Introducing a cut-stem inoculation method for fast evaluation of sunflower resistance to *Macrophomina phaseolina*

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Abstract The fast adaptation to different growing conditions of a fungus *Macrophomina phaseolina*, led to its becoming one of the sunflower (*Helianthus annuus* L.) disease causal agents in regions with a temperate climate. Methods currently used to determine sunflower resistance require laborious manual inoculation and confirmation of pathogen appearance, due to the late stage of testing. The paper proposes a cut-stem method for inoculating sunflower plants in the controlled conditions and the possibility of early-stage disease evaluation. A set of 15 sunflower inbred lines was inoculated using *M. phaselolina* isolate in the growth chamber and the obtained data were analysed using Cut-stem Disease Severity (CSDS) and compared with disease severity obtained from field experiments using traditional inoculation methods (toothpick, Unwounded Stem Base Inoculation (USBI) and non-inoculated plants). The results showed that, based on CSDS, inbred lines infected with the cut-stem inoculation method significantly differed regarding resistance to *M. phaseolina.* None of the inbred lines exhibited complete resistance but three lines could be proposed as a source of resistance to this pathogen. Ranking of inbred lines which was based on resistance to *M.phaseolina* was similar in all inoculation methods and in non-inoculated plants. There were highly significant correlations between the values obtained from growth chamber experiment and disease severity scores from field evaluations. Thus, the obtained results indicate that the cut-stem method could potentially complement field testing methods and be valuable tool in sunflower breeding for resistance to *M. phaseolina*.

Keywords: charcoal rot, disease progress, disease severity, early-stage evaluation, inbred lines,

Statements & Declarations

Conflicts of Interest: The authors declare no conflict of interest.

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1.Introduction

Sunflower (*Helianthus annuus* L.) production is adversely affected by numerous fungal diseases (Harveson et al., 2018). Disease occurrence and development depend on the host susceptibility, pathogen presence and environmental conditions. Some sunflower pathogens are widely distributed and considered a significant constraint to sunflower production, while others are of regional or minor importance. Among them, charcoal rot caused by a soil-borne fungus (*Macrophomina phaseolina* (Tassi) Goid), is gaining importance in the light of changing environments (Mah et al., 2012). *M. phaseolina* is a multi-host pathogen adapted to warm and dry environmental conditions. Fungal growth is fast under conditions of 30-35ºC and water deficit, when the infection can occur within 24 to 48 hours (Marquez 2021; Akhtar et al., 2011). These conditions cause significant damage to sunflower yield, exceeding 75% under the most favorable conditions for pathogen growth (Mahmoud, 2010). Furthermore, extreme cases of inoculation can reduce yield by 90% (Ijaz et al., 2013). Most of the climate prediction models have already indicated average rising surface temperatures, affecting global agricultural systems (IPCC 2018). Climate change impacts, such as extended dry periods that occur with very high average maximal temperatures, often above 30°C, favour *M. phaseolina* development (Iqbal & Mukhtar 2014). These weather conditions will particularly facilitate the wider geographical distribution of the fungus in countries with traditionally continental climate (Veverka et al., 2009).

A sustainable management strategy should be applied in order to prevent the decline in sunflower production, based on use of genetically resistant material to develop new genotypes is the best practice for disease management (Leiete. 2014). Although *M. phaseolina* is monotypic and no physiological races have been reported, it has high genetic variability. (Ijaz et al., 2013). To date, sunflower genotypes that are completely resistant to *M. phaseolina* have not been found, although several genotypes have been marked as highly or moderately resistant to *M. phaseolina* and therefore can be used as sources of resistance in breeding programs (Taha et al., 2018, Siddique et al., 2020). High level of resistance to *M. phaseolina* was observed in some populations of wild *Helianthus* species but has not been transferred into cultivated sunflower because of its horizontal and quantitatively nature. The susceptibility of sunflower to charcoal rot is clearly visible in genotypes with a short vegetation period and grown in arid climate conditions (Kaya 2016).

Precise determination and characterization of resistance reactions of crops against fungal pathogens are essential for finding resistance sources and selection of resistant genotypes (Mahlein et al., 2019). In the case of *M. phaseolina*, the large-scale phenotyping for resistance remains challenging due to late-stage testing methods, laborious manual inoculation and confirmation of pathogen appearance, as well as disease scoring procedure. Methods for the detection of sunflower resistance to *M. phaseolina* mainly consist of field experiments and artificial inoculation. Fast indoor methods that can speed up resistance evaluation are already used for different sunflower pathogens and represent a reliable alternative for field testing (Terzić et al., 2010; Larfeil et al., 2010). Therefore, the determination of a method that provides information on *M. phaseolina* resistance in earlier stages of sunflower plant development is imperative, as it would accelerate the selection of resistant genotypes and reduce cost of testing.

Cut-stem method used to evaluate soybean resistance to *M. phaseolina* gave reliable results enabling the determination of genotype resistance during early soybean development stages (Twizeyimana et al., 2012). This research aims to propose a new early stage, cut-stem inoculation method for inoculating sunflower seedlings with *M. phaseolina* under controlled conditions, based on data from research conducted in soybean. The method's reliability is verified by comparing with field testing experiments using standard methods of *M. phaseolina* inoculation evaluation. Additionally, the aim of this work was to select resistant inbred lines that have the potential for use in breeding for resistance to charcoal rot.

2. Material and methods

2.1 Plant material

In the experiment, 15 inbred lines of sunflower were selected for determination of *M. phaseolina* resistance. All inbred lines originate from the Institute of Field and Vegetable Crops Novi Sad (IFVCNS) gene pool which have large number of genotypes resistant to diseases (Anđelković et al., 2020). The main characteristics are represented in

(Table 1). Inbred lines were previously tested for *M. phaseolina* resistance and selected based on the exhibited level of resistance (data not presented).

