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Full Length Research Paper

Chemical components of the volatile and non-volatile extractives of *Croton* species and their microbial activities

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Essential oil compounds of *Croton pseudopulchellus* and *Croton gratissimus* were analysed using Gas Chromatography/Mass Spectrophotometry and screened for antimicrobial activity against *Bacillus pimilus* (ATCC 29212), *Bacillus cereus* (ATCC 10702), *Staphylococcus aureus* (ATCC 3983), *Streptococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 4983), *Klebsiella pneumoniae* (ATCC 2983) and *Pseudomonas aeruginosa* (ATCC 19582) using Agar gel disk diffusion test and minimum inhibitory concentrations. The susceptibilities of all isolates of different essential oil compounds were standardised by National Committee for Clinical Laboratory Standards (NCCLS 1998). The cytotoxicity test was also carried out to determine the toxicity levels of essential oil compounds. These plants were selected based on their use by traditional healers for treatment of upper respiratory tract, gastrointestinal tract and urinary tract infections. The essential oil compounds of *C. pseudopulchellus* and *C. gratissimus* were found to be active against all the test microorganisms, while the preliminary assessment of essential oil compounds from these plants exhibited low cytotoxic activity.

Key words: Essential oil, sesquiterpenes, chemical composition, antimicrobial activity.

INTRODUCTION

The emergence of multidrug resistance to antimicrobials, has become an important public health issue in many developing countries as treatment of ailments require the use of more expensive drugs for a longer treatment period (Buwa and Afolayan, 2009; Oyedeji et al., 2010). Therefore, this study focused on the use of essential oil compounds derived from *Croton pseudopulchellus* and *Croton gratissimus* as alternative therapeutic agents or new inexpensive antimicrobial drugs which are more effective and with less side effects for treatment of upper

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respiratory and gastrointestinal tract infections. Antimicrobial agents inhibit the growth of microorganisms by interfering with the specific physiological characters or metabolic functions of microorganisms (Ndip et al., 2008). Based on the ethnomedical information on these plants they were screened against four Gram-positive bacteria, namely, *Bacillus cereus* (ATCC 10702), *Bacillus pumilus* (ATCC 14884), *Staphylococcus aureus* (ATCC 3983), and *Streptococcus faecalis* (ATCC 29212). Gram-negative bacteria were *Escherichia coli* (ATCC 4983), *Klebsiella pneumoniae* (ATCC 2983) and *Pseudomonas aeruginosa* (ATCC 19582). The four Gram positive bacteria were included because of their opportunistic properties in the upper respiratory tract infections, while the other three Gram negative bacteria are custodians of the urinary and gastrointestinal tract infections. It is expected that essential oil compounds derived from *C. gratissimus* and *C. pseudopulchellus* showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens (Ndip et al., 2008). *C. gratissimus* and *C. pseudopulchellus*, though not widely distributed in Africa have shown in traditional medicine that their leaves can be used in treating several ailments; therefore its versatile use in traditional medicine necessitated this research.

Croton is a large family genus of Euphorbiaceae comprising about 700 species as trees, shrubs and herbs (Salatino et al., 2007). The leaves are glossy and aromatic in nature (Van Wyk, 2008; Compagnone et al., 2010). *C. pseudopulchellus* Pax is commonly known as small lavender fever – berry, a pale white-yellow-flowered, bark grey, smooth to roughish, reddish brown branchlets covered with hairy scales, shrubby, perennial plant up to 4 m tall with a sweet smell that attracts many insects, while *C. gratissimus* Burch is known as Lavender Croton. Both are found to grow widely in the North-Eastern regions of South Africa along the coastal belt, rocky hillside and along rivers and streams, from Kwazulu-Natal, extending further North through Swaziland to Mpumalanga and Limpopo province. Leaves and bark of *Croton* species are often used in folklore medicine for the treatment of syphilitic ulcers and chest-complaints. Root-decoction is also used for Asthma while powdered root is taken as snuff for head colds (Neves and da Camara, 2012).

According to the review of *Croton* spp. by Salatino et al. (2007), several *Croton* spp. are well known as medicinal plants in the traditional medicinal practices for the treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers and weight-loss across Africa, Asia and South America. Other studies reported that oil derived from *Croton* spp. was commonly used as a therapeutic tool to treat acne and skin infections (Bikanga et al., 2010; Mulholland et al., 2010). Mulholland et al. (2010) further reported that *Croton gratissimus* Burch (Lavender Croton) also contains organic compounds such as pumarane, kaurane, labdane, clerodane, cembrane, diterpenoides, isoquinoline, alkaloids and triterpenoides that enhance treatment of headache, coughs, fever, cold, syphilic ulcers and chest pains, while the leaf sap of *C. pseudopulchellus* is drunk to treat abdominal pains, painful respiratory conditions, bleeding gums, asthma, headache, coughs, fever and colds (Ndip et al., 2008). Decocted leafy twigs are drunk for the treatment of gonorrhea and cytotoxicity tests showed an ID_{50} of 64 µg/mL against vervet monkey cells (Langat et al., 2012).

Essential oil compounds derived from plants have been widely used in the treatment of respiratory tract, urinary tract, gastrointestinal tract infections as well as skin infections (Bikanga et al., 2010; Mulholland et al., 2010; Leite et al., 2015). Since the essential oil compounds of *Croton* spp. are of economic and medicinal value (Viljoen et al., 2006). The aim of this study was to investigate the chemical composition and antimicrobial activity of *C. psedopulchellus* and *C. gratissimus* essential oil compounds against four Gram positive and three Gram negative test microorganisms.

MATERIALS AND METHODS

Plant Collection

Croton pseudopulchellus samples were collected from Twin Stream Indigenous Nursery and Landscaping, Mtunzini, while *C. gratissimus* samples were collected from a private garden in Mtunzini, Kwazulu Natal. Mrs A. Hutching of the Botany Department, University of Kwazulu Natal authenticated the samples. Voucher specimens were deposited at the University of Zululand Herbarium. The Eastern Cape *Croton* spp. were collected from Flagstaff by Mr Iwopa of the Botany Department and Dr Morobe of Medical Microbiology Department, Walter Sisulu University and the plants were authenticated by Dr Immelman, KL, a taxonomist of the Department of Botany, Walter Sisulu University (WSU), Mthatha, Eastern Cape, South Africa. Voucher specimens were deposited at the Walter Sisulu University Herbarium.

Extraction of essential oil

One kilogram of fresh leaves of each species was subjected to hydro distillation in a Clevenger apparatus for 4 h. This technique is based on the evaporation of volatile compounds induced by steam. The essential oil was collected in amber vials after 4 h, weighed, sealed and stored in the refrigerator (4°C) until use (Oliveira et al., 2020).

