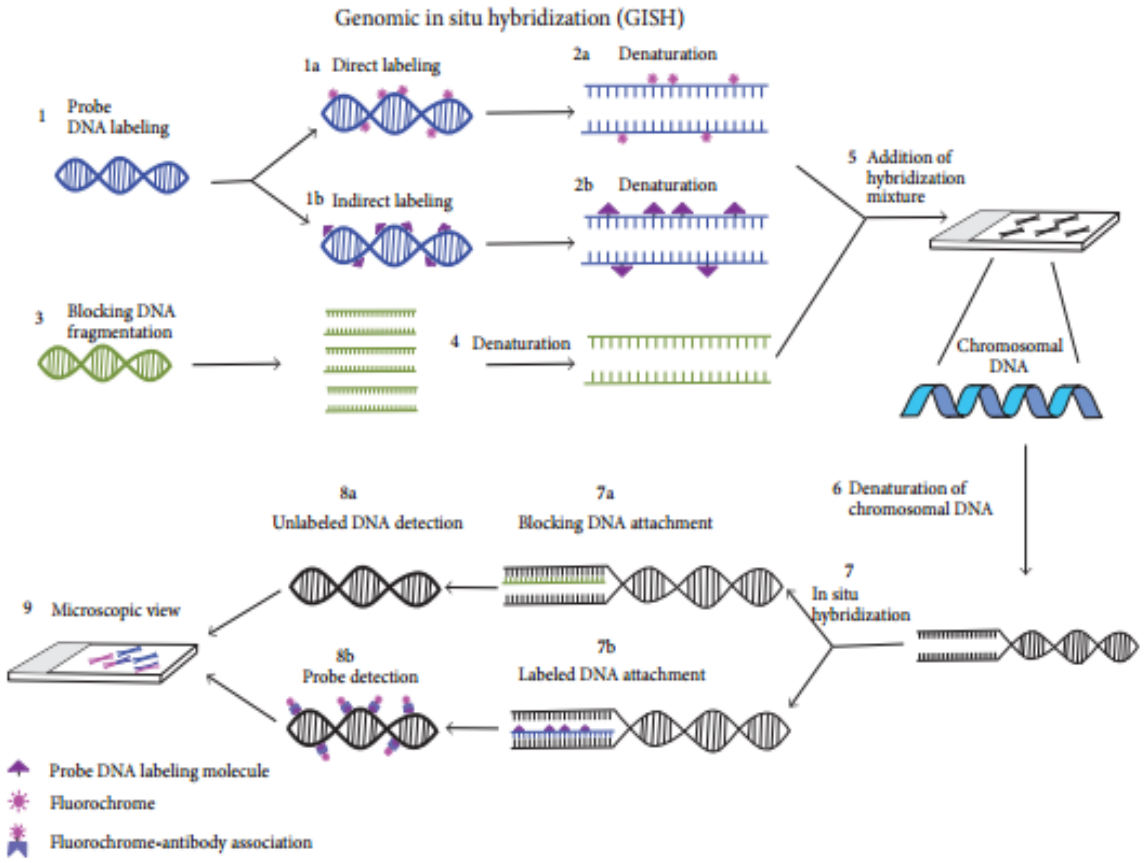

Figure 2: Genomic in situ hybridization (GISH) mechanism diagram (Pendinen *et al.*, 2012).
Source: https://www.majordifferences.com/2015/10/difference-between-fish-and-gish.html#.WxO4BTQvzIV.

Advantages and Disadvantages of FISH

FISH does not require the cultivation of the organisms or any technology-based gene amplification which can lead to false negatives and positives. In contrast to some other EMDs, FISH allows visualization of whole cells that are important to environmental remediation activities. FISH can thus provide complementary information to other EMDs, such as the morphology of the cells or the association of groups of microorganisms with relationship to one another. FISH can target several different genes simultaneously, for example, genes associated with specific degrading species of interest (e.g., Dehalococcoides) and broader microbial groups, such as methane-producing organisms. Depending on the species, and in combination with other appropriately validated activity-targeted approaches, FISH can provide general information about the activity of the organisms or populations of interest. FISH enables single-cell microbial studies and allows for subsequent studies, such as gene sequencing.
Source: https://www.itrcweb.org/documents/team_emd/FISH_Fact_Sheet.pdf.

