# Fiziološki odgovor genotipova pšenice ovisno o infekciji Fusarium vrstama i gnojidbi dušikom

**Matić, Magdalena**

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**Povjerenstvo za ocjenu:**

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**izv. prof. dr. sc. Ivna Štolfa Čamagajevac, profesorica Odjela za biologiju, Sveučilišta Josipa Jurja Strossmayera u Osijeku, član**

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**Fiziološki odgovor genotipova pšenice ovisno o infekciji *Fusarium* vrstama i gnojidbi dušikom**

**Magdalena Matić, mag. biol.**

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**Mentor: prof. dr. sc. Karolina Vrandečić**
**Komentor: izv. prof. dr. sc. Rosemary Vuković**

Fuzarijska palež klasa (FHB) i neadekvatna gnojidba dušikom mogu uzrokovati brojne biokemijske promjene u tkivu pšenice. Pšenica je jedna od najvažnijih žitarica na svijetu i od velikog je značaja razumjeti načine na koje reagira na stresne čimbenike okoliša. Neki od ciljeva ovog istraživanja bili su utvrditi utjecaj različite gnojidbe dušikom na pojavnost i intenzitet FHB-a te odrediti utjecaj inokulacije vrstama roda *Fusarium* i različite gnojidbe dušikom na fiziološki odgovor, tj. oksidacijski i antioksidacijski status lista zastavičara i klasa ozime pšenice. Poljski su pokusi provedeni tijekom tri vegetacijske godine (2017./2018., 2018./2019. i 2019./2020.), kao višefaktorijalni pokusi s tri glavna faktora: sorta, gnojidba dušikom i infekcija vrstama roda *Fusarium*. Tijekom 2017./2018. fokus je bio na istraživanju povezanosti oksidacijskog statusa i antioksidacijskog odgovora lista zastavičara i klasa. Mjereni su sljedeći pokazatelji: koncentracija vodikova peroksida, razina lipidne peroksidacije, koncentracija ukupnih topljivih fenola (PHE), sadržaj fotosintetskih pigmenata i aktivnosti antioksidacijskih enzima. Sva tri faktora istraživanja imala su utjecaj na vizualne simptome FHB-a te na mjerene pokazatelje. Najznačajniji utjecaj imala je niska razina dušika, a antioksidacijski odgovor bio je i sortno i tkivno-specifičan. U pokusima tijekom 2018./2019. i 2019./2020. fokus je bio na istraživanju biokemijskih mehanizama otpornosti koji uključuju PHE i enzime metabolizma PHE. U ovom se dijelu istraživanja mogu jasno uočiti razlike u ozbiljnosti FHB-a između dvije vegetacijske godine tijekom kojih su prevladavali različiti klimatski uvjeti. U obje vegetacijske godine inokulacija vrstama roda *Fusarium* uzrokovala je promjene koncentracije PHE u klasovima pšenice, što upućuje na uključenost PHE u obrambeni odgovor pšenice na infekciju. Oplemenjivanje sorti pšenice koje su nositelj poželjnog svojstva (pojačana sinteza PHE) mogla bi biti strategija u kontroli FHB-a.

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**Physiological response in wheat genotypes depending on *Fusarium* infection and nitrogen fertilization**

**Magdalena Matić, mag. biol.**

**Thesis performed at Faculty of Agrobiotechnical Sciences Osijek, University of Josip Juraj Strossmayer in Osijek**

**Supervisor: Prof. Karolina Vrandečić, PhD**
**Co-supervisor: Assoc. Prof. Rosemary Vuković, PhD**

Fusarium head blight (FHB) and inadequate nitrogen fertilization can cause numerous biochemical changes in wheat tissue. Wheat is one of the most important cereals in the world, and it is of great importance to understand the way it responds to environmental stressors. Some of the aims of this research were to determine the effect of different nitrogen fertilization levels on the occurrence and intensity of FHB, and to determine the effect of *Fusarium* inoculation and different nitrogen fertilization levels on the physiological response, i.e. oxidative and antioxidative status in flag leaves and spikes of winter wheat. The field trials were set up during three growing seasons (2017/2018, 2018/2019 and 2019/2020) as multifactorial trials with three main factors: variety, nitrogen fertilization level and *Fusarium* infection. During 2017/2018 the focus was on determining the relationship between oxidative status and antioxidative response in flag leaves and spikes. The following parameters were measured: concentration of hydrogen peroxide, level of lipid peroxidation, concentration of total soluble phenolics (PHE), content of photosynthetic pigments and activities of antioxidant enzymes. All three research factors had an impact on visual FHB symptoms and measured parameters. The most significant effect had a low nitrogen level and antioxidative response was both variety- and tissue-specific. During 2018/2019 and 2019/2020 the focus was on studying biochemical resistance mechanisms involving PHE and enzymes related to PHE metabolism. In this part of the research, differences in the FHB severity can be clearly observed between two growing seasons in which different climatic conditions prevailed. In both growing seasons, *Fusarium* inoculation altered PHE content in wheat spikes, indicating involvement of PHE in the defense response of wheat to infection. Breeding wheat varieties with enhanced PHE synthesis could be a promising strategy to control FHB.

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# KAZALO

# 1. UVOD

Pšenica (*Triticum aestivum* L. i *Triticum turgidum* L. var. *durum*) je jedna od najvažnijih žitarica na svijetu, kako zbog široke rasprostranjenosti i velike proizvodnje, tako i zbog nezamjenjivog značaja u prehrani ljudi. Budući da je pšenica sesilni organizam, trajno je izložena brojnim negativnim abiotičkim i/ili biotičkim stresnim čimbenicima koji mogu imati negativan utjecaj na pravilan rast te na prinos i kvalitetu zrna. Pojava fuzarijske paleži klasa (engl. *Fusarium head blight*, FHB) i neadekvatna gnojidba dušikom, kao abiotički i biotički stresni čimbenici, mogu uzrokovati brojne fiziološke i biokemijske promjene u tkivu pšenice.
Kako bi spriječile negativan utjecaj abiotičkih i biotičkih stresnih čimbenika, biljke su razvile niz obrambenih mehanizama, među kojima se ističe antioksidacijski obrambeni sustav koji čine brojni enzimi i neenzimski antioksidansi. Zbog rastuće svjetske populacije i sve većih potreba za hranom od velikog je značaja razumjeti načine na koje pšenica reagira na stresne čimbenike okoliša. Detaljnije poznavanje obrambenog odgovora pšenice doprinijelo bi preciznijem oplemenjivanju pšenice i uzgoju sorti pšenice otpornijih na negativne utjecaje okoliša.
Ova doktorska disertacija temelji se na tri znanstvena rada koji su objavljeni u recenziranim međunarodnim časopisima. Objedinjeni ciljevi ove doktorske disertacije jesu utvrditi utjecaj različite gnojidbe dušikom na pojavnost i intenzitet FHB-a, odrediti utjecaj inokulacije vrstama roda *Fusarium* i različite gnojidbe dušikom na oksidacijski status i antioksidacijski odgovor lista zastavičara i klasa različitih sorti ozime pšenice, te utvrditi odražava li se infekcija vrstama roda *Fusarium* u klasu na antioksidacijski odgovor lista zastavičara. Također, željeli smo odrediti potencijalne biokemijske mehanizme otpornosti pojedinih genotipova pšenice kako bismo što bolje razumjeli obranu pšenice na fiziološkoj razini, što može poslužiti kao mjera prevencije od zaraze FHB-om. Glavni cilj ove doktorske disertacije želja je za pronalaskom parametra koji bi mogao poslužiti kao dobar biomarker za daljnje oplemenjivanje i uzgoj otpornijih sorti pšenice na proučavane stresne uvjete.

# 2. PREGLED LITERATURE

Pšenica pripada porodici trava (Poaceae, Gramineae), rodu *Triticum* unutar kojega razlikujemo mnogo različitih vrsta, koje postoje kao divlji ili kultivirani oblici. Najvažniji predstavnik roda *Triticum* jest vrsta *Triticum aestivum* L., heksaploidna vrsta koja je većinom poznata pod nazivom obična, meka ili krušna pšenica. Veliko gospodarsko značenje ima i tetraploidna vrsta *T. turgidum* var. *durum,* poznata pod nazivom tvrda pšenica. Pšenica je jedna od najstarijih kultiviranih biljaka čija kultivacija počinje prije otprilike 8000 – 10 000 godina (Montenegro i sur., 2017.). U Hrvatskoj se pšenica 2020. godine uzgajala na površini od 147,8 tisuća hektara, a ukupna proizvodnja iznosila je 867,5 tisuća tona (FAO, 2020.). Prosječni prinos pšenice u petogodišnjem razdoblju (2016. – 2020.) iznosio je 5,7 t $ha^{-1}$, zbog čega je pšenica, iza kukuruza, žitarica s najvećim prinosom u Republici Hrvatskoj (Statistički ljetopis Republike Hrvatske, 2021.). Razlikujemo dvije forme pšenice - ozima i jara (proljetna) pšenica, između kojih postoje razlike ovisno o brojnim svojstvima, kao što su vrijeme sjetve, uzgojno područje, dužina stadija jarovizacije, otpornost na zimu, kvaliteta zrna i dr. Iako se pšenica prvenstveno smatra izvorom energije (ugljikohidrata), ona također sadrži i značajne količine niza komponenti koje su bitne i korisne za zdravlje, posebno proteina, dijetalnih vlakana, vitamina (osobito vitamina B), minerala i fitokemikalija (Shewry i Hey, 2015.). U proizvodnim uvjetima pšenica je često izložena brojnim negativnim abiotičkim i/ili biotičkim stresnim čimbenicima koji mogu imati negativan utjecaj na njezin rast i produktivnost (Duveiller i sur., 2007.).

Pšenica je često izložena pojavi FHB-a, ekonomski vrlo značajnoj bolesti pšenice koja može imati negativne posljedice na njezin prinos i kvalitetu, a uzrokuju je fitopatogene gljive roda *Fusarium* (Buerstmayr i sur., 2019.). Ekonomski značaj FHB-a ogleda se i u podatku da se globalni gubitci prinosa zrna pšenice zbog pojave pojedinačnih patogena i štetnika procjenjuju na 21,5 %, dok su gubitci prinosa uzrokovani pojavom FHB-a na drugom mjestu nakon gubitaka uzrokovanih pojavom uzročnika hrđa (*Puccinia* sp.) (Savary i sur., 2019.). Dvije najvažnije vrste koje uzrokuju FHB na području Europe jesu vrste *Fusarium graminearum* Schwabe (teleomorf *Gibberella zeae* Schwein. Petch.) i *Fusarium culmorum* (Wm. G. Sm.) Sacc., iako pojavnost tih vrsta može varirati tijekom vegetacijske godine (Španić i sur., 2021b). Naime, geografska distribucija i pojavnost navedenih *Fusarium* vrsta povezana je s klimatskim elementima, prije svega s temperaturom i količinom oborina. Vrsta *F. graminearum* povezuje se s toplijim i vlažnijim uvjetima, dok je vrsta *F. culmorum* prilagođena hladnijim i vlažnijim uvjetima (Xu i sur., 2008.). Vrste *F. graminearum* i *F. culmorum* održavaju se u tlu kao saprofit

ili kao paraziti na zaraženim biljnim ostatcima, u obliku nespolnih spora (konidija), micelija ili peritecija unutar kojeg kod vrste *F. graminearum* nastaju spolne spore (askospore). Obje vrste stvaraju i trajne spore hlamidospore, koje nastaju fragmentacijom micelija, a služe za održavanje patogena tijekom nepovoljnih uvjeta. Tijekom vegetacije vrste roda *Fusarium* primarnu zarazu klasa vrše konidijama. Konidije se do klasa pšenice šire vjetrom i kapljicama vode te pri povoljnim uvjetima kliju u micelij. Konidije kliju samo u prisutnosti vode, stoga je infekcija favorizirana visokom relativnom vlagom zraka i dovoljnom količinom oborina. Iako je pšenica najosjetljivija na infekciju FHB-om u fazi cvatnje, do zaraze pojedinih klasova može doći od faze cvatnje pa sve do faze sazrijevanja zrna (Bai i Shaner, 2004.; McMullen i sur., 2012.). Konidije kliju u micelij na prašnicima ili tučku te postupno inficiraju sve dijelove cvijeta, odnosno klasića, ometajući normalno formiranje zrna (Jurković i sur., 2016.). Prvi simptomi FHB-a postaju vidljivi na pljevicama klasova kao sitne zelenkastosmeđe, vodenaste pjege, a razvojem bolesti dolazi do blijeđenja pojedinih klasića ili pak blijeđenja cijelog klasa čiji izgled podsjeća na zreli klas. Gubitci prinosa kao rezultat infekcije FHB-om nastaju kao posljedica sterilnosti zaraženih cvjetova iz kojih se ne razvija zrno. Ukoliko do infekcije dođe u kasnijoj fazi razvoja zrna, infekcija FHB-om može uzrokovati slabije nalijevanje zrna, a zaražena su zrna sitnija, smežurana i prekrivena bijelom ili ružičastom prevlakom (McMullen i sur., 2012.). Osim na prinos infekcija FHB-om utječe i na kvalitetu pšenice. Uz sitnije i smežurano zrno infekcija FHB-om može negativno djelovati na količinu škroba, udio proteina te samim time na krajnje karakteristike brašna i tijesta (Bacala i sur., 2021.). Infekcija FHB-om utječe na smanjenje kvalitete pšenice i zbog činjenice da tijekom procesa infekcije brojne vrste roda *Fusarium* proizvode mikotoksine koji mogu biti štetni za zdravlje ljudi i životinja (Buerstmayr i sur., 2019.). Među brojnim mikotoksinima koje proizvode vrste roda *Fusarium* najzastupljeniji i najviše proučavani mikotoksini jesu deoksinivalenol (DON) i zearalenon (ZEA) (Bottalico i Perrone, 2002.). Pojavnost i intenzitet infekcije FHB-om i kontaminacije zrna mikotoksinima pod jakim su utjecajem klimatskih prilika tijekom vegetacijske godine (Czaban i sur., 2015.; Birr i sur., 2020.). Stoga ozbiljnost infekcije i stopa kontaminacije mikotoksinima znatno variraju od godine do godine. Budući da je pšenica najosjetljivija na infekciju FHB-om u fazi cvatnje, preklapanja cvatnje s vlažnim klimatskim uvjetima znatno pogoduje razvoju bolesti i povećanom stvaranju mikotoksina u zrnu (Parry i sur., 1995.; Bai i Shaner, 2004.; Marzec-Schmidt i sur., 2021.).

Dušik ima nezamjenjivu ulogu u razvojnom ciklusu biljaka, kao glavna komponenta biljnih stanica, proteina, peptida, nukleinskih kiselina i fotosintetskih pigmenata. Nadalje, asimilacija

dušika povezana je s ključnim fiziološkim i metaboličkim procesima kao što su fotosinteza, fotorespiracija, stanično disanje, sinteza aminokiselina i Krebsov ciklus (Sun i sur., 2020.). Dušik ima važnu ulogu u proizvodnji ozime pšenice kao ključni nutrijent za rast i razvoj te nutrijent koji osigurava visok i kvalitetan prinos. Pri uzgoju ozime pšenice dušična se prihrana može smatrati kritičnim agrotehničkim zahvatom jer je gotovo nemoguće dobiti visok i kvalitetan prinos bez adekvatne količine i pravovremene primjene prihrane dušikom (Vukadinović i Lončarić, 1998.). Adekvatna gnojidba utječe na povećanje fotosintetske aktivnosti i povećanje sadržaja proteina u zrnu, što rezultira kvalitetnijim i većim prinosom (Hawkesford, 2014.). Nedovoljna gnojidba može utjecati na smanjenje sinteze proteina, što rezultira nižim prinosom i manjom cijenom na tržištu, dok prekomjerna gnojidba može izazvati polijeganje i povećati osjetljivost pšenice na brojne uzročnike bolesti (Wagan i sur., 2003.). Kao reakcija na neadekvatne razine dušika u tkivu pšenice događaju se brojne fiziološke i metaboličke promjene, koje su znatno izraženije u uvjetima nedostatka dušika i uključuju promjenu sastava masnih kiselina, smanjenje sadržaja klorofila i pojavu oksidacijskog stresa (Liu i sur., 2020.).

Uloga dušika u interakcijama između različitih biljaka domaćina i patogena iznimno je složena i dalje nedovoljno razjašnjena. Ipak, poznato je kako dušik može utjecati na promjenu biokemijskog obrambenog odgovora biljke te povećati ili smanjiti osjetljivosti biljke na napad različitih patogena (Sun i sur., 2020.). U literaturi su dostupni često proturječni rezultati o utjecaju gnojidbe dušikom na pojavnost i intenzitet FHB-a te na kontaminaciju zrna mikotoksinima. Pojedini autori navode kako povećana opskrba dušikom, do određene razine, može dovesti do povećane pojave FHB-a i povećane koncentracije mikotoksina u zrnu pšenice (Lemmens i sur., 2004.; Heier i sur., 2005.). Lemmens i sur. (2004.) istraživali su utjecaj različitih tretmana prihrane dušikom (0, 40, 80, 120 i 160 kg N $ha^{-1}$) i različitih oblika dušika na razvoj i intenzitet FHB-a i na koncentraciju mikotoksina DON u zrnu jare i ozime pšenice. Povećanjem dušika od 0 do 80 kg N $ha^{-1}$ utvrdili su značajno povećanje intenziteta FHB-a i koncentracije DON-a. Pri većim koncentracijama dušika relevantnim u suvremenom uzgoju pšenice, intenzitet FHB-a i koncentracija DON-a ostali su pri konstantnim razinama. Zaključili su da različite prihrane dušikom u praktičnoj primjeni ne predstavljaju mogućnost u borbi protiv FHB-a. Slično prethodnom istraživanju, Krnjaja i sur. (2015.) istraživali su utjecaj različitih tretmana prihrane dušikom (0, 75 i 150 kg N $ha^{-1}$) na pojavu i intenzitet FHB-a te na koncentraciju mikotoksina DON i ZEA u zrnu ozime pšenice tijekom dvije vegetacijske godine. Razina gnojidbe dušikom nije značajno utjecala na FHB indeks i akumulaciju

mikotoksina tijekom prve vegetacijske godine. Tijekom druge vegetacijske godine najviša razina dušika (150 kg N $ha^{-1}$) uzrokovala je povećanje koncentracije mikotoksina DON i ZEA u zrnu pšenice. Autori zaključuju kako je navedeno povećanje mikotoksina više bilo favorizirano odgovarajućim klimatskim prilikama pogodnim za razvoj FHB-a nego razinom dušika. Veliki dio literaturnih podataka koji se odnose na utjecaj dušika na razvoj FHB-a teško je protumačiti zbog nedovoljno podataka o količini dušičnih gnojiva, vremenu primjene i obliku dušika te uvjetima klime i tla. Naime, različiti odgovor biljke na različite razine gnojidbe dušikom može biti posljedica upotrebe različitih oblika dušika (Huber i Watson, 1974.). Pojedinačna dušična gnojiva prema kemijskom obliku dušika dijelimo na: amonijska ($NH_4^+$, amonijev kation), nitratna ($NO_3^-$, nitratni anion), amonijsko-nitratna i amidna (Lončarić i Karalić, 2015.). Zbog različite kemijske strukture navedeni se oblici dušika ponašaju veoma različito u tlu te djelomično imaju različite asimilacijske i metaboličke puteve (Sun i sur., 2020.). Činjenica da gnojidba dušikom može stimulirati razvoj jedne bolesti, a s druge strane smanjiti razvoj neke druge bolesti, ukazuje na potrebu za detaljnim razumijevanjem što većeg broja čimbenika koji mogu utjecati na gnojidbu.