Table 1 Names and characteristics of inbred lines used in experiment and their specific traits

Inbred line	Characteristics
AB OR 8	Medium-early, broomrape resistance
AS 87	Medium-early, good combining ability
CMS 1 30	Medium-early
DF AB 2	Late, good combining ability
Ha 26	Medium-early, good combining ability
Ha 74	Medium-early, highly reistant to *Phomopsis* spp
IMI AB 12 PR	Late, tolerant to imidazolinone, *Pl6* gene
L 1	Medium-early, good combining ability
LIP P 98	Very-early, broomrape resistance
LIV 10	Medium-early, broompare resistant
MA SC 2	Medium-late, specific combining ability
ODESSA 4	Medium early
PB 21	Medium-early, rust resistance, *Pl6* gene
PL DI 25	Very early, *Pl6* gene
RUB 3	Medium early

2.2 Sampling, isolation procedure for *M. phaseolina*, pathogenicity test and inoculum preparation

During 2018, sunflower stems infected with *M. pahseolina* were collected from different locations in the Vojvodina region (North part of Serbia). The stem samples were placed in a paper bag, air dried at room temperature and stored at 4°C. Stem samples were washed under the running tap water for 30 minutes and left to dry on sterile filter papers. Small cuts from the stems with visible microsclerotia were surface sterilized first in 70% ethanol (C_2H_5OH) for three minutes and then with 1% sodium hypochlorite (NaOCl) for three minutes. Sterilized parts were plated onto potato dextrose agar (PDA) and incubated at 30ºC for 24 hours. The hyphal tip was separated and transferred on a new PDA to obtain new and pure fungal colony. In order to choose the most aggressive isolate of *M. phaseolina* a pathogenicity test was performed. A 4 mm mycelial disc from colony were placed on the centre of the PDA plate and incubated for four days. When PDA plate was completely with *M. phaseolina* colony, ten de-hulled sunflower seeds of inbred line Ha 26 were put on each colony. Isolate which first infected all seeds was used for further work. Based on pathogenicity test isolate labelled as MPIN-18, collected near town Indjija, Serbia (45°4'9.4074"N 20°3'22.536"E) was selected for further experiments.

2.3.1 Cut-stem method

These experiments were obtained during January and February 2021 and repeated test during December 2021 and January 2022. Experiments were conducted in same greenhouse but in different growth chambers. Sunflower seeds were germinated on filter paper for 48 hours at 23°C in order to avoid plant loss due to non-germinated seeds. Seeds were then de-hulled and sown in 4.1 l containers filled with a mixture of Klansmann-Deilmann substrate 1 and 10% perlite. Five sunflower seeds per inbred line were planted in the container and after one week, 2 plants from every container were removed. Experiment in controlled conditions was conducted in walk-in growth chamber with temperature day/night regime of 32/20°C. Plants were grown under artificial lighting (lamps PHILIPS HPI-T 1000W/543 e40) with a 16 h light duration. Containers were watered with 300 ml of water every day and relative humidity was maintained for 40%, and checked with a digital hygrometer.

The inoculation was done on the three plants per container using a modified method of Twizeyimana et al., (2012). The plants were inoculated by cutting the plants 1 cm above the last developed leaf. A broader end of 200 µl

pipette tip was immersed in with the edge of four days old fungal colony, and placed on the freshly made stem cut. Pipette tips were removed from the plants three days after the inoculation. The progress of the disease symptoms was recorded four times on days: 4, 5, 7, and 8 after inoculation by measuring the length of lesion on each stem.

AUDPC (Area Under Disease Progress Curve) was calculated separately for every inbred line on basis of consecutive lesion length data. For each inbred line, AUDPC was calculated with formulae:

$$AUDPC = \Sigma(xi + xi + 1)/(ti + 1 - ti)$$

where xi is lesion length of each plant at day i after inoculation, x_i+1 is lesion length of each plant at date i+1, and expression $t_i+1 - ti$ is number of days between scoring dates i and i+1 (Altinok et al., 2014)

Considering AUDPC for every inbred line cut-stem disease severity (CSDS) was calculated on the basis of AUDPC of every plant with formulae:

$$CSDS = (\Sigma Xi)/(m * n)$$

where X is disease score based on the AUDPC values 0-(0); 1-(1-10), 2-(10-50); 3-(50-100); 4-(100), ∑Xi is sum of scores, m is the highest score and n is number of examined plants (McKinney 1923). Since these two repeated experiments obtained in different growth chamber, and seeds were 11 months older in repeated than in first experiment, the obtained results will not be conjoined together.

2.3.2 Field experiments

The field experiments were conducted at the IFVCNS disease testing field, at Rimski Šančevi, Novi Sad, Serbia in order to compare the results with the cut-stem method. The experiments were designed as a randomized block design using 0.7 m inter-row and 0.3 m intra-row spacing, and three replications. Selection of 15 inbred lines was sown on April 18th 2019. and May 5th 2020. Seeds of each inbred line were sown in 3 rows with 12 plants per row. Weather data during June, July and August 2019/2020 were collected from meteorological station Rimski Šančevi- Novi Sad, (Table 2) (RHSS, 2020; RHSS, 2020; RHSS, 2020; RHSS, 2020).

Table 2 Weather data: monthly minimum and maximum average temperature and precipitation in Rimski Šančevi during June, July and August of 2019 and 2020

	June 2019	**July 2019**	**August 2019**	**June 2020**	**July 2020**	**August 2020**
Min.DTA[a] (°C)	17.4°C	16.4°C	17.6°C	15.5°C	15.2°C	17.2°C
Max.DTA[b] (°C)	28.6°C	29.9°C	31.7°C	26.2°C	27.9°C	30.0°C
Precipitation	64 mm	21 mm	79 mm	160 mm	76 mm	140 mm

a-Min.DTA(°C)- Average value of minimal daily temperature (°C);

b-Max.DTA (°C)- Average value of maximal daily temperature (°C)

Two artificial methods of inoculation were used to test sunflower resistance to *M. phaseolina:*
Unwounded Stem Base Inoculation (USBI)- This method was used by Jiménez-Díaz and Blanco-Lópaz, 1983. Inoculum was mix of sand, corn flour and microsclerotia. In mixture of 950 g of sand and 50 g of maize flour was added 100 cuts (4 mm diameter) of *M. phaseolina* with PDA, which were incubated for two weeks on 30°C. The 2 g of mixture was placed around sunflower stem base 30 days after the emergence. The first row with 12 plant per replication was inoculated by this method.

Toothpick method – This method was used by Mihaljčević, 1981. Wooden toothpick covered with the PDA substrate and fungal colony were used during flowering time by piercing the ground part of the stem and left there until the end of experiment. The second row of 12 plants per replication was used for this method.

The third row of 12 plants was not inoculated and spontaneous occurrence of disease was recorded (*non-inoculated plants*).

Plant reaction to *M. phaseolina* was recorded in the maturity phase R8 (Schneiter and Miller, 1981). Each stem was cut longitudinally and length of the stem part covered with microsclerotia was measured. Disease severity (DS) was calculated according to the equation

$$DS = (\Sigma Xi)/(m * n) * 100$$

where X is disease score based on the length of the stem part covered with microsclerotia (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), $\sum X_i$ sum is of plant grades, m is the highest grade of the scale and n was the total number of disease severity. Values were calculated separately for each inoculation method.

2.4 Data analysis

Kruskal-Wallis test was used in order to test existence of significant differences between inbred lines based on AUDPC values from cut-stem method and DS values from field experiments.