The detection limit of FISH is high (~106 cells/mL). However, in some cases, high detection limits can be corrected by sample concentration or cell extraction methods which lower the detection limits to a few hundred cells per concentrated sample. Validated probes and FISH procedures are

not available for a wide range of organisms within the bioremediation field. Additionally, standard protocols for sample collection and storage before FISH analysis have not yet been developed. FISH can also be used to target not only ribosomal genes (which indicate the type of organism) but also functional genes (via mRNA) relevant to bioremediation. These other genes indicate what the microorganisms can do with regards to contaminant biodegradation, for example, naphthalene dioxygenase or reductive dehalogenase. However, laboratory protocols are often time-consuming and complicated and not yet validated for field applications.

The FISH method is not widely commercially available. Currently, mainly specialized research laboratories are performing these analyses to explore and optimize the potential of FISH for validated and cost-effective applied studies. The FISH method is currently expensive because of the expertise and labor needed for the development of validated FISH protocols and direct microscopic counting. Once validated protocols have been developed, FISH can be automated to some extent by using flow cytometers to count target cells more efficiently, reducing the analytical costs. However, when using flow cytometers for cell counting, all information regarding spatial relationships (among and between the cells) is lost.

Source: https://www.itrcweb.org/documents/team_emd/FISH_Fact_Sheet.pdf.

SUMMARY

The recent developments in cytogenetic described above will provide new tools for the analysis of plant genomes. These techniques allow the study of the fine details of chromosome structure and will permit sophisticated analyses of chromosomal behavior. As more genomes become sequenced, tools to study chromosomal organization and behavior will play a greater role in investigating the function of those genomes. Chromatin staining for classical cytological studies still contributes much to the present appreciation of chromosomal diversity in wild species. This approach allows the detection of gross karyotype alterations such as changes in chromosome number and morphology and gives an overview of chromosome behavior in mitosis and meiosis.

The FISH and GISH techniques present an authentic model for analyzing the individual chromosome, chromosomal segments, or the genomes of natural and artificial hybrid plants. These have become the most reliable techniques for studying allopolyploids because most cultivated plants have been developed through the hybridization of polyploidization. FISH is the technique that relies on exposing chromosomes to a small DNA sequence (probe) that has a fluorescent molecule attached to it. GISH is an in-situ hybridization method that uses whole genomic DNA as a probe to study the relationship, divergence, and evolution of the genome between different species.

Cytogenetic research and chromosome analysis are the main aspects of genomics and genetic sciences. Molecular cytogenetic techniques, such as in situ hybridization methods, are admirable tools to analyze the genomic structure and function, chromosome constituents, recombination patterns, alien gene introgression, genome evolution, aneuploidy, and polyploidy (Andres et al., 2013 Younis et al., 2015).

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The Effect of Toxic Substances of Cigarette Filter on the Growth and Some Physiological Characteristics of Tall fescue (*Festuca arundinacea*) in Soil and Hydroponic Media

Leila Momeni and Sasan Mohsenzadeh

Abstract:
A greenhouse experiment was conducted to assess the impacts of discarded filters, either from smoked and non-smoked cigarette filters (shredded and unshredded filter) on fescue plant from poaceae family. This experiment was carried out on the seed germination in the petri dishes and cultivation in the pot and in the hydroponic media. The experiment consisted of four replications and five treatments. Laboratory tests including measuring the chlorophyll a, b and carotenoid, measuring the amount of proline, antioxidant properties were carried out. In the seed germination test, fresh weight of the shoot had significant decrease compared to the control. The amount of chlorophyll a, b and the carotenoid of the hydroponic culture was better influenced than pot culture for the better diffusion of toxic substances. All the cigarette filter treatments caused a significant increase in the antioxidant content in the pot and hydroponic culture, compared to the control. The treatments of shredded and unshredded smoked cigarette filter in pots and hydroponics caused a significant increase in the amount of proline compared to the control conditions. Proline is an osmotic regulator that shows the positive response of the plant under the stress conditions of toxic substances from these filters.

Keywords: cellulose acetate, cigarette filter, tall fescue, toxic substance, environmental impact

ENVIRONMENTAL IMPLICATION

Cigarette filters have a significant environmental impact, and not in a good way. 95% of cigarette filters are made of plastic material (cellulose acetate) that may appear like cotton. It is non-biodegradable and may remain toxic for up to ten years. Cigarette butts scattered along green spaces, sidewalks, roadsides and beaches. About 4.5 trillion cigarettes are littered each year. Littered cigarette butts leach toxic chemicals such as arsenic and lead, to name a few into the environment and can contaminate water.