GC/MS analysis

GC/MS analyses of the oils were performed on a Hewlett Packard Gas Chromatography HP 6890 interfaced with Hewlett Packard 5973 mass spectrometer system operating in EI mode at 70 eV, equipped with a HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The initial temperature of the column was 70°C

and was heated to 240°C at a rate of 5°C min^{-1}. Helium was usedas the carrier gas at a flow rate of 1 mL/min. The split ratio was 1:25. Scan time was 50 min with a scanning range of 35 to 450 amu. 1 µL of the diluted oil was injection for analysis. *n*-Alkane of C_8-C_{30} was run under the same condition for Kovat indices determination (Ndukwe and Okhiku, 2018). The components of the oils were identified by matching their spectra and retention indices (Kovat Index) with those of the authentic samples and literature values (Oyedeji et al., 2010).

Cytotoxic screening of seven essential oil compounds from *C. pseudopulchellus* and *C. gratissimus*

MAGGI CCR5+ cells were used for cytotoxic screening of the essential oil compounds. All cell lines were purchased from ATCC, Manassas, VA 20108, USA. Cell lines were cultured in Advanced Modified Eagle's Medium (DMEM) with 10% 5 Mm L-glutamine (Gibco BRL) and grown at 37°C in a 5% CO_2 humidified incubator (Thermo Fisher Scientific, Wakenyaku Co. Ltd, Japan). Cells were subcultured every 2 days after the confluent growth was observed, MAGI cells were then seeded into two 96 well µL plates with 10^4 cells/well in 100 µL of DMEM supplemented with 10% foetus bovine serum (FBS). 11 µL of oil was added into 2 wells of row B with final concentration of 1/20. Another 11 µL of mixture was removed from B to C and then to D, E, F and 10 µL was discarded from F. 100 µL of medium was added into each well from B to G. After 48 h, cells were observed and 150 µL of supernatant from each well was discarded and then 10 µL of MTT was added into each well. The plates were incubated at 37°C for 4 h. 100 µL of stop solution was added into each well and OD_{570} was checked and then CC_{50} were determined as previously reported (Morobe et al., 2012).

Biological activity

Antimicrobial assay

Agar gel disk diffusion: The essential oil compounds were tested for antibacterial activity using modified Kirby-Bauer agar gel disk diffusion test according to Kose et al. (2010) and the MIC breakpoints of all isolates were determined using the E-test strips (Morobe et al., 2013). The susceptibilities of all isolates of different essential oil compounds were standardised using National Committee for Clinical Laboratory Standards (NCCLS, 1998).

Microorganisms were grown overnight at 37°C in 20 mL of Müller-Hinton broth (Oxoid). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland No. 5 standard (1.0×10^8) CFU/mL. 90 mm Petri dishes (Merck, South Africa) containing 12 mL of sterilized Mueller-Hinton agar (Oxoid) were inoculated with these microbial suspensions. Sterile Whatmann No. 1 (6 mm) discs papers were individually placed on the surface of the seeded agar plates and 10 µL of essential oil compound in DMSO was applied to the filter paper disk. The plates were incubated at 37°C for 24 h and the diameter of the resulting zones of inhibition (mm) of growth was measured. All tests were performed in triplicates. Ampicillin (10 µg) and Chloramphenicol (10 µg) were used as positive controls, while hexane and DMSO served as negative controls.

The essential oil compounds were tested against seven reference bacterial strains obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice. Gram-positive bacteria: *B. cereus* (ATCC 10702), *B. pumilus* (ATCC 14884), *S. aureus* (ATCC 3983), and *S. faecalis* (ATCC 29212). Gram-negative strains were *Escherichia coli* (ATCC 4983), *K. pneumoniae* (ATCC 2983), and *P. aeruginosa* (ATCC 19582).

The stock cultures were maintained at 4°C in Mueller-Hinton agar (Oxoid) (Morobe et al., 2018).

Minimum inhibitory concentration of essential oil compounds

The minimum inhibitory concentrations (MICs) of the essential oil compounds were determined using 96-well µL dilution method as described by Oyedeji et al. (2010) and Eloff et al. (2011). Bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37°C and a 1:1 dilution of each culture in fresh MH broth was prepared prior to use in the micro dilution assay. Sterile water (100 µL) was pipetted into all wells of the µL plate, before transferring 100 µL of essential oil compound into DMSO. Serial dilutions were made to obtain concentrations ranging from 10 to 0.078 mg/mL. 100 µL of bacterial culture of approximate inoculums size of 1.0×10^8 CFU/mL was added to all well and incubated at 37°C for 24 h. After incubation, 40 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet (INT) solution was added to each well and incubated at 37°C. Plates were examined after 60 min of incubation. Microbial growth was indicated by the presence of a reddish colour which was produced when *p*-iodonitrotetrazolium violet (INT), a dehydrogenase activity detecting reagent, was reduced by metabolically active microorganisms to the corresponding intensely coloured formazan (Oyedeji et al., 2010). Solvent controls (DMSO and Hexane) and the standard antibiotics ampicillin (10 µg) and chloramphenicol (10 µg) were included in the assay.

RESULTS

Chemical analysis of essential oil compounds

In this study, the chemical profile of *C. pseudopulchellus* and *C. gratissimus* oils showed a high amount of monoterpenes and sesquiterpenes similar to that reported for samples collected worldwide. Analysis of the oils was performed using GC/MS (Table 1). The leaf oils of *C. pseudopulchellus* had germacrene (24.2%), β-phellandrene (17.4%), myrcene (13.4%) and β-caryophyllene (11.4%) as the prominent compounds. The chemical composition of the leaf oil of *C. gratissimus* was characterized by sabinene (14.6%), β-phellandrene (12.3%), α-pinene (6.0%) and germacrene D (5.9%), respectively.

Cytotoxic screening of seven essential oil compounds from *C. pseudopulchellus* and *C. gratissimus*

In this study, a systematic evaluation of cytotoxic activities of seven essential oil compounds from *C. pseudopulchellus* and *C. gratissimus* were conducted and exhibited a minimal toxic activity on cell lines (Table 2).

The results from both *Croton* spp. (Table 2) revealed germacrene (0.2 Cc_{50}) and β-phellandrene (0.2 Cc_{50}) as the most toxic oils and induced over 50% cell death, followed by α-phellandrene (0.19 Cc_{50}) and β-caryophellene (0.18 Cc_{50}), respectively. The oils that induced the least cell death were α-pinene (0.15 Cc_{50}),

Table 1. Percentage composition of essential oil compounds of *C. pseudopulchellus* and *C. gratissimus*.