Do danas je poznato kako svi abiotički i biotički stresni uvjeti induciraju ili uključuju pojavu oksidacijskog stresa do određenog stupnja, a sposobnost biljaka da kontroliraju razinu oksidansa povezana je s boljom tolerancijom stresnih uvjeta (Cheeseman, 2007.). Oksidacijski je stres složeni kemijski i fiziološki fenomen koji nastaje zbog neravnoteže između proizvodnje i uklanjanja reaktivnih kisikovih jedinki (engl. *reactive oxygen species*, ROS), kao što su vodikov peroksid ($H_2O_2$), superoksidni radikal ($O_2^{\bullet-}$) i hidroksilni radikal (•OH) (Suzuki i Katano, 2018.; Hasanuzzaman i sur., 2020.). U biljnim stanicama ROS mogu nastati i kao nusprodukti aerobnog metabolizma i tada mogu djelovati kao signalne molekule važne za regulaciju i koordinaciju brojnih fizioloških procesa (rast i razvoj biljaka, stanični ciklus, odgovor na abiotički stres i obrana od patogena) (Demidchik, 2015.). Osim ROS-a pojavu oksidacijskog stresa može uzrokovati i prekomjerno nakupljanje reaktivnih dušikovih jedinki (engl. *reactive nitrogen species*, RNS), kao što su dušikov oksid ($^{\bullet}NO$), peroksiinitrit (ONOO), dušik dioksid ($^{\bullet}NO_2$) i dinitrogen trioksid ($N_2O_3$) te je u tom slučaju pravilnije govoriti o nitro-oksidacijskom stresu (Corpas i Barroso, 2013.; Saddhe i sur., 2019.). Iako u prekomjernim količinama NO može biti reaktivna molekula, do danas je prepoznata uloga NO kao signalne molekule koja također ima važnu ulogu u brojnim razvojnim procesima biljaka te u mehanizmima tolerancije različitih stresnih uvjeta (Štolfa Čamagajevac i sur., 2019.). Prekomjerna proizvodnja ROS-a i RNS-a, odnosno pojava nitro-oksidacijskog stresa u biljnim

stanicama, može dovesti do lipidne peroksidacije (LPO), oštećenja proteina i nukleinskih kiselina, inhibicije aktivnosti antioksidacijskih enzima i aktivacije programirane stanične smrti (Ahmad i sur., 2019.; Hasanuzzaman i sur., 2020.). LPO je proces lančane oksidacije, odnosno razgradnje višestruko nezasićenih masnih kiselina, tijekom koje nastaju brojni primarni i sekundarni produkti. Među najvažnijim sekundarnim produktima ističe se malondialdehid (MDA) koji može služiti kao pokazatelj oksidacijskog stresa. Ravnoteža između proizvodnje i eliminacije ROS-a i RNS-a kritična je za održavanje stanične redoks homeostaze. Kako bi prevladale visoke razine ROS-a i RNS-a i držale ih u ravnotežnom stanju, biljke su razvile kompleksne enzimske i neenzimske antioksidacijske mehanizme (Apel i Hirt, 2004.). Enzimski sustav čine enzimi kao što su: superoksid-dismutaza (SOD), katalaza (CAT), askorbat-peroksidaza (APX), glutation-reduktaza (GR), gvajakol-peroksidaza (GPOD) i dr. (Hasanuzzaman i sur., 2020.). Uklanjanje prekomjerne količine ROS-a (posebice $H_2O_2$) djelovanjem antioksidacijskih enzima presudno je za zaštitu biljnih stanica i normalno odvijanje stanične signalizacije (Apel i Hirt, 2004.). CAT katalizira dismutaciju $H_2O_2$ u molekule $H_2O$ i $O_2$, a za razliku od ostalih antioksidacijskih enzima ne zahtijeva redukcijsko sredstvo za reakciju dismutacije (Mhamdi i sur. 2010.). APX je dio askorbat-glutationskog ciklusa u kojem ima ključnu ulogu u procesu kataliziranja pretvorbe $H_2O_2$ u $H_2O$, koristeći askorbat kao specifični donor elektrona (Caverzan i sur., 2012.). Važan enzim askorbat-glutationskog ciklusa jest i enzim GR, koji obnavlja unutarstaničnu razinu glutationa (GSH) redukcijom oksidiranog oblika GSH (GSSG) u prisutnosti NADPH kao donora elektrona (Noctor i sur., 2012.). Neenzimski antioksidacijski sustav čine biomolekule kao što su fenoli (PHE), pigmenti, askorbat, GSH i dr. (Hasanuzzaman i sur., 2020.). PHE su aromatski spojevi s jednom ili više hidroksilnih skupina vezanih na aromatski benzenski prsten. PHE se klasificiraju, uglavnom prema razlikama u svojoj kemijskoj strukturi, na flavonoide, fenolne kiseline, tanine, stilbene i lignane (Zhang i sur., 2022.). Također, PHE se mogu podijeliti u dvije skupine: (1) prethodno formirani PHE, koji se sintetiziraju tijekom normalnog razvoja i (2) inducirani PHE, koji se sintetiziraju pri odgovoru na različite abiotičke i biotičke stresne uvjete (Pratyusha, 2022.). Zbog svoje kemijske strukture PHE imaju snažnu antioksidacijsku aktivnost i izraženu aktivnost smanjivanja količine slobodnih radikala, koje se ispoljavaju različitim mehanizmima djelovanja (Zeb, 2020.; Kaur i sur., 2022.). Danas se za određivanje antioksidacijskog kapaciteta PHE u biljnom tkivu koriste različite metode iako se metoda određivanja antioksidacijske jakosti redukcijom željeza (engl. *ferric reducing antioxidant power*, FRAP) pokazala kao vrlo jednostavna i korisna metoda za provjeru ukupnog

antioksidacijskog kapaciteta (engl. *total antioxidant capacity*, TAC) različitih grupa fenolnih spojeva (Spiegel i sur., 2020.).

Dva su enzima važna u metabolizmu PHE: fenilalanin-amonij-lijaza (PAL; EC 4.3.1.24) i polifenol-oksidaza (PPO; EC 1.14.18.1) (Kaur i sur., 2022.). PAL je važan enzim u fenilpropanoidnom putu koji katalizira primarnu deaminaciju L-fenilalanina u trans-cimetnu kiselinu osiguravajući tako prekursore za sintezu sekundarnih spojeva kao što su PHE, lignin i salicilna kiselina, važnih u obrambenim reakcijama biljaka (Duba i sur., 2019.). Fenilpropanoidni put osigurava prekursore za širok raspon fenolnih spojeva i važna je regulacijska točka između primarnog i sekundarnog metabolizma biljaka (Huang i sur., 2010.). Poznato je kako je upravo pojačana sinteza i nakupljanje PHE u biljnim tkivima prvi odgovor biljke na različite abiotičke i biotičke stresne uvjete (Bhattacharya i sur., 2010.). Djelovanjem enzima PPO akumulirani se PHE mogu oksidirati u kinone, spojeve s jakim antimikrobnim djelovanjem (Taranto i sur., 2017.). PPO katalizira reakciju oksidacije PHE u kinone pri čemu koristi molekularni kisik kao akceptor elektrona. PPO su u biljnom tkivu uključene u brojne biološke funkcije, uzrokuju enzimsko tamnjenje usjeva i njihovih krajnjih proizvoda (Taranto i sur., 2017.) te su uključene u obrambene mehanizme protiv biljnih patogena i detoksikaciju ROS-a (Mohammadi i Kazemi, 2002.; Raj i sur., 2006.; Sorahinobar i sur., 2015.). Povećana sinteza i aktivnost PPO mogu se koristiti kao biokemijski pokazatelj stupnja otpornosti i/ili osjetljivosti biljaka na različite negativne abiotičke i biotičke stresne uvjete (Lebeda i sur., 2001.; Raj i sur., 2006.; Taranto i sur., 2017.).

U literaturi su dostupna istraživanja o utjecaju FHB-a na oksidacijski i antioksidacijski odgovor pšenice u kojima su utvrđene značajne sortne razlike u antioksidacijskom odgovoru (Sorahinobar i sur., 2015.; Khaledi i sur., 2016.; Španić i sur., 2017b; Marček i sur., 2018.; Španić i sur., 2020.). Sorahinobar i sur. (2015.) istraživali su utjecaj umjetne infekcije *Fusarium* vrstama na fiziološki odgovor dvije različite sorte ozime pšenice prema stupnju osjetljivosti na FHB (Sumai-3 – otporna i Falat – osjetljiva). Zaključuju kako povećana koncentracija $H_2O_2$ kao signalne molekule i brza indukcija aktivnosti antioksidacijskih enzima kod sorte Sumai-3 imaju važnu ulogu u otpornosti na FHB. U istraživanju Khaledi i sur. (2016.) korištene su sorte Gaskozhen – umjereno otporna i Falat – osjetljiva na FHB. Autori zaključuju kako je povećana aktivnost antioksidacijskih enzima (SOD, CAT, GPOD i APX) u listovima i klasovima sorte Gaskozhen povezana s njezinom boljom otpornošću na FHB. Španić i sur. (2017b) proveli su istraživanja utjecaja umjetne infekcije *Fusarium* vrstama na oksidacijski status i antioksidacijski odgovor ozime pšenice na tri različite sorte (Vulkan – otporna, Kraljica

– umjereno otporna i Golubica – osjetljiva). Zaključuju kako sorta Golubica na napad patogena reagira povećanjem aktivnosti enzima GPOD. U ranim fazama nakon infekcije kod sorata Vulkan i Kraljica dolazi do povećanja aktivnosti enzima APX i PPO. Zaključuju kako antioksidacijski odgovor pšenice na napad patogena može biti koristan pokazatelj pri selekciji na FHB otpornost. Marček i sur. (2018.) uočavaju kako je povećana aktivnost enzima APX i GPOD kod otporne sorte Apache uključena u otpornost na FHB. Na temelju procjene zaraze Španić i sur. (2020.) utvrdili su da su sorte Apache i U-1 otpornije na napad patogena od sorte Bezostaya-1. Autori zaključuju kako je brza aktivacija enzima GPOD i APX u sorte U-1 uključena u ograničavanje širenja patogena. S druge strane, obrambeni odgovor sorte Apache uključuje metabolizam PHE koji je induciran $H_2O_2$ kao signalnom molekulom. Autori također zaključuju kako slabija aktivacija antioksidacijskih enzima u sorte Bezostaya-1 onemogućuje obranu od napada i širenja patogena. Literaturni podatci o utjecaju različite gnojidbe dušikom na oksidacijski status pšenice vrlo su ograničeni. Pri nedovoljnoj gnojidbi dušikom dolazi do aktivacije obrambenih mehanizama u tkivu ozime pšenice, odnosno dolazi do povećanja koncentracije $H_2O_2$ i povećanja aktivnosti antioksidacijskih enzima (Mamenko, 2018.). S druge strane, prekomjerna upotreba dušika djeluje na smanjenje aktivnosti antioksidacijskih enzima i povećanje LPO (Kong i sur., 2017.). Istraživanja u sklopu ove doktorske disertacije pružaju uvid o oksidacijskom i antioksidacijskom odgovoru pšenice na kombinaciju utjecaja infekcije vrstama roda *Fusarium* i različite gnojidbe dušikom.

# 3. CILJEVI I HIPOTEZE ISTRAŽIVANJA

Ciljevi ovog istraživanja su:

1. utvrditi utjecaj različite gnojidbe dušikom na pojavnost i intenzitet FHB-a;
2. odrediti utjecaj inokulacije vrstama roda *Fusarium* i različite gnojidbe dušikom na fiziološki odgovor, tj. oksidacijski i antioksidacijski status lista zastavičara i klasa različitih sorti pšenice;
3. utvrditi odražava li se infekcija vrstama roda *Fusarium* u klasu na antioksidacijski odgovor lista zastavičara te postoji li povezanost fiziološkog odgovora lista zastavičara i klasa;
4. pronaći fiziološki parametar koji bi mogao poslužiti kao dobar biomarker za daljnje oplemenjivanje i uzgoj otpornijih sorti pšenice na proučavane stresne uvjete.

Glavne hipoteze ovog istraživanja su:

1. različita gnojidba dušikom uzrokovat će značajne razlike u pojavnosti i intenzitetu FHB-a;
2. različite sorte pšenice pokazuju sortno-specifični fiziološki odgovor na FHB i različitu gnojidbu dušikom;
3. klas i list zastavičar pšenice pokazat će sličan antioksidacijski odgovor;
4. fenolni će spojevi značajno doprinijeti obrambenom odgovoru pšenice na FHB.

# 4. MATERIJAL I METODE RADA

## 4.1. Poljski pokusi

### 4.1.1. Poljski pokus tijekom vegetacijske godine 2017./2018.

U svrhu ovog istraživanja postavljen je poljski pokus tijekom vegetacijske godine 2017./2018. na poljima Poljoprivrednog instituta Osijek. Pokus je postavljen prema *split-split plot* dizajnu kao kompletni randomizirani blok u tri ponavljanja, kao višefaktorijalni pokus s tri glavna faktora: sorta, gnojidba dušikom i infekcija vrstama roda *Fusarium* (prirodna i umjetna infekcija). Istraživanje je provedeno na devet sorti ozime pšenice različitog podrijetla (BC Mandica, BC Opsesija, Bezostaya-1, Felix, Ficko, Galloper, Ingenio, Isengrain i U-1) (Tablica 1).

**Tablica 1.** Sorte ozime pšenice i njihovo podrijetlo.

| Sorta | Zemlja podrijetla | Oplemenjivačka kuća | Godina priznavanja |
|---|---|---|---|
| BC Mandica | Hrvatska | Bc Institut d.d. Zagreb | 2015. |
| BC Opsesija | Hrvatska | Bc Institut d.d. Zagreb | 2016. |
| Bezostaya-1 | Rusija | Krasnodar Lukyanenko Research Institute | 1959. |
| Felix | Hrvatska | Poljoprivredni institut Osijek | 2007. |
| Ficko | Hrvatska | Poljoprivredni institut Osijek | 2007. |
| Galloper | Hrvatska | Poljoprivredni institut Osijek | 2014. |
| Ingenio | Francuska | CC Benoist SA | 2010. |
| Isengrain | Francuska | Florimond Desprez Veuve et Fils (FR) | 1997. |
| U-1 | Hrvatska | Poljoprivredni institut Osijek | 1936. |

Izvor: Matić i sur., 2021a

Osnovna je gnojidba bila jednaka za sve parcele i iznosila je 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$ i 120 kg $K_2O$ $ha^{-1}$ te je primijenjena dodatkom 100 kg $ha^{-1}$ Uree i 400 kg $ha^{-1}$ NPK (7 : 20 : 30). Tretman dušikom uključivao je dvije različite prihrane dušikom, bez prihrane 0 kg N $ha^{-1}$ (niska razina dušika; engl. *low nitrogen*, LN) i 100 kg N $ha^{-1}$ (visoka razina dušika; engl. *high nitrogen*, HN), koja je primijenjena u dva obroka po 50 kg N $ha^{-1}$ u fazi vlatanja (Zadoksova skala 23 – 25) te u fazi izduživanja stabljike (Zadoksova skala 33 – 35) (Tablica 2).

**Tablica 2.** Sadržaj dušika (N) u tlu (kg $ha^{-1}$) u Osijeku u vegetacijskoj godini 2017./2018.

| Lokacija | Tip tla | Godina | Rezidualni N u tlu (kg N $ha^{-1}$) | Osnovna N gnojidba (kg N $ha^{-1}$) | N prihrana (kg N $ha^{-1}$) | | Ukupni N (kg N $ha^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | LN | HN | LN | HN |
| Osijek | Eutrični kambisol | 2017./2018. | 20 | 74 | 0 | 50 + 50 | 94 | 194 |

LN – niska razina dušika/*low nitrogen level*; HN – visoka razina dušika/*high nitrogen level*. Izvor: Matić i sur., 2021a

Za inokulaciju pšenice korištena je konidijska suspenzija vrste *F. culmorum*, a za proizvodnju inokuluma korištena je modificirana metoda po Snijders i Van Eeuwijk (1991.). Mješavina zrna pšenice i zobi (3 : 1) ostavljena je preko noći u vodi, nakon čega je autoklavirana i inokulirana sporama gljive *F. culmorum* iz Kolekcije fitopatogenih gljiva Fakulteta agrobiotehničkih znanosti Osijek, Katedra za fitopatologiju. Inokulirana zrna inkubirana su tri tjedna na 25 °C, zaštićena od sunčeve svjetlosti. Nakon inkubacije, spore gljive *F. culmorum* isprane su sa zrna sterilnom $H_2O$ te su prebrojane pod mikroskopom pomoću Bürker-Türkove komorice. Konačna koncentracija spora u inokulumu podešena je na $1 \times 10^6$ spora $mL^{-1}$. Umjetna inokulacija ručnom prskalicom vršena je primarno na klas pšenice te samo na prvom $m^2$ svake parcele (150 mL suspenzije $m^{-2}$). Ostatak biljaka na parceli prepušten je prirodnoj infekciji. Inokulacija je izvršena pojedinačno na svakoj parceli kada se 50 % biljaka po parceli nalazilo u fenofazi cvatnje (Zadoksova skala 65) te je ponovljena nakon 48 sati. Za održavanje vlage za optimalne uvjete infekcije pšenica je nekoliko puta tijekom dana prskana vodom. Vizualna procjena simptoma FHB-a provedena je 18 dana nakon inokulacija, a postotak zaraženih klasova procijenjen je prema linearnoj skali (0 – 100 %) prema EPPO standardu.

### 4.1.2. Poljski pokusi tijekom vegetacijske godine 2018./2019. i 2019./2020.

Poljski pokusi postavljeni su tijekom dvije vegetacijske godine (2018./2019. i 2019./2020.) na Poljoprivrednom institutu Osijek. Pokusi su postavljeni prema *split-split plot* dizajnu kao kompletni randomizirani blokovi u dva ponavljanja, kao višefaktorijalni pokusi s tri glavna faktora: sorta, gnojidba dušikom i infekcija vrstama roda *Fusarium* (prirodna i umjetna infekcija). U istraživanju su korištene četiri sorte ozime pšenice koje su odabrane na temelju različite razine osjetljivosti na FHB: Srpanjka, Sofru, Apache i Graindor. Srpanjka je vrlo rana sorta, izuzetno niske stabljike, srednje osjetljiva na *Fusarium* sp. te najrasprostranjenija sorta u Hrvatskoj do 2014. godine. Sofru je rana, visokoprinosna sorta, osjetljiva na *Fusarium* sp., druga najraširenija sorta u Hrvatskoj danas. Apache je srednje kasna, vrlo prilagodljiva i

stabilna sorta s izvrsnom otpornošću na *Fusarium* sp. Graindor je srednje kasna do kasna sorta, visokoprinosna sorta s odličnom otpornošću na *Fusarium* sp.

Osnovna gnojidba od 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$ i 120 kg $K_2O$ $ha^{-1}$ primijenjena je dodatkom 100 kg $ha^{-1}$ Uree (udio dušika od 46 %, pri čemu je 100 % dušika u amidnom obliku) i 400 kg $ha^{-1}$ NPK (7 : 20 : 30; 8,5 % dušika u amonijskom i 6,5 % u nitratnom obliku) i bila je jednaka za sve parcele. Tretman dušikom uključivao je različite prihrane dušikom koja je primijenjena ručno dodatkom KAN gnojiva (udio dušika od 27 %) u fazi vlatanja (Zadoksova skala 23 – 25) te fazi izduživanje stabljike (Zadoksova skala 33 – 35) u razinama od 0, 35, 70 i 140 kg N $ha^{-1}$ po tretmanu (Tablica 3). KAN gnojiva sadrže dva oblika dušika, pri čemu je 13,5 % dušika u amonijskom i 13,5 % u nitratnom obliku.

**Tablica 3.** Sadržaj dušika (N) u tlu (kg $ha^{-1}$) u Osijeku u vegetacijskoj godini 2018./2019. i 2019./2020.

| Lokacija | Tip tla | Godina | Rezidualni N u tlu (kg N $ha^{-1}$) | Osnovna N gnojidba (kg N $ha^{-1}$) | N prihrana (kg N $ha^{-1}$) | Ukupni N (kg N $ha^{-1}$) |
|---|---|---|---|---|---|---|
| Osijek | Eutrični kambisol | 2018./2019. i 2019./2020. | 20 | 74 | 0 | 94 |
| | | | | | 35 | 129 |
| | | | | | 70 | 164 |
| | | | | | 140 | 234 |

Izvor: Matić i sur., 2022.

Unutar svake parcelice nasumično je odabrano 50 klasova pšenice za *Fusarium* inokulaciju, a 50 klasova prepušteno je prirodnoj infekciji. Unutar svake parcelice inokulirani i neinokulirani klasovi pšenice bili su prostorno odvojeni. Za proizvodnju inokuluma korištena je modificirana metoda po Snijders i Van Eeuwijk (1991.). Inokulum za infekciju pšenice sastojao se od suspenzije konidija dviju vrsta roda *Fusarium*, *F. graminearum* (IFA 65) i *F. culmorum*. Konačna koncentracija spora u inokulumu podešena je na $1 \times 10^6$ spora $mL^{-1}$. *Fusarium* inokulacija vršena je ručnom prskalicom na svakoj sorti pojedinačno kada se 50 % biljaka po parcelici nalazilo u fenofazi cvatnje (Zadoksova skala 65). Inokulacije su obavljene u prijepodnevnim satima te su ponovljene nakon 48 sati. Kako bi se održala optimalna vlažnost za infekciju, klasovi su tijekom 48 sati bili prekriveni polietilenskim vrećicama. Ozbiljnost FHB-a (točnije postotak zaraženih klasića po klasu) procijenjena je pomoću linearne skale (0 – 100 %) 10., 14., 18., 22., 26. i 30. dana nakon inokulacije. Na temelju dobivenih postotaka zaraze izračunato je područje unutar progresivne krivulje bolesti (engl. *area under the disease*

*progress curve*, AUDPC), kao integrirana jedinica za ukupni intenzitet bolesti odnosno, ukupnu otpornost na napad patogena.

## 4.2. Određivanje pokazatelja oksidacijskog stresa i antioksidacijskog odgovora pšenice

Za određivanje pokazatelja oksidacijskog stresa i antioksidacijskog odgovora pšenice uzorkovanje listova zastavičara ili klasova pšenice (ovisno o istraživanju) izvršeno je sedam dana nakon *Fusarium* inokulacije. Sakupljeni uzorci (prirodna i umjetna infekcija) odmah su smrznuti u tekućem dušiku i pohranjeni na -80 °C do daljnjih analiza. Uzorci listova zastavičara usitnjeni su pomoću tarionika i tučka uz pomoć tekućeg dušika, dok su klasovi pšenice usitnjeni pomoću homogenizatora TissueLyser (Qiagen, Hilden, Njemačka) tijekom 1 minute, pri vibracijskoj frekvenciji od 30 Hz. Usitnjeno tkivo listova zastavičara i klasova odvagano je u mikroepruvete za potrebe daljnjih analiza.

### 4.2.1. Određivanje pokazatelja oksidacijskog stresa pšenice

Kako bi odredili utjecaj FHB-a i različite gnojidbe dušikom na oksidacijski status pšenice, kao pokazatelji oksidacijskog stresa mjereni su razina LPO i koncentracija $H_2O_2$. Razina LPO određena je mjerenjem količine reaktivnih supstanci tiobarbiturne kiseline (TBARS), uglavnom MDA, metodom po Verma i Dubey (2003.). Produkti LPO ekstrahirani su pomoću 0,1 % trikloroctene kiseline (TCA) kako bi se denaturirali i centrifugiranjem uklonili proteini iz ekstrakta. Dobiveni su ekstrakti inkubirani s 0,5 % tiobarbiturnom kiselinom (TBA) u kiselom mediju (20 % TCA) i pri visokoj temperaturi, pri čemu MDA reagira s TBA, uslijed čega dolazi do pojave crvene boje čiji je intenzitet mjeren spektrofotometrijski pri 532 nm na UV-VIS spektrofotometru Perkin Elmer Lambda 25 (PerkinElmer, Waltham, SAD). Količina MDA određena je pomoću jednadžbe pravca standardnog dijagrama, a kao standard korišten je 1,1,3,3-tetrametoksipropan. Rezultati su izraženi u nanomolima po gramu svježe tvari (nmol $g^{-1}$ sv. tv.).

