THE POTENTIAL IMPACT OF FLOWER INFECTING BOTRYTIS BUD ROT (*BOTRYTIS CINEREA* PERS.) ON HEMP (*CANNABIS SATIVA* L.) SELECTIVE BREEDING

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Abstract

The aim of this paper is to examine the possible adverse phytopathological effects of the selection of hemp (*Cannabis sativa* L.) plants with altered inflorescence morphological characteristics. We have created the conditions for an accelerated method to judge the offspring of our hemp crosses. The late-autumn growing environment requires additional heating and ventilation. Despite all those controlled environmental conditions the incidence of flower bud rot increased. The application of fungicides is not recommended during breeding as it has a detrimental effect on the selection processes. Fungicides against flower and storage diseases at later stages can not be used in the final product either, due to food health regulations. So in case of our available plant material for variety production the expected rate of fungal infection was evaluated in a greenhouse environment. A preliminary study was conducted to assess the extent of natural resistance to artificial infection. We attempted to examine the critical plant phenological stages. No bud rot resistant individuals were found in our plant stock, although the severity of symptoms was very different.

Keywords: hemp breeding, *Botrytis cinerea*

Összefoglalás

Vizsgálatunk célja a kender (*Cannabis sativa* L.) növény egyedek virágzatának morfológiai szelekciója során bekövetkező káros növénykórtani hatások felderítése volt. Az utódnemzedékek gyorsított elbírásála érdekében egyedi környezeti feltételeket hoztunk létre. A késő őszi termesztési körülmények nem nélkülözhetik a kiegészítő fűtést és szellőztetést. Mindezek ellenére a virágzatok megbetegedésének mértéke növekedett. A nemesítés során nem javasolható gombaölőszerek felhasználása, hiszen károsan befolyásolja a szelekciós folyamatot. A késői fenológiai stádiumokban, a virágzatot érintő és a tárolási betegségek ellen alkalmazott fungicidek élelmezés-egészségügyi problámát jelentenek. A fenti okok miatt üvegházi környezetben kórtani szempontból vizsgáltuk a meglévő növényanyagunkat. A természetes rezisztenca elbírálása céljából egy előzetes vizsgálati módszert vontunk be a szelekciós folyamatba, mesterséges fertőzés alkalmazásával. Megpróbáltuk elemezni a fertőződés szempontjából kritikus fenológiai stádiumokat. A meglévő nemesítési anyagunkban rezisztens egyedeket nem találtunk, azonban a fertőzés tüneteinek mértéke nagyfokú eltérést mutatott az egyedek között.

Kulcsszavak: Kender nemesítés, *Botrytis cinerea*

Introduction

Hemp (*Cannabis sativa* L.) is an annual plant belonging to the *Cannabaceae* family. It is one of the first domesticated plants. According to our latest knowledge it was domesticated in early Neolithic times in East Asia and the common, ancestral gene pool of both drug type and non-

drug type cultivars diverged from Chinese landraces (Ren et al. 2021). The main utilization of *Cannabis sativa* in addition to seed and fibre production is the fact, that the glandular trichomes of this plant contain terpenes and phytocannabinoid compounds as secondary metabolites. Based on the ratio of those compounds *C. sativa* varieties can be divided into psychoactive, marijuana cultivars with high tetrahydrocannabinol (THC) content, and hemp cultivars with very low THC content (Small and Cronquist, 1976). The non-psychoactive medicinal substances (e.g. cannabidiol – CBD) are present also in hemp varieties. The growing demand for these cannabinoids resulted in the fact that the related cultivation and processing of hemp has become one of the fastest growing industries (Vergara et al., 2016).

A comprehensive literature review mentions almost a hundred different fungal pathogens on cannabis (McPartland et al., 2000).

The pathogens and molds affecting the production of cannabis inflorescence are *Penicillium olsonii* and *Penicillium copticola* (Penicillium bud rot), *Botrytis cinerea* (Botrytis bud rot) and *Fusarium solani*, *Fusarium oxysporum, Fusarium equiseti* (Fusarium bud rot) (Punja et al., 2019). Most often the pre- and post-harvest internal rot was associated with *Botrytis cinerea* which causes significant harvest losses in indoor or in greenhouse environment (Punja, 2018).

Botrytis cinerea is one of the most studied necrotrophic plant pathogens (van Kan, 2006). It is a very widespread generalist pathogen. The typical grey mould may appear on over 1400 plant species (Garfinkel, 2020; Garfinkel, 2021).

The most typical symptom is the decay of the infected plant part, and the ‘grey mold’ which is the visible sporulation of the pathogen (van Kan, 2006).

Environmental factors are very important for the infection. High humidity or free moisture is necessary for spore germination. Spontaneous or artificial injuries are the entry points for the pathogen (Bika et al. 2021). In controlled environments the sanitization methods and continuous monitoring and disposal of infected plants could reduce inoculum sources. The controlled

diversion from the environmental requirements of the pathogen is inevitable. In greenhouses keeping the temperature above 25 °C is recommended, supplemented with proper ventilation for low humidity (McPartland et al., 2000).