In order to group inbred lines, rank sum test was used to compare inbred lines inoculated with different inoculation methods and inoculation of non-inoculated plants. (Ohunakin et al., 2019). CSDS data from growth chamber experiment and DS from field experiments were ranked in ascending order. Rank positions of same methods were added together after which the grand mean of the rank position (X) was calculated. Deviation from this rank position grand mean (Xn X) was calculated for every inbred line. The value of deviation was used to determine resistance level of every inbred line for each inoculation method: (-10<) Resistant; (-10-0) Moderately Resistant; (0-10) Moderately Susceptible; (>10) Susceptible. Results were obtained for each inbred line using the toothpick, USBI method, and non-inoculated plants, adding ranks of measurements collected from inbred lines inoculated by the same method.

According to values obtained from two filed methods, non-inoculated plants in two years and two cut-stem test Spearman's correlation were used in order to inspect how much cut-stem method is similar to filed methods. Software PAST 3, and Microsoft Office Excel was used for all analyses.

3.RESULTS

3.1 Differences among inbred lines in different inoculation method

Using three different inoculation methods and non-inoculated plants, 15 sunflower inbred lines were tested for charcoal rot resistance. The cut-stem inoculation method was used in the walk-in growing chamber and calculated CSDS values ranged between 0.03 (PB 21 and L1) and 1 (Odessa 4). Kruskal-Wallis test was used to test difference between AUDPC of inbred lines in growth chamber. Inbred lines in first cut-stem method were statistically significant at level ($p < 0.05$, $\rho = 0.045$). Repeated cut-stem method also revealed that inbred lines in this experiment were statistically significant at level ($p < 0.01$, $\rho = 0.000$).

Resistance to charcoal rot of 15 inbred lines were also tested during two-year field experiment, using two inoculation method and resistance was noted at non-inoculated plants. Obtained values of DS from field experiments variated between 0 and 67.64. In year 2019 during the flowering stage and seed filling of sunflower (June to August), the average maximum temperature ranged between 28.6 and 31.7°C and rainfall was 164 mm. In 2020, on the other hand, the average maximum temperature ranged between 26.2 and 30.0°C with rainfall 376 mm during the flowering to seed filling (Table 2). Kruskal-Wallis test was done in field experiments on the base of DS (Table 5). DS values from different methods were obtained from every year separately. Significant differences were confirmed between inbred lines in respect to their level of resistance to charcoal rot. The Kruskal-Wallis test (Table 5) of 15 inbred lines, tested in years 2019 and 2020 in field conditions, showed that differences in view or resistance to charcoal rot were on level ($p < 0.01$).

Table 3 Kruskal-Wallis test of 15 inbred lines of sunflower according Disease severity (DS) values obtained during filed experiments for toothpick, Unwounded Stem Base Inoculation (USBI) methods and for non-inoculated plants in the years 2019 and 2020

	Toothpick method 2019	USBI[a] method 2019	Non-inoculated plants 2019	Toothpick method 2020	USBI method 2020	Non-inoculated plants 2020
Kruskal-Wallis H	37.871	32.241	28.756	36.886	37.278	37.443
Df	14	14	14	14	14	14
Asymp. Sig.	0.001**	0.004**	0.001**	0.001**	0.001**	0.001**

*Signification level marked with asterisks *and **indicate the significance at level P ≤ 0.05, 0.01.*

a-USBI- Unwounded Stem Base Inoculation

3.2 Spearman correlation

According to Spearman's correlation, all methods were in positive correlation (Table 4). Inoculation method which had the highest correlation with his own repeated trail is cut-stem method, ($r = 0.81$, $p < 0.01$). The biggest correlation was noticed between toothpick method and USBI method from the year 2019 and this correlation was highly significant ($r = 0.92$, $p < 0.01$). First and repeated cut-stem methods had the highest number of highly significant correlations with other inoculation methods. First and repeated cut-stem methods were in highly significant correlations to methods used in field experiments ($p < 0.01$) except with not-inoculated plants, but correlations of cut-stem methods with non-inoculated plants were significant on level $p < 0.05$. Non-inoculated plants had the lowest number of statistically significant correlation with other methods. Non-inoculated plants in years 2019 and 2020 were not corelated statistically significant. Correlation between non-inoculated plants from year 2019 and toothpick method from year 2020 and correlation between non-inoculated plants in year 2020 and USBI method from year 2019, also were not statistically significant. Correlation of cut-stem method with toothpick method was variated between first cut-stem method toothpick method in year 2019 (0.54), while the strongest was between toothpick method in year 2020 and repeated cut-stem method (0.86). Considering correlations between cut-stem methods and USBI method the weakest correlation was recorded in between USBI method in year 2019 and first cut-stem method (0.69) and the strongest was between repeated cut-stem method and USBI method in year 2020 (0.86).

Table 4 Spearman's correlation coefficients between different inoculation methods of sunflower inbred lines with *Macrophomina phaseolina*. Correlation coefficients from cut-stem disease severity (CSDS) obtained from the first and repeated cut-stem method, disease severity (DS) obtained from toothpick, Unwounded Stem Base Inoculation (USBI) method, and non-inoculated plants in years 2019 and 2020 were compared

	Cut-stem method	Repeated cut-stem method	Toothpick method 2019	Toothpick method 2020	USBI[a] method 2019	USBI method 2020	Non-inoculated plants 2019	Non-inoculated plants 2020
Cut-stem method	1	0.809**	0.786**	0.538*	0.847**	0.692**	0.556*	0.498*
Repeated cut-stem method	0.809**	1	0.858**	0.610**	0.863**	0.680**	0.784**	0.478*
Toothpick method 2019	0.786**	0.858**	1	0.585*	0.922**	0.692**	0.766**	0.493*
Toothpick method 2020	0.538*	0.610**	0.585*	1	0.589*	0.897**	0.297	0.775**
USBI method 2019	0.847**	0.863**	0.922**	0.589*	1	0.709**	0.772**	0.419
USBI method 2020	0.692**	0.680**	0.692**	0.897**	0.709**	1	0.447*	0.746**
Non-inoculated plants 2019	0.556*	0.784**	0.766**	0.297	0.772**	0.447*	1	0.223
Non-inoculated plants 2020	0.498*	0.478*	0.493*	0.775**	0.419	0.746**	0.223	1

**Significant at the 0.05 probability level, **significant at the 0.01 probability level.*

a-USBI- Unwounded Stem Base Inoculation

3.3 Comparation of different inoculation methods

In order to test results from growth chamber experiments and field experiments, CSDS and DS values were ranked (Table 5) and analysed by Rank sum method (Fig 1). In (Table 5) for every inbred line is represented eight observed values (CSDS or DS) and eight ranks. Two CSDS and two ranks for cut-stem experiments, two DS and two ranks for toothpick method in years 2019 and 2020, two DS and two ranks for USBI method in both years and two DS and two ranks for non-inoculated plants also in both years. Minimal recorded value of DS for inbred lines was 0 in both years, but maximal DS for every method was higher in year 2020, than in year 2019. The highest value for DS in year 2019 had toothpick method (59.95) and in 2020 USBI method had the highest recorded value (67.64).

