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A comprehensive Assessment of Sunflower Genetic Diversity Against *Macrophomina phaseolina*

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ABSTRACT
The sunflower is a significant oil crop that can be cultivated in various environmental conditions. Due to the changing climate, the pathogen profile has been altered, posing a threat to sunflower production. Among the various threats, charcoal rot, caused by the soil-borne fungus *Macrophomina phaseolina* (Tassi) Goid, is one of the most significant pathogen. This study aimed to investigate the resistance of 80 sunflower inbred lines to this pathogen using two inoculation methods and naturally infested area under field conditions in two years, 2019 and 2020. The results showed that both inoculation methods and occurrence of disease in naturally infested area (DNI) effectively differentiated between resistant and susceptible inbred lines, with the toothpick method being the most effective. Thirteen inbred lines were resistant according to all inoculation methods, and the others were moderately resistant moderately susceptible or susceptible regarding to inoculation method. The study identified five inbred lines (Ha 74, L1, LIV 10, MA SC 2 and PB 21) as the most resistant, making them important sources for breeding sunflower hybrids resistant to *M. phaseolina*. Their resistance was confirmed in 2020, highlighting their potential to combat the impact of climate change on sunflower production. This study represents a valuable insight into the control of *M. phaseolina* using sunflower resistant genotypes, especially since resistance findings have been lacking in other plant species.

Keywords: Charcoal rot, Disease severity, Inbred lines, Inoculation methods, Sunflower

1. Introduction

Sunflower (*Helianthus annuus* L.) is an important oil crop that is grown on more than 28 million hectares worldwide, primarily in temperate, semi-dry regions (Miklič 2022). The major producers of sunflower seeds are Russia, Ukraine, the European Union, and Argentina, which collectively account for over 75% of total sunflower seed production (USDA 2023). Sunflower has advantages over other oil crops due to its adaptation, i.e., its ability to grow in different agroecological conditions, and its moderate drought tolerance attributed to a well-developed root system (Debaeke et al. 2017). However, the changing climate is affecting the pathogen profile, jeopardizing sunflower production. One of the severe threats to sunflower production is charcoal rot caused by a soil-borne fungus, *Macrophomina phaseolina* (Tassi) Goid. Unlike most pathogens, *M. phaseolina* prefers in warm and dry conditions, and can spread within the host within 24 to 48 hours after infection (Khan 2007), especially in conditions of water deficient yields can be significantly reduced (Özelçi et al. 2022). The severity of the disease can be extremely high, and the changing climate has led to the emergence of this pathogen in other European countries with continental climates. Isolates from these countries are more adapted to lower temperatures than those from tropical and subtropical regions (Veverka 2008). Moreover, *M. phaseolina* is a worldwide crop pathogen that can affect over 700 plant species and has a broad geographic distribution (Schroeder et al. 2019; Dell'Olmo et al. 2022).

In field production of sunflower, symptoms of *M. phaseolina* infestation can be visible after seed filling, resulting in premature ripening and complete yield loss (Mahmoud 2010; Chattopadhyay et al. 2015). The infestation can cause a reduction in seed yield of up to 20% (Jordaan et al. 2019; Qamar & Ghazanfar 2019), and in severe cases, yield can be reduced by 75% or 90% (Mahmoud 2010; Ijaz et al. 2013). The stems of affected plants lose their green colour, and grey discoloration appears from the lower part of the stem and spreads to the upper parts, making it challenging to intervene in crops towards the end of the vegetation period (Bokor 2007). Other symptoms on sunflower that can confirm presence of disease are, reduced head diameter, premature ripening, absence or compression of pith in the lower part of the stem, and microsclerotia presence in the middle area

of stem and on the main root (Mahmoud & Budak 2011). Therefore, it is essential to develop suitable field screening methods for *M. phaseolina* to obtain accurate information on its impact on sunflower plants.

Controlling the pathogen is a difficult task due to its ability to survive in various conditions, and no existing fungicides are available. Therefore, the most effective way to control the disease is by using resistant genotypes (Cotuna et al. 2021). To ensure ecologically acceptable sunflower production, it is necessary to use highly resistant hybrids to economically important pathogens (Seiler et al. 2017). The production of high-quality sunflower genetic material requires constant monitoring of the interactions between the sunflower as a host, economically significant diseases, and the environment, as well as the type of existing resistance (Škorić 2016; Tančić-Živanov et al. 2021). Successful breeding for disease resistance involves monitoring the interactions between the sunflower, specific pathogens, and the environment, assessing the stability of sunflower resistance to certain pathogens, and applying general principles of resistance breeding. However, genetics of resistance against *M. phaseolina* has not been fully elucidated and different findings have been reported. Talukdar et al. (2009) reported a continuous distribution of soybean reaction to *M. phaseolina*, ranging from highly susceptible through moderately resistant, to highly resistant. This suggests that disease resistance is influenced by multiple loci, thus making breeding for resistance difficult. Khan (2007) reported that sunflower tolerance to *M. phaseolina* has been horizontal, controlled by polygenes and completely resistant genes do not exist. All commercial sunflower cultivars are susceptible, especially expressed among hybrids with short vegetation and for genotypes in arid areas (Kaya 2016). Thus, only a moderate resistance level has been found in cultivated sunflower germplasm (Tančić et al. 2012; Ijaz et al. 2013; Jalil et al. 2013) and the wild relatives (Tančić et al. 2012; Seiler et al. 2017; Warburton et al. 2017; Shehbaz et al. 2018).

The rising abiotic and biotic stresses associated with global climate change necessitate the development of climate-ready sunflower capable of withstanding stress and providing stable yields (Radanović et al. 2022). Climate changes deliver unpredictable rainfall patterns resulting in more extended and more frequent periods of drought (Masalia et al. 2018). Environmental factors are limiting for the disease appearance caused by *M. phaseolina*, and high humidity immediately after infection could completely stop the disease development, while dry and warm period will boost formation of microsclerotia in the stem and finally lead to symptoms appearing at the plant maturity stage.