INTRODUCTION

Tall fescue is known also as Festuca arundinacea from poaceae family. This is a perennial plant with thick and deep main roots. Tall fescue has a short underground stem and is considered a tall plant. The leaves of this plant are dark green or shiny. Its panicle is 10 to 30 cm long and there are 5 to 7 seeds in each panicle (Hannaway et al., 1999). Tall fescue is a perennial plant, which can tolerate a wide range of environmental stressors, including strongly acid to alkaline conditions, and its deep root system allows it to grow in wetlands and other low-lying areas. This plant is an introduced grass found in the Pacific Ocean, northwest wetlands, reclaimed tidal lands, and the mid-west coast of South Korea (Lee et al., 2022). This plant has positive characteristics such as high fodder yield, long grazing season, relatively good seed production, adaptability to a wide

range of soil conditions, suitable cold resistance and soil protection. Due to its thick, strong, deep and wide fibrous filaments, tall fescue reduces soil erosion (Hannaway et al., 1999).

Cellulose acetate filters were used in cigarettes in the 1950s following the increasing number of smokers. There was convincing scientific evidence that smoking results into lung cancer and other serious diseases (Novotny et al., 2009). Cigarette contains higher levels of a cancercausing substance. The heavy metals and trace elements in cigarette butt leachate are the cause of toxicity in the organisms. Although the presence of various compounds in cigarettes and their filters has been extensively studied, few reports have been presented on the amount leached into aquatic medium. The occurrence of heavy metals in cigarettes is largely due to the soil conditions in which tobacco is being cultivated. Apart from that, using pesticides, insecticides and herbicides, the addition of casing materials to tobacco leaves and dried leaves, and the use of brightening agents on wrapping paper produce most of the new chemicals (Michael et al., 2022). Cigarette filter contains dangerous substances including chromium, nickel, cadmium, arsenic and lead, which are carcinogenic and dangerous materials for humans and animals (Hazbehiean et al., 2022). Cigarette filters cause pollution in different areas, swallowing by birds, animals and sea creatures can result into death or some problems. The washing of cigarette filters by rainfall and passing through waterways causes the release of toxic chemicals such as nickel, lead acetone, cadmium and arsenic in the water and soil affects organisms. (Hazbehiean et al., 2022).

MATERIALS AND METHODS

Plant Growth and Treatments

For this research, pots and hydroponic medium were treated in the research greenhouse of the biology department of Shiraz University, in the winter 2021 and the spring 2022, and the related experiments were also conducted in the laboratory. Based on the performed studies, the preparation of samples and their treatment was as follows.

To test seed germination, 50 seeds of tall fescue in petri dish with paperfilter under and above the seeds were used in the laboratory. There were 5 treatments including control, unsmoked unshredded cigarette filter, unsmoked shredded, smoked unshredded, and shredded smoked cigarette filter each with 4 replicates. Each petri dish had five cigarette filters. The seeds were wet with distilled water and number of seed germinated were calculated on the 10th day.

In pot cultivation, 20 pots (each 2 kg) with the suitable soil (sand, garden soil and leaf fertilizer with the same amount) were used and the same size of 10 seedlings were planted in each pot. Totally 30 cigarette filters were added to each pot in three times 10th, 20th and 40th days after seedlings planting and on the 54th day of planting, the seedlings were collected.

In hydroponic culture the germinated seeds were used. The containers of the same size and shape with the capacity of four liters of water were choose. The eight holes on the top of the containers were formed and the surroundings of the containers painted with black color so that the roots of the plant were placed in the darkness. Twenty containers were filled each with four litters of water, then added one milliliter of micronutrients solution, and ten milliliters of macronutrients solution for every liter of water. The seedlings were placed in the holes at the head of the containers so that the root was at the bottom and the stem was at the top. The glass wool was used to fix the plant. The eight plants were put in each hole so 3 that each container had 64 plants. Like the previous experiment, five treatments and four repetitions were used. The treatments were added the in two stages, 20 cigarette filters on the 2nd day of hydroponic culture, and 30

cigarette filters on the 6th day of hydroponic culture. The containers were refilled with Hoagland solution whenever they were empty. The plants were collected on the 28th day of experiment.

Measurement of Shoot and Root Fresh Weight
the shoot of the plant was cut from pot and hydroponic cultures and also the root from hydroponic culture. Then measured their weight before placing in liquid nitrogen.