Inbred line L1 had the lowest rank in all filed methods except in repeated cut-stem method and at non-inoculated plants from the year 2019, it these two methods, this inbred line had the second lowest rank. DS values for inbred line L1 variated between (0 and 1.39). Odessa 4 had rank 13-15 in all inoculation method except at non-inoculated plants in year 2019. Also values for DS of Odessa 4 were high (47.05-63.05) in toothpick and USBI methods, comparing to values from non-inoculated plants where these values were lower (4.17-25.6). Inbred lines had similar ranks in the same method, except inbred lines RUB 3 and DF AB 2. Inbred line RUB 3 was the most susceptible inbred line in the first year of field experiments and had rank 15 for toothpick and USBI method. In experiment from the year 2020, rank values were 6 for toothpick method and 7 for USBI method. The higher level of susceptibility in year 2019 than in year 2020 was also recorded for inbred line DF AB 2. According to toothpick method this inbred line had rank value 9 in year 2019 and in year 2020 rank value was 2. At non-inoculated plants differences between ranks of this inbred line were higher, DF AB 2 had rank 15 at year 2019 and rank 3 at year 2020.

Table 5 Ranks of selected sunflower inbred lines inoculated with *M. phaseolina* according to their disease severity (DS) measured in growth chamber experiments (CSDS) in first and repeated cut-stem method and according to disease severity obtained from toothpick method, Unwounded Stem Base Inoculation (USBI) method and non-inoculated plants during years 2019 and 2020

	First cut-stem method		Repeated cut-stem method		Toothpick method 2019		Toothpick method 2020		USBI[a] method 2019		USBI method 2020		Non-inoculated plants 2019		Non-inoculated plants 2020	
	CSDS[b]	Rank	CSDS	Rank	DS[c]	Rank	DS	Rank	DS	Rank	DS	Rank	DS	Rank	DS	Rank
AB OR 8	0.47	14	0.95	12.5	52.15	14	42.23	13	34.09	11	53.82	13	12.23	10	38.19	15
AS 87	0.17	5	0.90	10	45.23	11	15.42	9	22.08	8	12.31	10	19.15	11	0.76	7
CMS 1-30	0.28	10	0.80	8	27.08	8	29.34	11	27.08	10	20.83	12	4.17	7.5	0	3
DF-AB-2	0.28	10	0.95	12.5	29.63	9	0.52	2	26.62	9	1.67	5	45.89	15	0	3
Ha 26	0.08	3.5	0.50	4.5	13.69	7	34.03	12	4.10	6	15.63	11	3.65	6	8.33	12
Ha 74	0.19	6.5	0.45	3	0.83	4	0	1	2.65	5	0.46	3	0	2	0	3
IMI-AB-12-PR	0.25	8	0.95	12.5	47.43	12	15.96	10	43.07	12	9.85	9	42.94	14	3.70	11
L1	0.03	1.5	0.40	2	0	2	1.39	3.5	0	1.5	0	1.5	0.42	4.5	0	3
LIP P 98	0.28	10	0.70	7	9.67	6	9.17	7	13.87	7	8.65	8	11.81	9	3.47	10
Liv 10	0.19	6.5	0.65	6	3.37	5	11.81	8	0.35	3	0.76	4	0	2	3.07	8
MA-SC-2	0.08	3.5	0.25	1	0	2	1.39	3.5	0	1.5	1.85	6	0	2	3.33	9
Odessa 4	0.53	15	1.00	15	49.65	13	49.48	14	47.05	14	63.05	14	4.17	7.5	25.6	13
PB 21	0.03	1.5	0.50	4.5	0	2	6.25	5	0.69	4	0	1.5	0.42	4.5	0	3
PL-DI-25	0.39	13	0.95	12.5	41.27	10	61.20	15	43.53	13	67.64	15	29.17	12	36.68	14
RUB-3	0.33	12	0.85	9	59.96	15	7.38	6	48.83	15	3.41	7	38.19	13	0.52	6

a-USBI- Unwounded Stem Base Inoculation

b- CSDS – cut-stem disease severity

c - DS – disease severity

3.4 Rank sum method

Based on rank sum method, values from every inoculation method and non-inoculated plants were compared to each other. Inbred lines in all inoculation methods showed similar reaction (Fig 1). Cut-stem method and toothpick method distinguished two inbred lines AB OR 8 and Odessa 4 as susceptible compared to other inbred lines, while USBI method showed PL DI 25 and also Odessa 4 as susceptible. Non-inoculated plants had zero inbred lines which were classified as susceptible. Inbred lines L1 and MA SC 2, and PB 21 were resistant inbred lines according to cut-stem inoculation, L1, MA SC 2 and Ha 74 were resistant inbred lines in toothpick inoculation. Inbred line L1 was also resistant according USBI method along with PB 21. Inbred lines: LIP P 98, Ha 26, DF-AB-2, CMS 1 30 and AS 87 depending on method of inoculation, had reaction categorised as moderately resistant and moderately susceptible. Cut-stem and toothpick method showed three inbred lines as resistant, USBI method two inbred lines, and non-inoculated plants one inbred line. Four moderately resistant inbred lines were in cut-stem method and toothpick method, and five inbred lines according to USBI method and non-inoculated plants. Six moderately susceptible inbred lines was recorded according to cut-stem, toothpick and USBI methods and nine inbred lines with non-inoculated plants. Two susceptible inbred lines were classified by cut-stem, toothpick and USBI method, while according to non-inoculated plants there was weak disease occurrence and it was not possible to determine susceptible inbred line.

Fig 1 Graphical presentation of inbred line's RANK SUM values inoculated with different methods. On the down side of the graph are names of inbred lines, on the left side of the graph are values which represents rank sum deviation from average value. With dotted horizontal lines are marked borders between resistant, and moderately resistant area and between moderately susceptible and susceptible area. With black horizontal line on the middle of the graph is border between moderately resistant and moderately susceptible area. Vertical bar colour represents inoculation method, blue bar is cut-stem method, red bar is toothpick method, grey bar is Unwounded stem base inoculation (USBI) method and green bar represents non-inoculated plants. For every inbred line, vertical bars are lined up from left to right: 1st cut-stem method, 2nd toothpick method, 3rd USBI method and 4th non-inoculated plants.