The aim of this study was to assess the response of 80 sunflower inbred lines to *M. phaseolina* infection, by analyzing disease symptoms, disease incidence, and McKinney index. Two primary goals were to identify potential sources of resistance to *M. phaseolina* and to evaluate different inoculation methods during two-year trial to determine the most effective approach. The identification of sunflower inbred lines with resistance to *M. phaseolina* would enable the timely improvement of preferred hybrids through resistance introgression, leading to the production of high-quality sunflower.

2. Material and Methods

2.1.Plant material

Eighty sunflower inbred lines were selected from the sunflower broad gene pool at the Institute of Field and Vegetables Crops (IFVC) Novi Sad, Serbia (Anđelković et al. 2020). Ensuring divergence among the lines in terms of various characteristics such as origin, maturity, morphological traits, disease tolerance, type, and general agronomic properties (Table 1). Some of the inbred lines were previously evaluated for resistance to *M. phaseolina*, and only those exhibiting a certain level of resistance were selected for this study (Tančić-Živanov et al. 2021).

Table 1- List of 80 sunflower inbred lines inoculated in Rimski Šančevi Novi Sad, 2019, with three inoculation method and evaluated for resistance to *Macrophomina phaseolina*

No	*Inbred lines*	*Traits of interest*	*No*	*Inbred lines*	*Traits of interest*
1	AB-OR-8*	Medium early, OR	41	IMI AB 24 PR	IMI
2	AB-OR-ST-50	Medium early	42	KINA-B-5	Medium late
3	AB-OR-ST-62	Medium early	43	KINA-H-25	Late
4	AR-KOR-10	Medium early	44	L1 *	Medium early, good GCA
5	AR-7	Bright leaves	45	LIP P 16	Early
6	AS 1 PR	HO	46	LIP P 32	resistant to *O. cumana*
7	AS 87*	Medium early, good GCA	47	LIP P 98*	ultra-early, resistant to *O. cumana*
8	AS 95 PR	High 1000 seed mass	48	LIV 10	Medium early, OR
9	AZDO-2	Late	49	LIV 17	OR
10	BT-VL-24	OR	50	MA-SC-2*	Medium late, good GCA
11	BT-VL-17-SU	SU	51	NS BW 3	White seed colour, birds
12	CMS 1-90	Good GCA, PH	52	NS KOD 10	OR
13	CMS1 122	Late	53	OD-DI-32	Early
14	CMS1 30*	Medium early	54	OD-DI-47	Early
15	CMS-III-8	PH	55	OD-DI-49	Medium early
16	DEJ-10	Dwarf	56	OD-DI-80	Good GCA
17	DF AB 2 *	Late, good GCA	57	OD-DI-83	Good GCA
18	DI-42	Medium late	58	ODESSA 4*	Medium early
19	DM 3	Resistance to rust	59	OR 26 PL	OR
20	DOP 27 08	HO	60	PB-21*	Medium early, resistant to rust
21	DOP 32 08	HO, tolerant to *Phoma macdonaldi*	61	PH BC1 92	PH
22	FE 49	Late	62	PH BC2 67	PH
23	FE 54	OR	63	PL-DI-25*	Early, good GCA, *Pl6* gene
24	FE 7	OR	64	POP 3	Resistance to rust
25	Ha 22	PH, good GCA	65	PR-ST-3	PH, good GCA
26	HA 26*	Medium early, good GCA	66	PR ST 28	Late
27	Ha 26 OL ARG	HO	67	PR-2648-2	Good GCA
28	Ha 267	OR	68	RNS P 10	Ultra-early
26	Ha 412 HO	HO	69	RNS P 2	Ultra-early
30	Ha 431	Resistant to rust	70	RS O 2	Ultra-early
31	HA 441	Tolerant to *Sclerotinia spp.*	71	RUB-3 *	Medium early
32	HA 444	High oleic	72	SAM-INTER-3	Dwarf
33	HA 465	High tolerant to *Sclerotinia spp.*	73	SAN 3	Ultra-early maturity
34	Ha 48	Late	74	SAN 35	Ultra-early maturity
35	Ha 74*	Medium early, PH	75	SC MI 4	Good GCA
36	Ha R 3	Resistant to rust	76	SU-AB-4-PR	SU
37	Ha 458	HO	77	UK 58 ST	HO
38	Ha-98	PH	78	V 8931-3-4-OL	HO
39	IMI AB 12 PR*	Late, IMI	79	VL A 8 PR	OR
40	IMI AB 14 PR	IMI	80	VL-3	Early

GCA-general combining ability; IMI-tolerant to imidasolinone herbicides; SU-tolerant to sulphonyl urea herbicide; HO - high oleic OR-resistant to *O. cumana*; PH-tolerant to *Phomopsis spp.*; HO-high oleic line

2.2. Isolate characteristics

The isolate of *M. phaseolina*, was selected from collection of 50 isolates based on pathogenicity test. Infected plant tissue was placed in paper bag for air drying, after that plant samples were stored at 4 °C. Before of testing the isolate, the stems were washed under running tap water for half our and left to dry on sterile filter papers. Subsequently, small cuts from stems exhibiting

visible microsclerotia underwent surface sterilization in two steps: in 70% ethanol (C_2H_5OH) for three minutes, and in 1% sodium hypochlorite (NaOCl) for an extra three minutes. These sterilized stem sections were plated onto potato dextrose agar (PDA) and incubated at 30 ºC for 24 hours. The isolated hyphal tips were then transferred to new PDA plates to establish fresh, uncontaminated fungal colonies.

To determine the most aggressive *M. phaseolina* isolate, a pathogenicity test was conducted. A 4 mm mycelial disc from a colony was placed at the center of a PDA plate and allowed to incubate for four days. Once the PDA plate was entirely colonized by *M. phaseolina*, ten de-hulled sunflower seeds from the inbred line Ha 26 were introduced onto each colony. After six day seed were evaluated according to Rayatpanah et al. (2012) scale: 0= completly healthy seed, 1= seedling discoloration on the contact with the mycelium, 2= seed teguments covered with and healthy seedling, 3= seed teguments cowered with microsclerotia and infected seedlings 4= bouth tegument and seedling infected 5= infected seed, not germinated.