Compound	KI	Percentage composition of essential oil compound			
		CpECP	CgECP	CpKZP	CgKZP
α-thujene	936	-	-	-	1.2
α-pinene	943	4.5	-	3.7	6.0
sabinene	977	-	-	-	14.6
1-octen-3-ol	983	-	-	6.7	-
myrcene	993	11.3	4.6	13.4	2.4
α-phellandrene	1003	1.0	15.5	-	12.3
α-terpinene	1019	0.5	0.7	-	1.5
β-phellandrene	1037	9.2	5.0	17.4	T
trans-β-ocimene	1040	1.0	1.1	-	2.8
γ-terpinene	1069	1.4	1.6	-	2.1
cis-sabinene hydrate	1097	-	1.0	-	1.4
α-terpinolene	1098	-	3.0	-	1.9
linalool	1101	1.0	0.3	1.2	4.1
α-terpineol	-	1.8	0.2	-	-
α-cubebene	-	1.5	0.5	-	-
Eugenol	-	0.2	2.0	-	-
α-copaene	1376	3.2	5.3	2.2	2.5
β-bourbonene	1387	1.0	1.8	1.2	0.7
β-elemene	1391	4.0	1.0	3.6	0.5
β-caryophyllene	1442	10.2	12.9	11.7	4.2
β-cubebene	-	-	0.6	-	-
Viridiflorene	-	-	0.8	-	-
α-humulene	1460	3.0	2.7	3.6	1.1
Aromadendrene	1470	1.3	1.9	0.4	2.0
germacrene D	1481	28.1	16.0	24.2	5.9
Bicyclogermacrene	1497	4.0	2.6	3.1	1.6
γ–cadinene	1518	-	1.9	-	1.0
γ–muurolene	-	-	1.6	-	-
δ–cadinene	1526	1.0	1.3	2.3	0.9
α–cadinene	1538	0.9	-	1.0	-
germacrene-D-4-ol	1574	2.7	7.8	0.9	-
caryophyllene oxide	1586	1.6	1.4	1.3	2.4
Spathulenol	1589	1.0	0.8	0.8	T
Total % (no. of cpd)	-	95.3 (24)	96.0 (28)	78.8 (18)	82.1 (24)

KI = Kovat indices; t = trace amount; - = not detected, CpECP = *C. pseudopulchellus* Eastern Cape Province, CpKZP = *C. pseudopulchellus* KwaZulu-Natal Province, CgECP = *C. gratissimus* Eastern Cape Province, CgKZP = *C. gratissimus* KwaZulu-Natal Province.
Source: Authors

cytotoxic activity of 21%.

Antimicrobial activity of essential oil compounds of *C. pseudopulchellus* and *C. gratissimus* against seven microrganisims

In this study, results obtained (Table 3) revealed the varying levels of the antimicrobial activity of *C. pseudopulchellus* and *C. gratissimus* essential oil compounds against bacterial isolates studied.

The essential oil compounds of *C. pseudopulchellus* and *C. gratissimus* were tested for antibacterial activity against seven microorganisms using agar gel disc diffusion test. The essential oil compounds of the two *Croton* spp. showed activity against all test microorganisms (Table 3) and the zones of inhibition of essential oil compounds varied from 0 to 12 mm and the largest zone of inhibition was obtained for *E. coli* (12 mm) and the lowest for *B. pimilus* (2 mm).

Table 2. Cytotoxic screening of seven essential oil coumpounds from *C. pseudopulchellus* and *C. gratissimus*.

Compound	Essential oil compound concentration (Cc50)	
	C. pseudopulchellus	***C. gratissimus***
A-phellandrene	0.16	0.19
β-phellandrene	0.14	0.2
Germacrene	0.17	0.2
β-caryophellene	0.15	0.18
α-pinene	0.13	0.15
Myrcene	0.14	0.13
Sabinene	0.09	0.13

Source: Authors

Table 3. The zones of inhibition of essential oil compounds of two Croton species against seven microorganisms.

Compound	Inhibition zones of essential oil compound against seven microorganisms (mm)						
	S. aureus	***S. faecalis***	***B. cereus***	***B. pumilus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***
Germacrene	9	7	3	2	12	11	8
A-phellandrene	6	5	3	2	10	9	11
β-phellandrene	5	6	4	3	5	7	9
β-caryophyllene	6	6	2	2	7	5	5
α-pinene	3	2	2	2	4	3	2
Myrcene	9	7	3	3	8	8	7
Sabinene	5	5	2	2	5	3	2
Ampicillin (10 µg)	30	25	20	19	18	28	19
Chloramphenicol (10 µg)	21	20	15	14	26	17	8

Source: Authors

Minimum inhibitory concentration of essential oil compounds

The Minimum Inhibition Concentration method showed that essential oil compounds of the two *Croton* spp. were active against all test microorganisms (Table 4).

The MIC values of the essential oil compounds ranged from 2 to 19 µg/mL (Table 4), with the most prominent being *E. coli* (19 µg/mL), *K. pneumoniae* (17 µg/mL), *S. aureus* (16 µg/mL) and *P. aeruginosa* (15 µg/mL), with the least active being *B. cereus* (2 µg/mL).

DISCUSSION

Leaves of *Croton* spp. are used in traditional medicine for the treatment of syphilitic ulcers and chest-complaints (Compagnone et al., 2010). In this study, the three major compounds among sesquiterpene were germacrene (24.2%) and phellandrene (17.4%). The chemical profile of the oils had germacrene (5.0-28.1%) and β-caryophyllene (4.2-12.9%) as the two most prominent compounds in all the oil extracts. α-phellandrene was found in trace amount in the oil extract of *C. gratissimus* from Kwazulu-Natal province, while other oil samples had significant amount of the compound (5.0- 17.4%).

In previous studies on the essential oil compounds from other samples of sesquiterpene hydrocarbons, Oliveira et al. (2007) showed germacrene as a major compound (66.0%), while the monoterpene hydrocarbons (phellandrenes) were present only as trace constituents (1.1%). The essential oil from the leaves of *C. gratissimus* gave fenchyl acetate (25.3%), β-caryophyllene (20.7%), α-selinene (12.8%) and β-bourbene (9.3%) as major constituents. In contrast to the aforementioned findings, in the present study germacrene was identified as a major compound in the essential oil of South African *Croton* spp. from Kwazulu-Natal and the Eastern Cape provinces. These findings strongly suggest that the germacrene content in the sample analysed in this study was due to environmental conditions, since the seasonal, climate and soil conditions are different in various geographical areas, supporting the existence of two different chemotypes for germacrene and phellandrene. Furthermore, this suggests that there are different chemotypes for these species. However, it is also known

Table 4. The Minimum Inhibition Concentration values of essential oil compounds against seven microorganisms.

Compound	MIC values of essential oil compounds against seven microorganisms (µg/ml)						
	S. aureus	***S. faecalis***	***B. cereus***	***B. pumilus***	***E. coli***	***K. pneumoniae***	***P. aeruginosa***
Germacrene	16	7	2	3	18	17	10
A-phellandrene	13	5	2	3	19	10	15
β-phellandrene	12	8	3	5	17	13	9
β-caryophyllene	11	7	4	4	14	10	11
α-pinene	2	6	2	2	9	7	6
Myrcene	13	7	3	5	12	17	12
Sabinene	3	4	2	2	5	2	4
Ampicillin (10 µg)	6	6	6	6	9.4	9.4	9.4
Chloramphenicol (10 µg)	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Source: Authors

that cultivation conditions can affect secondary metabolite production (Edris, 2007). The preliminary bioassay assessment of *C. pseudopulchellus* and *C. gratissimus* essential oils exhibited low cytotoxic activity 21%.