In the event of disease onset the use of fungicides is not recommended. Perhaps the only exception is the application of naturally occurring beneficial microorganisms as biocontrol agents (Balthazar et al., 2020).

Susceptibility tests have also recently appeared. Cannabis strains with a higher total number of bract leaves have a higher bud rot incidence. The volatile terpenoid compounds accumulating in the inflorescence can also influence the pathogenicity. In addition, little is known about the role of glandular trichomes and the compounds they produce in altering susceptibility to bud rot pathogens (Punja and Ni, 2021).

The aim of our study is to determine the susceptibility of our hemp breeding material to botrytis bud rot.

Material and method

An accelerated, conventional high CBD hemp breeding project takes place in a block-system polytunnel in Hédervár on the property of Lajtamag Ltd. Adequate heating, ventilation and blackout systems have been incorporated. This growing environment allows seed harvesting four times a year and significantly speeds up the evaluation of selection material and crossbreeding progenies. Until the autumn-winter growing cycle only aphids and mites appeared and damaged the hemp plantation. As far as fungal pathogens a negligible number of mold-like diseases were observed in the seedling growth room. In November 2021 the first symptoms of bud rot became visible only in the poorly ventilated areas. The climate of those places allows the condensation to take place at lower temperatures at night which may favour the development of infections. However, the symptoms of bud rot did not affect plant

individuals equally. The first sampling was followed by microscopic identification. *Botrytis cinerea* inoculum was obtained from our hemp plants with visible symptoms and conidia. The conidia of the pathogen were propagated in 2% malt extract agar. The petri dishes were incubated at 20 °C with 12 h day-night cycle. After 2 weeks, when sporulation was evident, the dishes were flooded with 10 mL of water. The concentration of the conidium suspension was min. $1x10^5/cm^3$ which has been set in a haemocytometer. The inoculation was performed by using a micropipette. 0.1 mL suspension was pipetted directly onto the bud surface. As a preliminary test we used only 5 plants with very different morphological properties. The plants were previously 'topped', the apical buds were removed so that at least three identical side shoots can develop. Table 1 contains the most important morphological features of the 5 plants which may affect the susceptibility to bunch rot. The final length is short when the mature plant is not taller than 1 meter. Tall plant means that the final length is more than 3 meters. Main flower is dense if the contiguous inflorescence is longer than 0,3 m and the sugar leaves are almost touching each other (<0,5 cm). Main flower is rare if the contiguous inflorescence is shorter than 0,3 m, the inflorescences are 'airy'. Medium flower means a compact, long enough habit, although the bract floors are located at a greater distance. The tendency to branching without pruning is very branching if more than 5, at least 0,5 m long side shoots are located on the plant with promising flowers. A hemp plant is not branched if no side shoots are detected (in a 0,4 m * 0,4 m square, where the plant is in the center).

Table 1 The main morphological differences between the tested individuals

Breeding material	Final length	Main flower density	Tendency to branching
PAK	Short	Dense	Very branching
K21KF1	Medium	Rare	Branching
CAN20	Medium	Rare	Very branching
C21KF1	Medium	Medium	Not branched
K21EF1	Tall	Dense	Branching

Different shoots of the same plant represented the 3 replications. The plants were placed in a cool and humid environment (20 °C – 23 °C with 90% relative humidity) for 2 weeks. After that the inoculated flowers were covered with a veil-plastic foil bag. One day later the bags were removed. The inflorescences were not disinfected at the beginning of the experiment. Conidia were not activated with carrot extract. Disease ratings were made after 5 and 7 days using a scale of 1–5 as shown in Table 2.

Table 2 The scale used for assessing the severity of bud rot symptoms (Punja and Ni, 2021)

Scale value	Disease severity
0	healthy bud, no mycelium visible, tissues green
1	less than 10% mycelial growth on bud surface and/or browning tissues
2	mycelial growth in one or multiple spots with 10-30% coverage
3	30– 50% of the bud surface covered with mycelial growth
4	50–80% of the bud surface covered with mycelial growth
5	the entire bud covered with mycelium

The above-mentioned method is very labour intensive. We repeated the susceptibility test in a chamber previously used for isolated pollination experiments. Clones of the same individual plants were used in artificial infection (2-month-old cuttings at the end of flowering stage with

first visible immature seeds at the bottom of the shoots) previously shifted into the flowering stage by using short daylength illumination. These cuttings were placed in an isolation chamber covered with veil-plastic foil (PP 17 g/m^2). Very high humidity (90%) and a complete lack of air movement were provided for the plants while they were exposed to the fungal infection. A previously infected plant was placed in the centre of the chamber. The observed cannabis individuals were incubated in the chamber for 3 weeks.

Software for analysis of the results was ARM Revision 2019.4 from Gylling Data Management. Data were analysed using analysis of variance (ANOVA) on untransformed data.

The probability of no significant differences occurring between treatment means was calculated as the F probability value (Treatment Prob(F)). Student-Newman-Keuls (S-N-K) tests were applied when treatment differences were identified on the basis of the ANOVA test.