2.3. Trial setup and plant material

The experiments were conducted at the Sunflower Department's disease testing nursery of the Institute of Field and Vegetable Crops in Rimski Šančevi, Novi Sad, in 2019 and 2020. On April 18th, 2019, the 80 selected inbred lines from the sunflower gene pool at the IFVC Novi Sad, Serbia were planted. However, inbred line PR-ST-28 was eliminated due to its low emergence, leaving 79 inbred lines for *M. phaseolina* resistance screening at the end of the growing season. Based on their resistance levels, 15 inbred lines were chosen for the following year's trial, which were planted on May 5th, 2020. The meteorological data analysis for the two-year weather conditions is presented in Figure 1 (RSRHZ 2023).

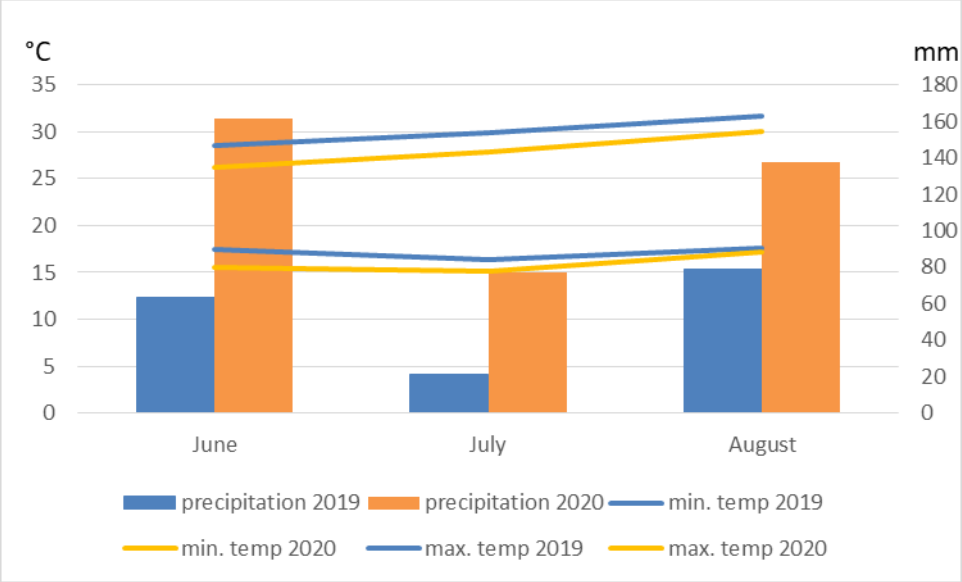

Figure 1- Meteorological data for the vegetation season (june-august) 2019 and 2020 in Novi Sad, Serbia
Min t - minimal temperature, Max t - maximal temperature

2.4. Inoculation methods

Two inoculation methods, the Unwounded Stem Base Inoculation (USBI) method and the toothpick (TP) method, were used to artificially inoculate the sunflower inbred lines. These two inoculation methods are different from each other since toothpick method represents an aggressive method, which requires artificial tissue penetration, and plants show the reaction to stop pathogen which is already in the plant. The USBI method is less aggressive and this method doesn't require artificial plant injury, and pathogen itself struggles autonomously to penetrate the plant tissue. The trials were set up as a Complete Randomized Block Design (CRBD), with each inbred line planted in three replications and each replication comprising three rows, resulting in a total of 36 plants per replication (3 x 12 plants). The plants were spaced at 0.7 m between rows and 0.3 m within rows, and no irrigation was applied.

In the first group (12 plants per inbred line and replication), the USBI method was applied 30 days after emergence, and each plant was inoculated by digging area around plant where is 2g mixture of maize flour and microsclerotia evenly distributed all around the plant stem. This mixture was contains: maize flour and sand medium in a ratio of 1:20, which was also contain 5 discs (4 mm in diameter) from the edge of the 4 day old *M. phaseolina* colony grown on PDA incubated at 30ºC. In order to obtain quality distribution of microsclerotia mixture was shaken every second day, until 14th day, when mixture was prepared for use (Mihaljčević 1980). The second group (12 plants per inbred line and replication) was inoculated using the TP method by inserting infected toothpicks into the stem tissue 1 cm above the plant's first node (Jiménez -Díaz et al. 1983). Toothpicks were

sterilized and placed in laboratory glass cup and then PDA was a poured all over toothpicks. This glass was incubated on 30 ºC in order to microclerotinia cover all toothpicks. Incubation of toothpicks lasted for 7 days. The third group (12 plants per inbred line and replication) was grown in natural conditions, marked as a disease in naturally infested area (DNI). In this group of plants disease occurrence without artificial inoculation was observed.

2.5. Data anlyses

At the maturity stage (R8) of sunflower, symptoms of *M. phaseolina* infection were evaluated (Schneiter & Miller 1981). Each plant stem was longitudinally cut (photo 1)., and the length of the tissue with visible microsclerotia was measured and evaluated with grades from 0 to 8: (0-(0-5 cm); 1-(5-10 cm), 2-(10-20 cm); 3-(20-30 cm); 4-(30-40 cm), 5-(40-50 cm); 6-(50-60 cm); 7-(60-70 cm): 8-(more than 70 cm). Any plants displaying symptoms of other diseases were removed before the end of the growing season.

Photo 1: Longitudinally cut of sunflower stem with visible multiple microsclerotia and damages of infection with *Macrophomina phaseolina*

Disease incidence and McKinney index were calculated for each inbred line in each year, method, and DNI (McKinney 1923). Disease incidence (I) was calculated as the proportion of diseased plants, which represents percents of plants with symptoms:

$$I = \frac{x}{N} \times 100$$

The number of plants with symptoms (x) divided by the total number of plants examined (N), multiplied by 100.

McKinney index is estimated as a value on the interval scale and has been used to determine a disease severity index on a percentage basis. (M) was calculated according to the formula:

$$M = \frac{\varepsilon xi}{nin} \times 100$$

Where; x represents the disease grade according to the area covered with microsclerotia ∑xi represents the sum of every plant's grade; ni represents the highest grade of the scale – 8, and n represents the total number of diseased plants evaluated. The results were expressed as a percentage (%).

The inbred line reaction to *M. phaseolina* was evaluated MPR (*M. phaseolina* reaction) using the proposed scale for each tested method. The obtained data were used to classify inbred lines McKinney index (Table 2).