The essential oil compounds were tested for antibacterial activity by the agar gel disc diffusion method (Kose et al., 2010). Disc diffusion is one of the most common assays used in the evaluation of antimicrobial activity of essential oil compounds. In this study antimicrobial activity by disc diffusion method showed that the essential oil compounds of the *C. gratissimus* and *C. pseudopulchellus* were active against *E. coli* followed by *K. pneumoniae* and *P. aeruginosa*. The zones of inhibition of essential oil compounds varied from 2 to 12 mm. The largest zone of inhibition was obtained for *E. coli* (12 mm) and the lowest for *B. pimilus* (2 mm).

The MIC values of the essential oil compounds ranged from 2 to 19 µg/mL (Table 4), with the most prominent being *E. coli* (19 µg/mL), *K. pneumoniae* (17 µg/mL), *S. aureus* (16 µg/ml) and *P. aeruginosa* (15 µg/mL), with the least active being *B. cereus* (2 µg/mL). According to Burt (2004), both chemotypes (germacrene and phellandrene) appear to make the cell membrane permeable and are able to disintegrate the outer membrane of Gram negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to adenotriphosphate (ATP). Furthermore, in this study Minimum Inhibition Concentration method showed that the essential oil compounds of the two *Croton* spp. were active against all test organisms.

Therefore essential oil compounds from *C. gratissimus* and *C. pseudopulchellus* may be suitable for treatment of infections caused by designated pathogens and this is consistent with a previous finding (Morobe et al., 2012). According to Nanyonga et al. (2013), the antimicrobial activity of essential oil compounds is linked to its chemical composition. The essential oil compounds of *C. gratissimus* had a broader inhibitory effect of the bacteria, compared to the essential oil compound of *C. pseudopulchellus*. However, the antimicrobial activity of *C. gratissimus* and *C. pseudopulcellus* are slightly related to the major compounds of the essential oil compounds of germacrene and phellandrene.

Conclusion

The essential oil compounds from *C. pseudopulchellus* and *C. gratissimus* leaves exhibited variable activities against seven different microorganisms tested in this study and in some cases showed equivalent or better activities than some antibiotics. The potency of these compounds against test microorganisms and on cell lines suggests their potential to be used as a source of alternative medicine, new pharmaceutical and health care product that can be used as a therapeutic agent in the face of antibiotic resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of the grain yield performance of 5 soybean genotypes in Mozambique using the GGE Biplot method

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Soybean is a peculiar crop due to its photoperiod sensitivity. The introduction of this crop to a new production region requires a detailed study of its adaptability to select the best genotypes with high production stability for the region. In Mozambique, the demand for soybean grain has been growing. However, national production is low due to little knowledge of the performance of genotypes. The objective of this study is to evaluate the grain yield of 5 soybean genotypes and evaluate genotype-by-environment (GxE) interactions by the GGE biplot method. The trials were performed at the stations of Namapa (District of Eráti, province of Nampula), Ribáuè (District of Ribáuè, Province of Nampula), and Montepuez (District of Montepuez, Province of Cabo Delgado) in 3 seasons from 2017 to 2020. A randomized complete block design was adopted with 4 replications and 5 treatments: Wima, Wámini, 10E, Safari, and Zamboane cultivars. The results of the joint analysis of variance ($p<0.05$) showed a complex GxE. According to the GGE biplot method, the 10E genotype was the ideotype. Wámini was the worst genotype. PC1 was 89.64 and PC2 was 8.26. Thus the GGE biplot methodology proved to be efficient since the sum of the first two principal components (PCs) was 98.26%.

Key words: Soybean, adaptability, stability, genotype + genotype + environment (GGE) biplot methodology.

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is the 4th most widely grown crop in the world. It is a source of oil and protein for human and animal production and raw material for different products (Silva et al., 2017). Currently, Brazil is the main global soybean producer and exporter (USDA, 2020), although other countries, such as EUA, China, and India, also have high soybean production. Soybean is cultivated from low to high latitudes (Liu et al., 2017). Because of its high nutritional value, soybean has become one of the most consumed foods in the world; it

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reduces the risk of cardiovascular disease and cholesterol levels and acts as a good alternative for people with hypertension (Santos et al., 2013; Zakir and Freitas, 2015). Soybean oil and bran are consumed worldwide as food and animal feed, respectively (Thoenes, 2016). Soybean can be used in fresh or processed human food, animal feed as bran, and concentrates (Silva et al., 2011). Among the African countries with soybean, Nigeria, South Africa, Malawi, Zambia, Uganda and Benin account for approximately 88% of the planted area on the continent, with average productivity of approximately 1.052 $kg.ha^{-1}$ (FAOSTAT, 2019). Nigeria and South Africa produced 1.32 and 0.73 million tons, respectively, based on data from 2017 (FAOSTAT, 2019). Nevertheless, soybean production in Africa represented approximately 1% of the global production in 2017 (FAOSTAT, 2019).

In Mozambique, soybean production has grown considerably in the last 10 years, since the beginning of the “Feed the Future” Program of the USAID in Mozambique in 2008/2009 and the Tropical Legumes Project, by the Bill and Melinda Gates Foundation, in 2007/2008, R&D (Walker and Cunguara, 2016). According to Walker and Cunguara (2016), in the northern and central regions of the country, most identify themselves as the main producers of this crop, and they stand out as having favorable soil, favorable climates, and availability of land. Rapidly growing chicken consumption is the main determinant of the growing demand for soybean meal in Mozambique (Technoserve, 2018).

Although interest in soybean cultivation has grown and projects have been designed to promote production, these initiatives have not yielded results (Technoserve, 2011). The low yields result from several factors such as lack of adapted varieties, poor agronomic practices, and infertile soils. Despite the current productivity limitations, Foyer et al. (2019) suggests that Africa holds tremendous potential for increasing sustainable soybean production, even in the face of a changing global climate. Multi-location trials are a key component of selection for stable and best-performing genotypes in different environments (Ahmadi et al., 2012; Oral et al., 2018; Tekdal and Kendal, 2018).

Among the most recent methodologies, there are 2 main types: Additive Main Effect and Multiplicative Interaction (AMMI) model and the GGE biplot analysis, which have been observed and reported to accurately capture the majority of sum-of-squares interactions, isolate major and interacting components and facilitate visualization of genotypic fitness in various environments (Kumara et al., 2020). The GGE biplot has the advantage of higher discriminative ability and representativeness of the GGE biplot than the AMMI biplot (Singh et al., 2019).

On the other hand, the GGE biplot analysis considers both genotype and GEI effects and graphically displays GEI in a two-way table (Yan et al., 2000). The GGE biplot is a data visualization tool used to evaluate environments due to its discriminative ability and representativeness of GGE patterns, giving it an advantage over the AMMI biplot analysis (Aktas, 2016). These graphical options facilitate the identification of high-yielding, stable genotypes, particularly in multi-environment trials. Moreover, yield stability and wide adaptation are increasingly important, since the climate at specific locations has become more variable over the years (Singh, 2019). In the GGE methodology, the cosine of the angle between the two environments corresponds to their genetic correlation, and the other types of biplots do not have this property (Yan, 2007). Thus, this methodology is more efficient than other techniques based on biplots (Yan et al., 2011). The grain yield, the final product of any crop, is determined by the genotypic potential (G), environmental effect (E), and the genotype × environment (GE) interaction (Yan and Rajcan, 2002).