Fig 2 Symptoms of disease on susceptible sunflower inbred line ODESSA 4 on 8th day after the cut-stem inoculation with *Macrophomina phaseolina*

4. Discussion

Experiments conducted in controlled conditions can provide valuable information about disease development and progress and help better understanding of pathogen-host interaction in conditions conductive for disease development (Rotem, 1988). Up to a certain point, they also provide better accuracy of the resistance testing and by using some of them, efficacy in terms of number of tested samples and testing duration is increased (Retig et al., 1973). In sunflower, there were several laboratory experiments for estimation of resistance to *M. phaseolina*, but mainly providing information about number of dumped off or infected plants (Hussien et al., 2018; Taha et al.,2018). This kind of experiments can give information about the percent of plants with developed symptoms but cannot determine complexed information such as disease severity or progress of disease in specific plant. Most of experiments aimed on disease severity or disease progress in controlled conditions, but last till the maturity phase (Jordaan et al., 2019; Siddique et al., 2020). Twizeyimana et al., (2012) developed the method for evaluation of soybean resistance to *M. phaseoilna* in controlled conditions, which has proven to be rapid and reliable. In this work the cut-stem method was adjusted for sunflower and used to examine *M. phaseolina* resistance of 15 inbred sunflower lines under controlled conditions. Ghimire et al., (2019) based on soybean testing concluded that the cut-stem method is better for soybean resistance testing than toothpicks since this method shortens the experiment time and can give results similar to the toothpick method.

Considering the Spearman's correlations it can be noticed that cut-stem method had the highest correlation between all repeated experiments on the level $p < 0.01$. Evans et al., (1999) confirmed that data from two repeated greenhouse experiments with significance level $p < 0.01$ can be used as single experiment. Cut-stem method was in high correlation with field inoculation methods. Correlation of cut-stem method with filed inoculation methods variated between 0.54 and 0.86. Several authors confirmed that indoor disease tests are valid if they are corresponding to filed test around 60% (Foolad et al., 2000, Hashmi et al., 2005; Neto et al., 2008;). The percentage of great correspondence obtained in this work is considered sufficient to mark cut-stem method as method which can give us similar information as information obtained from field experiments. Two different field trail methods in year 2020, toothpick and USBI method are in strong positive correlation (0.92). It was concluded that diseases caused with *M. phaseolina* are strongly correlated with environmental conditions, and in favorable conditions differences between

aggressive and nonaggressive inoculation method will not be large as in less favorable climate conditions (de Sousa Linhares et al., 2020).

Although the higher average temperatures were during year 2019, the highest range between the most resistant and the most susceptible inbred line was in year 2020 in view of toothpick and USBI method. For non-inoculated plants the highest range between the most resistant and the most susceptible inbred line was in year 2019. In both years of field experiments average maximum temperatures were in optimum range for *M. phaseolina* development 25-35°C (Csöndes et al., 2012). Favourable climate conditions were enough to show variability among inbred lines, and that none of inbred lines was completely resistant, indicating partial resistance that could be quantitatively inherited. The polygenic, horizontal resistance to this pathogen was reported in previous research (Kaya, 2016). Inbred line L1 was the most resistant inbred line, based on results after using cut-stem, toothpick and USBI method. While inbred line MA SC 2 was resistant after using toothpick method, so as PB 21 with USBI method, the resistance of these two inbred lines also confirmed with cut-stem method. Resistant lines can offer various opportunities to introduce *M. phaseolina* resistance into cultivated sunflower, which was proven by (Shehbaz et al., 2018). One of the approaches that could be used in order to obtain sustainable resistance is gene pyramiding by combining different resistance sources, as it is proposed for other constraints on sunflower (Cvejić et al., 2020). Although most of the inbred lines had similar rank levels through the years, inbred lines DF AB 2 and RUB 3 showed differences in view of resistance during field experiments. It was noticed that these two inbred lines had much more severe symptoms of charcoal rot in year 2019, than in 2020. According to both cut-stem method these two inbred lines were more susceptible than the most of the inbred lines in experiments and these two inbred lines did not variate as much as in field experiments. As it already told, resistance to charcoal rot is inherited polygenic, so the level of resistance to charcoal rot can be different in different environment conditions (Kumar et al., 2016). García-Olivares et al., (2012) also proved that in common beans is possible to have non stabile genotypes through changed environments in view of charcoal rot. Non-inoculated plants in both years showed lower level of inoculation at all inbred lines. However non-inoculated plants had the narrowest interval between the most resistant and the most susceptible inbred line. In this group of plants, it was not recorded susceptible inbred lines, which was expected since the inoculation is affected by unevenly distributed amount of inoculum in soil.

According to rank sum method, it can be concluded that inbred lines were ranked similarly regardless of inoculation method. Cut-stem method made the most detailed division according to charcoal rot resistance from -12.5 for inbred line L1, to 14 for ODESSA 4. Other inoculation methods toothpick and USBI method had similar but slightly narrowed interval. Similar conclusion had Ghimire et al., (2019) which have found that using cut-stem and toothpick method can result in greater disease severity at soybean inoculation with different *Diaporthe* species. On the other side Souza et al., (2021) reviled that toothpick method is more effective in inoculation of cowpea with *M. phaseolina* than cut-stem method. Different conclusion about effectiveness of cut-stem and toothpick method with *M. phaseolina* can be occur due to different hosts (Talapov et al., 2021). Although non-inoculated plants had the narrowest interval between the most resistant and the most susceptible inbred lines, this method showed variation among inbred lines. This confirms strong presence of *M. phaseolina* isolates in soil of Serbia. Large amount of *M. phaseolina* inoculum in Serbia was proven by several fresh reports of new hosts of *M. phaseolina* in Serbia such as immortelle, blueberry and chickpea (Pavlović et al., 2015; Popović et al., 2018; Živanov et al., 2019).

Comparing to all methods it can be concluded that cut-stem method is reliable method for sunflower resistance testing to charcoal rot, allthought filed tests are inreplaceble since it impossible to completly imitate field conditon in indoor experiments. Cut-stem method can be suitable specially for premlimiraly testing in order to make preliminary selection for filed testing. Twiteyezama et al., (2012) consider that this method can: clearly distinguished plants based on the resistance to charcoal rot and to distinguished *M. phaseolina* isolates based on aggressiveness. However, field experiments especially non-aggressive ones are irreplaceable, because it is very hard to completely imitate all outdoor factors. Use of aggressive methods which damaging the tissue, together with the non-destructive methods could help researches to distinguish between pre-formed barriers to infection and physiological mechanisms of resistance (Botha et al., 2009). Control of indoor conditions, physiological differences between genotypes, resistance mechanisms, uniform concentrations of inoculum, and inoculation location on the plant should be considered to help and correlate future research efforts (Coser et al., 2017).

5. CONCLUSIONS

In our work, we have confirmed that the cut-stem method can provide similar information as information obtained from field experiment methods for testing sunflower inbred lines for *M. phaseolina* resistance. This method showed great potential to accelerate *M. phaseolina* resistance testing in sunflower and make it more cost-effective, less laborious because experiment lasts 40 days. Furthermore, it is possible to use similar rating scales in the field

experiments and controlled conditions and categorized inbred lines as resistant, moderately resistant, moderately susceptible and susceptible. Sunflower inbred lines L 1, MA SC 2, and PB 21 were identified as a potential source of resistance to *M. phaseolina* with low disease severity. Therefore, the cut-stem method could be fast and reliable method for successfully distinguished differences among sunflower inbred lines for resistance to *M. phaseolina*, as well as for identification of new resistance sources, thus contributing to control of this emerging pathogen.