Measurement of Chlorophyll and Carotenoid Concentration
Leaf tissue (0.01 g) was taken and ground with 80% acetone, then poured into the falcon and reached to a volume of 10 ml. The solution was centrifuged for 10 minutes at 6000 rpm. The upper layer was used to measure chlorophyll and carotenoid.

To measure the absorbance of the solutions, the spectrophotometer (SHIMADZU 160A, Japan) was used with the 663 nm for chlorophyll a, 645 nm for chlorophyll b, and 470 nm for carotenoid (Arnon, 1967).

The following formulas were applied to calculate chlorophyll a, b and carotenoids.
Chlorophyll a = 12.7 × A663 – 2.69 × A645 (mgg-1)
Chlorophyll b = 22.9 × A645 – 4.68 × A663 (mgg-1)
Carotenoid = [(1000 × A470 – 1.82 × Chl a – 85.02 × Chl b) /198] (mgg-1)

Measurement of Proline in the Shoot
Proline content in the shoot was measured using ninhydrin acid in accordance with Bates method (Bates et al., 1973). Increased absorption is directly associated with the amount of proline. The shoot (0.05 g) was weighed and placed in a mortar, 10 ml of sulfosalicylic acid 3% was gradually added to it and ground well to create a uniform solution. The solution was poured into the falcon and placed in the centrifuge at 1500 rpm for 10 minutes. The upper layer was separated and it was considered as the base extract for proline measurement. Two ml of base extract, plus two ml of pure glacial acetic acid, with two ml of ninhydrin reagent were poured into the test tube and shaken to mix well.

The test tube was placed in a BainMarie with aluminum cover at a temperature of 95°C. After one hour, the samples were removed from the water bath and transferred directly to the ice bath to be cool, and the reaction was terminated. After cooling, it was transferred to room temperature, then four ml of toluene were added to the solution and mixed by vortex for 30 seconds, this caused the two phasing of the contents of the tube. After 20 minutes, the optical absorbance of the upper layer was read at of 520 nm using a spectrophotometer (SHIMADZU 160A, Japan). The amount of proline was calculated by the standard curve, as milligrams per liter, but the final data was presented by changing the unit using the following calculation in µmoles proline per gram.

[(µg proline/ml × ml toluene) / 115.5 µg/µmole] / [(g sample) / 5 = µmoles proline/g of fresh weight

Antioxidant Measurement
Antioxidant potential was determined using the stable DPPH radical in accordance to the method of Shimada et al. (1992). First, 150 µL of the plant base extract was added to each foil wrapped falcon containing 2850 µL of a 0.004% DPPH solution, and there was a control solution that contained no extract and only contained 150 µL of 100% methanol and 285 microliter solution of

0.004% DPPH. The control solution and the sample solution were placed in the dark for one hour and then its absorbance was read at 517 nm by a spectrophotometer (SHIMADZU 160A, Japan).

RESULTS AND DISCUSSION

The Impacts of Toxic Substances of Cigarette Filter on the Seed Germination

The percentage of seed germination under the treatments of shredded unsmoked cigarette filter, shredded smoked cigarette filter and shredded smoked cigarette filter has decreased significantly compared to the control. The reasons of this culture are growth inhibitory stressed of heavy metals and other toxic components of tobacco. Cigarette tobacco proved that it is toxic at the seed germination stage, and could be toxic at both vegetative and reproductive stages of plant growth (Haq et al., 2018). Cigarette filters are made of cellulose acetate, which is a plastic product (Novotny et al., 2009). Cellulose acetate present in cigarette filter mitigates the seed germination (Fig. 1).

The Impacts of Toxic Substances of Cigarette Filter on the Fresh Weight

In the pot, the fresh weight of tall fescue in cigarette filter treatments had decreased compared to control conditions. This reduction was observed from unshredded unsmoked cigarette filter, unshredded smoked cigarette filter, shredded unsmoked cigarette filter and shredded smoked cigarette filter, respectively. In the hydroponic medium, all the treatments had a significant reduction compared to the control conditions. The fresh weight of the unshredded unsmoked cigarette filter, the shredded unsmoked cigarette filter, the unshredded smoked cigarette filter and the shredded smoked cigarette filter showed significant decrease compared to the control conditions, respectively. The stress of toxic substances and heavy metals leads to plant cell damage and the accumulation of metal ions in the plant, which disrupts the ionic cell homeostasis (Yadav, 2010). Heavy metal toxicity reduces the movement of sucrose and water and increases oxidative damage (Haider et al., 2021). Also, cellulose acetate in cigarette filter causes negative effects on plant growth (Fig. 2).