In the GGE methodology, the cosine of the angle between the two environments corresponds to their genetic correlation, and the other types of biplots do not have this property (Yan et al., 2007). Thus, this methodology is more efficient than other techniques based on biplots (Yan, 2011). Few studies have evaluated the grain yield performance of soybean genotypes in Mozambique by using the GGE biplot method. The objective of this study is to evaluate the grain yield performance of 5 soybean genotypes grown in different environments by assessing G × E interactions using the GGE biplot method.

MATERIALS AND METHODS

Location and seasons

The experiment was set up at stations in Namapa (District of Eráti) and Ribáuè in Nampula Province and Montepuez in Cabo Delgado Province. In total, 7 environments were considered by combining location and year (Table 1). The soils of the Namapa and Montepuez locations are medium to heavy in texture, deep, well to moderately drained with variations from brown to yellowish-brown moderately well drained with clay. The average temperature varies between 20 and 25°C (MAE, 2005). The Ribáuè Region has a humid tropical climate characterized by 2 annual seasons: a dry and cold season from May to December, which is usually without precipitation with temperatures above 26°C, and a rainy and hot season from December to May with precipitation above 1500 mm. Most soils vary in color from brown to yellowish-brown and are moderately well drained with clay (MAE, 2005).

Experimental design

All experiments were conducted in a randomized complete block design. A summary of the genetic material, G1 (Wámini), G2 (Wima), G3 (10E), G4 (Safari), and G5 (Zamboane), used to establish the experiments is provided in Table 2. The cultivars were not inoculated so that the study could approximate the technological conditions of local farmers. Each plot had a total area of 10 m^2 with dimensions of 4 × 2.5 m and 1 m spacing. Five (4 m) long lines constituted the parcel, of which only the 3 central lines

Table 1. Geographical locations.

Environment	Season	Site	Latitude S	Longitude W	Altitude (m)	Rainfall (mm)
E1	2017/2018	Namapa	13°30'	39°30'	250-500	800 to 1200
E2	2017/2018	Montepuez	13°07'	38°59'	550	800 to 1200
E3	2018/2019	Namapa	13°30'	39°30'	250-500	800 to 1200
E4	2018/2019	Montepuez	13°07'	38°59'	550	800 to 1200
E5	2018/2019	Ribáuè	14°34'	38°19'	511	> 1500
E6	2019/2020	Namapa	13°30'	39°30'	250-500	800 to 1200
E7	2019/2020	Montepuez	13°07'	38°59'	550	800 to 1200

Source: Author's computations
The 7 environments were named E1_Namapa, E2_Montepuez, E3_Namapa, E4_Montepuez, E5_ Ribaué, E6_ Namapa, and E7_ Montepuez.

were used for sample collection; the 0.5 m ends of the lines were neglected.

Genotypes' characteristics

The tgx 1740-2f trade name Wámini is a determined erect genotype, with oval leaf and violet flowers. This genotype is resistant to the witch's broom. The recommended production areas in Mozambique are the center and north regions. The physiological characteristics of the tgx 1908-8F, trade name Wima, can be summarized as follows: the growth habit is undetermined, and the leaf shape is oval, with violet flowers. This genotype is also resistant to the witch's broom. It is recommended to the center and north regions of the country. Whereas the tgx 1904-6f, with the trade name of Zamboane, is a genotype with a determined semi-erect growth habit; the leaf shape is also oval and the flower color is pink. This genotype is also resistant to the witch's broom, and the production area is the center and north of the country. The grain color of all these genotypes is dark brown (Boahen, 2017).

Management and evaluation of variables

The sowing compass was 0.5 × 0.1 m, with a sowing density of 2 seeds per hole. Thinning was performed 3 weeks after sowing, leaving 1 plant per hole. Manual sowing of the trial was performed from November to December when the rains were sufficient. Weeds were controlled by a hoe whenever necessary. Sprayings were performed using 200 $g.L^{-1}$ cypermethrin with a 16 L dorsal sprayer.

The yield of each variety in any environment is the sum of the environment (E) main effect, genotype (G) main effect, and genotype-by-environment interaction GE or GEI (Farshadfar et al., 2013). The grain yield (kg ha^{-1}) was obtained by individual harvesting of each plot, weighing and correction for 13% moisture, and extrapolation of the obtained value for the number of kg harvested in one ha (Gesteira et al., 2018). The plants from each experimental plot were harvested one week after 95% of the pods were mature at the R8 stage (Carvalho et al., 2013).

Statistical analysis

Before the analysis of variance (ANOVA), the data were submitted to tests of homogeneity of variances and normality (Bartlett, 1937; Shapiro and Wilk, 1965) to ensure the feasibility of ANOVA (Hartley, 1950). For individual ANOVAs, every combination of location and season/year was considered an environment (Ramalho et al., 2000). Before the combined ANOVA, the homogeneity of the residual variances of the environments was assessed using Hartley's Fmax test (Cruz and Regazzi, 2001) at a 5% probability to ensure the feasibility of the combined analysis of variance.

The combined ANOVA was conducted after the residual variances of all the environments were considered homogeneous ($p > 0.05$), where the effect of genotype was considered fixed and the effects of the environment and block were random (Cruz and Regazzi, 2001). When an interaction is verified, adaptability and stability analysis through the GGE biplot method can be used (Gesteira et al., 2018). After the existence of a GxE interaction had been verified, the GGE biplot method was applied. Graph preparation and individual and combined ANOVAs were performed in the R environment (Cruz and Regazzi, 2001). The GGE biplot methodology is as follows:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \Phi_{ij} \quad (1)$$

Where Y_{ij} is the expected performance of genotype i in environment j; μ is the general average of the observations; α_i is the main effect of genotype i; β_j is the main effect of environment j, and Φ_{ij} is the interaction between genotype i and environment j. Therefore, in this analysis, phenotypic variation is the result of the genotypic effect (α_i), environment (β_j), and interaction between genotype and environment (Φ_{ij}) (Olivoto and Lúcio, 2020). In this methodology, only the main effects of the genotype and GE are important and should be concurrently considered.

According to Gesteira et al. (2018), since the data were unbalanced and the analysis was based on a mixed model, a joint deviance analysis was performed considering all the environments (site-year combination) following the model:

$$y_{ijk} = \mu + g_i + a_k + b_{j(k)} + ga_{ik} + \varepsilon_{ijk} \quad (2)$$

Where y_{ijk} is the phenotypic observation of line i in block j in environment k, μ is the overall average, g_i is the effect of line i (fixed nature), a_k is the effect of environment k (random effect), $b_{j(k)}$ is the effect of block j in environment k (random effect),

Table 2. Agronomic characteristics.