Koncentracija $H_2O_2$ određena je metodom po Mukherjee i Choudhuri (1983.). $H_2O_2$ je iz tkiva listova zastavičara ekstrahiran pomoću 1 mL ledeno-hladnog acetona, a dobiveni je homogenat centrifugiran. Dobiveni je supernatant kvantitativno prebačen u drugu tubicu te mu je dodano 400 µL otopine titan-sulfata i 500 µL koncentriranog amonijevog hidroksida ($NH_4OH$). Reakcija između titan-sulfata i $NH_4OH$ egzotermna je reakcija pri kojoj dolazi do nastanka i taloženja kompleksa titan-peroksida. Istaloženi kompleks titan-peroksida odvojen je

centrifugiranjem, a dobiveni je bijeli talog zatim otopljen s 1 mL 2 M otopine $H_2SO_4$. Apsorbancija dobivene otopine mjerena je pri valnoj duljini od 415 nm na UV-VIS spektrofotometru Perkin Elmer Lambda 25 (PerkinElmer, Waltham, SAD). Koncentracija $H_2O_2$ izračunata je iz jednadžbe pravca standardnog dijagrama s poznatom koncentracijom $H_2O_2$ kao standardom. Rezultati su izraženi u nanomolima po miligramu svježe tvari (nmol $mg^{-1}$ sv. tv.).

### 4.2.2. Određivanje pokazatelja antioksidacijskog odgovora pšenice

Antioksidacijski odgovor određen je spektrofotometrijski mjerenjem aktivnosti antioksidacijskih enzima CAT, APX i GR. Za mjerenje aktivnosti antioksidacijskih enzima, proteinski su ekstrakti pripremljeni homogeniziranjem tkiva sa 100 mM kalij-fosfatnim puferom (pH=7,0, 1 mM EDTA + polivinilpirolidon (PVP)) te centrifugiranjem. PVP služi za uklanjanje fenolnih spojeva iz biljnog ekstrakta koji bi mogli ometati mjerenje aktivnosti enzima. Aktivnosti antioksidacijskih enzima određene su spektrofotometrijski na UV-VIS spektrofotometru Perkin Elmer Lambda 25 (PerkinElmer, Waltham, SAD). Aktivnost enzima CAT određena je metodom po Aebiju (1984.). Reakcijska smjesa za mjerenje enzima CAT sastojala se od 0,036 %-tne otopine $H_2O_2$ u 50 mM kalij-fosfatnom puferu (pH 7,0). Pad apsorbancije praćen je spektrofotometrijski pri valnoj duljini 240 nm svakih 10 sekundi tijekom 3 minute. Aktivnost APX-a određena je metodom koju su opisali Nakano i Asada (1981.). Reakcijska smjesa za kinetičko mjerenje sastojala se od 0,5 mM askorbinske kiseline i proteinskog ekstrakta u 50 mM kalij-fosfatnom puferu s 0,1 mM EDTA (pH 7,0). Nakon ekvilibracije smjese, reakcija je potaknuta dodatkom $H_2O_2$ u konačnoj koncentraciji 0,12 mM. Pad apsorbancije, uslijed razgradnje askorbata, praćen je na 290 nm svakih 15 sekundi tijekom 3,5 minute. Aktivnost GR-a određena je metodom po Halliwell i Foyer (1978.), koja se temelji na redukciji GSH koju katalizira GR koristeći NADPH. Reakcijska smjesa za mjerenje aktivnosti GR-a sastojala se od 1 mM GSSG-a i proteinskog ekstrakta u 100 mM kalij-fosfatnom puferu s 1 mM EDTA (pH 7,5). Nakon ekvilibracije reakcija je započeta dodatkom NADPH konačne koncentracije 0,1 mM. Pad apsorbancije, do kojeg dolazi uslijed oksidacije NADPH, praćen je na 340 nm svakih 30 sekundi tijekom 4 minute. Specifična aktivnost pojedinog antioksidacijskog enzima izražena je kao količina (μmol) razgrađenog supstrata po minuti po miligramu proteina, odnosno u jedinicama aktivnosti enzima po miligramu proteina (U $mg^{-1}$ proteina; U = μmol $min^{-1}$ proteina). Koncentracija topljivih proteina određena je metodom po Bradfordu (1976.). Metoda se temelji na nespecifičnom vezanju anionskog oblika

boje za bazične i aromatske bočne ogranke proteina, uslijed čega dolazi do stvaranja kompleksa proteina i boje, čija se apsorbancija mjeri pri valnoj duljini od 595 nm. Metoda je prilagođena za mjerenje na čitaču mikrotitarskih pločica Tecan Spark 10M (Tecan, Männedorf, Švicarska), a koncentracija topljivih proteina određena je pomoću jednadžbe pravca standardnog dijagrama, pri čemu je kao standard korišten albumin goveđeg seruma.

Kao neenzimski biomarkeri antioksidacijskog statusa određene su koncentracije ukupnih topljivih PHE, sadržaj fotosintetskih pigmenata te ukupni antioksidacijski kapacitet. Ekstrakcija PHE provedena je u 80 %-tnom etanolu, pri 80 °C. Koncentracija ukupnih fenola određena je metodom po Folin-Ciocalteu (Folin i Ciocalteu, 1927.). Metoda se temelji na reakciji Folin-Ciocalteu reagensa (kompleks fosfomolibdenskefosfovolframske kiseline) s fenolnim spojevima pri čemu dolazi do nastanka plavo obojenog produkta čiji se intenzitet mjerio spektrofotometrijski pri 765 nm na UV-VIS spektrofotometru Perkin Elmer Lambda 25 (PerkinElmer, Waltham, SAD). Koncentracija ukupnih PHE određena je pomoću jednadžbe pravca standardnog dijagrama, a kao standard korištena je galna kiselina (engl. *gallic acid,* GA). Rezultati su izraženi u mikrogramima ekvivalenata galne kiseline (engl. *gallic acid equivalent*, GAE) po gramu svježe tvari (μg GAE $g^{-1}$ sv. tv.).

Fotosintetski pigmenti (klorofila a, klorofila b i karotenoidi) ekstrahirani su ledeno-hladnim acetonom, a apsorbancija ekstrakata mjerena je pri valnim duljinama od 470, 645 i 662 nm na UV-VIS spektrofotometru Perkin Elmer Lambda 25 (PerkinElmer, Waltham, SAD). Koncentracije fotosintetskih pigmenata, kao i koncentracije ukupnog klorofila (Chl a+b), izračunate su formulama po Lichtenthaleru (Lichtenthaler, 1987.) te su izražene u miligramima po gramu svježe tvari (mg $g^{-1}$ sv. tv.).

Ukupni antioksidacijski kapacitet tkiva pšenice određen je metodom FRAP (engl. *ferric reducing antioxidant power*) (Benzie i Strain, 1996.), koja se temelji na sposobnosti antioksidansa da reducira žuti kompleks feri željeza ($Fe^{3+}$) s 2,4,6-tris(2piridil)-s-triazina (TPTZ) u plavo obojeni kompleks $Fe^{2+}$-TPTZ, čiji se intenzitet mjeri na 593 nm. Intenzitet boje proporcionalan je redukcijskoj sposobnosti antioksidansa. Navedena je metoda modificirana za potrebe mjerenja apsorbancije u 96-mikrotitarskim pločicama. Reagens za FRAP analizu sastojao se od 0,5 mM TPTZ-a, 1 mM $FeCl_3 \times 6\ H_2O$ u acetatnom puferu (pH 3,6). Apsorbancija reakcijske smjese koja se sastojala od reagensa i etanolnog ekstrakta mjerena je na čitaču mikrotitarskih pločica Tecan Spark 10M (Tecan, Männedorf, Švicarska) nakon inkubacije na sobnoj temperaturi tijekom 3,5 minute. Ukupni antioksidacijski kapacitet

određen je pomoću jednadžbe pravca standardnog dijagrama, a kao standard korišten je Trolox (sintetički vitamin E) u koncentracijama od 0,25 do 2 mM. Rezultati su izraženi u miligramima ekvivalenata Troloxa po miligramu svježe tvari (mg Trolox $mg^{-1}$ sv. tv.).

### 4.2.3. Određivanje aktivnosti enzima PAL i PPO uključenih u metabolizam fenola

Proteinski ekstrakti za određivanje aktivnosti enzima PAL i PPO pripremljeni su homogenizacijom klasova pšenice s odgovarajućim puferima za ekstrakciju (1 : 5, w/v). Tako je za ekstrakciju PAL-a korišten 150 mM Tris-HCl pufer (pH 8,5) s 0,2 % (w/v) PVP-a, dok je za ekstrakciju PPO korišten 100 mM kalij-fosfatni pufer (pH 7,0) s 1 mM EDTA i 0,2 % (w/v) PVP-a. Homogenizirani su uzorci centrifugirani, a dobiveni supernatanti korišteni su za spektrofotometrijsko određivanje aktivnosti enzima PAL i PPO.

Aktivnost PAL-a određena je prema modificiranoj metodi po Havir i Hanson (1970.). Reakcijska se smjesa sastojala od 2 mM L-fenilalanina kao supstrata i proteinskog ekstrakta u 150 mM Tris-HCl puferu (pH 8,5) u konačnom volumenu od 1,5 mL. Povećanje apsorbancije zbog proizvodnje trans-cimetne kiseline praćeno je pri valnoj duljini od 270 nm tijekom 5 minuta, svakih 30 sekundi na 40 °C na UV-VIS spektrofotometru Perkin Elmer Lambda 25 (PerkinElmer, Waltham, SAD). Aktivnost PAL-a izračunata je koristeći ekstinkcijski koeficijent ($\varepsilon$ = 19,73 mM $cm^{-1}$) te je izražena kao količina (μmol) nastalog produkta po minuti po miligramu proteina, odnosno u jedinicama aktivnosti enzima po miligramu proteina (U $mg^{-1}$ proteina; U = μmol $min^{-1}$ proteina).

Aktivnost PPO određena je metodom koju su opisali Marumo i Waite (1986.). Navedena je metoda modificirana za potrebe mjerenja apsorbancije u 96-mikrotitarskim pločicama. Metoda se temelji na reakciji spajanja derivata benzokinona, nastalog tijekom oksidacije L-3,4-dihidroksifenilalanina (L-DOPA) posredovanjem enzima PPO, i L-askorbinske kiseline čijim spajanjem nastaje dehidroaskorbinska kiselina. Reakcijska se smjesa sastojala od 0,17 mM L-3,4-dihidroksifenilalanina, 0,07 mM L-askorbinske kiseline, 0,002 mM EDTA i proteinskog ekstrakta u 50 mM kalij-fosfatnom puferu (pH 6,5) u konačnom volumenu od 0,3 mL. Spektrofotometrijsko određivanje aktivnosti PPO provedeno je na čitaču mikrotitarskih pločica Tecan Spark 10M (Tecan, Männedorf, Švicarska). Smanjenje apsorbancije praćeno je pri valnoj duljini od 265 nm tijekom 3 minute, svakih 15 sekundi na sobnoj temperaturi. Aktivnost PPO izračunata je koristeći ekstinkcijski koeficijent ($\varepsilon$ = 2,5 mM $cm^{-1}$) te je izražena u

jedinicama aktivnosti enzima po miligramu proteina (U $mg^{-1}$ proteina; U = μmol $min^{-1}$ proteina).

### 4.3. Određivanje koncentracije mikotoksina deoksinivalenola (DON) i zearalenona (ZEA)

Tijekom vegetacijske godine 2019./2020. sakupljeni su neinokulirani i inokulirani klasovi pšenice. Klasovi su ručno ovršeni u laboratoriju, a dobivena su zrna korištena za određivanje koncentracije mikotoksina DON i ZEA. Analize mikotoksina obavljene su u akreditiranom laboratoriju (Eurofins Croatiakontrola, Zagreb, Hrvatska). Mikotoksini su iz uzoraka zrna ekstrahirani deioniziranom vodom, a dobiveni su ekstrakti pročišćeni korištenjem imunoafinitetnih kolona DONStar® IAC (Romer Labs Diagnostic GmbH, Tulln, Austrija). Koncentracija mikotoksina određena je korištenjem tekućinske kromatografije visoke djelotvornosti (engl. *high-performance liquid chromatography*, HPLC) s detektorom s nizom dioda (engl. *diode array detector,* DAD) (HPLC-DAD). Kromatografsko odvajanje provedeno je na Agilent Zorbax C18 kolonama (150 mm × 4,6 mm, 5 μm). Rezultati koncentracije mikotoksina izraženi su u mikrogramima po kilogramu (μg $kg^{-1}$).

# 5. REZULTATI ISTRAŽIVANJA S RASPRAVOM

## 5.1. Oksidacijski status i antioksidacijski odgovor na *Fusarium* infekciju i različite razine gnojidbe dušikom u klasovima ozime pšenice

Neadekvatna gnojidba dušikom i zaraza fitopatogenim gljivama roda *Fusarium* predstavljaju abiotičke i biotičke stresne čimbenike u proizvodnji pšenice. Trenutno je u literaturi dostupan malen broj istraživanja koja se odnose na utjecaj FHB-a i različite gnojidbe dušikom na fiziološki odgovor, odnosno oksidacijski i antioksidacijski status pšenice. U ovom smo istraživanju, u uvjetima na polju, ispitali utjecaj *Fusarium* tretmana i tretmana dušikom na pojavu FHB-a, na oksidacijski stres i antioksidacijski odgovor klasova devet sorti ozime pšenice. Općenito, *Fusarium* tretman i tretman dušikom imali su utjecaj na sve ispitivane biokemijske pokazatelje.

Trofaktorijalnom analizom varijance (ANOVA) utvrđen je značajan utjecaj sva tri faktora istraživanja (sorta, tretman dušikom i *Fusarium* tretman) na vizualnu procjenu simptoma FHB-a (Tablica 4). Najveći utjecaj na vizualnu procjenu simptoma FHB-a imali su *Fusarium* tretman i sorta pšenice ($p \leq 0,001$) te u manjoj mjeri tretman dušikom ($p \leq 0,05$). Neinokulirane sorte pšenice nisu pokazivale simptome FHB-a, stoga nisu računate niti prikazane razlike između inokuliranih i neinokuliranih biljaka.

**Tablica 4.** Trofaktorijalna analiza varijance (ANOVA) za vizualnu procjenu simptoma FHB-a i pet različitih biokemijskih parametara pod utjecajem različitog tretmana dušikom i *Fusarium* tretmana u klasovima devet sorti ozime pšenice.

| | | Sredina kvadrata | | | | | |
|---|---|---|---|---|---|---|---|
| **Izvor varijacije** | **Stupnjevi slobode** | **VS[1]** | **TBARS** | **PHE** | **CAT** | **APX** | **GR** |
| **Sorta**/*Variety* (V) | 8 | 0,08*** | 59,92*** | 108,02*** | 1700,63*** | 1,295*** | 719,05*** |
| **Tretman dušikom**/*Nitrogen treatment* (N) | 1 | 0,04* | 43,73** | 20,51 ns | 1215,25** | 4,227*** | 180,06 ns |
| ***Fusarium* inokulacija**/*Fusarium inoculation* (F) | 1 | 23,02*** | 11,62 ns | 190,55*** | 669,20* | 0,001 ns | 733,05* |
| V×N | 8 | 0,14*** | 17,14*** | 38,75* | 163,39 ns | 0,571*** | 176,72 ns |
| V×F | 8 | 0,08*** | 6,78 ns | 25,96 ns | 801,97*** | 0,307*** | 299,44 ns |
| N×F | 1 | 0,04* | 2,31 ns | 153,85** | 1769,33*** | 0,338* | 808,47* |
| V×N×F | 8 | 0,14*** | 7,16 ns | 27,53 ns | 579,37*** | 0,439*** | 321,27 ns |

ns – nije statistički značajno/*not significant*, *, ** i *** – značajno na razini $p \leq 0,05$, 0,01 i 0,001. VS, vizualna procjena/*visual scoring*; TBARS, reaktivne supstance tiobarbiturne kiseline/*thiobarbituric acid reactive substances*; PHE, fenoli/*phenolics*; CAT, katalaza/*catalase*; APX, askorbat-peroksidaza/*ascorbate peroxidase*; GR, glutation-reduktaza/*glutathione reductase*.
[1]Za normalizaciju rezultata vizualne procjene korištena je logaritamska transformacija podataka. Izvor: Matić i sur., 2021a

Razlike u procjeni vizualnih simptoma FHB-a u inokuliranim sortama pšenice pri niskim i visokim razinama dušika, za svaku sortu pojedinačno, prikazane su u Tablici 5. Visoka je razina dušika, u usporedbi s niskom razinom dušika, uzrokovala povećanje vizualnih simptoma FHB-a u sortama Ficko, Galloper i Felix. Suprotno tomu, visoka je razina dušika u sorti U-1 uzrokovala smanjenje vizualnih simptoma FHB-a, što ukazuje na sortno-specifični odgovor pšenice. S obzirom na različite eksperimentalne uvjete, u literaturi su dostupni brojni, ponekad proturječni podatci o utjecaju dušika na pojavnost i intenzitet FHB-a. Prema pojedinim autorima, visoka razina dušika povećava pojavnost i intenzitet bolesti (Lemmens i sur., 2004.; Suproniene i sur., 2011.; Vrandečić i sur., 2019.), dok prema drugim autorima, visoki unos dušika nema značajnog utjecaja (Yoshida i sur., 2008.; Van der Burgt i sur., 2011.; Krnjaja i sur., 2015.). Međutim, kako bismo sa što većom sigurnošću mogli utvrditi utjecaj različitih razina dušika na pojavnost i intenzitet FHB-a, u daljnja je istraživanja potrebno uključiti širi raspon gnojidbe dušikom te su potrebne opsežnije mikološke i mikotoksikološke analize.

**Tablica 5.** Vizualna procjena simptoma fuzarijske paleži klasa u devet inokuliranih sorti ozime pšenice pri dvije različite razine dušika.

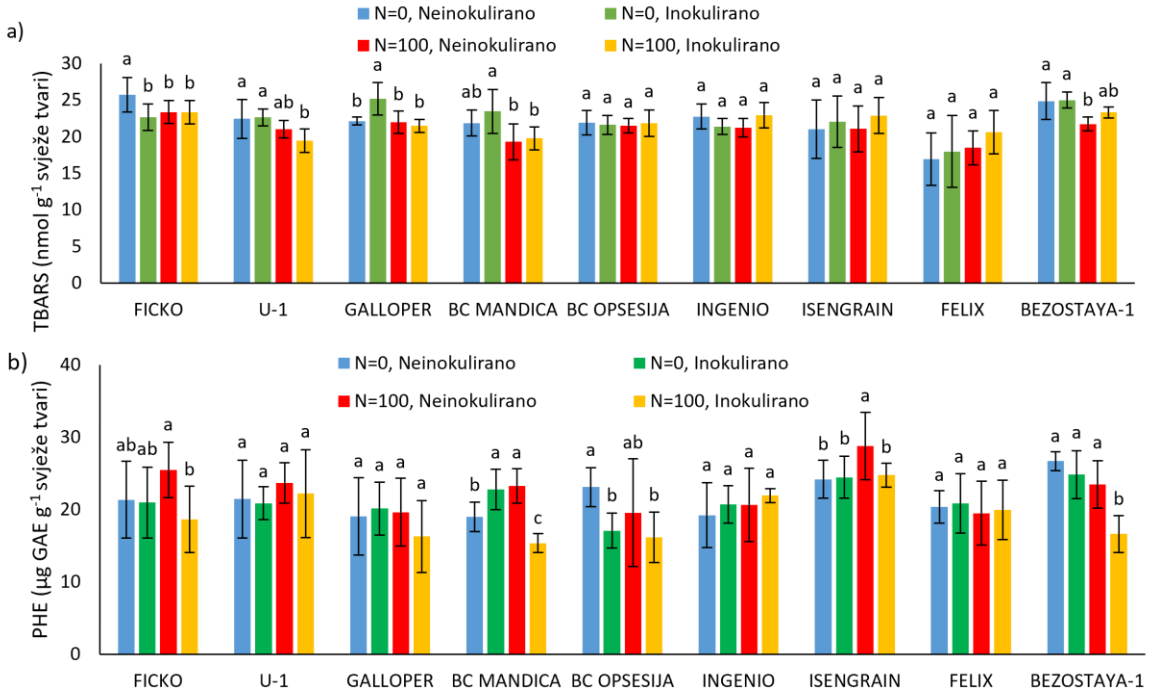
| | Vizualna procjena (u postotcima zaraženih klasova) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Razina dušika** | **Ficko** | **U-1** | **Galloper** | **BC Mandica** | **BC Opsesija** | **Ingenio** | **Isengrain** | **Felix** | **Bezostaya-1** |
| LN | 6,67 ± 1,53b | 8,00 ± 1,00a | 3,33 ± 1,53b | 9,00 ± 5,29a | 20,00 ± 10,00a | 10,33 ± 4,51a | 10,00 ± 0,00a | 8,33 ± 2,89b | 5,00 ± 0,00a |
| HN | 25,00 ± 5,00a | 3,33 ± 0,58b | 10,00 ± 0,00a | 7,00 ± 2,65a | 4,33 ± 0,58a | 10,33 ± 4,51a | 8,33 ± 2,89a | 32,67 ± 2,52a | 8,00 ± 2,00a |

Vrijednosti su prikazane kao srednja vrijednost tri replike ± standardna devijacija. Različita slova označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). LN – niska razina dušika/*low nitrogen level*; HN – visoka razina dušika/*high nitrogen level*. Izvor: Matić i sur., 2021a

Trofaktorijalna ANOVA utjecaja sorte, tretmana dušikom i *Fusarium* tretmana na mjerene biokemijske parametre prikazana je u Tablici 4. Sorta pšenice imala je značajan utjecaj na sve ispitivane biokemijske parametre ($p \leq 0,001$), dok je tretman dušikom značajno utjecao na količinu TBARS-a, aktivnost enzima CAT i APX. *Fusarium* tretman utjecao na koncentraciju PHE ($p \leq 0,001$), a u manjoj mjeri na aktivnost enzima CAT i GR ($p \leq 0,05$). Interakcija između sorte i tretmana dušikom bila je značajna za količinu TBARS-a, koncentraciju PHE i aktivnost APX-a. Interakcija između tri glavna faktora bila je značajna za aktivnost enzima CAT i APX.