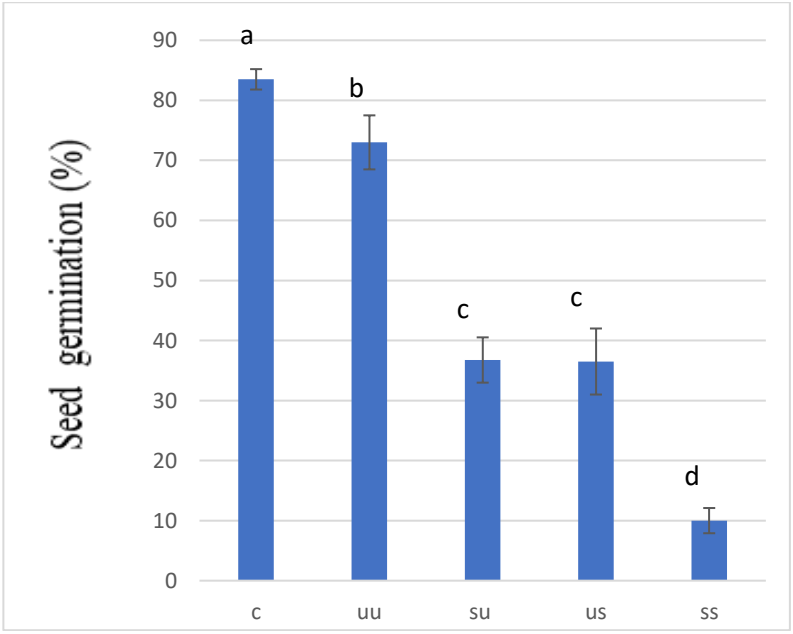

Figure 1- The impacts of toxic substances of cigarette filter on the germination of tall fescue seeds. Each number is the average of four repetitions of ±SE. Different letters indicate a significant difference at level p

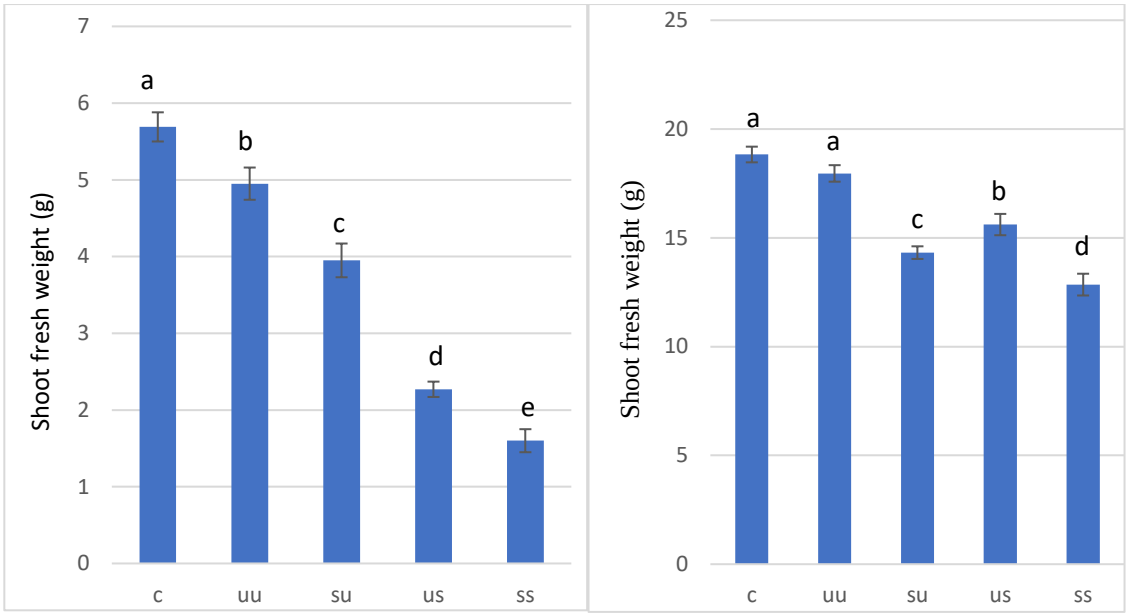

Figure 2- The impacts of toxic substances of cigarette filter on the fresh weight of the shoot of tall fescue in hydroponics (left) and pots (right) media. Each number is the average of four repetitions of ±SE. Different letters indicate a significant difference at level p