Genotypes	Code	Yeld (Kg.ha^{-1})	Repening cycle	Growth habit	Origin
Wámini	G1	3000	Precocious	Determinant	IITA
Wima	G2	3500	Median	Indeterminant	IITA
10E	G3	-	-	-	IITA
Safari	G4	-	-	-	IITA
Zamboane	G5	3500	Precocious	Semi-erect	IITA

Source: Author's computations
G1_Wamini, G2_Wima, G3_10E, G4_Safari, and G5_Zamboane; IITA_International Institute for Tropical Agriculture.

ga_{ik} is the effect of the interaction of line i and environment k (random effect), and ε_{ijk} is the error associated with the observation of line i in block j in environment k.

Tests for normality and homogeneity of variances

Through the *metan* package, graphs were obtained, and all individual and combined ANOVAs were performed using R software (Olivoto and Lúcio, 2020). Shapiro-Wilk (Hartley, 1950) and Bartlett (Bartlett, 1937) tests were performed for soybean yield before the individual ANOVA in each of the 7 environments. Then, Hartley's Fmax test (Shapiro and Wilk, 1965) indicated homogeneous error variances among the evaluated environments, which enabled us to conduct the combined ANOVA. The assumption of homogeneous variances and normality of the error was proven, so the ANOVA could be validated (Maleia et al., 2017).

RESULTS AND DISCUSSION

Table 3 summarizes the joint analysis of variance with mean squares and significance for the F test. A significant difference was observed for the genotype and environment interaction (GxE) of a complex nature due to fluctuations in the ranking of the productive performance of the genotypes when grown in the tested environments. According to Kedir and Letta, (2022), the significant GxE interaction indicated that the performance of the genotypes in quality traits was not consistent over environments; some genotypes performed well at some locations but poorly at other locations. The significance of these effects was also found by Silva et al (2016) when evaluating 12 soybean genotypes, and by Soares et al. (2017) who observed a (GxE) interaction of a complex type on the grain yield of soybean genotypes. The large occurrence of GXE interactions causes the relative rankings of genotypes to change from location to location (Kedir and Letta, (2022).

When the existence of interaction is verified, adaptability and stability analysis can be conducted using the genotype + genotype + environment (GGE) biplot method. Several studies have reported the use of this method, especially for grain yield. The GGE biplot analysis was used to identify the best line in each environment and assess the stability of the lines. The most attractive feature of GGE biplots is the 'which-won-where' analysis, in which crossover GE interaction, mega-environment differentiation, and specific genotype adaptation are graphically represented (Rakshit et al., 2014; Oral et al., 2018).

GGE biplot analysis

The eigenvectors principal component 1 (PC1) and principal component 2 (PC2) cannot be directly plotted before singular values are partitioned into genotypes and environments (Silva and Benin, 2012). The first main component (PC1) indicates the adaptability of the genotypes, thus being highly correlated with grain yield (Yan et al., 2000). According to Yan (2001), the GGE biplot analysis is considered satisfactory when the sum of the 2 main components PC1 and PC2 explains more than 74% of the total variance due to G+GE. The results obtained for PC1 and PC2 (Figure 1) were 89.61 and 8.2%, respectively and the first 2 PCs explained 97.81% of the total variation in G+GE in grain yield; thus, the analysis of the genotype x environment interaction (GEI) using the GGE biplot was efficient. These results corroborate those obtained by Amira et al. (2013) who found in trials with early soybean strains that the first 2 PCs explained 86.6% of the interaction effect. Gesteira et al. (2018) studied the selection of early soybean inbred lines using multiple indices and reported that the first 2 PCs explained 77.39 and 4.84 of the variation, respectively.

According to Silva and Benini (2012), when the cosine of the angle between the genotype and the environment is analyzed, there is greater accuracy in the identification of positive associations, as it is no longer visual and is analyzed mathematically. Genotypes that are located in the same quadrant as environments are positively associated with those environments; the smaller the distance observed between the genotype and the environmental marker, the genotype will be strongly associated (adapted).

As shown in Table 4, the genotype G1 was found to have a strong (0.7) association (correlation) in the environments E1 and E3. However, the same genotype had a different behaviour in the environment E6 with a

Table 3. Summary of the combined ANOVA of soybean grain yield (kg.ha^{-1}).

Source of variation	DF	Mean square
ENV	6	2392.8*
REP (ENV)	21	573.9
GEN	4	34.7^{ns}
GEN x ENV	24	27.7*
Residue (Error)	84	14.7
Total	139	
Overall average		9.79
CV (%)		39.2

Source: Author's computations
*Significant at a 1% probability, ** Significant at a 5% probability, ns: Non-significant, DF: Degrees of freedom.

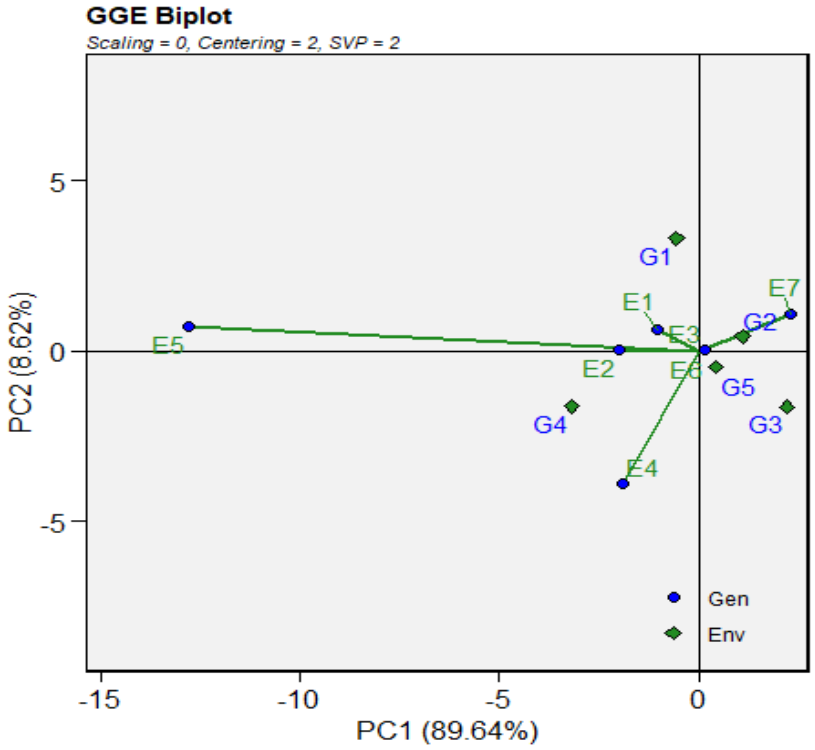

Figure 1. GGE biplot with the effects of the first two principal components (PC 1 vs. PC 2) for the 5 genotypes evaluated in 7 environments.
The genotypes are represented by the blue coloration, whereas the environments are represented by the green coloration. E1_Namapa, E2_Montepuez, E3_Namapa, E4_Montepuez, E5_ Ribaué, E6_ Namapa, and E7_ Montepuez.

very strong correlation of (0.9). The genotype G2 had a strong correlation of (0.7) only with the environment E7, a different behavior was verified with the genotype G3, where a moderate correlation (0.5) with the environment E4 was observed. On the other hand, the genotype G3 had a strong correlation (0.8) with the environment E7.