Table 2- Proposed scale of sunflower inbred lines to *Macrophomina phaseolina* reaction according to McKinney index

MPR of inbred lines	*McKinney index*
Resistant (R)	0-5%
Moderately resistant (MR)	5.1-20%
Moderately susceptible (MS)	20.1-40%
Susceptible (S)	40.1-100%

MPR -*Macrophomina phaseolina* reaction;

The Kruskal-Wallis test together with pot hoc Dunn's test was used to analyze statistical differences in the resistance of inbred lines to *M. phaseolina.* In order to control the familywise error rate, it was used Holm stepwise adjustment (Dinno 2015). To visualize the experimental results, a UPGMA cluster analysis of previously standardized data by z-score was performed by using IBM SPSS 25, and PAST 4.10. Cluster graphic was followed by a heat map that categorized the inbred lines into according to resistance, implicating that inbred lines with dark blue colour are more resistant than others, while brighter colours are representing lower level of resistance and eventually red colour represent sensitive inbred lines. Levels of resistance to *M. phaseolina* and highlighted differences between inoculation methods and disease in naturally infested area. Additionally, a Kurskal-Wallis test was conducted to compare the values of McKinney index in 2019 and 2020 for the 15 selected lines.

3. Results and Discussion

3.1. Macrophomina phaseolina characteristic and isolate pathogenicity

In order to choose the most aggressive *M. phaseolina* isolate for the field experiments, we tested 50 isolates collected from sunflower production areas in Serbia. According to the pathogenicity test, the isolate with the highest grade (Indjija, Serbia 45°4′8"N, 20°3′21"E – named MPIN18) of aggressivenes was chosen for further work (Ćuk et al. 2022). This isolate was obtain grade 5 for every examinated seed, which is maximal grade (data not shown).

3.2. Weather conditions

Relying on natural infestation and pathogen attack for testing breeding material is unreliable due to dependence on environmental conditions and non-homogeneous inoculum distribution in the soil (Van der Heyden et al. 2021). In that context, the two-year weather conditions varied during the vegetation season, having an unusual relationship between rainfall distribution and average daily temperature. Average temperatures were lower in 2020, followed by heavy rains but distributed in fewer days. The temperature range for the optimal development of *M. phaseolina* can vary between 25 to 35 ºC, so the temperatures for *M. phaseolina* were optimal for both years (Parmar et al. 2018). However, higher temperatures in 2019 and less rainfall were more suitable for *M. phaseolina* development, affecting the sunflower's infestation level of inbred lines.

3.3. Filed experiment in 2019

Inbred line PR-ST-3 was eliminated due to its low emergence, leaving 79 inbred lines for *M. phaseolina* resistance screening at the end of the growing season. According to every plant disease grade, the variability of these inbred lines to *M. phaseolina* was

confirmed using Kruskal-Wallis test showing significant differences for both tested methods and DNI (for all methods, $p<0.01$). However, the reaction of inbred lines varied due to the inoculation method. Therefore, it is necessary to examine in more detail the reaction of inbred lines depending on the infection of a particular method.

In this study, 79 sunflower inbred lines were evaluated for their resistance to *M. phaseolina*, and disease incidence and McKinney index of every inbred line were calculated (Table 3). According to the strictest MPR, that inbred lines obtain in table 3. Thirteen inbred lines were found to be resistant, while 16 were susceptible, and the remaining lines were classified as moderately resistant or moderately susceptible in year 2019. Inbred lines (FE 49, HA 74, HA 458, L1, LIV 10, LIV 17, MA-SC-2, PB 21, PH BC 1 92, SAM INTER 3, SAN 35, AR-7, VL A 8 PR) were resistant and they also showed low disease incidence and low McKinney index value, indicating high resistance level or slowed disease progress due to the complexity of interaction between host and pathogen. The biotrophy-necrotrophy switch in pathogen evokes differential response results, showing that the host tailored its defence strategy to meet the changing situation (Chowdhury et al. 2017).

Table 3- Disease incidence and McKinney index for *M. phaseolina* resistance of 79 sunflower inbred lines inoculated using Toothpick (TP) method and Unwounded Stem Base Inoculation (USBI) method and in disease in naturally infested area (DNI) obtained in Rimski šančevi, Novi Sad in 2019