6. REFERENCES

Akhtar, K.P., Sarwar, G., & Arshad, H.M I. (2011). Temperature response, pathogenicity, seed infection and mutant evaluation against *Macrophomina phaseolina* causing charcoal rot disease of sesame. *Archives of phytopathology and plant protection*, *44*(4), 320-330. https://doi.org/10.1080/03235400903052945

Altinok, H. H., Can, C., Boyaci, H. F., & Topcu, V. (2013). Genetic variability among breeding lines and cultivars of eggplant against *Fusarium oxysporum f. sp. melongenae* from Turkey. *Phytoparasitica,*, 42(1), 75–84. https://doi.org10.1007/s12600-013-0340-y

Anđelković, V., Cvejić, S., Jocić, S., Kondić-Špika, A., Jeromela, A. M., Mikić, S., Prodanović, S., Radanović, A., Savić Ivanov, M., Trkulja, D.& Miladinović, D. (2020). Use of plant genetic resources in crop improvement–example of Serbia. *Genetic Resources and Crop Evolution*, 1-14. https://doi.org/10.1007/s10722-020-01029-9

Botha, C., McLaren, N. W. & Swart, W. J. (2009). Evaluation of greenhouse inoculation techniques used to screen for *Sclerotinia* stem rot resistance in soybeans. *South African Journal of Plant and Soil,* 26(1),48-50. https://doi.org/10.1080/02571862.2009.10639932

Coser, S.M., Chowda Reddy, R.V., Zhang, J., Mueller, D., Mengistu, A., Wise, K. A., Allen, T.W., Singh, A., & Singh, A.K. (2017). Genetic architecture of charcoal rot (*Macrophomina phaseolina*) resistance in soybean revealed using a diverse panel. *Frontiers in plant science*, 8: 1626. https://doi.org/10.3389/fpls.2017.01626

Csöndes, I., Cseh, A., Taller, J., & Poczai, P. (2012). Genetic diversity and effect of temperature and pH on the growth of *Macrophomina phaseolina* isolates from sunflower fields in Hungary. *Molecular biology reports*, *39*(3), 3259-3269. https://doi.org/10.1007/s11033-011-1094-6**Error! Hyperlink reference not valid.**

Cvejić, S., Radanović, A., Dedić, B., Jocković, M., Jocić, S. & Miladinović, D. (2020). Genetic and genomic tools in sunflower breeding for broomrape resistance. *Genes*, 11.2: 152. https://doi.org/10.3390/genes11020152

De Sousa Linhares, C. M., Ambrósio, M. M. Q., Castro, G., Torres, S. B., Esteras, C., de Sousa Nunes, G. H., & Picó, B. (2020). Effect of temperature on disease severity of charcoal rot of melons caused by *Macrophomina phaseolina*: implications for selection of resistance sources. *European Journal of Plant Pathology 158*(2), 431-441. https://doi.org/10.1007/s10658-020-02083-w

Evans, C. K., Hunger, R. M., & Siegerist, W. C. (1999). Comparison of greenhouse and field testing to identify wheat resistant to tan spot. *Plant disease*, *83*(3), 269-273. https://doi.org/10.1094/PDIS.1999.83.3.269

Foolad, M.R, Ntahimpera, N, Christ, B.J. & Lin, G.Y. (2000). Comparison of field, greenhouse, and detached-leaflet evaluations of tomato germ plasm for early blight resistance. *Plant disease*, *84*(9), 967-972. https://doi.org/10.1094/PDIS.2000.84.9.967

Ghimire, K., Petrović, K., Kontz, B. J, Bradley, C.A., Chilvers, M.I., Mueller, D.S., Smith, D.L., Wise K.A. & Mathew F.M. (2019). Inoculation method impacts symptom development associated with *Diaporthe aspalathi, D. caulivora*, and *D. longicolla* on soybean (*Glycine max*). *Plant disease*, *103*(4), 677-684. https://doi.org/10.1094/PDIS-06-18-1078-RE

García-Olivares, J. G., López-Salinas, E., Cumpián-Gutiérrez, J., Cantú-Almaguer, M. A., Zavala-García, F., & Mayek-Pérez, N. (2012). Grain yield and charcoal rot resistance stability in common beans under terminal drought conditions. *Journal of Phytopathology*, *160*(2), 98-105. https://doi.org/10.1111/j.1439-0434.2011.01864.x

Harveson, R., Mathew, F., Gulya, T., Markell, S., Block, C. & Thompson, S. (2018). Sunflower stalk diseases initiated through leaf inoculations. *Plant Health Progress*, *19*(1), 82-91. https://doi.org/10.1094/PHP-12-17-0083-DG

Hashmi, R.Y., Bond, J.P., Schmidt, M.E., & Klein J.H. (2005). A temperature-controlled water bath method for evaluating soybean reaction to sudden death syndrome (SDS). *Plant Health Progress*, *6*(1), 5. https://doi.org/10.1094/PHP-2005-0906-01-RS

Hussien, Z.N, Ibrahim, M.M, Ahmed, M, & Khalil, A.A. (2018). Thermotherapy of Sunflower Seeds Controlling Charcoal-Rot Caused by *Macrophomina phaseolina*. *Egyptian Journal of Phytopathology*, *46*(2), 179-194. . https://doi.org/10.21608/ejp.2018.115842

Ijaz S., Sadaqat, H.A., Khan, M.N. (2013). A review of the impact of charcoal rot (*Macrophomina phaseolina*) on sunflower. *The Journal of Agricultural Science*, *151*(2), 222-227 . https://doi.org/10.1017/S0021859612000512

IPCC, (2018). Global Warming of 1.5°C.An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)].

Iqbal, U. & Mukhtar, T. (2014). Morphological and pathogenic variability among *Macrophomina phaseolina* isolates associated with mungbean (*Vigna radiata* L.) Wilczek from Pakistan. *The Scientific World Journal*, 1-9. https://doi.org/10.1155/2014/950175

Jiménez-Díaz, R.M. & Blanco-López, M.A. (1983). Incidence and Distribution of Charcoal Rot of Sunflower Caused by *Macrophomina phaseolina* in Spain. *Plant Disease*, 67: 1033-1036.