According to Goa et al. (2022), environments within the same sector had a small angle (<90°) between themselves and had a high positive correlation with each other. Genotypes at the vertex of a sector had the highest positive GxE interaction with environments in that sector and the largest in absolute value negative GEI with environments on the opposite side (at 180° with them). Environments at 90° from each other are uncorrelated while an angle of > 90° indicates a negative correlation between the environments. The genotype G4 had a strong correlation of (0.7) with the environments E2, E4, and E7. Finally, the genotype G5 had a negligible

Table 4. Description of genotypes and environments correlations.

Genotype	Code	Environment
Wámini	G1	E1, E3, and E6
Wima	G2	E7
10E	G3	E4 and E7
Safari	G4	E2, E4, and E6
Zamboane	G5	E4 and E7

Source: Author's Computation
G1_Wámini, G2_Wima, G3_10E, G4_Safari, and E1_Namapa, E2_Namapa,E3_Namapa, E4_Montepuez, E5_Ribáuè, E6_Montepuez, E7_Montepuez.

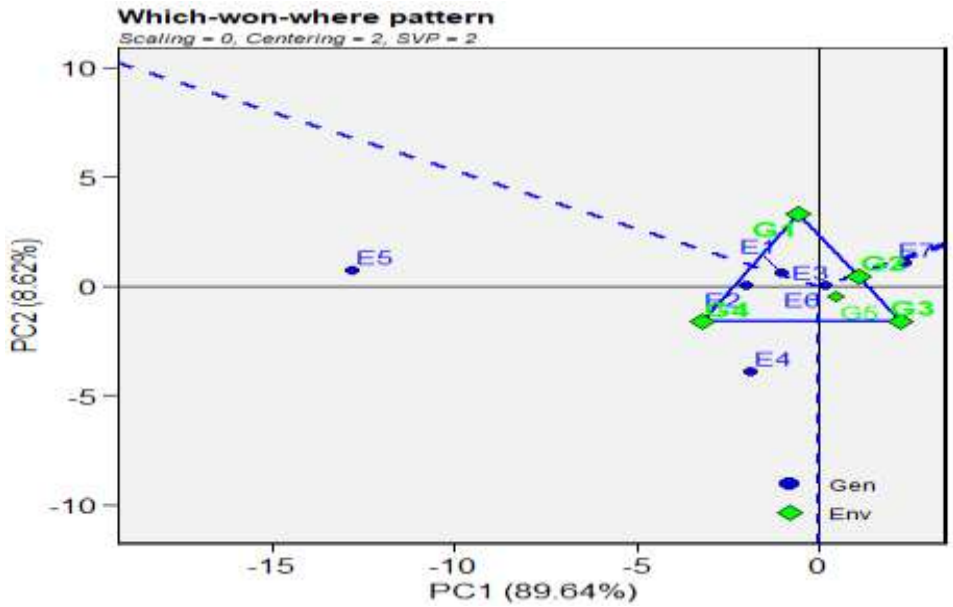

Figure 2. GGE Biplot ("Which-won-where") for data on grain yield characteristics of five soybean genotypes in eight environments.
G1_Wamini, G2_Wima, G3_10E, G4_Safari, G5_Zamboane. E1_Namapa, E2_Montepuez, E3_Namapa, E4_Montepuez, E5_ Ribaué, E6_ Namapa, and E7_ Montepuez.

correlation with the environment E4 and a moderate correlation of (0.5) with the environment E7. The environment E5 did not correlate with any genotype.

Mean vs stability of genotypes

The most attractive feature of GGE biplots is the "which-won-where'' analysis, where the GxE interaction, mega-environment differentiation, and specific genotype adaptation are graphically represented (Rakshit et al., 2014; Oral et al., 2018). The visualization of a "which-won-where" pattern in multi-environment trials is essential to studying the possible existence of different mega-environments in a region (Yan and Tinker, 2006). In Figure 2, the 5 soybean genotypes are labeled G1 to G5, and the 7 environments are labeled E1 to E7. At the vertex of the polygon, the genotypes with the best average performance for GY (grain yield) are indicated, and the G1, G4, and G3 genotypes are the vertexes of the polygon in the sector that contains environments E1, E3, E5, E2, E4, and E6. Genotype G3 is the vertex of the sector where environment E5 is placed, so it has the best performance in this environment. The same is true for genotype G1 in environments E1, E3, and E7 and genotype G4 in environments E2, E4, and E6. According to Singh et al (2019), the vertex genotypes are located at the greatest distance from the biplot origin. The genotypes with the best or the poorest performance in one or all environments were considered responsive (Yan and Tinker, 2006) and fell within the sectors.

Genotypes G2, G3, and G5 had the highest

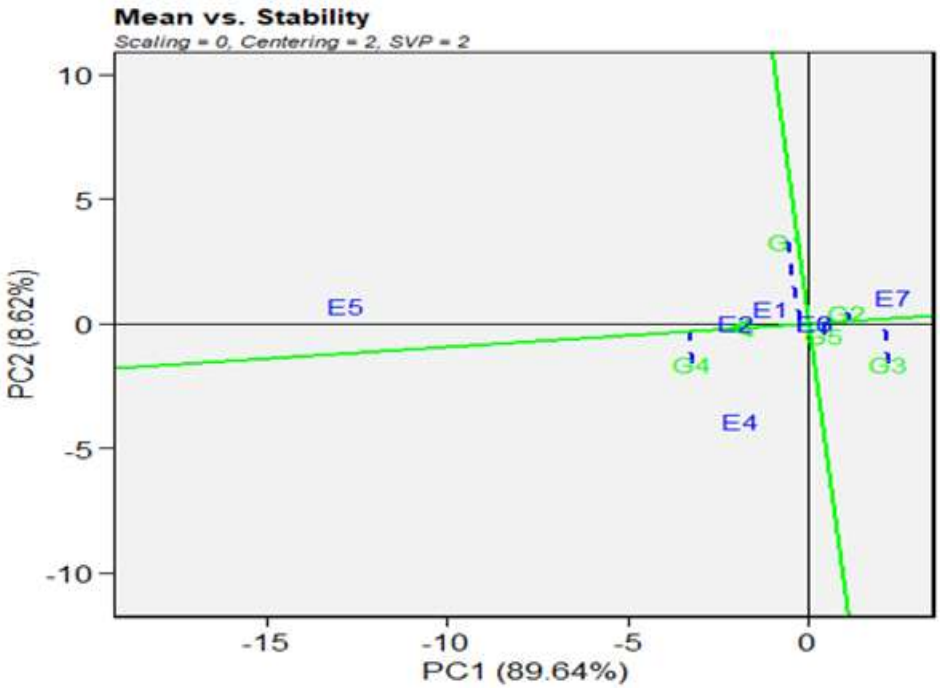

Figure 3. The GGe biplot ("Average versus Stability") with environment axis (EAM) shows the average performance and stability of the five genotypes.

left indicates lower performance. Stability is visualized on the y-axis, where farther from the center (o) of the biplot indicates lower stability (Gabriel, 1971; Kaya et al., 2002).