Razina LPO, kao pokazatelj oksidacijskog stresa, može biti koristan alat za procjenu osjetljivosti različitih sorti pšenice na FHB (Khaledi i sur., 2018.). Na količinu TBARS-a značajno su utjecali sorta pšenice i tretman dušikom (Tablica 4). U sorti Ficko, pri niskoj razini dušika, *Fusarium* tretman uzrokovao je smanjenje količine TBARS-a, dok je u sorti Galloper količina TBARS-a bila povećana (Slika 1a). Povećana količina TBARS-a u sorti Galloper može se objasniti osjetljivošću navedene sorte na FHB. U uvjetima visoke razine dušika,

*Fusarium* tretman nije uzrokovao značajne promjene u razini LPO. Različit odgovor može biti posljedica različite reakcije biljaka na pojedinačni stres u usporedbi s reakcijom na kombinaciju više stresnih uvjeta (Jiang i Huang, 2001.; Rampino i sur., 2012.; Haworth i sur., 2018.). U većine neinokuliranih sorti, niska je razina dušika uzrokovala trend povećanja količine TBARS-a, iako je značajan porast utvrđen samo u sortama Ficko i Bezostaya-1. Različiti oblici abiotičkog stresa dovode do prekomjerne proizvodnje i neuravnotežene detoksikacije ROS-a, što dovodi do pojave LPO (Singh i sur., 2018.; Hasanuzzaman i sur., 2020.). U ovom su istraživanju dobiveni slični rezultati jer je niska razina dušika, kao abiotički stresni čimbenik, uzrokovala povećanje LPO u većine neinokuliranih sorti. Ipak, značajno je povećanje LPO pronađeno samo u neinokuliranim sortama Ficko i Bezostaya-1 koje su također imale i smanjenu aktivnost APX-a, što ukazuje na važnost ovog enzima u uklanjanju ROS-a.

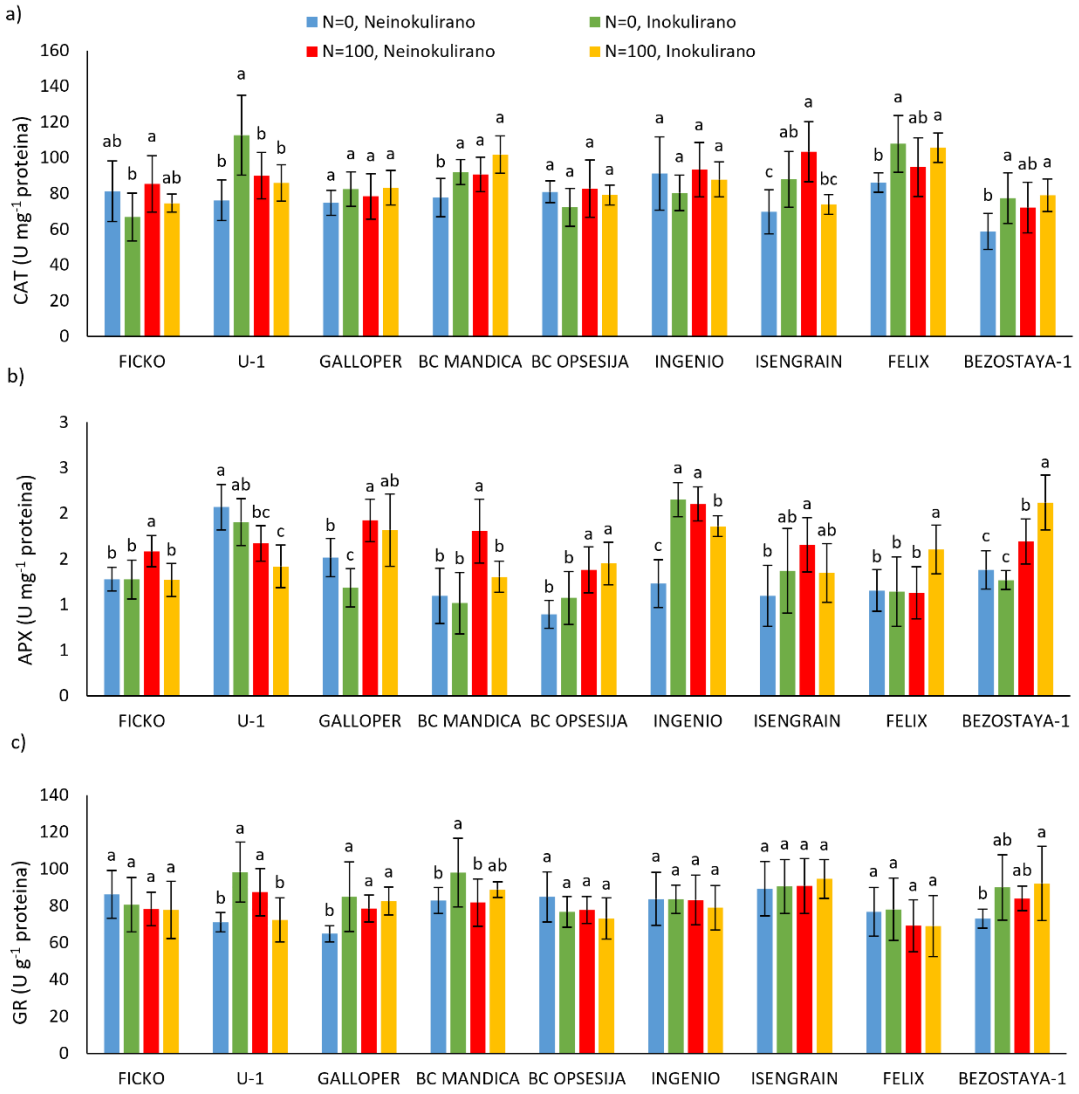



**Slika 1.** Količina reaktivnih supstanci tiobarbiturne kiseline (TBARS; a) i koncentracija topljivih fenola (PHE; b) u klasovima devet sorti pšenice pri različitim prihranama dušikom (0 kg N $ha^{-1}$ i 100 kg N $ha^{-1}$) i tretmanom vrstom *Fusarium culmorum* (neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2021a

Na koncentraciju PHE, najjači su utjecaj imali *Fusarium* tretman i sorta pšenice (Tablica 4). Brojna su istraživanja pokazala kako u uvjetima abiotičkog i biotičkog stresa u biljnom tkivu dolazi do povećanja koncentracije polifenola, kao što su fenolne kiseline i flavonoidi, u svrhu

prevladavanja štetnih posljedica stresnih čimbenika (Datta i Lal, 2012.; Sharma i sur., 2019.). U ovom je istraživanju, *Fusarium* tretman pri niskoj razini dušika uzrokovao povećanje koncentracije PHE u sorti BC Mandica, dok je u sorti BC Opsesija koncentracije PHE bila smanjena (Slika 1b). Povećana koncentracija PHE u sorti BC Mandica upućuje na moguću uključenost PHE u obrambeni odgovor ove sorte protiv napada i širenja patogena. U uvjetima visoke razine dušika, *Fusarium* tretman utjecao je na smanjenje koncentracije PHE u sortama Ficko, BC Mandica, Isengrain i Bezostaya-1 (Slika 1b). U većine neinokuliranih sorti, niska je razina dušika uzrokovala smanjenje koncentracije PHE, iako je značajno smanjenje zabilježeno samo u sortama BC Mandica i Isengrain. Ovaj rezultat nije u skladu s ostalim istraživanjima, u kojima je pri ograničenim uvjetima dušika utvrđeno povećano nakupljanje fenolnih komponenti u biljnom tkivu zbog povećanja C:N omjera unutar biljaka (Ibrahim i sur., 2011.; Munene i sur., 2017.; Deng i sur., 2019.). S druge strane, u istraživanju Matić i sur. (2021b), uzgoj pšenice pri niskoj razini dušika uzrokovao je povećanje koncentracije PHE u listovima zastavičarima neinokuliranih biljaka, što upućuje na tkivno-specifični odgovor pšenice.

Na aktivnost enzima CAT značajno su utjecala sva tri faktora istraživanja: sorta pšenice ($p \leq 0,001$), tretman dušikom ($p \leq 0,01$) i *Fusarium* tretman ($p \leq 0,05$; Tablica 4). U prosjeku je niska razina dušika uzrokovala smanjenje aktivnosti enzima CAT za 5,46 %, u usporedbi s visokom razinom dušika. Analizirajući promjene aktivnosti enzima CAT unutar svake sorte pojedinačno, u sortama U-1, BC Mandica, Isengrain, Felix i Bezostaya-1, *Fusarium* tretman uzrokovao je povećanje aktivnosti enzima CAT pri niskoj razini dušika (Slika 2a). U uvjetima visoke razine dušika, *Fusarium* tretman uzrokovao je smanjenje aktivnosti enzima CAT u sorti Isengrain. U većine neinokuliranih sorti, niska je razina dušika uzrokovala smanjenje aktivnosti enzima CAT, iako je značajno smanjenje utvrđeno samo u sortama BC Mandica i Isengrain.




**Slika 2.** Aktivnosti antioksidacijskih enzima: katalaze (CAT; a), askorbat-peroksidaze (APX; b) i glutation-reduktaze (GR; c) u klasovima devet sorti pšenice pri različitim prihranama dušikom (0 kg N $ha^{-1}$ i 100 kg N $ha^{-1}$) i tretmanom vrstom *Fusarium culmorum* (neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2021a

Na aktivnost APX-a najviše su utjecali tretman dušikom i sorta pšenice (Tablica 4). U prosjeku je niska razina dušika uzrokovala smanjenje aktivnosti APX-a za 17,26 %, u odnosu na visoku razinu dušika. Analizom promjene aktivnosti APX-a unutar svake sorte pojedinačno u sorti Galloper, pri niskoj razini dušika, *Fusarium* tretman uzrokovao je smanjenje aktivnosti APX-a, dok je u sorti Ingenio aktivnost ovog enzima bila povećana (Slika 2b). U uvjetima visoke razine dušika, *Fusarium* tretman uzrokovao je smanjenje aktivnosti APX-a u sortama Ficko, BC Mandica i Ingenio, dok je u sortama Felix i Bezostaya-1 aktivnost APX-a bila povećana.

U većine neinokuliranih sorti (Ficko, Galloper, BC Mandica, BC Opsesija, Ingenio, Isengrain i Bezostaya-1) niska je razina dušika uzrokovala značajno smanjenje aktivnosti APX-a, u usporedbi s visokom razinom dušika. Na aktivnost GR-a najznačajnije je utjecala sorta pšenice ($p \leq 0,001$), a u manjoj mjeri *Fusarium* tretman ($p \leq 0,05$; Tablica 4). *Fusarium* tretman pri niskoj razini dušika uzrokovao je povećanje aktivnosti GR-a u sortama U-1, Galloper i BC Mandica, dok je u uvjetima visoke razine dušika djelovao na smanjenje aktivnosti GR-a samo u sorti U-1 (Slika 2c). U odnosu na visoku razinu dušika, niska je razina dušika uzrokovala značajno smanjenje aktivnosti GR-a u neinokuliranih sorti U-1 i Galloper.

Zaključno, u uvjetima niske razine dušika, *Fusarium* tretman utjecao je na značajno povećanje aktivnosti nekih mjerenih antioksidacijskih enzima (CAT, APX ili GR) u većine sorti. Iznimka je pronađena u sorti Galloper, jedinoj sorti s povećanom razinom LPO, koja ima smanjenu aktivnost APX-a, što ukazuje na važnost ovog enzima u obrambenom odgovoru te sorte. Indukcija enzimskih obrambenih mehanizama povezana je s otpornošću na FHB, pri čemu tolerantnije sorte aktiviraju antioksidacijske enzime brže i ranije u procesu infekcije (Sorahinobar i sur., 2015.; Khaledi i sur., 2016.). S druge strane, u uvjetima visoke razine dušika aktivnosti antioksidacijskih enzima bile su smanjene u većine inokuliranih sorti pšenice. Iako u uvjetima visoke razine dušika nisu utvrđene značajne promjene u količini TBARS-a, smanjena se aktivnost antioksidacijskih enzima mogla odraziti na povećanu učestalost i intenzitet FHB-a. Različit utjecaj *Fusarium* tretmana na antioksidacijski odgovor u uvjetima visoke i niske razine dušika može biti posljedica različitog stvaranja i uklanjanja ROS-a u uvjetima pojedinačnog stresa (*Fusarium* infekcija) i u uvjetima kombiniranih stresnih čimbenika (niska razina dušika i *Fusarium* infekcija). U većini neinokuliranih sorti, niska je razina dušika uzrokovala smanjenje aktivnosti antioksidacijskih enzima. Brojna su druga istraživanja utvrdila smanjenje aktivnosti enzima CAT, APX i GR u tkivu biljaka uzgajanih u uvjetima nedostatne ili smanjene razine dušika (Kandlbinder i sur., 2004.; Lin i sur., 2011.). Smanjena aktivnost antioksidacijskih enzima mogla bi biti povezana sa smanjenom sintezom aminokiselina i proteina u uvjetima nedostatka dušika, u usporedbi s uvjetima visoke razine dušika.

Rezultati trofaktorijalne analize varijance (ANOVA) utvrdili su značajan utjecaj sorte pšenice, *Fusarium* tretmana i tretmana dušikom na sadržaj mjerenih fotosintetskih pigmenata (Tablica 6). Sorta pšenice i tretman dušikom imali su značajan utjecaj na sve mjerene fotosintetske pigmente: klorofil a (Chl a), klorofil b (Chl b), karotenoide (Car), ukupni klorofil (Chl a + b) i

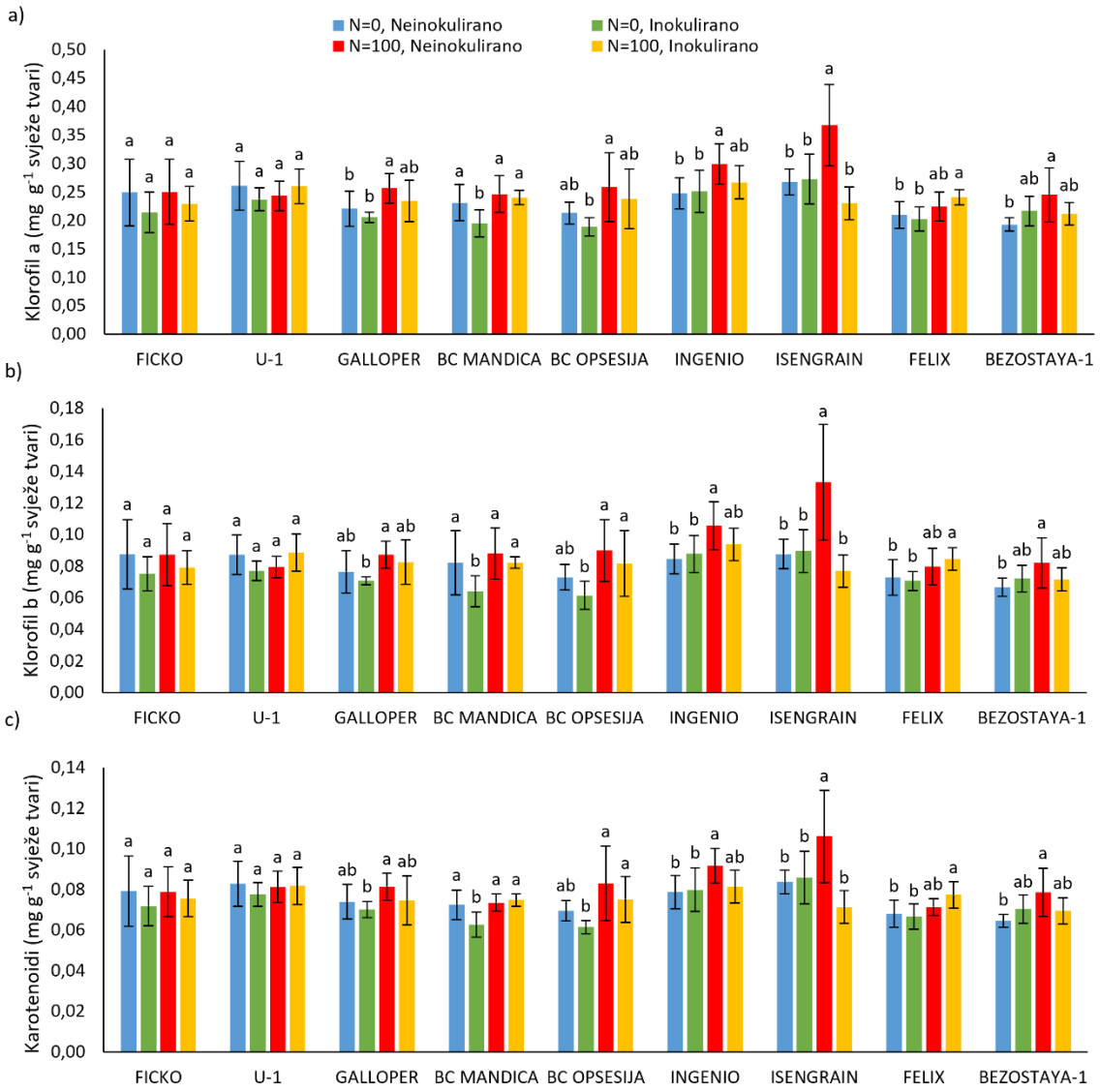
omjer klorofila a i b (Chl a/Chl b) u klasovima pšenice. *Fusarium* tretman značajno je utjecao na gotovo sve fotosintetske pigmente osim na Chl a/Chl b.

**Tablica 6.** Trofaktorijalna analiza varijance (ANOVA) za različite fotosintetske pigmente pod utjecajem različitog tretmana dušikom i *Fusarium* tretmana u klasovima devet sorti ozime pšenice.

| | | Sredina kvadrata | | | | |
|---|---|---|---|---|---|---|
| **Izvor varijacije** | **Stupnjevi slobode** | **Chl a** | **Chl b** | **Chl a + b** | **Car** | **Chl a/Chl b** |
| **Sorta**/*Variety* (V) | 8 | 0,0128*** | 0,0015*** | 0,0228*** | 0,0008*** | 0,0745** |
| **Tretman dušikom**/*Nitrogen treatment* (N) | 1 | 0,0363*** | 0,0058*** | 0,0712*** | 0,0019*** | 0,1192* |
| ***Fusarium* inokulacija**/*Fusarium inoculation* (F) | 1 | 0,0204*** | 0,0033*** | 0,0402*** | 0,0014*** | 0,0592 ns |
| V×N | 8 | 0,0011 ns | 0,0002 ns | 0,0022 ns | 0,0001 ns | 0,0217 ns |
| V×F | 8 | 0,0025* | 0,0004* | 0,0050* | 0,0002 ns | 0,0258 ns |
| N×F | 1 | 0,0028 ns | 0,0003 ns | 0,0049 ns | 0,0002 ns | 0,0059 ns |
| V×N×F | 8 | 0,0049*** | 0,0008*** | 0,0097*** | 0,0003*** | 0,0229 ns |

ns – nije statistički značajno/*not significant*, *, ** i *** – značajno na razini $p \leq 0,05$, 0,01 i 0,001. Chl a, klorofil a/*chlorophyll a*; Chl b, klorofil b/*chlorophyll b*; Chl a+b, ukupni klorofil/*total chlorophyll*; Car, karotenoidi/*carotenoids*; Chl a/Chl b, omjer klorofila a i b/*chlorophyll a and b ratio*. Izvor: Matić i sur., 2021a

U prosjeku je niska razina dušika, u odnosu na visoku razinu, uzrokovala smanjenje sadržaja Chl a, Chl b, Chl a + b i Car u klasovima pšenice, dok je Chl a/Chl b bio povećan. Sorta pšenice također je imala snažan utjecaj na sadržaj fotosintetskih pigmenata u klasovima pšenice. U prosjeku za sve sorte, sorte Ingenio i Isengrain imale su najveći sadržaj Chl a, Chl b, Chl a + b i Car u klasovima pšenice, dok je najmanji sadržaj fotosintetskih pigmenata utvrđen u sorti Bezostaya-1 (podatci nisu prikazani). Analizom sadržaja fotosintetskih pigmenata unutar svake sorte pojedinačno, *Fusarium* je tretman pri obje razine dušika uzrokovao smanjenje sadržaja Chl a, Chl b i Car u sortama BC Mandica i Isengrain (Slika 3a – c). Navedeno je u skladu s istraživanjem Lobato i sur. (2010.) u kojem je utvrđen značajan gubitak fotosintetskih pigmenata u tkivu biljaka izloženih infekciji patogenom. U usporedbi s visokom razinom dušika, niska je razina dušika uzrokovala značajno smanjenje sadržaja Chl a, Chl b, Chl a + b i Car u klasovima pšenice većine neinokuliranih sorti. Međutim, značajno smanjenje utvrđeno je u sortama Ingenio, Isengrain i Bezostaya-1 za sadržaj Chl a, Chl b i Car, te u sorti Galloper za sadržaj Chl a. Navedeni su rezultati u skladu s prethodnim istraživanjima u kojima je utvrđen smanjen sadržaj fotosintetskih pigmenata u uvjetima nedostatka dušika (Laza i sur., 1993.; Liu i sur., 2020.) Naime, niska razina dušika uzrokuje inhibiciju fotosinteze i smanjuje fotosintetski kapacitet, što posljedično inhibira rast i razvoj biljaka (Boussadia i sur., 2010.; Prinsi i sur., 2020.).