Mean comparison performed only when AOV Treatment P(F) is significant at level selected. Results obtained where indicated by a letter-treatment means with no letters in common are significantly different in accordance with a S-N-K conducted at a 95% confidence level.

Results

Microscopic identification

The previously collected samples were evaluated based on the symptoms and the microscopic identification. Twentyfour of the 25 collected bud rot samples were infected with *Botrytis cinerea* (Figure 1 and Figure 2).

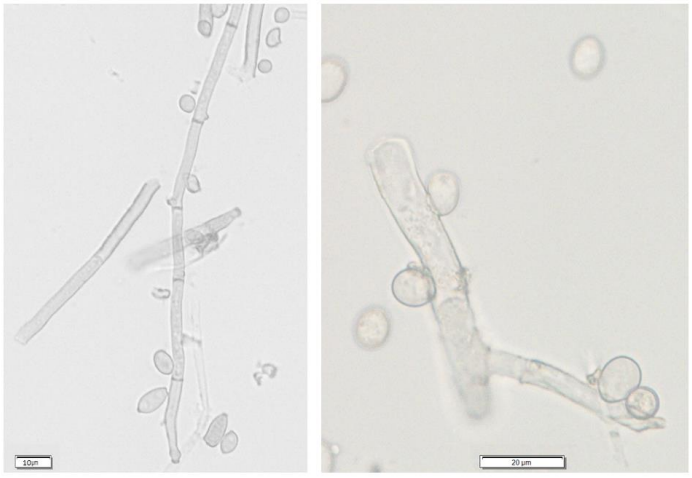

Figure 1 The microscopic image of Botrytis cinerea Pers. conidiophores and conidia

Only one sample without the sporulating 'grey mold' was identified as *Fusarium sp.* with macro and microconidia which caused very similar flower decay. As the rest of this study is about the tolerance to *Botrytis cinerea* Fusarium bud rot was not further assessed.

Figure 2 The increasing severity of Botrytis bud rot observed in our breeding material

Artificial infection

Significant differences could be declared in susceptibility to *Botrytis cinerea* (Table 3). Visible symptoms did not occur to the same extent.

Table 3 The severity scores of the different hemp breeding materials in case of artificial inoculation (severity scores 0-5 – 5 represents the most severe symptoms caused by Botrytis cinerea according to Punja and Ni, 2021)

Breeding material	**Replication 1 (Severity score 0-5)**	**Replication 2 (Severity score 0-5)**	**Replication 3 (Severity score 0-5)**	**Average of 3 replicates**
PAK	5	4	4	4,3 (a)
K21KF1	2	3	2	2,3 (b)
CAN20	2	2	3	2,3 (b)
C21KF1	2	2	2	2,0 (b)
K21EF1	3	4	3	3,3 (ab)
LSD (P=.05)	1,06			
Standard Deviation	0,56			
CV	16,88			

Means followed by same letter do not significantly differ (P=.05, Student-Newman-Keuls)

Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Natural infection in the isolation chamber

In case of the isolation chamber assessments with longer incubation time statistical significant difference could be found (Table 4).

Table 4 The severity scores of the different hemp breeding materials in case of natural infection in the isolation chamber (severity scores 0-5 – 5 represents the most severe symptoms caused by Botrytis cinerea according to Punja and Ni, 2021)

Breeding material	Replication 1 (Severity score 0-5)	Replication 2 (Severity score 0-5)	Replication 3 (Severity score 0-5)	Average of 3 replicates
PAK	5	5	5	5 (a)
K21KF1	2	3	3	2,6 (b)
CAN20	3	3	2	2,6 (b)
C21KF1	3	3	3	3,0 (b)
K21EF1	3	4	4	3,0 (b)
LSD (P=.05)	0,23			
Standard Deviation	0,12			
CV	6,36			

Means followed by same letter do not significantly differ (P=.05, Student-Newman-Keuls)

Mean comparisons performed only when AOV Treatment P(F) is significant at mean comparison OSL.

Discussion

From our results, we concluded that it is very important to take pathogen susceptibility into account during the breeding process. Our preliminary results in accordance with literature data suggested that a dense inflorescence, with a large frequency of bract leaves promoted gray mold infection.

During morphological selection the greater and more dense flowers are preferred with a large number of visible glandular trichomes. Because of this we incorporate the simplified ‘isolation chamber’ sensitivity test into the selection process. Although that process exposes the plants to excessive infection pressure, the risk of susceptibility is much more overestimated than under normal growing conditions. But we get an idea of which individuals need to be tested more thoroughly. It is necessary to ensure precisely regulated environmental conditions and to avoid infection by shaping the inflorescence with pruning and thinning techniques.

After the occurrence of visible *Botrytis cinerea* infection it also becomes impossible to extract the seeds for further propagation because the plants dry out before seed maturing. The most effective feature preservation procedure is cloning. Although, transferring cuttings that are already contaminated into the greenhouse greatly facilitates the appearance of cannabis bud rot. Nevertheless, the complete lack of genetic diversity inevitably causes severe risk of plant decay in the entire plant population. In the future it would be necessary to study breeding materials from very distant origin and to examine the issue of susceptibility based on phytochemical composition.

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