Effects of Toxic Substances of Cigarette Filter on Photosynthetic Pigments

In pots, cigarette filter treatments did not have a significant impact on the content of chlorophyll a and b, but the amount of carotenoids decreased significantly under the conditions of shredded and unshredded smoked cigarette filter treatments compared to the control (Fig. 3). It shows the effect of toxic substances of smoked cigarette filter. In hydroponic medium, the treatments of shredded unsmoked cigarette filter, unshredded smoked cigarette filter and shredded smoked cigarette filter significantly decreased their chlorophyll a content compared to the control. The content of chlorophyll b in the treatments of unshredded smoked cigarette filter and shredded smoked cigarette filter compared to the control conditions was significantly decreased. The amount of carotenoids in the treatment of shredded smoked cigarette filter and unshredded smoked cigarette filter has been significantly reduced compared to the control (Fig. 4). The difference between the content of chlorophyll a and b in pot and hydroponic media can be related to better diffusion of toxic substance in hydroponic medium from cigarette filter and absorption to the plant. The toxicity of toxic substances and heavy metals in photosynthesis can prevent the activity of different photosynthetic enzymes and chlorophyll biosynthesis, damage the chloroplast membrane system, and interfere with the process of photosynthetic electron transport (Aggarwal et al., 2012).

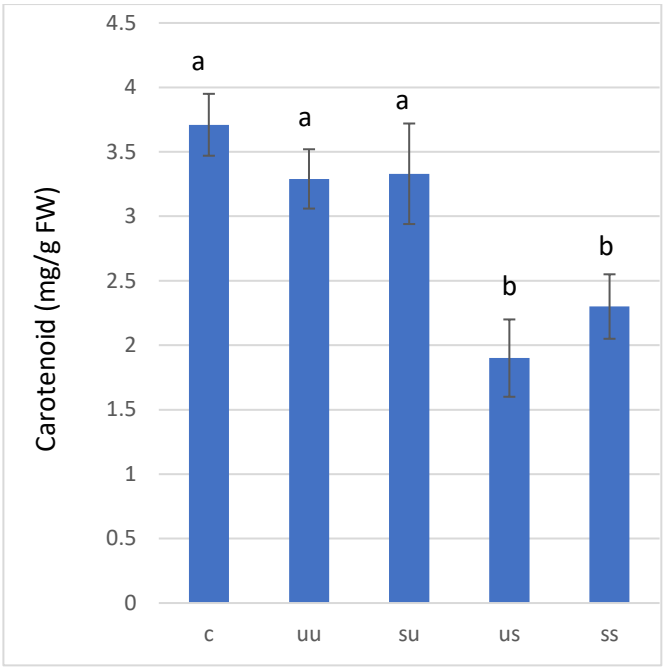

Figure 3- Effects of toxic substances of cigarette filter on carotenoids of tall fescue in pots. Each number is the average of four repetitions of ±SE. Different letters indicate a significant difference at level p