Jordaan, E., Van der Waals, J. E., & McLaren, N. W. (2019). Effect of irrigation on charcoal rot severity, yield loss and colonization of soybean and sunflower. *Crop Protection*, *122*, 63-69. . https://doi.org/10.1007/s10658-020-02083-w

Kaya, Y. (2016). Sunflower *In* Gupta S. (Eds.), *Breeding oilseed crops for sustainable production* (1st., pp. 55-88) Elsevier Inc. DOI: 10.1016/B978-0-12-801309-0.00004-5

Kumar, B., Hooda, K. S., Singh, V., Sekhar, J. C., Kumar, V., Parihar, C. M., Jat, L.S., Singh, A.K., Kaul, J. Kaur, H., Kaur, H. & Yadav, O. P. (2016). Multi-environment field testing to identify stable sources of resistance to charcoal rot (*Macrophomina phaseolina*) disease in tropical maize germplasm. *Maydica*, *62*(2) 1-7.

Larfeil, C, Barrault G. & Dechamp-Guillaume G. (2010). Assessment of sunflower genotype tolerance to *Phoma macdonaldii*. *OCL17*(3), 161-166. https://doi.org/10.1051/ocl.2010.0304

Leiete, R. (2014). Disease menagment in suflower. In Arribas J. (Eds.), *Sunflowers, Growth and Development, Environmental Influences and Pests/Diseases*. (1st,ed., 165-185). Nova Science.

Mah K.M, Uppalapati, S.R., Tang, Y, Allen, S. & Shuai, B. (2012). Gene expression profiling of *Macrophomina phaseolina* infected Medicago truncatula roots reveals a role for auxin in plant tolerance against the charcoal rot pathogen. *Physiological and molecular plant pathology*, *79*, 21-30. https://doi.org 10.1016/j.pmpp.2012.03.004

Mahlein, A.K., Kuska, M.T., Thomas, S., Wahabzada, M., Behmann, J., Rasher, U. & Kersting, K. (2019). Quantitative and qualitative phenotyping of disease resistance of crops by hyperspectral sensors: seamless interlocking of phytopathology, sensors, and machine learning is needed! *Current opinion in plant biology*, *50*, 156-162. https://doi.org10.1016/j.pbi.201906.007

Mahmoud A. (2010). Molecular and biological investigations of damping-off and charcoal-rot diseases in sunflower. Diseration, Sabanci University

Marquez, N., Giachero, M.L, Declerck S. & Ducasse D.A. (2021). *Macrophomina phaseolina*: general characteristics of pathogenicity and methods of control. *Frontiers in Plant Science*, *12*. https://doi.org10.3389/fpls.2021.634397

McKinney, H.H. (1923). Influence of soil, temperature and moisture on infection of wheat seedling by *Helminthosporium sativum*.*Journal of Agricultural Research.*, 31(9) 827-840

Mihaljčević, M. (1983). Pronalaženje izvora otpornosti prema *Sclerotiumbataticola* Taub. kod inbred linija i hibrida suncokreta. Bachelor tesis, Faculty of Agriculture University of Novi Sad

Neto, F.A.L.D, Schmidt, M., Hartman, G.L, Shuxian, L. & Diers, B.W. (2008). Inoculation methods under greenhouse conditions for evaluating soybean resistance to sudden death syndrome. *Pesquisa Agropecuária Brasileira*, *43*(11), 1475-1482. https://doi.org10.1590/S0100-204X2008001100005

Ohunakin, A.O., Odiyi, A.C. & Akinyele B.O. (2019). Comparison of rank sum and Area under Disease Progress Curve (AUDPC) as determinant for relative resistance status of maize populations to Northern leaf blight disease of maize. Advanced Plants & Agricultural Research.;9(3):395-400. https://doi.org10.15406/apar.2019.09.00455

Pavlović, S., Ristić, D., Aleksić, G., Milošević, D., Stević, T., & Starović, M. (2015). The first report of *Macrophomina phaseolina* of Immortelle (*Helichrysum italicum*) in Serbia. *Plant Disease*, *99*(9), 1279-1279.

Pawlowski, M.L, Hill, C.B. & Hartman, G.L. (2015). Resistance to charcoal rot identified in ancestral soybean germplasm. *Crop Science*, 55.3: 1230-1235. https://doi.org10.2135/cropsci2014.10.0687

Popović, T., Blagojević, J., Aleksić, G., Jelušić, A., Krnjajić, S., & Milovanović, P. (2018). A blight disease on highbush blueberry associated with *Macrophomina phaseolina* in Serbia. *Canadian Journal of Plant Pathology*, *40*(1), 121-127. https://doi.org/10.1080/07060661.2017.1415977

Retig, N., Rabinowitch, H.D. & Kedar, N. A. (1973). Simplified method for determining the resistance of tomato seedlings to*Fusarium* and *Verticillium* wilts. *Phytoparasitica* , ***1(2)***111–114 https://doi.org/10.1007/BF02981040

RHSS, (2020). Mesečni bilten za Srbiju, Avgust 2020. Godine, Odeljenje za monitoring klime i klimatske prognoze, Sektor Nacionalnog centra za klimatske promene, razvoj klimatskih modela i ocenu rizika elementarnih nepogoda Available from: http://www.hidmet.gov.rs/podaci/meteorologija/latin/Avgust_l.pdf. [assessed 21.11.2020].

RHSS, (2020). Mesečni bilten za Srbiju, Jul 2020. Godine, Odeljenje za monitoring klime i klimatske prognoze, Sektor Nacionalnog centra za klimatske promene, razvoj klimatskih modela i ocenu rizika elementarnih nepogoda Available from: http://www.hidmet.gov.rs/podaci/meteorologija/latin/Jul_l.pdf [assessed 21.11.2020].

RHSS, (2020). Mesečni bilten za Srbiju, Jun 2020. Godine, Odeljenje za monitoring klime i klimatske prognoze Sektor Nacionalnog centra za klimatske promene, razvoj klimatskih modela i ocenu rizika elementarnih nepogoda. Available from: http://www.hidmet.gov.rs/podaci/meteorologija/latin/Jun_l.pdf [assessed 21.11.2020]

RHSS, (2020). Meteorološki godišnjak 1 Klimatološki podaci 2019, Republika Srbija, Beograd Available from: http://www.meteologos.rs/mesecni-meteoroloski-podaci/ [assessed 21.11.2020]

Retig, N., Rabinowitch, H.D. & Kedar, N. A. (1973). Simplified method for determining the resistance of tomato seedlings to*Fusarium* and *Verticillium* wilts. *Phytoparasitica* **1,** 111–114 https://doi.org/10.1007/BF02981040

Rotem, J. (1988). Techniques of Controlled-Condition Experiments In Kranz, J., & Rotem, J. (Eds.), *Experimental techniques in plant disease epidemiology. Springer Science & Business Media*, (1st, ed., 19-31). Springer-Verlag https://doi.org10.1007/978-3-642-95534-1_3