**Slika 3.** Sadržaj klorofila a (a), klorofila b (b) i karotenoida (c) u klasovima devet sorti pšenice pri različitim prihranama dušikom (0 kg N $ha^{-1}$ i 100 kg N $ha^{-1}$) i tretmanom vrstom *Fusarium culmorum* (neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2021a

## 5.2. Oksidacijski status i antioksidacijski odgovor na *Fusarium* infekciju i različite razine gnojidbe dušikom u listu zastavičaru ozime pšenice

U ovom je radu analiziran utjecaj abiotičkog stresa uzrokovanog deficitom dušika i biotičkog stresa izazvanog fitopatogenom gljivom *F. culmorum* na biomarkere oksidacijskog stresa u listu zastavičaru devet sorti ozime pšenice. Sorta pšenice, tretman dušikom i inokulacija izolatom *F. culmorum* imali su značajan utjecaj na ispitivane biomarkere oksidacijskog stresa u listu zastavičaru. Rezultati su također prokomentirani u kontekstu prethodnog istraživanja gdje su pri istim eksperimentalnim uvjetima iste analize provedene na klasu pšenice (Matić i sur., 2021a), koji se najčešće i koristi u biokemijskim analizama utjecaja zaraze FHB-om. Iako se FHB prvenstveno javlja na klasu, infekcija klasa može uzrokovati promjene pojedinih metaboličkih puteva u ostalim biljnim organima, npr. promjene u stopi fotosinteze lista zastavičara (Yang i sur., 2016., Španić i sur., 2017a). U ovom smo istraživanju željeli utvrditi odražava li se zaraza u klasu i na antioksidacijski odgovor lista zastavičara, te postoji li povezanost u antioksidacijskom odgovoru između lista zastavičara i klasa. Iako bi korelacija u mjerenim biokemijskim parametrima omogućila upotrebu lista zastavičara za detaljniju analizu biomarkera oksidacijskog stresa uzrokovanog FHB-om, rezultati ovog istraživanja nisu pokazali značajno jaku korelaciju između lista zastavičara i klasa u mjerenim parametrima (rezultati Pearsonova testa korelacije nisu prikazani). Na temelju dobivenih rezultata može se zaključiti kako je antioksidacijski odgovor bio tkivno-specifičan.

Trofaktorijalnom analizom varijance (ANOVA) utvrđen je značajan utjecaj sorte na sve ispitivane biomarkere oksidacijskog stresa u listovima zastavičarima pšenice (Tablica 7). Značajan utjecaj sorte na biomarkere stresa utvrđen je i u klasu pšenice pri istim eksperimentalnim uvjetima (Matić i sur., 2021a). Tretman dušikom značajno je utjecao na koncentraciju $H_2O_2$ i koncentraciju PHE te na aktivnost antioksidacijskih enzima CAT, APX i GR. Inokulacija izolatom *F. culmorum* značajno je utjecala na količinu TBARS-a, koncentraciju $H_2O_2$ te koncentraciju PHE ($p \leq 0{,}001$). Za razliku od lista zastavičara, inokulacija izolatom *F. culmorum* je u klasu imala učinak na većinu mjerenih biomarkera (PHE, CAT, GR, Car) (Matić i sur., 2021a). Interakcija između tri glavna faktora bila je značajna za sve ispitivane biomarkere osim za koncentraciju PHE.

**Tablica 7.** Trofaktorijalna analiza varijance (ANOVA) utjecaja sorte, tretmana dušikom i inokulacije vrstom *Fusarium culmorum* i njihovih interakcija na ispitivane biomarkere oksidacijskog stresa u listu zastavičaru pšenice.

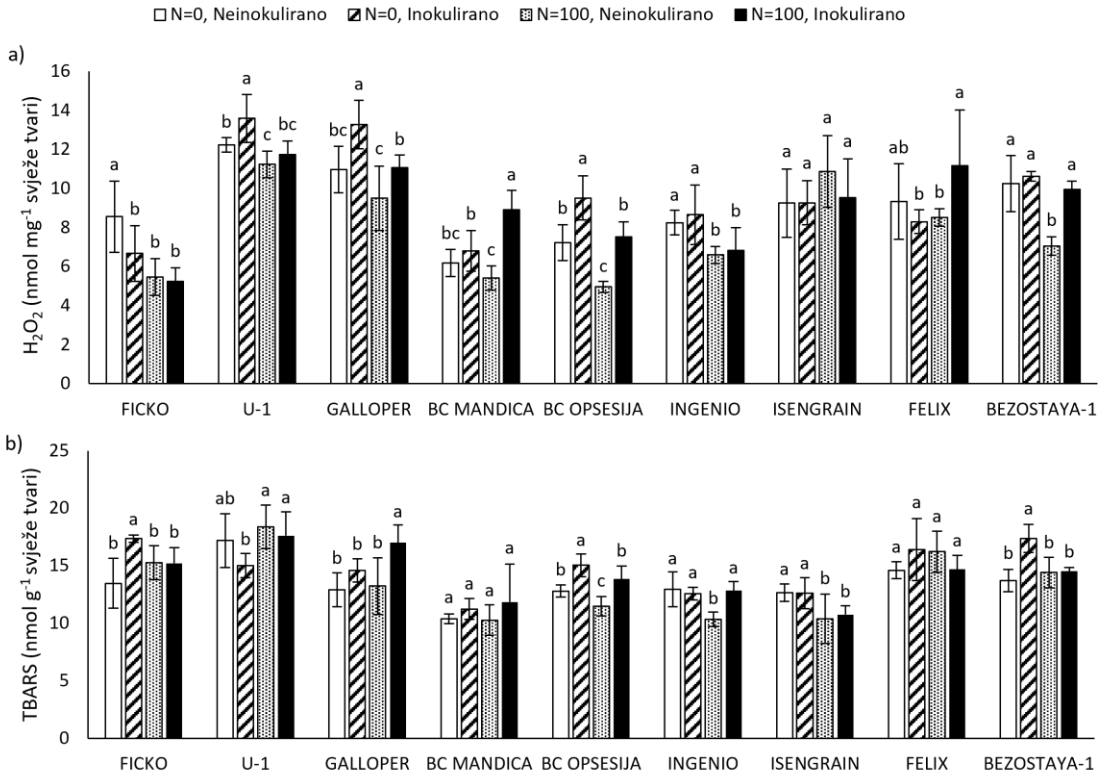
| | | Sredina kvadrata | | | | | |
|---|---|---|---|---|---|---|---|
| **Izvor varijacije** | **Stupnjevi slobode** | HP | TBARS | PHE | CAT | APX | GR |
| **Sorta**/*Variety* (V) | 8 | 95,36*** | 99,96*** | 2115,80*** | 1300,17* | 0,29*** | 787,75*** |
| **Tretman dušikom**/*Nitrogen treatment* (N) | 1 | 50,63*** | 3,53 ns | 2199,62*** | 19041,20*** | 0,13* | 3764,30*** |
| ***Fusarium* inokulacija**/*Fusarium inoculation* (F) | 1 | 47,00*** | 65,25*** | 422,01*** | 1491,89 ns | 0,04 ns | 421,11 ns |
| V×N | 8 | 11,85*** | 9,97*** | 40,39 ns | 1598,01** | 0,16*** | 124,06 ns |
| V×F | 8 | 8,87*** | 10,32*** | 77,31* | 1480,71** | 0,04 ns | 70,60 ns |
| N×F | 1 | 10,41** | 1,67 ns | 53,49 ns | 1306,70 ns | 0,07 ns | 2,11 ns |
| V×N×F | 8 | 5,11*** | 9,97*** | 48,36 ns | 1899,69*** | 0,10*** | 221,61* |

ns – nije statistički značajno/*not significant*, *, ** i *** – značajno na razini $p \leq 0,05$, 0,01 i 0,001. HP, vodik peroksid/*hydrogen peroxide*; TBARS, reaktivne supstance tiobarbiturne kiseline/*thiobarbituric acid reactive substances*; PHE, fenoli/*phenolics*; CAT, katalaza/*catalase*; APX, askorbat-peroksidaza/*ascorbate peroxidase*; GR, glutation-reduktaza/*glutathione reductase*. Izvor: Matić i sur., 2021b

U sortama U-1, Galloper i BC Opsesija (pri niskoj razini dušika) inokulacija patogenom *F. culmorum* uzrokovala je povećanje koncentracije $H_2O_2$, dok je u uvjetima visoke razine dušika koncentracija $H_2O_2$ bila povećana u sortama Galloper, BC Mandica, BC Opsesija, Felix i Bezostaya-1 (Slika 4a). Nedostatak prihrane dušikom u neinokuliranih biljaka uzrokovao je trend povećanja koncentracije $H_2O_2$ u gotovo svim sortama, a značajan rast utvrđen je u sortama Ficko, U-1, BC Opsesija, Ingenio i Bezostaya-1. Budući da je nedostatak prihrane dušikom u neinokuliranih biljaka većinom uzrokovao povećanje količine TBARS-a te smanjenje aktivnosti antioksidacijskih enzima, možemo zaključiti kako je u ovom slučaju $H_2O_2$ djelovao kao promotor oksidacijskog stresa. Mamenko (2018.) je također utvrdio povećanje koncentracije $H_2O_2$ u listovima pšenice pri nedovoljnoj gnojidbi dušikom. Ipak, autor je utvrdio i povećanje aktivnosti antioksidacijskih enzima pa zaključuje kako je $H_2O_2$ djelovao kao signalna molekula koja je pri nedovoljnoj gnojidbi utjecala na aktivaciju obrambenih mehanizama pšenice.

Brojna su istraživanja pokazala kako pri različitim abiotičkim i/ili biotičkim stresnim uvjetima dolazi do pojave LPO uslijed čega dolazi do oksidacijskog oštećenja strukturnih komponenti biljaka (Španić i sur., 2017b.; Hasanuzzaman i sur., 2020.). Pojava LPO pri napadu patogena i/ili nedovoljnoj gnojidbi dušikom utvrđena je u nekim sortama i u ovom istraživanju. U sortama Ficko, BC Opsesija i Bezostaya-1 pri niskoj razini dušika inokulacija izolatom *F. culmorum* uzrokovala je povećanje količine TBARS-a (Slika 4b), dok je u uvjetima visoke razine dušika količina TBARS-a bila povećana u sortama Galloper, BC Opsesija i Ingenio. U

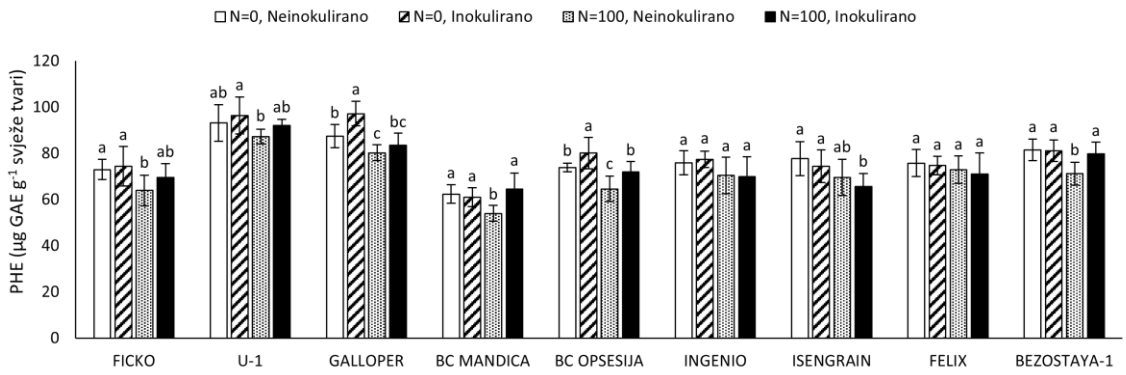
prosjeku, sam tretman dušikom nije značajno utjecao na količinu TBARS-a u listovima zastavičarima pšenice (Tablica 7). Međutim, nedostatak prihrane dušikom u neinokuliranih biljaka uzrokovao je povećanje količine TBARS-a u sortama BC Opsesija, Ingenio i Isengrain. Iako je razina LPO sortno i tkivno-specifična, u listu zastavičaru izraženiji je porast LPO, tj. prisutan je u znatno većem broju sorti pri različitim tretmanima, u odnosu na klas (Matić i sur., 2021a).




**Slika 4.** Koncentracija vodika peroksida ($H_2O_2$; a) i količina reaktivnih supstanci tiobarbiturne kiseline (TBARS; b) listova zastavičara devet sorti pšenice pri različitim prihranama dušikom (0 kg N ha$^{-1}$ i 100 kg N ha$^{-1}$) i *Fusarium culmorum* tretmanom (neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2021b

Sva tri faktora istraživanja (tretman dušikom, sorta i inokulacija izolatom *F. culmorum*) imala su značajan utjecaj na koncentraciju ukupnih topljivih PHE ($p \leq 0,001$) (Tablica 7). Najznačajniji utjecaj imao je sam tretman dušikom, a u prosjeku je niska razina dušika uzrokovala porast koncentracije PHE za 8,82 % u usporedbi s visokom razinom dušika. Inokulacija izolatom *F. culmorum* uzrokovala je porast koncentracije PHE u sortama Galloper i BC Opsesija pri niskoj razini dušika (Slika 5). Trend povećanja koncentracije PHE uslijed inokulacije izolatom *F. culmorum* uočen je i pri visokoj razini dušika, a značajan rast utvrđen je u sortama BC Mandica, BC Opsesija i Bezostaya-1. U većine neinokuliranih sorti, niska je

razina dušika uzrokovala trend povećanja koncentracije topljivih PHE, a statistički značajan porast zabilježen je u sortama Ficko, Galloper, BC Mandica, BC Opsesija i Bezostaya-1. Dobiveni rezultat u skladu je s drugim istraživanjima u kojima je utvrđeno kako pri uvjetima nedostatka dušika, zbog povećanja C:N omjera unutar biljaka, dolazi do pojačanog stvaranja i nakupljanja fenolnih spojeva (Ibrahim i sur., 2011., Munene i sur., 2017., Deng i sur., 2019.). Fenolni su spojevi najznačajniji i najrašireniji sekundarni metaboliti biljaka koji imaju važnu ulogu u obrani biljaka u uvjetima abiotičkog i/ili biotičkog stresa (Kulbat, 2016.; Sharma i sur., 2019.). Dušična je prihrana stoga od velike važnosti jer može utjecati na metabolizam i koncentraciju primarnih i sekundarnih biljnih metabolita (Chen i sur., 2011.). U istraživanju Matić i sur. (2021a) uzgoj pšenice pri niskoj razini dušika uzrokovao je smanjenje koncentracije PHE u klasovima neinokuliranih biljaka, što upućuje na tkivno-specifični odgovor pšenice.



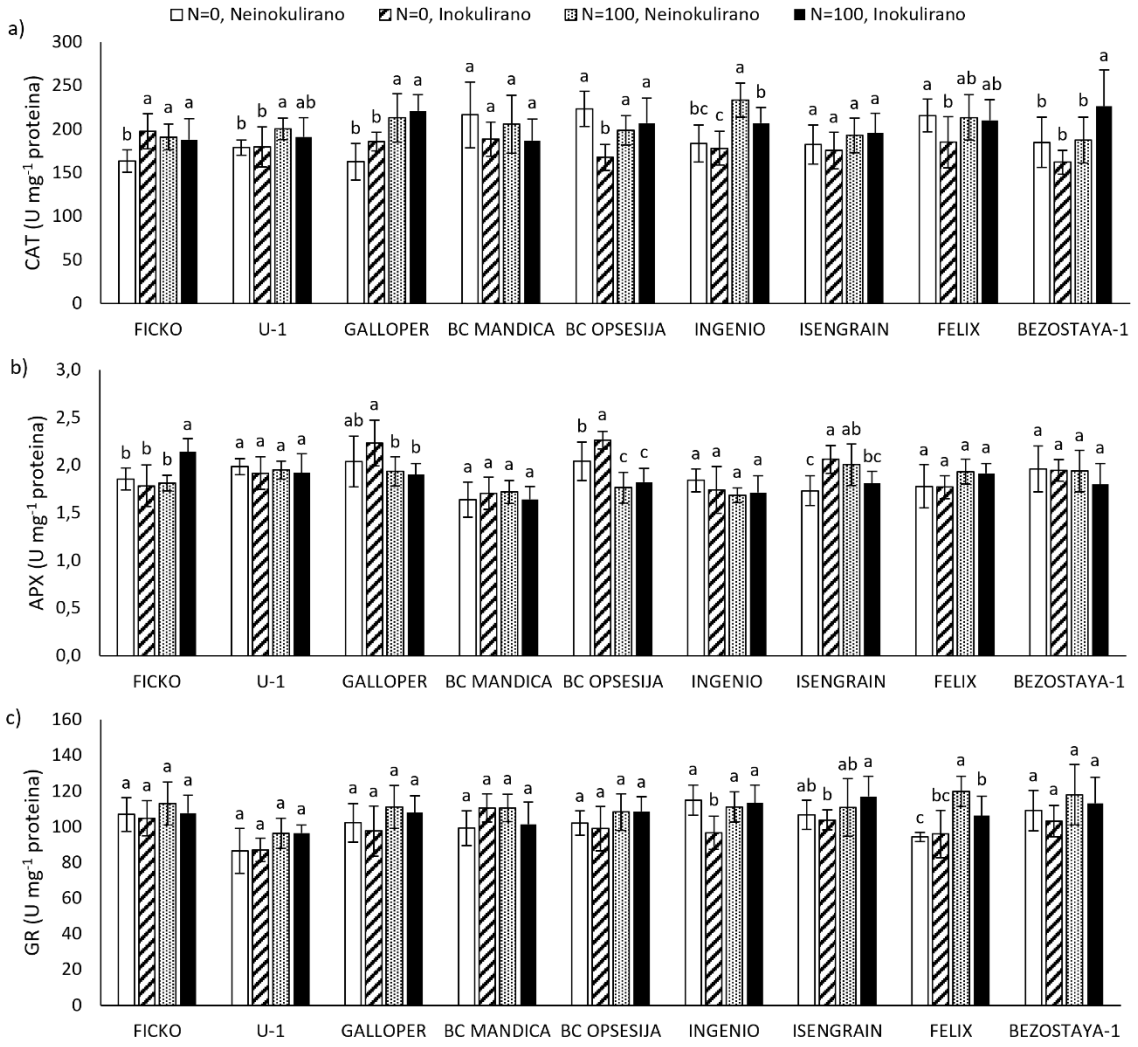

**Slika 5.** Koncentracija topljivih fenola (PHE) listova zastavičara devet sorti pšenice pri različitim prihranama dušikom (0 kg N $ha^{-1}$ i 100 kg N $ha^{-1}$) i *Fusarium culmorum* tretmanom (neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2021b

Tretman dušikom i sorta pšenice imali su značajan utjecaj na aktivnost antioksidacijskih enzima CAT, APX i GR (Tablica 7). U prosjeku je niska razina dušika, u usporedbi s visokom razinom, uzrokovala smanjenje aktivnosti antioksidacijskih enzima CAT i GR listova zastavičara pšenice. Smanjenje aktivnosti antioksidacijskih enzima bilo je redom 9,21 % za CAT i 7,63 % za GR.

U prosjeku, inokulacija izolatom *F. culmorum* nije značajno utjecala na aktivnost antioksidacijskih enzima listova zastavičara pšenice (Tablica 7). Ipak, analizom utjecaja inokulacije izolatom *F. culmorum* svake sorte pojedinačno, vidljive su značajne promjene u aktivnosti enzima koje su sortno-specifične (Slika 6). Inokulacija izolatom *F. culmorum* je u

uvjetima niske razine dušika, uzrokovala povećanje aktivnosti enzima CAT u sorti Ficko, dok je u sortama BC Opsesija i Felix uzrokovala smanjenje aktivnosti enzima CAT (Slika 6a). Pri visokoj razini dušika, inokulacija izolatom *F. culmorum* uzrokovala je povećanje aktivnosti enzima CAT u sorti Bezostaya-1, a smanjenje aktivnosti u sorti Ingenio. U uvjetima niske razine dušika, inokulacija izolatom *F. culmorum* uzrokovala je povećanje aktivnosti APX-a u sortama BC Opsesija i Isengrain, dok je u uvjetima visoke razine dušika aktivnost APX-a bila povećana u sorti Ficko (Slika 6b). Inokulacija izolatom *F. culmorum* uzrokovala je trend smanjenja aktivnosti enzima GR u gotovo svim sortama pri obje razine dušika. Međutim, značajno smanjenje aktivnosti enzima GR utvrđeno je u sorti Ingenio pri niskoj razini dušika te u sorti Felix pri visokoj razini dušika (Slika 6c). Osim što je odgovor antioksidacijskih enzima na inokulaciju izolatom *F. culmorum* ovisio o sorti, usporedbom rezultata analize lista s prijašnjom analizom klasa (Matić i sur., 2021a) uočava se i tkivno-specifični odgovor.

U neinokuliranim sortama Ficko, U-1, Galloper i Ingenio, niska razina dušika utjecala je na smanjenje aktivnosti enzima CAT, dok je u sorti Isengrain utjecala na smanjenje aktivnosti enzima APX. Također, u odnosu na visoku razinu dušika, niska je razina dušika uzrokovala trend smanjenja aktivnosti GR-a u gotovo svih neinokuliranih sorti, iako je statistički značajan pad aktivnosti utvrđen samo u sorti Felix. Prema Matić i sur. (2021a) nedostatna prihrana dušikom uzrokovala je smanjenje aktivnosti antioksidacijskih enzima i u klasovima većine neinokuliranih sorti. Smanjena aktivnost antioksidacijskih enzima u uvjetima nedostatka dušika može biti povezana sa smanjenom sintezom aminokiselina i proteina u uvjetima niske opskrbljenosti biljke dušikom.