There were formed 2 mega-environments (Figure 2): i) E1, E3, and E7 and ii) E2, E4, E5, and E6. The environments located in the same sector constituted a mega-environment (Yan et al., 2007). According to Yan et al (2000), a mega-environment can be defined as a positively correlated group of environments or sub-regions where a genotype or a group of genotypes has specifically adapted and achieved better performance. In mega environment 1: i) E1, E3, and E7, the genotype G4 was specifically adapted and achieved better performance. On the other hand, in mega-environment 2: ii) E2, E4, and E6, the genotypes G2, G3, and G5 were specifically adapted and achieved better performance. However, the genotype G1 was not located in any of the 2 mega-environments, being the worst genotype.

Gonçalves et al. (2020) evaluated 16 soybean genotypes in 8 environments in the 2015/2016 and 2016/2017 agricultural seasons using the GGE biplot method and found 2 mega-environments: I) E1, E4, E6, and E3 and II) E2, E5, and E8. Singh et al. (2019) found the same results, where 50 wheat genotypes were evaluated at 9 diverse locations in India. The test locations were partitioned into 2 mega-environments: i) E1, E8, E3, E4, and E7 and ii) E5, E2, and E6. Regardless of species, the GGE biplot analysis manages to highlight at least one year or environment that presents a different influence on the performance of the progenies (Hongyu et al., 2015).

Identification of the ideal genotype based on GGE

The GGE-biplot allows the detection of genotypes close to the ideal genotype (Kaya et al., 2002). In Figure 3, the genotypes are classified according to their average grain yield as follows: G1<G4<Average<G5<G2<G3. Genotype G4 was highly unstable but had good performance compared to that of the other genotypes.

The cultivar to the right of this axis has a greater yield, and the line perpendicular to this line with 2 arrows measures the stability or instability of the cultivars. Any genotype closer to this line will be more stable (Hongyu et al., 2015). An ideal genotype is a genotype with a high mean yield and exhibits very little yield change in different environments. Therefore, stability analyses are an important part of the breeding programs (Ilker et al., 2018). Although such an ideal genotype may not exist in reality, it can be used as a reference for genotype evaluation because a genotype that is located closer to the ideal genotype is more desirable (Kaya et al,, 2002).

Genotype G3 was the ideotype, followed by G2 and G5. Although G4 had a higher average yield, this genotype proved to be highly unstable and was the worst genotype. Genotypes closer to the ideal genotype and at the same time closer to zero by PC2 of the GGE biplot are considered to be the most stable ones; while genotypes far from the ideal genotype and far from

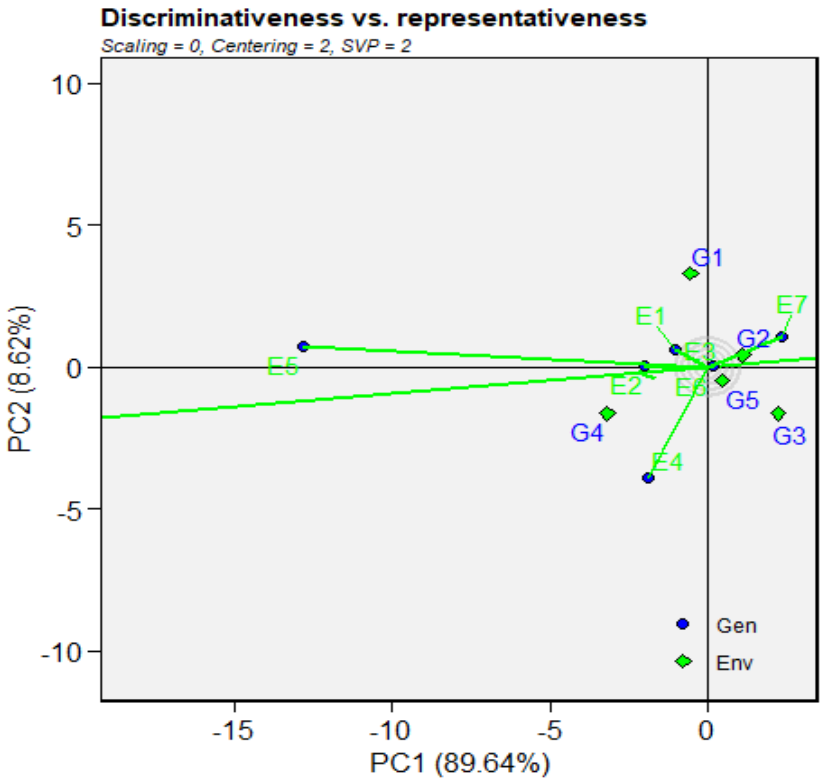

Figure 4. The GGE biplot "discrimination and representativeness" of eight test environments, based on the grain yield of five soybean genotypes. E1_Namapa, E2_Montepuez, E3_Namapa, E4_Montepuez, E5_ Ribaué, E6_ Namapa, and E7_ Montepuez.

PC2=0 in both directions are considered to be unstable (Kedir and Letta, (2022).

Test environment assessment

The relationship among test environments was studied based on environment-centered (centering 2) and environment-metric preserving (SVP, 2) without a scaling option (Singh et al., 2019). The "Discrimination vs. Representativeness" biplot is an effective tool for defining the best environments in which to evaluate genotypes (Frutos et al., 2014). Therefore, a specifically adapted genotype to a particular environment could be conveniently described by employing this type of graphical representation (Plavsin et al., 2021).

The objective of test environment evaluation is to effectively select superior genotypes for a mega-environment; the selection of a test environment must be based on greater discrimination of genotypes and representativeness (Hongyu et al., 2015). Figure 4 is a ranking biplot for comparison of the environments with the ideal environment. Discriminating and representativeness are the most important parameters of the GGE biplot when evaluating an environment. In Yan and Thinker (2006) model, along environmental vector had a high discriminating ability and a short one had low discrimination.

According to Ansarifard et al. (2020), the best environment is the one that has the closest distance from the ideal environment (concentric circles) and the most undesired one is the environment with the farthest distance to the ideal environment. As shown in Figure 4, environments E1 and E2 were the closest, being the most representative. However, the discriminating but not representative environments were E4 and E5 environments, respectively.

The best environment was the environment E3 for the selection of genotypes. The same results were observed by Peprah et al. (2016), for cassava productivity, where environments with vectors longer than the genotypes were discriminating, and no genotype was more discriminating environments. According to the discrimination and representativeness, the preferable genotypes for each environment are classified, as G1 is preferable in environments E1, E3, and E7; G4 in environments E2, E4, and E6; G2 in environments E7; the genotypes G3 in environments E4 and E7, and genotype G5 in environments E4 and E7. However, none of the genotypes were preferable for environment E5.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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