	Disease incidence (%)			*McKinney index (%)*					
Inbred lines	*TP*	*USBI*	*DNI*	*TP*	*grade*	*USBI*	*grade*	*DNI*	*grade*
AB-OR-8	92.59	80.57	42.90	52.15	**S**	34.09	MS	12.23	MR
AB-OR-ST-50	80.30	58.96	45.79	46.74	**S**	40.94	**S**	25.40	MS
AB-OR-ST-62	79.17	35.25	4.76	42.71	**S**	8.20	MR	1.19	R
AR-7	0.00	6.36	0.00	0.00	**R**	2.01	**R**	0.00	**R**
AR-KOR-10	25.76	12.12	3.03	9.70	**MR**	2.27	R	0.76	R
AS 87	83.33	53.33	38.74	45.23	**S**	22.08	MS	19.15	MS
AS 95 PR	61.95	68.89	25.00	26.30	**MS**	26.18	**MS**	7.19	MR
AS-1-PR	51.85	25.62	22.22	26.60	**MS**	9.56	MR	9.72	MR
AZDO 2	29.55	43.33	24.09	20.50	**MS**	29.93	**MS**	13.40	MR
BT VL 24	18.52	14.11	0.00	1.39	R	5.56	**MR**	0.00	R
BT-VL-17-SU	38.55	37.68	14.44	11.03	**MR**	16.53	**MR**	4.79	R
CMS 1-90	69.26	52.42	50.34	37.04	**MS**	30.18	**MS**	25.69	**MS**
CMS1 122	72.22	66.69	88.89	45.83	**S**	28.47	MS	18.06	MR
CMS1 30	100	77.77	33.33	27.08	**MS**	27.08	**MS**	4.17	R
CMS-3-8	34.43	23.23	15.34	11.40	**MR**	11.33	**MR**	3.74	R
DEJ-10	52.02	5.56	5.81	22.38	**MS**	1.39	R	2.56	R
DF AB 2	57.15	46.67	73.16	29.63	MS	26.62	MS	45.89	**S**
DI-42	28.24	25.45	28.55	12.96	**MR**	5.19	**MR**	8.23	**MR**
DM 3	70.37	51.82	64.88	27.43	**MS**	6.48	MR	41.36	S
DOP 27 08	30.95	33.59	31.82	13.10	**MR**	17.80	**MR**	16.77	**MR**
DOP 32 08	43.81	32.07	12.12	24.48	**MS**	9.34	MR	3.79	R
FE 49	14.95	9.09	2.78	4.84	**R**	2.35	**R**	0.69	**R**
FE 54	70.00	43.33	32.59	30.31	**MS**	16.59	MR	15.32	MR
FE 7	12.50	15.76	0.00	5.24	**MR**	3.11	R	0.00	R
Ha 22	85.71	38.85	36.72	44.40	**S**	11.99	MR	12.54	MR
HA 26	38.96	22.41	18.33	13.69	**MR**	4.10	R	3.65	R
Ha 26 OL ARG	47.62	29.63	43.96	16.07	**MR**	10.19	**MR**	13.76	**MR**
Ha 267	76.67	24.07	55.00	38.47	**MS**	6.89	MR	28.54	**MS**
HA 412 HO	66.45	43.45	51.85	37.17	**MS**	21.21	**MS**	22.57	**MS**
HA 431	65.74	30.81	17.5	17.49	**MR**	9.09	**MR**	0.83	**MR**
HA 441	35.21	33.81	19.47	13.66	MR	22.26	**MS**	7.29	MR
HA 444	23.54	4.17	5.56	6.71	**MR**	1.04	R	2.08	R
HA 465	30.97	36.29	3.03	6.02	**MR**	10.77	**MR**	0.38	R
Ha 48	50.51	21.06	24.05	27.78	**MS**	10.43	MR	22.94	**MS**
Ha 74	6.67	3.03	0.00	0.83	**R**	2.65	**R**	0.00	**R**
HA458	12.50	13.33	0.00	4.17	**R**	1.67	**R**	0.00	**R**
Ha-98	95.83	48.11	45.96	56.04	**S**	16.12	MR	14.74	MR
HA-R-3	67.58	81.53	73.57	33.03	MS	54.80	**S**	42.00	**S**
IMI AB 12 PR	69.44	61.82	51.91	47.43	**S**	43.07	**S**	42.94	**S**
IMI AB 14 PR	31.06	25.4	25.00	9.28	**MR**	12.57	**MR**	13.72	MR
IMI AB 24 PR	47.81	23.08	38.89	21.00	**MS**	8.97	MR	10.56	MR
KINA-B-5	88.90	83.33	48.26	73.49	**S**	53.54	**S**	30.79	MS
KINA-H-25	65.99	49.49	44.44	25.59	**MS**	12.25	MR	22.69	**MS**
L1	0.00	0.00	26.67	0.00	**R**	0.00	**R**	0.42	**R**
LIP P 16	81.67	64.47	56.75	33.75	**MS**	23.70	**MS**	13.75	MR
LIP P 32	27.94	9.09	7.04	8.29	**MR**	2.27	R	1.81	R
LIP P 98	72.62	57.43	91.11	9.67	**MR**	13.87	**MR**	11.81	**MR**
LIV 10	23.91	5.56	0.00	3.37	**R**	0.35	**R**	0.00	**R**

Table 3 (Continue)- Disease incidence and McKinney index for *M. phaseolina* resistance of 79 sunflower inbred lines inoculated using Toothpick (TP) method and Unwounded Stem Base Inoculation (USBI) method and in disease in naturally infested area (DNI) obtained in Rimski Šančevi, Novi Sad in 2019

	Disease incidence (%)			*McKinney index (%)*					
Inbred lines	*TP*	*USBI*	*DNI*	*TP*	*grade*	*USBI*	*grade*	*DNI*	*grade*
LIV 17	12.12	0.00	2.78	1.52	**R**	0.00	**R**	0.00	**R**
MA-SC-2	0.00	0.00	4.76	0.00	**R**	0.00	**R**	0.00	**R**
NS KOD 10	83.33	50.00	62.50	47.01	**S**	23.96	MS	36.98	MS
NS W 3	63.30	46.27	36.10	31.31	**MS**	16.46	MR	12.99	MR
OD-DI-32	40.68	29.6	6.48	9.09	**MR**	9.25	**MR**	4.40	R
OD-DI-47	31.61	16.67	4.76	12.49	**MR**	5.21	**MR**	1.79	R
OD-DI-49	68.35	49.95	67.09	41.2	**S**	24.39	MS	28.91	MS
OD-DI-80	76.72	53.41	43.81	22.29	**MS**	16.38	MR	11.79	MR
OD-DI-83	60.42	18.84	24.44	22.12	**MS**	5.54	MR	14.31	MR
ODESA 4	86.11	100	66.67	49.65	**S**	48.05	**S**	4.17	R
OR 26 PL	69.44	22.42	33.94	36.67	**MS**	9.36	MR	10.34	MR
PB-21	0.00	2.78	3.70	0.00	**R**	0.69	**R**	0.42	**R**
PH BC1 92	9.72	10.74	3.70	1.35	**R**	1.85	**R**	0.00	**R**
PH BC2 67	31.67	5.56	22.23	6.88	**MR**	0.52	R	0.00	R
PL-DI-25	84.40	79.55	67.58	41.27	**S**	43.53	**S**	29.17	MS
POP 3	72.22	54.88	35.00	40.97	**S**	24.37	MS	16.67	MR
PR ST 28	8.33	19.45	0.00	1.04	R	5.90	**MR**	0.00	R
PR-2648-2	64.65	58.12	26.01	25.13	**MS**	5.38	MR	11.55	MR
RNS P 10	53.59	42.07	39.29	31.19	**MS**	23.10	**MS**	15.43	**MS**
RNS P 2	44.17	18.84	0.00	14.38	**MR**	3.95	R	0.00	MR
RS O 2	78.15	9.09	6.67	29.89	**MS**	0.76	R	1.25	R
RUB-3	96.3	75.76	62.5	59.96	**S**	48.83	**S**	38.19	MS
SAM-INTER-3	42.41	29.07	12.96	3.45	**R**	2.57	**R**	0.69	**R**
SAN 3	60.00	57.41	63.89	15.21	**MR**	7.64	**MR**	12.15	**MR**
SAN 35	9.52	7.41	14.03	2.00	**R**	0.00	**R**	0.00	**R**
SC MI 4	27.27	8.33	11.11	5.68	**MR**	0.35	R	0.00	R
SU-AB-4-PR	31.11	36.11	47.42	11.20	**MR**	14.58	**MR**	16.23	**MR**
UK 58 ST	40.53	18.65	16.67	17.29	**MR**	10.42	**MR**	7.29	**MR**
V 8931-3-4-OL	25.00	41.33	37.9	5.00	**MR**	7.40	**MR**	4.03	**MR**
VL A 8 PR	8.89	3.03	0.00	1.25	**R**	0.76	**R**	0.00	**R**
VL-3	67.22	42.73	36.57	31.11	**MS**	15.80	MR	13.78	MR
Min	0.00	0.00	0.00	0.00		0.00		0.00	
Max	100.00	100.00	91.10	73.49		54.80		45.89	
CV	55.96	68.81	79.84	78.21		97.26		105.76	