**Slika 6.** Aktivnosti antioksidacijskih enzima: katalaze (CAT; a), askorbat-peroksidaze (APX; b) i glutation-reduktaze (GR; c) listova zastavičara devet sorti pšenice pri različitim prihranama dušikom (0 kg N $ha^{-1}$ i 100 kg N $ha^{-1}$) i različitim *Fusarium culmorum* tretmanom (neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2021b

Rezultati trofaktorijalne analize varijance (ANOVA) pokazali su kako su i sorta pšenice i tretman dušikom imali utjecaj na sadržaj fotosintetskih pigmenata listova zastavičara pšenice ($p \leq 0,001$; Tablica 8). S druge strane, inokulacija izolatom *F. culmorum* nije imala učinak na sadržaj fotosintetskih pigmenata u listu zastavičaru (Tablica 8), dok je u klasu pšenice inokulacija imala značajan učinak na sadržaj pigmenata (Matić i sur., 2021a). U svojem su istraživanju na velikom broju genotipova pšenice Molero i Reynolds (2020.) uočili nedostatak korelacije između razine intenziteta fotosinteze lista zastavičara i klasa, što upućuje na njihovu neovisnost.

U prosjeku je niska razina dušika, u usporedbi s visokom razinom, uzrokovala smanjenje sadržaja Chl a, Chl b, Chl a + b i Car listova zastavičara pšenice. Smanjeni sadržaj fotosintetskih pigmenata pri uvjetima smanjene dušične prihrane pronađen je i u klasovima pšenice (Matić i sur., 2021a). Kao makroelement dušik je sastavni dio stanica, proteina, nukleinskih kiselina, enzima i fotosintetskih pigmenata biljaka. Manjak dušika tijekom vegetacije može uzrokovati smanjenje sadržaja fotosintetskih pigmenata što posljedično može uzrokovati inhibiciju fotosinteze i smanjenje fotosintetskog kapaciteta (Boussadia i sur., 2010., Prinsi i sur., 2020.). Smanjeni intenzitet fotosinteze pak može negativno utjecati na prinos i kvalitetu usjeva.

**Tablica 8.** Trofaktorijalna analiza varijance (ANOVA) utjecaja sorte, tretmana dušikom i inokulacije izolatom *Fusarium culmorum* i njihovih interakcija na sadržaj fotosintetskih pigmenata u listu zastavičaru pšenice.

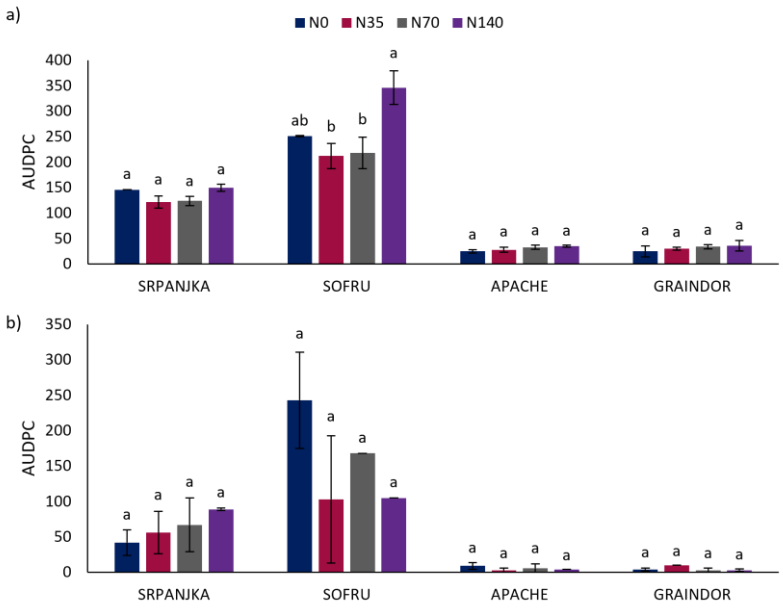
| | | **Sredina kvadrata** | | | |
|---|---|---|---|---|---|
| **Izvor varijacije** | **Stupnjevi slobode** | **Chl a** | **Chl b** | **Chl a + b** | **Car** |
| **Sorta**/*Variety* (V) | 8 | 0,1836*** | 0,0297*** | 0,3547*** | 0,0090** |
| **Tretman dušikom**/*Nitrogen treatment* (N) | 1 | 1,7471*** | 0,3130*** | 3,5392*** | 0,0966*** |
| ***Fusarium* inokulacija**/*Fusarium inoculation* (F) | 1 | 0,0004 ns | 0,0012 ns | 0,0031 ns | 0,0013 ns |
| V×N | 8 | 0,0279 ns | 0,0053 ns | 0,0483 ns | 0,0025 ns |
| V×F | 8 | 0,0441 ns | 0,0088 ns | 0,0904 ns | 0,0040 ns |
| N×F | 1 | 0,0239 ns | 0,0063 ns | 0,0547 ns | 0,0035 ns |
| V×N×F | 8 | 0,0057 ns | 0,0046 ns | 0,0143 ns | 0,0009 ns |

ns – nije statistički značajno/*not significant*, ** i *** – značajno na razini p ≤ 0,01 i 0,001. Chl a, klorofil a/*chlorophyll a*; Chl b, klorofil b/*chlorophyll b*; Chl a+b, ukupni klorofil/*total chlorophyll*; Car, karotenoidi/*carotenoids*. Izvor: Matić i sur., 2021b

## 5.3. Obrambeni odgovor na *Fusarium* infekciju u klasovima pšenice, različite osjetljivosti na FHB, uzgojene pod različitim razinama gnojidbe dušikom

Vizualna procjena simptoma FHB-a provedena je samo na inokuliranim sortama pšenice, budući da neinokulirane sorte nisu pokazivale simptome FHB-a. Ozbiljnost FHB-a (točnije postotak zaraženih klasića po klasu) procijenjena je pomoću linearne skale (0 – 100 %) 10., 14., 18., 22., 26. i 30. dana nakon inokulacije. Na temelju dobivenih postotaka zaraze izračunato je područje unutar progresivne krivulje bolesti (engl. *area under the disease progress curve*, AUDPC), kao integrirana jedinica za ukupni intenzitet bolesti. Ozbiljnost

FHB-a, izražena kao AUDPC vrijednost varirala je ovisno o sorti, tretmanu dušikom i vegetacijskoj godini (Slika 7). Prosječne vrijednosti AUDPC-a po sorti (bez obzira na razinu dušika) u 2018./2019. iznosile su redom: 257 (Sofru), 136 (Srpanjka), 31 (Graindor) i 30 (Apache). Sličan poredak bio je prisutan i u 2019./2020. gdje su prosječne vrijednosti AUDPC-a po sorti (bez obzira na razinu dušika) iznosile redom: 155 (Sofru), 64 (Srpanjka), 6 (Apache) i 5 (Graindor). U ovom je istraživanju sorta Sofru imala najveće vrijednosti AUDPC-a u obje vegetacijske godine, što ukazuje na veću osjetljivost ove sorte na FHB. Više vrijednosti AUDPC-a u sorti Sofru mogu biti povezane s prisutnošću osja s obzirom da je sorta Sofru jedina sorta s osjem korištena u ovom istraživanju. U svojem je istraživanju Mesterhazy (1995.) utvrdio osjetljivost sorti pšenice s osjem na FHB u uvjetima prirodne infekcije, dok prisutnost osja nije imala utjecaj na ozbiljnost FHB-a u uvjetima umjetne inokulacije vrstama roda *Fusarium*. U odnosu na sortu Sofru, niže prosječne vrijednosti AUDPC-a uočene su u sorti Srpanjka u obje vegetacijske godine. Stoga je sorta Srpanjka u ovom istraživanju klasificirana kao umjereno osjetljiva sorta na FHB. Sorte Apache i Graindor klasificirane su kao djelomično otporne sorte na FHB zbog najnižih prosječnih vrijednosti AUDPC-a u obje vegetacijske godine. Dobiveni rezultati u skladu su s prethodnim studijama koje su procijenile i okarakterizirale otpornost istih sorti na FHB (Španić i sur., 2021a; Španić i sur., 2021b).




**Slika 7.** Područje unutar progresivne krivulje bolesti (engl. *area under the disease progress curve*, AUDPC) za ukupnu otpornost na FHB u 2018./2019. (a) i 2019./2020. godini (b). Rezultati su prikazani kao srednja vrijednost dvije replike ± standardna pogreška. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između različitih razina dušika unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2022.

Meteorološki podatci za područje Osijeka dobiveni su od Državnog hidrometeorološkog zavoda. Godišnje količine oborina tijekom vegetacijskih godina 2018./2019. i 2019./2020. iznosile su redom 531,3 i 408,6 mm, a prosječne godišnje temperature 10,9 odnosno 11,1 °C (dostupno na: https://www.mdpi.com/article/10.3390/agronomy12081746/s1). U svibnju, tijekom faze formiranja zrna i mliječne zriobe, ukupna količina oborina iznosila je 150,8 mm u 2019. i 53,3 mm u 2020. godini, dok je prosječna dnevna temperatura bila 14,0 °C u 2019. i 15,3 °C u 2020. godini. U lipnju, u fazi zriobe, ukupna količina oborina iznosila je 112,8 mm u 2019. i 73,5 mm u 2020. godini, dok je prosječna dnevna temperatura iznosila 23,1 °C u 2019. i 20,2 °C u 2020. godini.

Pšenica je najosjetljivija na FHB u fazi cvatnje, a toplo i vlažno vrijeme tijekom faze cvatnje dodatno potiče razvoj bolesti (Birr i sur., 2020.). U ovom se istraživanju mogu uočiti razlike u ozbiljnosti FHB-a između dvije vegetacijske godine tijekom kojih su prevladavale različite klimatske prilike (Slika 7). U prosjeku, za sve sorte i sve razine dušika, veće vrijednosti AUDPC-a utvrđene su u vegetacijskoj godini 2018./2019. u usporedbi s 2019./2020. Veće vrijednosti AUDPC-a, odnosno ozbiljniji simptomi FHB-a u vegetacijskoj godini 2018./2019., mogu biti posljedica obilnih oborina i povezane veće vlažnosti zraka u svibnju 2019. U svibnju, tijekom faze formiranja zrna i mliječne zriobe, ukupna količina oborina bila je gotovo tri puta veća u 2019. nego u 2020. godini (dostupno na: https://www.mdpi.com/article/10.3390/agronomy12081746/s1). Slične rezultate potvrđuju i Krnjaja i sur. (2015.) navodeći klimatske uvjete, posebice intenzivne oborine tijekom faze cvatnje uzrokom višeg FHB indeksa i veće pojavnosti fitopatogenih gljiva koje uzrokuju FHB. U 2018./2019. godini više vrijednosti AUDPC-a zabilježene su u inokuliranim sortama pšenice uzgojenih pri najvećoj razini dušika (N140). Povećana gnojidba dušikom može neizravno povećati intenzitet FHB-a uzrokujući promjene u karakteristikama usjeva, posebno povećanjem gustoće sklopa i mijenjanjem mikroklime sklopa (Lemmens i sur., 2004.). Gušći sklop biljaka ima vlažniju mikroklimu koja pogoduje razvoju bolesti. Međutim, u obje vegetacijske godine primjena različitih razina gnojidbe dušikom nije značajno utjecala na ozbiljnost FHB-a. U našem prethodnom istraživanju visoka je razina dušika u nekih sorti (Ficko, Galloper i Felix) uzrokovala povećanje vizualnih simptoma FHB-a , dok je u sorti U-1 uzrokovala smanjenje simptoma FHB-a (Matić i sur., 2021a), što ukazuje na sortno-specifični odgovor na različitu gnojidbu dušikom. U literaturi su dostupni brojni, ponekad proturječni podatci, o utjecaju dušika na zarazu patogenima i razvoj bolesti u interakcijama između različitih biljaka i patogena (Huber i Watson, 1974.; Sun i sur., 2020.). Međutim, proturječni

rezultati možda nisu iznenađujući, jer je utjecaj dušika tijekom interakcije između različitih biljaka i patogena iznimno složen i ovisi o vrsti biljke domaćina i vrsti patogena, kao i o različitoj količini i kemijskom obliku dušika. Različiti kemijski oblici dušika, amonijev ($NH_4^+$) i/ili nitratni ($NO_3^-$) oblik, mogu imati različit utjecaj na otpornost biljaka, djelomično iz razloga što koriste različite asimilacijske i metaboličke puteve (Sun i sur., 2020.). U ovom su istraživanju korištena oba oblika dušika ($NH_4^+$ i $NO_3^-$) kao dio standardne agronomske prakse u primjeni mineralnih gnojiva u proizvodnji pšenice u Hrvatskoj pa nije bilo moguće utvrditi kako je svaki od pojedinih oblika dušika utjecao na ozbiljnost zaraze FHB-om. Iako su za pšenicu dostupna brojna i proturječna istraživanja, pojedini autori ističu kako različite razine gnojidbe dušikom nemaju značajan utjecaj na FHB i koncentraciju mikotoksina i zaključuju kako različite prihrane dušikom u praktičnoj primjeni ne predstavljaju mogućnost u borbi protiv FHB-a (Lemmens i sur., 2004.; Krnjaja i sur., 2015.).

Koncentracije mikotoksina DON i ZEA određene su samo iz uzoraka pšenice uzgajane tijekom vegetacijske godine 2019./2020. Analiza mikotoksina učinjena je dodatno kako bi se testirao utjecaj tri glavna faktora (sorta pšenice, *Fusarium* tretman i tretman dušikom) na koncentracije mikotoksina. Koncentracije mikotoksina u zrnu neinokuliranih sorti pšenice bile su ispod granice detekcije od 100 μg $kg^{-1}$ za DON i 8 μg $kg^{-1}$ za ZEA. Stoga su u Tablici 9 prikazane samo koncentracije mikotoksina DON i ZEA u zrnima inokuliranih sorti pšenice. Neovisno o tretmanu utvrđene su visoke koncentracije DON-a u svim inokuliranim uzorcima pšenice. Točnije, koncentracije DON-a premašile su najveću dopuštenu koncentraciju od 1250 μg $kg^{-1}$ za neprerađenu pšenicu koju je odredila Europska komisija (Europska komisija, 2006.). U prosjeku je najvišu koncentraciju DON-a imala sorta Sofru (1680,40 ± 157,10), zatim Srpanjka (1668,03 ± 130,71), Apache (1667,03 ± 97,28) i Graindor (1532,05 ± 232,35). Navedene vrijednosti koncentracije DON-a u skladu su s utvrđenom ozbiljnošću zaraze FHB-om. U prosjeku je najveća koncentracija DON-a zabilježena u inokuliranim sortama pšenice koja je uzgajana pri N0 (1724,48 ± 37,27), što je bilo u skladu s najvećom ozbiljnošću zaraze FHB-om pri N0 u 2019./2020. godini.

**Tablica 9.** Koncentracije mikotoksina deoksinivalenola (DON) i zearalenona (ZEA) u zrnima inokuliranih sorti pšenice uzgajane tijekom vegetacijske godine 2019./2020.

| Inokulirane sorte pšenice | Tretman dušikom | Koncentracija DON-a (µg kg$^{-1}$) | Koncentracija ZEA-a (µg kg$^{-1}$) |
|---|---|---|---|
| Srpanjka | N0 | 1691,30 | <8 |
| | N35 | 1504,80 | <8 |
| | N70 | 1822,50 | 16,30 |
| | N140 | 1653,50 | 19,90 |
| Sofru | N0 | 1769,90 | <8 |
| | N35 | 1623,10 | 49,80 |
| | N70 | 1487,90 | 17,30 |
| | N140 | 1940,70 | <8 |
| Apache | N0 | 1739,90 | <8 |
| | N35 | 1761,60 | <8 |
| | N70 | 1590,90 | <8 |
| | N140 | 1575,70 | <8 |
| Graindor | N0 | 1696,80 | <8 |
| | N35 | 1702,00 | <8 |
| | N70 | 1206,70 | <8 |
| | N140 | 1522,70 | <8 |

Izvor: Matić i sur., 2022.

Što se tiče ZEA, koncentracije mikotoksina u svim uzorcima inokuliranih sorti pšenice nisu premašile granicu tolerancije od 100 µg kg$^{-1}$ koju je postavila Europska komisija (Europska komisija, 2006.). Naime, koncentracije ZEA bile su ispod granice detekcije u gotovo svim uzorcima inokuliranih sorti pšenice. Prosječno najveće koncentracije ZEA utvrđene su u sortama Srpanjka i Sofru, što je u skladu s najvećom ozbiljnošću zaraze FHB-om u tim sortama.

ANOVA utjecaja sorte, tretmana dušikom i *Fusarium* tretmana na mjerene parametre u vegetacijskim godinama 2018./2019. i 2019./2020. prikazana je u Tablicama 10 i 11. U vegetacijskoj godini 2018./2019. (Tablica 10) sorta pšenice imala je značajan utjecaj na sve ispitivane biokemijske parametre ($p \leq 0,001$), dok je tretman dušikom imao značajan utjecaj samo na aktivnost enzima PPO ($p \leq 0,05$). *Fusarium* tretman utjecao je na koncentraciju PHE ($p \leq 0,001$) i TAC ($p \leq 0,01$). Interakcija između sorte i tretmana dušikom bila je značajna za koncentraciju PHE, te za aktivnost enzima PAL i PPO. Interakcija između sorte i *Fusarium* tretmana bila je značajna za sve ispitivane parametre. Interakcija između tretmana dušikom i *Fusarium* tretmana bila je značajna samo za koncentraciju PHE. Interakcija između sva tri faktora istraživanja bila je značajna za koncentraciju PHE i aktivnost enzima PPO.

**Tablica 10.** Trofaktorijalna analiza varijance (ANOVA) za mjerene biokemijske parametre pod utjecajem različitih tretmana dušikom i *Fusarium* tretmana u klasovima četiri različite sorte ozime pšenice u vegetacijskoj godini 2018./2019.

| | | Sredina kvadrata | | | |
|---|---|---|---|---|---|
| **Izvor varijacije** | **Stupnjevi slobode** | **PHE** | **TAC** | **PAL** | **PPO** |
| **Sorta**/*Variety* (V) | 3 | 5,31*** | 36,95*** | 75,43*** | 354684,34*** |
| **Tretman dušikom**/*Nitrogen treatment* (N) | 3 | 0,01 ns | 0,04 ns | 0,64 ns | 2586,77* |
| ***Fusarium* inokulacija**/*Fusarium inoculation* (F) | 1 | 14,08 *** | 1,66** | 0,27 ns | 688,89 ns |
| V × N | 9 | 0,15** | 0,21 ns | 2,67*** | 2957,68*** |
| V × F | 3 | 2,01*** | 2,62*** | 3,05** | 15940,55*** |
| N × F | 3 | 0,30*** | 0,19 ns | 0,02 ns | 1789,18 ns |
| V × N × F | 9 | 0,31*** | 0,11 ns | 1,56 ns | 1941,68* |

ns – nije statistički značajno/*not significant*, *, ** i *** – značajno na razini $p \leq 0,05$, 0,01 i 0,001. PHE, fenoli/*phenolics*; TAC, ukupni antioksidacijski kapacitet/*total antioxidant capacity*; PAL, fenilalanin-amonij-lijaza/*phenylalanine ammonia-lyase*; PPO, polifenol-oksidaza/*polyphenol oxidase*. Izvor: Matić i sur., 2022.

**Tablica 11.** Trofaktorijalna analiza varijance (ANOVA) za mjerene biokemijske parametre pod utjecajem različitih tretmana dušikom i *Fusarium* tretmana u klasovima četiri različite sorte ozime pšenice u vegetacijskoj godini 2019./2020.

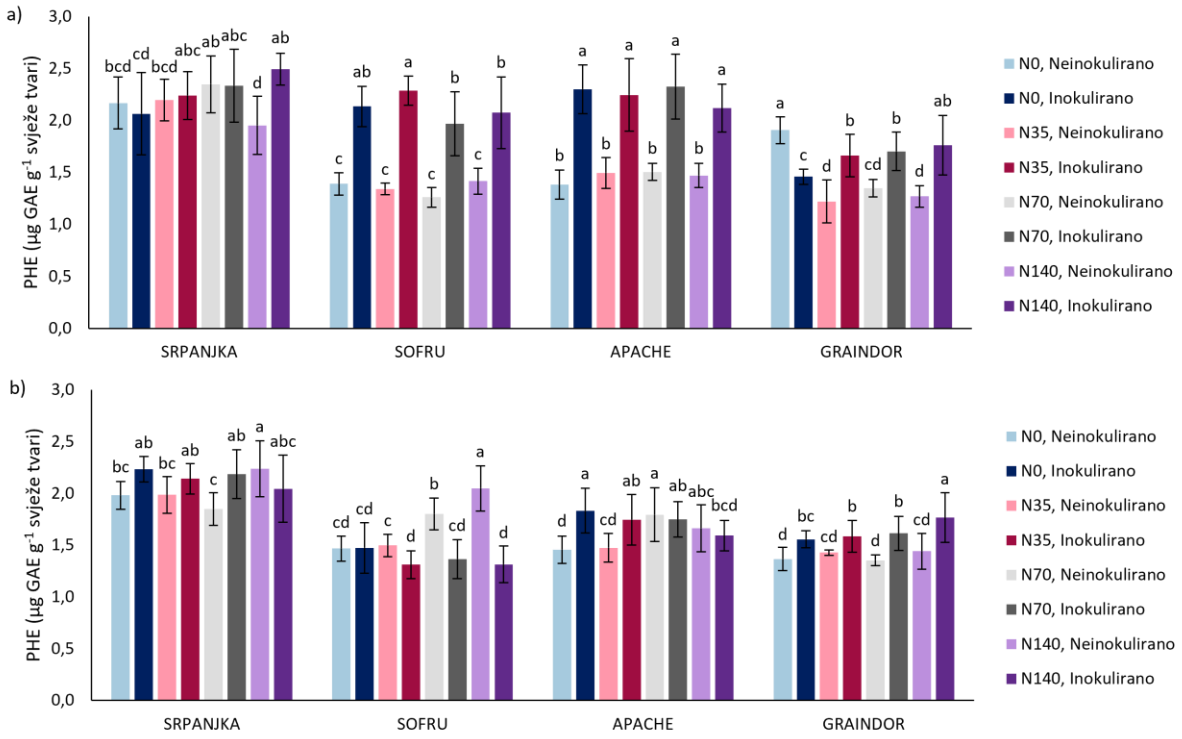
| | | Sredina kvadrata | | | |
|---|---|---|---|---|---|
| **Izvor varijacije** | **Stupnjevi slobode** | **PHE** | **TAC** | **PAL** | **PPO** |
| **Sorta**/*Variety* (V) | 3 | 4,46*** | 15,53*** | 26,35*** | 238480,99*** |
| **Tretman dušikom**/*Nitrogen treatment* (N) | 3 | 0,17** | 0,12 ns | 7,88*** | 13153,02*** |
| ***Fusarium* inokulacija**/*Fusarium inoculation* (F) | 1 | 0,11 ns | 6,70*** | 13,50** | 34039,71*** |
| V × N | 9 | 0,09** | 0,23* | 1,89 ns | 8876,24*** |
| V × F | 3 | 1,05*** | 0,66*** | 237,92*** | 56574,48*** |
| N × F | 3 | 0,40*** | 1,99*** | 4,86* | 4996,04 ns |
| V × N × F | 9 | 0,15*** | 0,27* | 14,94*** | 11892,92*** |

ns – nije statistički značajno/*not significant*, *, ** i *** – značajno na razini $p \leq 0,05$, 0,01 i 0,001. PHE, fenoli/*phenolics*; TAC, ukupni antioksidacijski kapacitet/*total antioxidant capacity*; PAL, fenilalanin-amonij-lijaza/*phenylalanine ammonia-lyase*; PPO, polifenol-oksidaza/*polyphenol oxidase*. Izvor: Matić i sur., 2022.