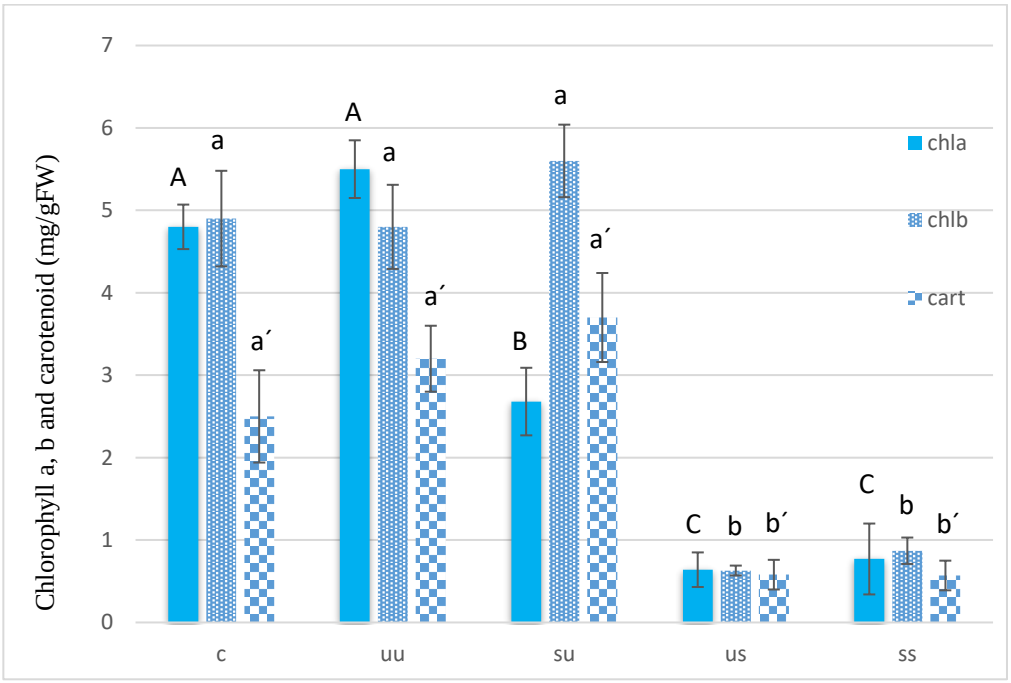

Figure 4- Effects of toxic substances of cigarette filter on chlorophyll a, chlorophyll b and carotenoids of tall fescue in hydroponics. Each number is the average of four repetitions of ±SE. Different letters indicate a significant difference at level p

Effects of Toxic Substances of Cigarette Filter on the Proline Content

Both in pots and in hydroponics, the treatments of shredded smoked cigarette filter and unshredded smoked cigarette filter have significantly increased compared to the control of the proline content (Fig. 5). It shows the effect of toxic substances in smoked filter. Plants accumulate large amounts of different types of compatible solutes in response to various plant stresses.

Compatible solutes are low molecular weight, highly soluble organic compounds, which are usually non-toxic at high cellular concentrations. These solutes protect plants against stress and adjust cellular osmosis, detoxify ROS, protect membrane integrity, stabilize enzymes and proteins. These include proline, sucrose, polyols, and ammonium compounds such as glycine (Hayat et al., 2012).

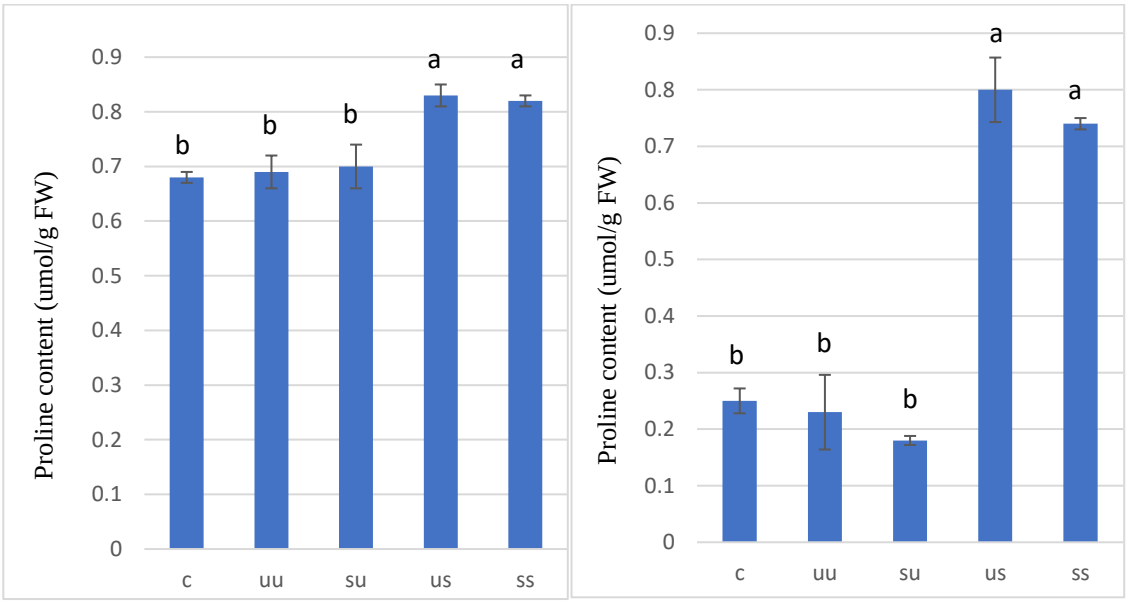

Figure 5- Effects of cigarette filter toxic substances on the proline content of tall fescue in hydroponics (left) and pots (right) media. Each number is the average of four repetitions of ±SE. Different letters indicate a significant difference at level p