The most severe disease severity score level obtain from disease severity of TP and USBI method and from DNI is bolded; R – resistant; MR - moderately resistant; MS - moderately susceptible; S - susceptible; Min - minimal value; Max - maximal value; CV - Coefficient of variation

Both the USBI and TP methods were employed for testing sunflower inbred lines for resistance to *M. phaseolina*, in order to avoid the uncertainties that may arise from adverse weather conditions or inadequate inoculum distribution in the field. While the USBI method mimics the natural path of pathogen infestation and is easy to handle, there is always a risk of failure due to unfavorable environmental conditions (Tančić Živanov et al. 2021). The TP method, on the other hand, produces a high infection rate but does not imitate the pathogen's natural infestation path and require artificial wounding of the plant, which can lead to disease incidence skipping and increased plant severity. According to Mc Kinney index, only one inbred line, MA SC 2, exhibited complete resistance, while the other resistant lines showed slight infestation in one of the artificial inoculation methods or even in DNI. Among resistant inbred lines, the highest McKinney index was observed in inbred lines FE 49, HA458, LIV 10, SAM-INTER 3, and SAN 35 when tested using the TP method. Overall, it can be concluded that the TP method is more reliable in obtaining high disease incidence even under unfavorable environmental conditions for the pathogen development. While artificial inoculation methods are expected to be highly efficient, natural disease occurrence can be influenced by various factors and may be highly variable, as demonstrated in the study of Dedić et al. (2011) in the inoculation of sunflower with *Sclerotinia sclerotiorum* where natural infection did not occur due to environmental factors. Additionally, it was observed that the TP method elicited the most aggressive reaction in the tested inbred lines. This was expected, as this method requires tissue injury, making it the most aggressive and most suitable inoculation method which require growing sunflower through whole vegetation (Tančić et al. 2012; Tančić-Živanov et al. 2021; Aydoğdu et al. 2022).

3.4. Cluster analysis

Additionally, a cluster analysis of disease severity followed by a heat map was used to better classify the 79 tested sunflower inbred lines based on the inoculation method (Figure 1). The resistant and moderately resistant inbred lines were represented by shades of blue, while susceptible inbred lines were indicated by red, orange, and yellow colours. The inbred lines Ha 74, L1, MA SC 2, PB 21 PH-BC 1 92, SAN 35, LIV 17, AR-7 and VL-A 8-PR were found to be similar to each other, with a dark blue

colour indicating that they were the most resistant inbred lines, not only to *M. phaseolina* but also for other desirable traits such as resistance to *Phomopsis* spp. (Ha 74 and PH BC 1-92), *Pucinnia* spp. (PB 21), broomrape resistance (LIV 17 and VL-A-8-PR), earliness (SAN 35), and good combining abilities (L1, MA-SC-2). Combining desirable traits in one genotype can make the breeding process more efficient and less time-consuming (Qi & Ma 2022). The cluster graph also showed that the TP and USBI methods were more similar to each other than DNI, which was expected as the contact of inoculum and sunflower plants was provided. However, using different inoculation methods resulted in significant variations in colour for certain inbred lines, indicating that the method of inoculation can affect the level of resistance observed in different sunflower inbred lines (Figure 2).