Schneiter, A. & Miller, J.F. (1981). Description of Sunflower Growth Stages. *Crop Science*, 21,901-903. https://doi.org10.2135/cropsci1981.0011183X002100060024x

Shehbaz, M., Rauf, S., Al-Sadi, A. M., Nazir, S., Bano, S., Shahzad, M., & Hussain, M. M. (2018). Introgression and inheritance of charcoal rot (*Macrophomina phaseolina*) resistance from silver sunflower (Helianthus argophyllus Torr. & A. Gray) into cultivated sunflower (*Helianthus annuus* L.). *Australasian Plant Pathology*, *47*(4), 413-420. https://doi.org/10.1007/s13313-018-0573-9

Siddique, S., Shoaib, A., Khan, S.N. & Mohy-Ud-Din, A. (2020). Screening and histopathological characterization of sunflower germplasm for resistance to *Macrophomina phaseolina*. *Mycologia*, 113 (1)1-16. https://doi.org 10.1080/00275514.2020.1810516

Souza, E. M. D. S., Silva, L. A. B. D., Pinto, F. Á. C., Borel, J. C., Capucho, A. S., & Ishikawa, F. H. (2021). Inoculation methods and agressiveness of *Macrophomina phaseolina* isolates in cowpea. *Ciência Rural*, *52*. https://doi.org/10.1590/0103-8478cr20210072

Taha, M.M, Mahmoud, A.F, Hassan, M.A, Mahmoud, A.M, & Sallam, M.A. (2018). Potential resistance of certain sunflower cultivars and inbred lines against charcoal rot disease caused by *Macrophomina phaseolina* (Tassi) Goid. *Journal of Phytopathology and Pest Management*, 5(3) 55-66.

Talapov, T., Yuceer, S., Dedecan, O., Demirel, O., & Can, C. (2021). Comparison of *Macrophomina phaseolina* inoculation techniques for screening sunflower and soybean germplasm in a controlled environment. *Canadian Journal of Plant Pathology*, 43(6), 859-870. https://doi.org/10.1080/07060661.2021.1937321

Terzić, S, Dedić, B, Atlagić, J, Jocić S. & Tančić, S. (2010). Screening wild sunflower species and F1 interspecific hybrids for resistance to broomrape. *Helia*, *33*(53), 25-30. https://doi.org10.2298/HEL1053025T

Twizeyimana, M, Hill, C.B, Pawlowski, M, Paul, C. & Hartman, G.L. (2012). A cut-stem inoculation technique to evaluate soybean for resistance to *Macrophomina phaseolina*. *Plant disease*, *96*(8), 1210-1215. 10.1094/PDIS-02-12-0126-REVeverka, K., Palicová, J., & Křížková, I. (2009). The incidence and spreading of *Macrophomina phaseolina* (Tassi) Goidanovich on sunflower in the Czech Republic. *Plant Protection Science*, *44*(4), 127-137.doi: 10.17221/31/2008-PPS

Živanov, D., Živanov, S. T., Nagl, N., Savić, A., Katanski, S., & Milić, D. (2019). First report of *Macrophomina phaseolina* causing dry root rot of chickpea (Cicer arietinum) in Serbia. *Plant Disease*, *103*(10), 2685. https://doi.org/10.1094/PDIS-03-19-0652-PDN

Declarations

Author contributions *All authors contributed to the study conception and design* "Conceptualization, S.C. and V.M.; methodology, B.D.; software, N.Ć.; validation, S.C., B.D. and V.M.; formal analysis, N.Ć.; investigation, N.Ć., B.B; resources, S.J., V.M; data curation, N.Ć.; writing—original draft preparation, N.Ć and B.B.; writing—review and editing, N.Ć, and S.C.; visualization, B.B.; supervision, D.M.; project administration, D.M.; funding acquisition, V.M. All authors have read and agreed to the published version of the manuscript.

Competing interests The authors declare no competing financial interests.

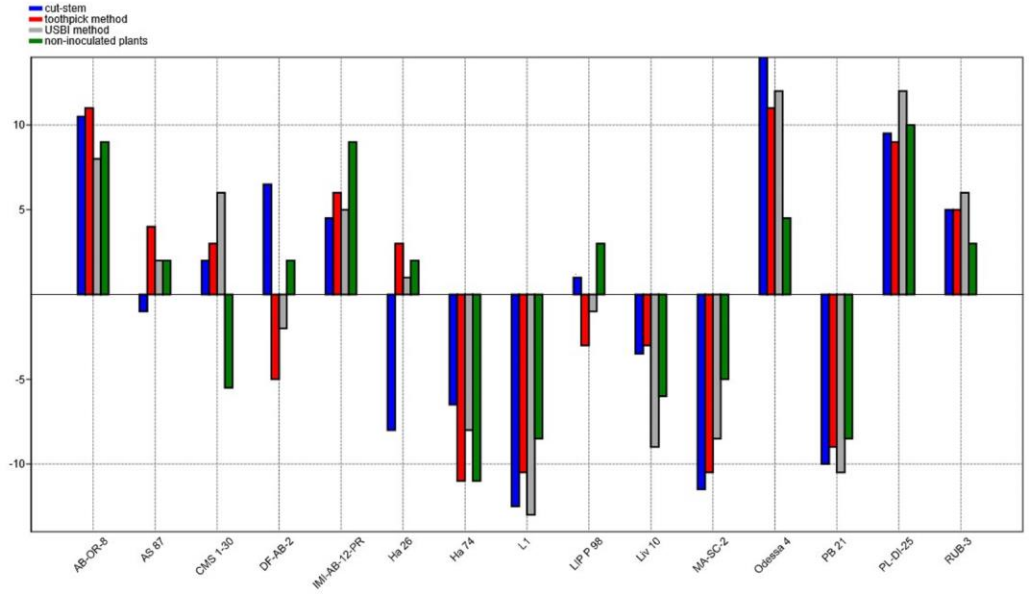

Figure 1. Graphical presentation of inbred line's RANK SUM values inoculated with diferent methods. On the down side of the graph are names of inbred lines, on the left side of the graph are values which represents rank sum deviation

566 from average value. With dotted horizontal lines are marked borders between resistant, and moderately resistant area
567 and between moderately susceptible and susceptible area. With black horizontal line on the middle of the graph is
568 border between moderately resistant and moderately susceptible area. Vertical bar colour represents inoculation
569 method, blue bar is cut-stem method, red bar is toothpick method, grey bar is Unwounded stem base inoculation (USBI)
570 method and green bar represents non-inoculated plants. For every inbred line, vertical bars are lined up from left to
571 right: 1st cut-stem method, 2nd toothpick method, 3rd USBI method and 4th non-inoculated plants
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573
574
575 Figure 2. Symptoms of disease on susceptible sunflower inbred
576 line ODESSA 4 on 8th day after the cut-stem inoculation with
577 Macrophomina phaseolina