The Impact of Toxic Substances of Cigarette Filter on the Antioxidant Content

In the pots, the amount of antioxidants has increased significantly in all cigarette filter treatments, compared to the control. In the treatment of unshredded unsmoked cigarette filter, shredded unsmoked cigarette filter, and smoked unshredded and shredded smoked cigarette filter, the amount of antioxidants increased significantly compared to the control. In the hydroponics, all the treatments from unshredded unsmoked cigarette filter, shredded unsmoked cigarette filter, unshredded smoked cigarette filter, and shredded smoked cigarette filter had a significant increase in their antioxidant content compared to the control (Fig. 6). Stress produces reactive oxygen species (ROS) that the plant must deal with. Thus, the plant uses enzymatic and non-enzymatic antioxidants, and antioxidant defense is of great importance to protect the plant from oxidative stress. Under stress conditions, antioxidant defense is increased and accumulation of secondary metabolites is occurring as a defense mechanism (Hasanuzzaman and Fujita, 2012).

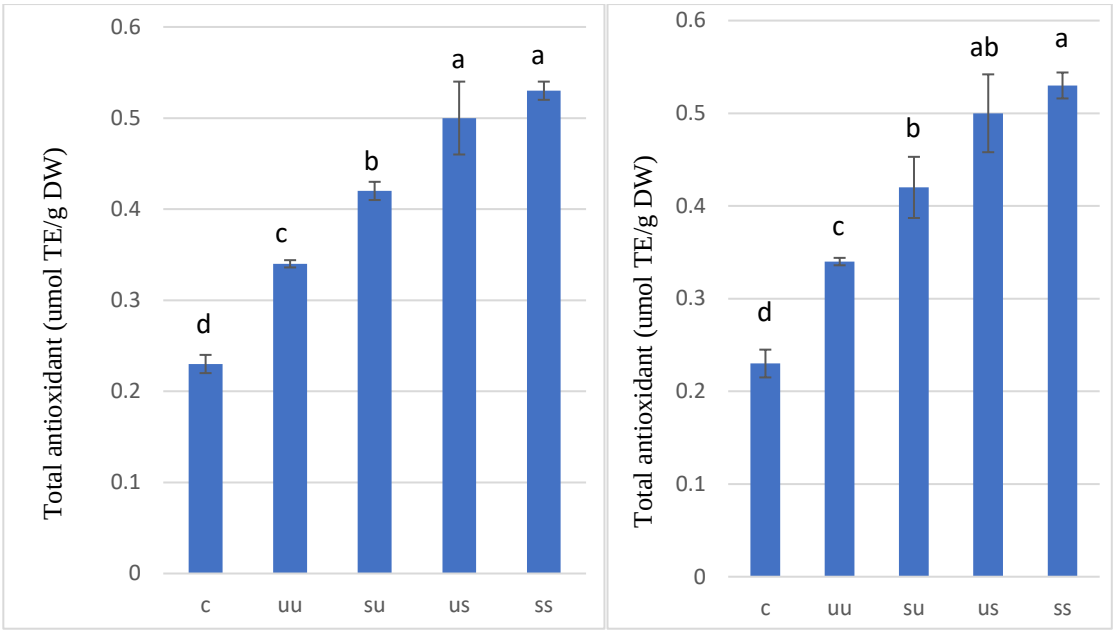

Figure 6- Effects of cigarette filter toxic substances on the antioxidant content of tall fescue in pots (left) and hydroponics (right). Each number is the average of four repetitions of ±SE. Different letters indicate a significant difference at level p

CONCLUSION

In the seed germination test, fresh weight of the shoot of the plant in the hydroponic culture and the fresh weight of the shoot of the tall fescue in the pot the treatments had significant decrease compared to the control. The amount of chlorophyll a, b and the carotenoid of the hydroponic culture was better influenced than pot culture for the better diffusion of toxic substances. All the cigarette filter treatments caused a significant increase in the antioxidant content in the pot and hydroponic culture of the tall fescue, compared to the control, which indicates that the plant has shown a positive response under stress conditions to cope with the stress. The treatments of shredded and unshredded smoked cigarette filter in pots and hydroponics caused a significant increase in the amount of proline compared to the control conditions. Proline is an osmotic regulator that shows the positive response of the plant under the stress conditions of toxic substances from these filters.

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