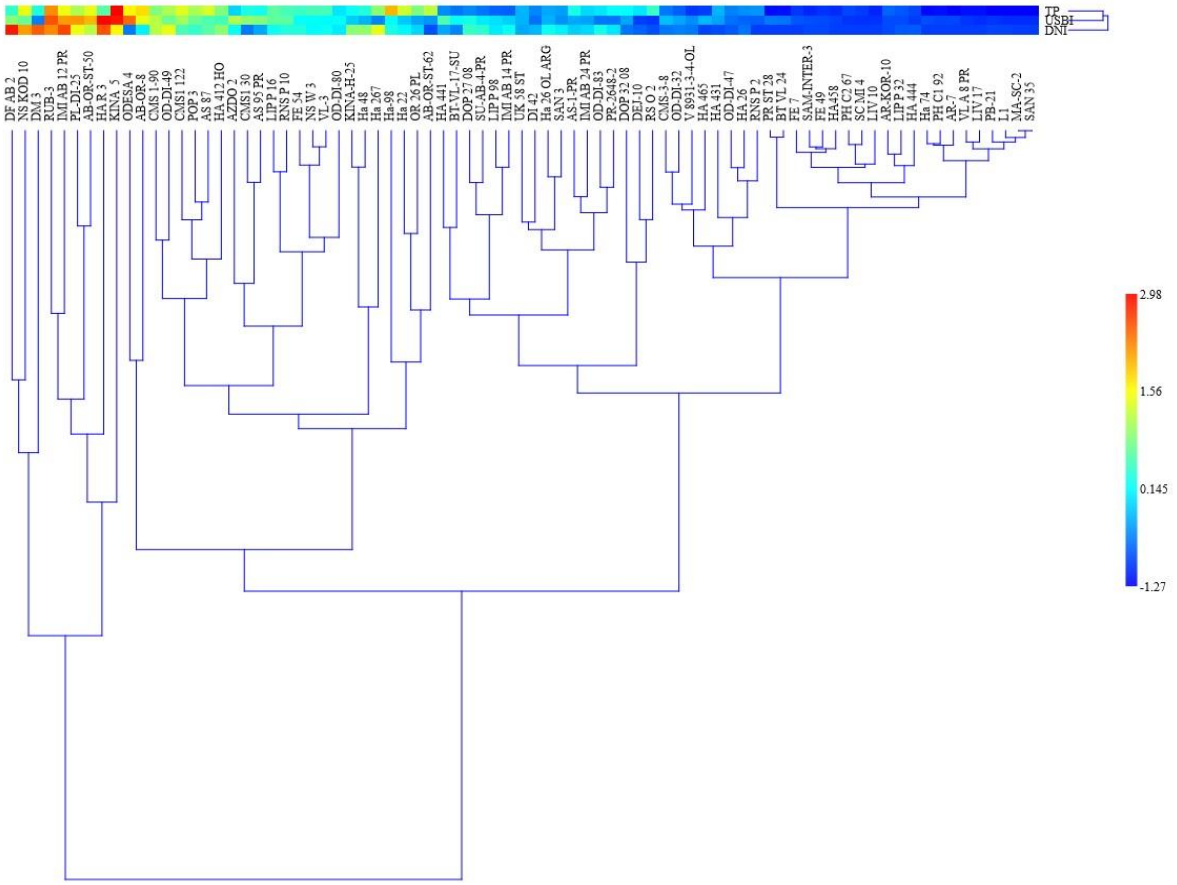

Figure 2- UPMGA cluster analysis followed by a heat map showing relationships among 79 sunflower inbred lines inoculated with *M. phaseolina* isolate using two different inoculation methods toothpick (TP) and Unwounded Stem Base Inoculation (USBI) method and disease in naturally infested area (DNI) without artificial inoculation based on McKinney index

3.5. Comparison of field trials in 2019 and 2020

Fifteen sunflower inbred lines were retested in 2020 for their resistance to *M. phaseolina* (Table 4). In order to check variability among chose examinated inbred lines, it was noticed that in 2019, inbred lines Ha 74, MA SC 2, L1, LIV 10, PB 21 were the most resistant among every inoculation method according to disease incidence and McKinney index. These lines showed low McKinney index, indicating high resistance which candidate them as resistance sources for further breeding (Laidig et al. 2021).

Table 4- Disease incidence and McKinney indexfor *M. phaseolina* resistance of 15 sunflower inbred lines inoculated using Toothpick (TP) method and Unwounded Stem Base Inoculation (USBI) method and in disease in naturally infested area (DNI) obtained in Rimski Šančevi, Novi Sad in 2020

	Disease incidence			*McKinney index*					
Inbred lines	*TP*	*USBI*	*DNI*	*TP*	*grade*	*USBI*	*grade*	*DNI*	*grade*
AB OR 8	87.71	83.33	47.78	42.23	S	53.82	S	38.19	MR
AS 87	70.00	59.60	20.45	15.42	MR	12.31	MR	0.76	R
CMS 1-30	94.44	45.83	0	29.34	MR	20.83	MS	0	R
DF-AB-2	20.83	3.33	0	0.52	R	1.67	R	0	R
Ha 26	55.56	25.00	16.67	34.03	MS	15.63	MR	8.33	MR
Ha 74	9.52	28.04	0	0	R	0.46	R	0	R
IMI-AB-12-PR	22.73	15.15	37.04	15.96	MR	9.85	MR	3.70	R
L1	9.76	3.33	0	1.39	R	0	R	0	R
LIP P 98	68.89	58.15	22.22	9.17	MR	8.65	MR	3.47	R
Liv 10	55.96	15.25	28.24	11.81	MR	0.76	R	3.07	R
MA-SC-2	27.78	8.33	13.33	1.39	**R**	1.85	**R**	3.33	**R**
Odessa 4	91.67	88.64	77.78	49.48	**S**	63.05	**S**	25.60	MS
PB 21	64.82	0	0	6.25	**MR**	0	R	0	R
PL-DI-25	100.00	89.26	64.29	61.20	**S**	67.64	**S**	36.68	MS
RUB-3	20.95	21.21	8.33	7.38	**MR**	3.41	R	0.52	**R**
Min	9.76	0	0	0		0		0	
Max	100	89.26	77.78	61.20		67.64		38.19	
CV	60.7	88.53	101.21	102.90		137.63		163.93	

By comparing these inbred lines to each other they did not have statistically significant difference in all years regarding to inoculation method (Figure 3). Similar reaction of these inbred lines was confirmed also in 2020. Inbred lines which were sensitive, AB OR 8, ODESSA 4 and PL-DI 25 were also similar to each other. Comparing these inbred lines regrading to reaction in 2019 and 2020, resistant inbred lines did not show statistically different reaction in these two years, while sensitive inbred lines AB OR 8, ODESSA 4 and PL-DI 25, had statistically different reaction regarding at least one inoculation method. Sharma et al. (2016) also confirms that resistant genotypes of pigeon pea have more stable reaction to Fusarium wilt than sensitive ones. Different reaction to *M. phaseolina* confirms that climate conditions had large impact on disease progress in these two years, since location of the trial was the same (Veverka 2008). Beside these inbred lines, other lines showed similar reactions in 2020 as in 2019 (Table 5).