U vegetacijskoj godini 2019./2020. (Tablica 11) sorta pšenice značajno je utjecala na sve mjerene biokemijske parametre ($p \leq 0,001$). Tretman dušikom imao je značajan utjecaj na koncentraciju PHE ($p \leq 0,01$), aktivnost enzima PAL i PPO ($p \leq 0,001$), dok je *Fusarium* tretman imao utjecaj na TAC, aktivnost enzima PPO ($p \leq 0,001$) i PAL ($p \leq 0,01$). Interakcija između sorte i tretmana dušikom bila je značajna za koncentraciju PHE, TAC i aktivnost

enzima PPO, dok je interakcija između sorte i *Fusarium* tretmana bila značajna za sve mjerene parametre ($p \leq 0{,}001$). Interakcija između tretmana dušikom i *Fusarium* tretmana bila je značajna za koncentraciju PHE, TAC i aktivnost enzima PAL. Interakcija između tri glavna faktora bila je značajna za sve mjerene parametre.

U 2018./2019. najznačajniji utjecaj na koncentraciju ukupnih topljivih PHE imali su *Fusarium* tretman i sorta pšenice (Tablica 10). Trend povećanja koncentracije PHE u inokuliranim biljkama, u usporedbi s neinokuliranim pri gotovo svim razinama dušika, nađen je u sortama Sofru, Apache i Graindor (Slika 8a). Značajno povećanje koncentracije PHE uslijed *Fusarium* tretmana u sorti Sofru kretalo se od 46 % pri N140 do 71 % pri N35, u sorti Apache od 44 % pri N140 do 67 % pri N0, te u sorti Graindor od 26 % pri N70 do 39 % pri N140. U sorti Srpanjka, *Fusarium* tretman uzrokovao je značajno povećanje koncentracije PHE samo pri N140.



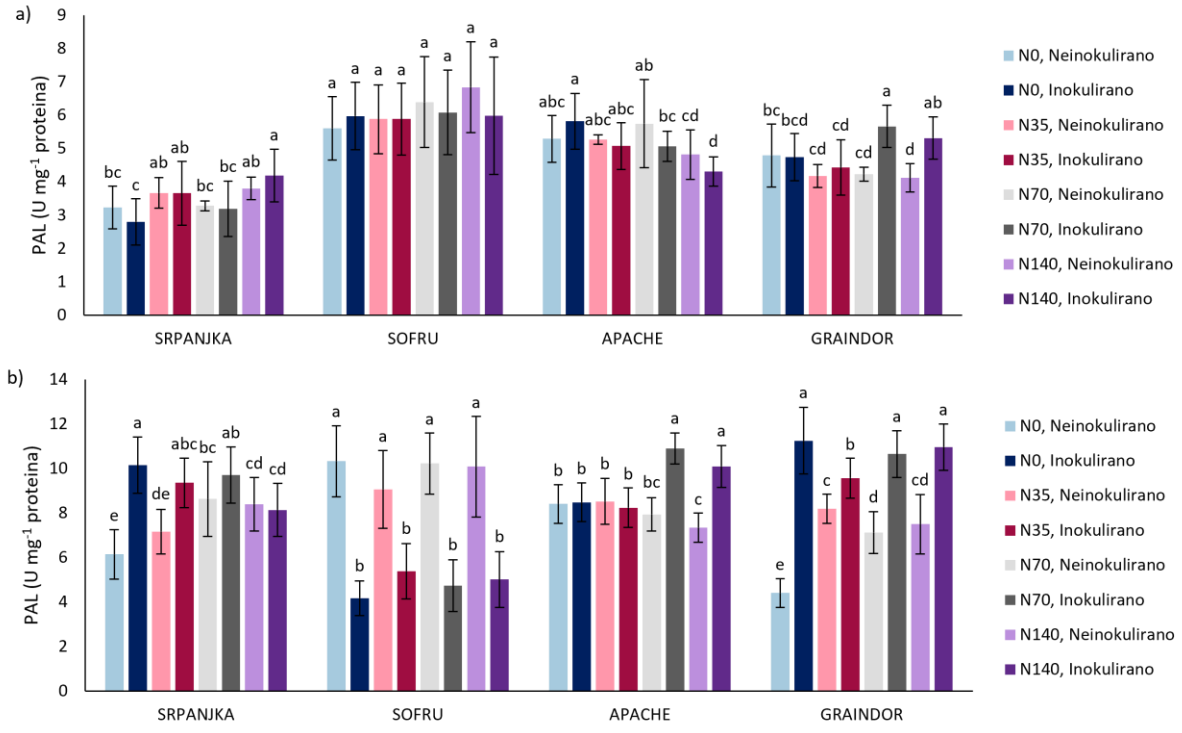

**Slika 8.** Koncentracija topljivih fenola (PHE) u klasovima četiri sorte pšenice pod različitim *Fusarium* tretmanom (neinokulirani i inokulirani klasovi) i tretmanom dušika (0, 35, 70 i 140 kg N $ha^{-1}$) u 2018./2019. (a) i 2019./2020. (b). Rezultati su prikazani kao srednja vrijednost osam replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0{,}05$). Izvor: Matić i sur., 2022.

U 2019./2020. značajan utjecaj na koncentraciju ukupnih topljivih PHE imali su sorta pšenice i tretman dušikom, iako su pronađene i značajne razlike za utjecaj *Fusarium* tretmana (Tablica

11). U sorti Srpanjka, inokulacija vrstama roda *Fusarium* uzrokovala je značajno povećanje koncentracije PHE samo pri N70 (Slika 8b). U sorti Apache, *Fusarium* tretman uzrokovao je značajno povećanje koncentracije PHE za 25 % i 19 % redom pri N0 i N35. Trend povećanja koncentracije PHE u inokuliranim biljkama u usporedbi s neinokuliranim, pri svim razinama dušika, utvrđen je u sorti Graindor. Povećanje koncentracije PHE kretalo se u rasponu od 10 % pri N35 do 23 % pri N140. Za razliku od prethodno navedenih sorti, u sorti Sofru, *Fusarium* tretman uzrokovao je značajno smanjenje koncentracije PHE redom za 13 %, 24 % i 36 % pri N35, N70 i N140. U ovom istraživanju utjecaj dušika na koncentraciju PHE bio je znatno izraženiji u 2019./2020. godini. Prilikom ispitivanja razlike između N0 i N140 te razlike između neinokuliranih i inokuliranih biljaka, N0 je uzrokovao smanjenje koncentracije PHE u sortama Srpanjka, Sofru i Apache u 2019./2020. godini. Slični rezultati dobiveni su u prethodnom istraživanju, gdje je niska razina dušika uzrokovala smanjenje koncentracije PHE u dvije sorte (BC Mandica i Isengrain) (Matić i sur., 2021a). Do sada navedeni rezultati istraživanja ukazuju na velik utjecaj klimatskih uvjeta i same sorte na koncentraciju PHE. U obje vegetacijske godine, *Fusarium* tretman utjecao je na promjenu koncentracije PHE u klasovima pšenice, što ukazuje na uključenost PHE u obrambeni odgovor pšenice na napad *Fusarium* patogena. Mnoga istraživanja potvrđuju uključenost PHE u obrambene mehanizme zaštite protiv FHB-a te navode kako povećana sinteza PHE u biljnom tkivu ukazuje na bolju prilagodljivost i toleranciju (Gunnaiah i Kushalappa, 2014.; Chrpová i sur., 2021.; Pratyusha, 2022.). Chowdhary i sur. (2021.) navode kako se obrambeni mehanizam biljke protiv napada patogena odvija u dvije faze: u prvoj fazi dolazi do brzog nakupljanja PHE na mjestu infekcije, što usporava rast patogena, a u drugoj fazi biljka sintetizira specifične tvari povezane sa stresom (jednostavne fenole, fitoaleksine, hidroksicimetnu kiselinu itd.) koje dodatno ograničavaju rast i širenje patogena od mjesta infekcije. U ovom istraživanju, tijekom obje vegetacijske godine, infekcija vrstama roda *Fusarium* izazvala je povećanje koncentracije PHE u djelomično otpornim sortama (Apache i Graindor), što ukazuje na uključenost PHE u obrambene reakcije i bolju toleranciju na bolest kod otpornijih sorti. Ovi su rezultati u skladu s rezultatima koje su objavili Gunnaiah i Kushalappa (2014.). Naime, autori su otkrili da je otpornost u sorti Sumai-3 povezana s nakupljanjem metabolita fenilpropanoidnog puta, koji su pak negativno djelovali na širenje patogena uzrokujući povećano zadebljanje stanične stijenke domaćina (Gunnaiah i Kushalappa, 2014.). Također, metaboliti fenilpropanoidnog puta uzrokovali su smanjenje rasta patogena zbog svojih antifungalnih i antioksidacijskih svojstava, što je neposredno djelovalo i na smanjenu sintezu mikotoksina. S druge strane, koncentracije PHE se u sorti Sofru razlikuju

između vegetacijskih godina. U 2018./2019. godini, koncentracija PHE bila je povećana u inokuliranim klasovima pšenice, dok je u 2019./2020. godini koncentracija PHE u inokuliranim, u usporedbi s neinokuliranim biljkama, pri gotovo svim razinama dušika bila smanjena. U obje vegetacijske godine, sorta Sofru imala je najizraženije simptome FHB-a u usporedbi s ostalim inokuliranim sortama. Stoga, u sorti Sofru, PHE nisu uspjeli spriječiti širenje *Fusarium* patogena i razvoj bolesti.

Uspoređujući dvije vegetacijske godine, izraženije promjene u aktivnosti PAL-a vidljive su u 2019./2020. Zbog povoljnih klimatskih uvjeta za razvoj bolesti (veća ukupna količina oborina) tijekom 2018./2019. visoki infekcijski pritisak FHB-a onemogućio je diferencijaciju sorti ovisno o razlikama u aktivnosti PAL-a. U 2018./2019. na aktivnost PAL-a značajno je utjecala samo sorta pšenice, iako su pronađene i određene značajne razlike za utjecaj *Fusarium* tretmana (Tablica 10). Značajne su razlike pronađene u sorti Graindor, gdje je infekcija vrstama roda *Fusarium* uzrokovala značajno povećanje aktivnosti PAL-a za 34 % i 29 % pri N70 i N140 (Slika 9a).




**Slika 9.** Aktivnost enzima fenilalanin-amonij-lijaze (PAL) u klasovima četiri sorte pšenice pod različitim *Fusarium* tretmanom (neinokulirani i inokulirani klasovi) i tretmanom dušika (0, 35, 70 i 140 kg N $ha^{-1}$) u 2018./2019. (a) i 2019./2020. (b). Rezultati su prikazani kao srednja vrijednost osam replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2022.

U 2019./2020. značajan utjecaj na aktivnost PAL-a imala su sva tri faktora istraživanja: sorta pšenice, *Fusarium* tretman i tretman dušikom (Tablica 11). U sorti Srpanjka, infekcija vrstama roda *Fusarium* uzrokovala je značajno povećanje aktivnosti PAL-a za 65 % i 31 % pri N0 i N35 (Slika 9b). U sorti Apache, *Fusarium* infekcija uzrokovala je značajno povećanje aktivnosti PAL-a za 37 % pri N70 i N140. Povećanje aktivnosti PAL-a u inokuliranih biljaka, u usporedbi s neinokuliranim, pri svim razinama dušika utvrđeno je u sorti Graindor (Slika 9b), gdje se povećanje aktivnosti PAL-a kretalo između 17 % pri N35 i 156 % pri N0. U sorti Graindor, klasificiranoj kao djelomično otpornoj sorti, povećana aktivnost PAL-a i manji simptomi zaraze mogu ukazivati na važnost ovog enzima u obrambenom odgovoru. S druge strane, u sorti Sofru, aktivnost PAL-a bila je smanjena u inokuliranim biljkama pri svim razinama dušika, a smanjenje se kretalo u rasponu od 41 % (N35) do 60 % (N0). U 2019./2020., sorta Sofru imala je najizraženije simptome zaraze FHB-om, a snažna infekcija mogla je uzrokovati inhibiciju aktivnosti PAL-a. Riaz i sur. (2014.) proučavanjem interakcije pšenice i fitopatogene vrste *Puccinia triticina* navode kako je aktivnost PAL-a prisutna i u rezistentnih i u osjetljivih sorti pšenice, ali je izraženija aktivnost PAL-a zabilježena u sortama otpornijim na infekciju patogenom. PAL je ključni enzim u metabolizmu fenilpropanoida i uključen je u sintezu nekoliko sekundarnih metabolita, uključujući fenole (kumarini, flavonoidi, lignini), fenolne derivate i lignin (Kaur i sur., 2022.). Inhibicija aktivnosti PAL-a posljedično može dovesti do smanjene sinteze molekula uključenih u obrambene mehanizme i povećane osjetljivosti biljaka na patogene (Huang i sur., 2010.). S obzirom na navedena teorijska znanja o mehanizmu djelovanja PAL-a pretpostavlja se kako bi aktivnost PAL-a i koncentracija PHE trebali biti u pozitivnoj korelaciji. Međutim, u ovom je istraživanju u 2018./2019. godini pronađena slaba negativna korelacija između koncentracija PHE i aktivnosti PAL-a (Tablica 12), dok je u 2019./2020. godini utvrđena slaba pozitivna korelacija (Tablica 13).

**Tablica 12.** Pearsonov koeficijent korelacije (r) i odgovarajuće razine značajnosti između mjerenih biokemijskih parametara u 2018./2019.

| | **PHE** | **TAC** | **PAL** | **PPO** |
|---|---|---|---|---|
| **PHE** | 1 | | | |
| **TAC** | 0,53*** | 1 | | |
| **PAL** | -0,21*** | -0,44*** | 1 | |
| **PPO** | -0,48*** | -0,53*** | 0,17** | 1 |

** i *** – značajno na razini $p \leq 0,01$ i 0,001. PHE, fenoli/*phenolics*; TAC, ukupni antioksidacijski kapacitet/*total antioxidant capacity*; PAL, fenilalanin-amonij-lijaza/*phenylalanine ammonia-lyase*; PPO, polifenol-oksidaza/*polyphenol oxidase*. Izvor: Matić i sur., 2022.

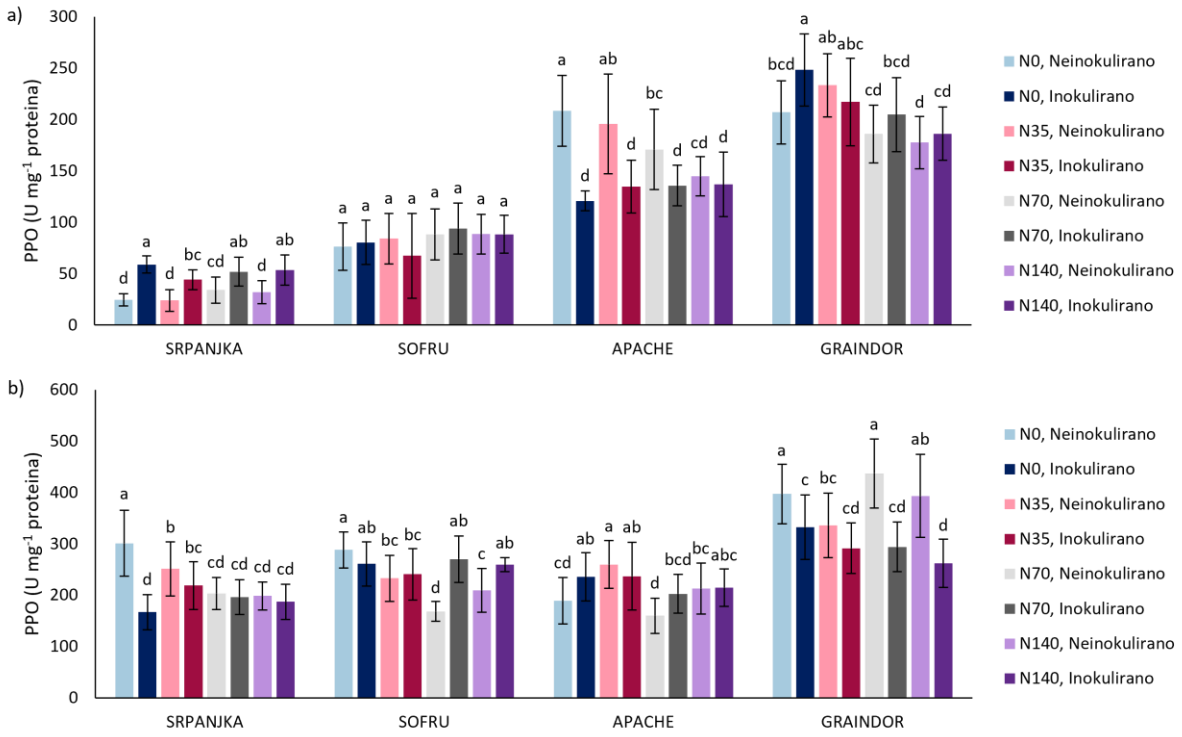
**Tablica 13.** Pearsonov koeficijent korelacije (r) i odgovarajuće razine značajnosti između mjerenih biokemijskih parametara u 2019./2020.

| | PHE | TAC | PAL | PPO |
|---|---|---|---|---|
| **PHE** | 1 | | | |
| **TAC** | 0,57*** | 1 | | |
| **PAL** | 0,33 *** | 0,16* | 1 | |
| **PPO** | -0,44*** | -0,20** | -0,30*** | 1 |

*, ** i *** – značajno na razini p ≤ 0,05, 0,01 i 0,001. PHE, fenoli/*phenolics*; TAC, ukupni antioksidacijski kapacitet/*total antioxidant capacity*; PAL, fenilalanin-amonij-lijaza/*phenylalanine ammonia-lyase*; PPO, polifenol-oksidaza/*polyphenol oxidase*. Izvor: Matić i sur., 2022.

Korelacijskom analizom između koncentracije PHE i aktivnosti PAL-a unutar svake sorte pojedinačno, u sorti Sofru u 2018./2019. godini utvrđena je pozitivna korelacija ($r = 0,40$, $p \leq 0,01$). U 2019./2020. godini utvrđena je pozitivna korelacija između koncentracije PHE i aktivnosti PAL-a u sortama Sofru ($r = 0,57$, $p \leq 0,001$) i Graindor ($r = 0,63$, $p \leq 0,001$). U 2019./2020. godini, infekcija vrstama roda *Fusarium* uzrokovala je smanjenje aktivnosti PAL-a u sorti Sofru, što je bilo povezano s nižom koncentracijom PHE. Može se pretpostaviti kako je snažna infekcija FHB-om u sorti Sofru uzrokovala inhibiciju PAL-a, što je posljedično rezultiralo smanjenom sintezom PHE. Tijekom obje vegetacijske godine u sorti Graindor pronađene su pozitivne korelacije između koncentracije PHE i aktivnosti PAL-a. Povećana aktivnost PAL-a posljedično je dovela do povećane sinteze PHE. Dakle, i PAL i PHE, kao produkti aktivnosti PAL-a, doprinose većoj toleranciji na FHB u otpornijih sorti. U sorti Srpanjka, koja je u ovom istraživanju klasificirana kao umjereno osjetljiva sorta, nisu utvrđene značajne promjene ni u koncentraciji PHE ni u aktivnosti PAL-a.

U 2018./2019. godini značajan utjecaj na aktivnost enzima PPO imali su sorta pšenice i tretman dušikom, iako su pronađene i značajne razlike za utjecaj *Fusarium* infekcije (Tablica 10). Analizom aktivnosti enzima PPO unutar svake sorte pojedinačno, u sorti Srpanjka, infekcija vrstama roda *Fusarium* uzrokovala je povećanje aktivnosti enzima PPO pri svim razinama dušika, a povećanje se kretalo u rasponu od 53 % pri N70 do 140 % pri N0 (Slika 10a). U sorti Graindor, infekcija vrstama roda *Fusarium* uzrokovala je značajno povećanje aktivnosti enzima PPO samo pri N0. Suprotno tomu, u sorti Apache utvrđena je smanjena aktivnost enzima PPO, odnosno infekcija vrstama roda *Fusarium* uzrokovala je značajno smanjenje od 42 %, 31 % i 21 % redom pri N0, N35 i N70.