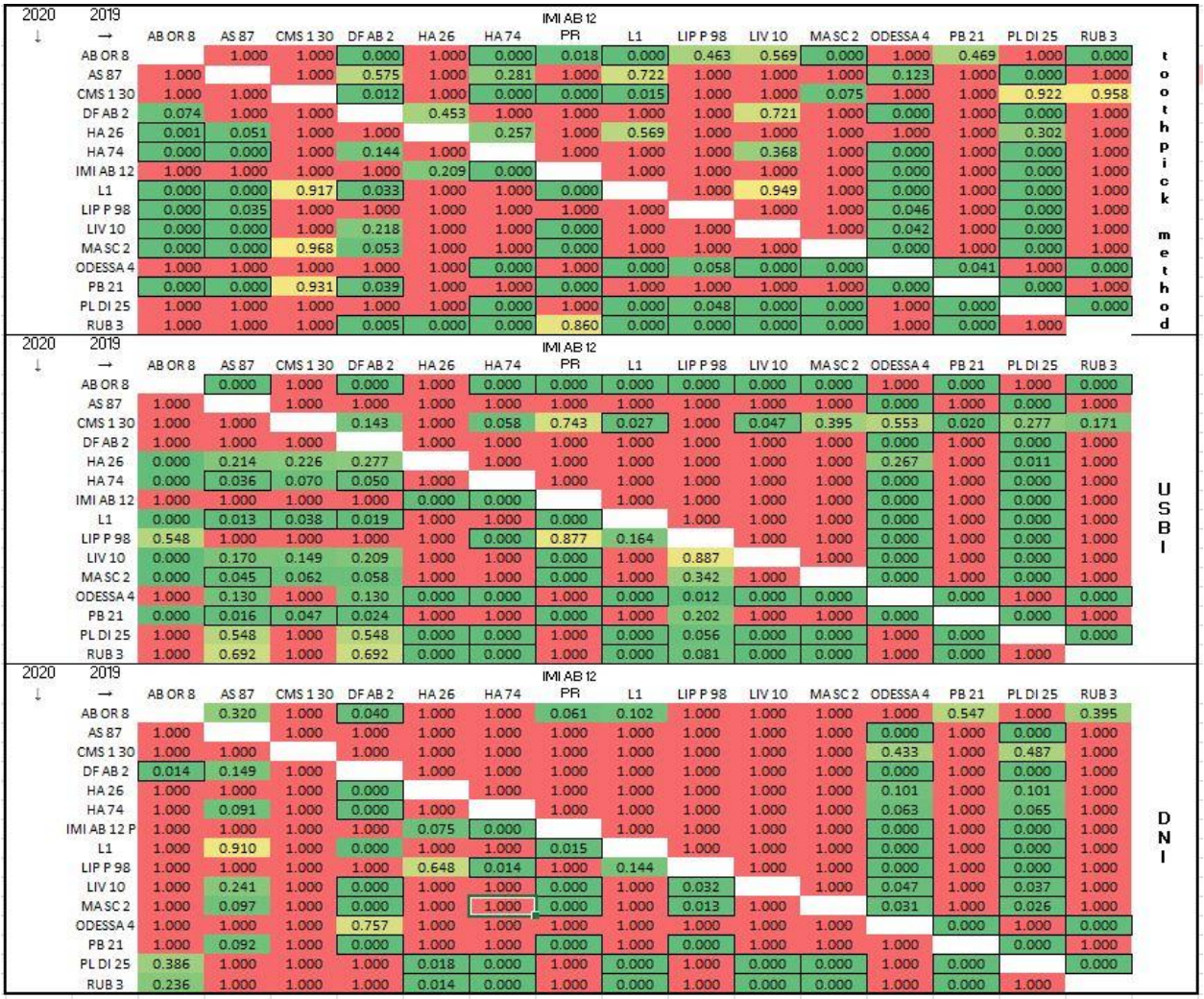

Figure 3- Pairwise comparisons of disease grade of inbred lines tested with Kruskal Wallis and Dunn's test, in 2019 and 2020. Intensity of colour implicate the significance of the association among inbred lines, only dark green and squared associations are statistically significant (P<0.05)

Table 5- Differences between *M. phaseolina* reactions (MPR) inoculated with toothpick (TP) method and Unwounded Stem Base Inoculation (USBI) method and disease in naturally infested area (DNI) of selected sunflower inbred lines in 2019 and 2020 using Kruskal Wallis test

Sunflower inbred lines	*TP*	*USBI*	*DNI*
AB-OR 8	0.28	0.13	0.05*
AS 87	0.05*	0.51	0.05*
CMS 1-30	0.66	0.83	0.11
DF-AB 2	0.05*	0.05*	0.04*
Ha 26	0.13	0.66	0.82
Ha 74	0.32	0.8	1.00
IMI AB 12 PR	0.05*	0.13	0.05*
L1	0.13	1.00	0.32
LIP-P-98	0.83	0.28	0.26
LIV 10	0.28	0.35	0.12
MA-SC 2	0.32	0.32	0.32
ODESSA 4	0.05*	0.268	0.121
PB 21	0.32	0.32	0.32
PL-DI 25	0.13	0.05*	0.52
RUB 3	0.05*	0.05*	0.05*

*Significance at level α=0.05

The significant differences in disease incidence and McKinney index in all inoculation methods between 2019 and 2020 were observed for inbred lines DF-AB-2 and RUB-3. Inbred line RUB-3 was highly susceptible in 2019 but much more resistant in

2020, while inbred line DF-AB-2 showed a high level of susceptibility, especially in the DNI method, in 2020. In 2019, inbred line DF-AB-2 was positioned with highly resistant inbred lines, while inbred line RUB 3 showed a noticeably lower level of susceptibility in 2020.

Although some of the tested inbred lines showed high levels of resistance in certain inoculation methods, previous studies have not found any sunflower genotypes that are completely resistant to *M. phaseolina* (Beg 1992; Aboutalebi et al. 2014; Taha et al. 2018; Siddique et al. 2020). However, other authors (Tančić-Živanov et al. 2021) have found hybrids and inbred lines that did not develop symptoms of *M. phaseolina*. The differences in these results may be due to the large variability among *M. phaseolina* isolates (Aboshosha et al. 2007; Tančić et al. 2012).

4. Conclusions

The study identified four sunflower inbred lines (Ha 74, L1, LIV 10, MA-SC 2 and PB 21) as potential sources of resistance to *M. phaseolina*, with the ability to limit the spread of infection even under high disease pressure. Both inoculation methods (TP and USBI) were effective in differentiating between resistant and susceptible inbred lines, with the TB method being the most aggressive and precise. Further research is needed to understand the mechanisms underlying resistance in these inbred lines and to develop novel resistant lines for sunflower breeding programs.

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