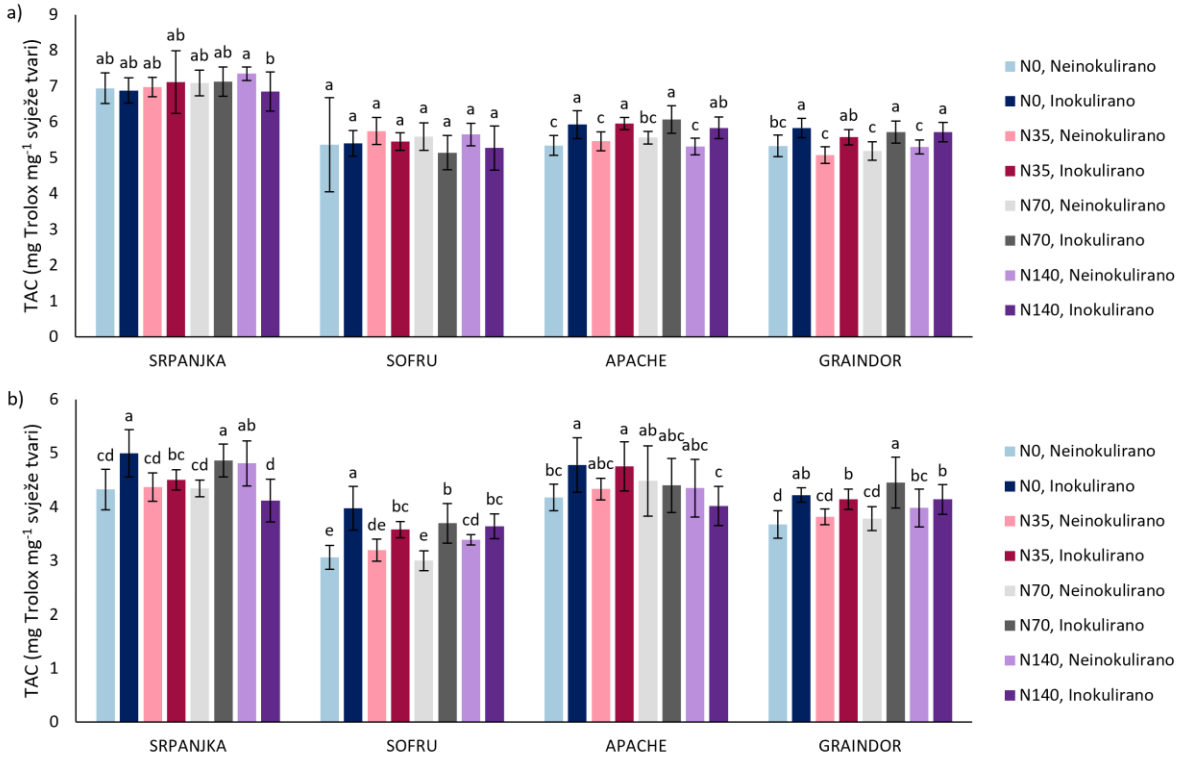

**Slika 10.** Aktivnost enzima polifenol-oksidaze (PPO) u klasovima četiri sorte pšenice pod različitim *Fusarium* tretmanom (neinokulirani i inokulirani klasovi) i tretmanom dušika (0, 35, 70 i 140 kg N $ha^{-1}$) u 2018./2019. (a) i 2019./2020. (b). Rezultati su prikazani kao srednja vrijednost osam replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2022.

U 2019./2020. godini značajan utjecaj na aktivnost enzima PPO imala su sva tri faktora istraživanja: sorta pšenice, *Fusarium* tretman i tretman dušikom (Tablica 11). Infekcija vrstama roda *Fusarium* uzrokovala je tendenciju smanjenja aktivnosti enzima PPO u sorti Srpanjka iako je značajno smanjenje utvrđeno samo pri N0. Trend smanjene aktivnosti enzima PPO također je pronađen u sorti Graindor, gdje je infekcija vrstama roda *Fusarium* uzrokovala značajno smanjenje za 16 %, 33 % i 33 % redom pri N0, N70 i N140. Za razliku od prethodno navedenih sorti, u sortama Sofru i Apache, infekcija vrstama roda *Fusarium* uzrokovala je značajan porast aktivnosti enzima PPO. Točnije, u sorti Sofru, infekcija vrstama roda *Fusarium* uzrokovala je značajno povećanje aktivnosti enzima PPO za 60 % pri N70 i 24 % pri N140, dok je u sorti Apache povećanje aktivnosti enzima PPO utvrđeno samo pri N0. Iako je do danas prepoznata uloga enzima PPO u obrani biljaka od napada i širenja patogena, točni podatci o mehanizmu djelovanju enzima još su uvijek nepoznanica. Predloženo je nekoliko različitih mehanizama djelovanja: (1) izravna toksičnost kinona, (2) alkilacija staničnih proteina biljke domaćina čime se smanjuje bioraspoloživost proteina kao izvora hranjivih tvari patogenu, (3) unakrsno

povezivanje kinona s proteinima ili fenolima stanične stijenke domaćina, čime se stvara fizička barijera koja onemogućava prodor i širenje patogena i (4) proizvodnja ROS-a koji imaju važnu ulogu u obrambenim mehanizmima (Taranto i sur., 2017.).

U 2018./2019. godini sorta Graindor imala je najveću aktivnost enzima PPO pri svim razinama dušika, dok je najmanja aktivnost enzima PPO zabilježena u sorti Srpanjka (četiri do pet puta niža ovisno o razini dušika). Slični rezultati zabilježeni su u drugim istraživanjima, gdje je aktivnost enzima PPO bila veća u klasovima sorti pšenice otpornih na *F. graminearum* u odnosu na osjetljive sorte (Mohammadi i Kazemi, 2002.; Sorahinobar i sur., 2015.). Povećana aktivnost enzima PPO, izazvana napadom patogena, često je povezana s povećanom otpornošću na patogene, što naglašava ulogu enzima PPO u obrani biljaka od napada patogena (Chen i sur., 2000.; Deborah i sur., 2001.; Raj i sur., 2006.). U ovom je istraživanju, u obje vegetacijske godine, utvrđena negativna korelacija između koncentracije PHE i aktivnosti enzima PPO (Tablice 12 i 13). Negativna korelacija između ove dvije varijable mogla bi se objasniti činjenicom da tijekom različitih stresnih uvjeta PPO katalizira oksidaciju fenolnih spojeva u visoko reaktivne kinone, čime se smanjuje koncentracija PHE.

U 2018./2019. godini značajan utjecaj na TAC imali su sorta pšenice i *Fusarium* tretman (Tablica 10). U inokuliranih sorti Apache i Graindor, pri svim razinama dušika, utvrđeno je značajno povećanje TAC-a zbog infekcije vrstama roda *Fusarium* (Slika 11a). U sorti Apache, povećanje TAC-a kretalo se od 9 % pri N35 i N70 do 11 % pri N0, dok se u sorti Graindor kretalo od 8 % pri N140 do 10 % pri N35 i N70. Za razliku od sorti Graindor i Apache, u sorti Srpanjka, infekcija vrstama roda *Fusarium* uzrokovala je značajan pad TAC-a pri N140, dok u sorti Sofru nisu uočene značajne promjene TAC-a (Slika 11a).




**Slika 11.** Ukupni antioksidacijski kapacitet u klasovima četiri sorte pšenice pod različitim *Fusarium* tretmanom (neinokulirani i inokulirani klasovi) i tretmanom dušika (0, 35, 70 i 140 kg N $ha^{-1}$) u 2018./2019. (a) i 2019./2020. (b). Rezultati su prikazani kao srednja vrijednost osam replika ± standardna devijacija. Različita slova iznad pojedinog stupca označavaju statistički značajne razlike između tretmana unutar svake sorte pojedinačno prema Fisher LSD testu ($p \leq 0,05$). Izvor: Matić i sur., 2022.

U 2019./2020. godini značajan utjecaj na TAC imali su sorta pšenice i *Fusarium* tretman (Tablica 11). Trend povećanja TAC-a, u inokuliranim biljkama u usporedbi s neinokuliranim, pri gotovo svim razinama dušika, pronađen je u sortama Sofru i Graindor (Slika 11b). U sorti Sofru značajno povećanje TAC-a kretalo se od 12 % pri N35 do 30 % pri N0, dok se u sorti Graindor kretalo od 8 % pri N35 do 18 % pri N70. U sorti Apache, infekcija vrstama roda *Fusarium* uzrokovala je značajan porast TAC-a za 14 % samo pri N0. U sorti Srpanjka, infekcija vrstama roda *Fusarium* uzrokovala je značajno povećanje TAC-a za 15 % pri N0 i 12 % pri N70 te značajno smanjenje TAC-a od 14 % pri N140. U obje vegetacijske godine pronađena je pozitivna korelacija između koncentracije PHE i TAC-a (Tablice 12 i 13). Značajna korelacija između koncentracije PHE i TAC-a ukazuje na visok doprinos PHE antioksidacijskom kapacitetu ozime pšenice izložene stresnim uvjetima okoliša. Ovaj je rezultat u skladu s istraživanjem Atanasova-Penichon i sur. (2016.), u kojemu se naglašava glavna uloga PHE u doprinosu ukupnom antioksidacijskom kapacitetu žitarica.

# 6. ZAKLJUČCI

- Nije utvrđen značajan utjecaj dušika na pojavnost i ozbiljnost FHB-a. Značajan utjecaj na pojavnost i ozbiljnost FHB-a imali su prevladavajući klimatski uvjeti tijekom vegetacijske godine, posebice obilne količine oborina tijekom faze cvatnje pšenice. Utvrđen je sortno-specifični odgovor ozime pšenice na FHB tijekom uzgoja pri različitim razinama gnojidbe dušikom.
- Utvrđen je sortno-specifični antioksidacijski odgovor pšenice na FHB i različitu gnojidbu dušikom. Najznačajniji utjecaj na mjerene pokazatelje oksidacijskog stresa i antioksidacijskog odgovora imao je uzgoj pšenice u uvjetima niske razine dušika. Naime, niska je razina dušika uzrokovala povećanje razine LPO i smanjenje aktivnosti antioksidacijskih enzima u listovima i klasovima većine neinokuliranih sorti. Dobiveni rezultati upućuju kako niska razina dušika predstavlja abiotički stresni faktor, pri uzgoju pšenice, koji uzrokuje mjerljive promjene u fiziološkom odgovoru pšenice.
- Pri istim eksperimentalnim uvjetima, nije utvrđena značajno jaka veza između mjerenih pokazatelja oksidacijskog stresa i antioksidacijskog odgovora lista zastavičara i klasa. Na temelju dobivenih rezultata može se zaključiti kako je antioksidacijski odgovor bio tkivno-specifičan.
- Inokulacija vrstama roda *Fusarium* utjecala je na promjenu koncentracije PHE u klasovima pšenice, što ukazuje na uključenost PHE u obrambeni odgovor pšenice na napad fitopatogenih gljiva roda *Fusarium*. Povećana sinteza PHE u biljnom tkivu ukazuje na bolju prilagodljivost i toleranciju. Pronađene su značajne korelacije između koncentracije PHE i ukupnog antioksidacijskog kapaciteta, što ukazuje na visok doprinos PHE antioksidacijskom kapacitetu ozime pšenice podvrgnute različitim stresnim uvjetima okoliša. Oplemenjivanje sorti pšenice koje su nositelj poželjnog svojstva (pojačana sinteza PHE) mogla bi biti jedna od strategija u kontroli FHB-a.

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**Autori:** Magdalena Matić, Rosemary Vuković, Karolina Vrandečić, Ivna Štolfa Čamagajevac, Jasenka Ćosić, Ana Vuković, Kristina Sabljić, Nikolina Sabo, Krešimir Dvojković i Dario Novoselović

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# Oxidative Status and Antioxidative Response to *Fusarium* Attack and Different Nitrogen Levels in Winter Wheat Varieties

**Magdalena Matić [1], Rosemary Vuković [2,*], Karolina Vrandečić [1,3,*], Ivna Štolfa Čamagajevac [2], Jasenka Ćosić [1], Ana Vuković [2], Kristina Sabljić [2], Nikolina Sabo [2], Krešimir Dvojković [4] and Dario Novoselović [3,4]**

1 Faculty of Agrobiotechnical Sciences, Josip Juraj Strossmayer University of Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia; maticm@fazos.hr (M.M.); jcosic@fazos.hr (J.Ć.)
2 Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia; ivna@biologija.unios.hr (I.Š.Č.); ana.vukovic@biologija.unios.hr (A.V.); kristina.sabljic@biologija.unios.hr (K.S.); nikolina.sabo@biologija.unios.hr (N.S.)
3 Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), 10000 Zagreb, Croatia; darion@poljinos.hr
4 Department for Cereal Breeding and Genetics, Agricultural Institute Osijek, Južno Predgrađe 17, 31000 Osijek, Croatia; kresimir.dvojkovic@poljinos.hr
* Correspondence: rosemary@biologija.unios.hr (R.V.); kvrandecic@fazos.hr (K.V.)

**Abstract:** Abiotic and biotic stresses, such as mineral nutrition deficiency (especially nitrogen) and *Fusarium* attack, pose a global threat with devastating impact on wheat yield and quality losses worldwide. This preliminary study aimed to determine the effect of *Fusarium* inoculation and two different nitrogen levels on oxidative status and antioxidative response in nine wheat varieties. Level of lipid peroxidation, activities of antioxidant enzymes (catalase, ascorbate peroxidase, glutathione reductase), phenolics, and chloroplast pigments content were measured. In general, wheat variety, nitrogen, and *Fusarium* treatment had an impact on all tested parameters. The most significant effect had a low nitrogen level itself, which mostly decreased activities of all antioxidant enzymes and reduced the chloroplast pigment content. At low nitrogen level, *Fusarium* treatment increased activities of some antioxidative enzymes, while in a condition of high nitrogen levels, antioxidative enzyme activities were mostly decreased due to *Fusarium* treatment. The obtained results provided a better understanding on wheat defense mechanisms against *F. culmorum*, under different nitrogen treatments and can serve as an additional tool in assessing wheat tolerance to various environmental stress conditions.

**Keywords:** *Triticum aestivum*; *Fusarium*; nitrogen; oxidative stress; antioxidant system



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## 1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops worldwide. In field conditions, wheat is confronted with both abiotic and biotic stresses that have a great impact on its growth and productivity [1]. Nitrogen (N) is one of the major nutritional elements in wheat production, and it is necessary to achieve high yields and grain quality [2]. Wheat quickly perceives and responds to nitrogen deficiency via a large number of physiological and metabolic events, such as the changes in fatty acid composition, reduction in chlorophyll content, and occurrence of oxidative stress [3]. During cultivation, wheat is often exposed to Fusarium head blight (FHB), an economically devastating disease that may exert an adverse impact on the wheat yield and quality [4]. Global yield losses due to individual pathogen and pests are estimated at 21.5% for wheat, whereas yield losses due to FHB ranked second after leaf rust [5]. FHB is caused by species of fungi in the genus *Fusarium*, of which *Fusarium graminearum* Schwabe (*Gibberella zeae* Schwein. Petch.) and *Fusarium culmorum* (Wm. G. Sm.) Sacc. are the most common and most virulent in Croatia [6].

It is now well established that all abiotic and biotic stresses induce or involved oxidative stress to some degree, and the ability of plants to control oxidant levels is highly correlated with stress tolerance [7]. Oxidative stress is a complex chemical and physiological phenomenon that arises due to excessive production and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [8,9]. The balance between production and elimination of ROS and RNS is critical to maintaining cellular redox homeostasis. Excessive production of ROS and RNS can induce a nitro-oxidative stress in plant cells, which causes lipid peroxidation, damages proteins and nucleic acids, inhibits antioxidant enzyme activities and activates the programmed cell death pathway [8,10]. In order to overcome the high levels of ROS and RNS, plants developed an efficient antioxidant defense system constitutes both enzymatic and nonenzymatic components. Enzymatic components that are involved in the detoxification of reactive species include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), while the non-enzymatic components include small molecules such phenolics (PHE), flavonoids, pigments, ascorbate, and glutathione [8,11].

Plant tolerance to different stress factors can be achieved by plant breeding or cultural practices that reduce levels of stress, which is in turn accomplished by the understanding of the plant's response to its stressors and how they affect individual plants and plant processes [12]. This study aimed to determine the effect of two different nitrogen levels and *Fusarium* inoculation on the biomarkers of oxidative and antioxidative status (lipid peroxidation, PHE, and activities of CAT, APX, and GR) measured in the spikes of nine wheat varieties. Although numerous studies have analyzed wheat's physiological response to *Fusarium* stress itself [13–15], this preliminary study will give insight into wheat's physiological response to combined nitrogen and *Fusarium* stress conditions.

## 2. Results

Three-way ANOVA showed significant differences in visual FHB scoring for all the main factors (Table 1). The visual scoring for FHB was affected mainly by *Fusarium* treatment and wheat variety ($p \leq 0.001$) and to a lesser extent by nitrogen treatment ($p \leq 0.05$). Non-inoculated wheat varieties did not show any FHB symptoms; thus statistical differences between inoculated and non-inoculated plants are not shown. Differences in symptoms of inoculated wheat varieties at low and high nitrogen levels for each variety separately are shown (Table 2). In Ficko, Galloper, and Felix varieties high nitrogen level caused an increase in visual symptoms. Contrarily, in variety U-1 high nitrogen level caused a decrease in visual symptoms.

**Table 1.** Three-way ANOVA for visual scoring and five different biochemical parameters under different nitrogen and *Fusarium* treatments in spikes of nine winter wheat varieties.

| | | MS | | | | | |
|---|---|---|---|---|---|---|---|
| **Source of Variation** | **df** | **VS[2]** | **TBARS** | **PHE** | **CAT** | **APX** | **GR** |
| VARIETY (V) | 8 | 0.08 *** | 59.92 *** | 108.02 *** | 1700.63 *** | 1.295 *** | 719.05 *** |
| N LEVEL (N) | 1 | 0.04 * | 43.73 ** | 20.51 ns | 1215.25 ** | 4.227 *** | 180.06 ns |
| FUSARIUM (F) | 1 | 23.02 *** | 11.62 ns | 190.55 *** | 669.20 * | 0.001 ns | 733.05 * |
| V×N | 8 | 0.14 *** | 17.14 *** | 38.75 * | 163.39 ns | 0.571 *** | 176.72 ns |
| V×F | 8 | 0.08 *** | 6.78 ns | 25.96 ns | 801.97 *** | 0.307 *** | 299.44 ns |
| N×F | 1 | 0.04 * | 2.31 ns | 153.85 ** | 1769.33 *** | 0.338 * | 808.47 * |
| V×N×F | 8 | 0.14 *** | 7.16 ns | 27.53 ns | 579.37 *** | 0.439 *** | 321.27 ns |

ns—not significant, *, ** and ***—significant at the level of probability $p \leq 0.05$, 0.01, and 0.001, respectively. Df, degrees of freedom; MS, mean sum of squares; VS, visual scoring (in percentage inoculated spikes); TBARS, thiobarbituric acid reactive substances; PHE, phenolics; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase. [2] A log transformation of the visual scores was used to normalize the data.

**Table 2.** Visual scores for Fusarium head blight (FHB) of nine inoculated winter wheat varieties under two different nitrogen levels.

| | Visual Scores (in Percentage Inoculated Spikes) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **N level** | **Ficko** | **U-1** | **Galloper** | **BC Mandica** | **BC Opsesija** | **Ingenio** | **Isengrain** | **Felix** | **Bezostaya-1** |
| LN | 6.67 ± 1.53 b | 8.00 ± 1.00 a | 3.33 ± 1.53 b | 9.00 ± 5.29 a | 20.00 ± 10.00 a | 10.33 ± 4.51 a | 10.00 ± 0.00 a | 8.33 ± 2.89 b | 5.00 ± 0.00 a |
| HN | 25.00 ± 5.00 a | 3.33 ± 0.58 b | 10.00 ± 0.00 a | 7.00 ± 2.65 a | 4.33 ± 0.58 a | 10.33 ± 4.51 a | 8.33 ± 2.89 a | 32.67 ± 2.52 a | 8.00 ± 2.00 a |

Values are means of three replicates ± standard deviation (SD). Different letters indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different nitrogen treatments in each variety separately. LN—low nitrogen; HN—high nitrogen.

Three-way ANOVA revealed a significant variety, nitrogen and *Fusarium* treatment effects for tested biochemical parameters (Table 1). Wheat variety significantly affected all tested biochemical parameters ($p \leq 0.001$), while nitrogen treatment significantly affected thiobarbituric acid reactive substances (TBARS) content, CAT and APX activity. *Fusarium* treatment mostly affected PHE content ($p \leq 0.001$), and to a lesser extent, CAT and GR activity ($p \leq 0.05$). Variety by nitrogen treatment interaction was significant for TBARS, PHE accumulation, and APX activity. Three-factor interaction between the variety, nitrogen, and *Fusarium* treatment was significant only for CAT and APX activity.

TBARS content was significantly influenced by the wheat variety and nitrogen treatment (Table 1). In the Ficko variety at low nitrogen level, *Fusarium* treatment caused a decrease in TBARS content, while in the Galloper variety, the content of TBARS was increased (Figure 1a). In conditions of high nitrogen level, *Fusarium* treatment did not cause any significant changes in TBARS content. In most non-inoculated varieties, low nitrogen level tended to increase TBARS content, although a significant increase was only found in the Ficko and Bezostaya-1 varieties.

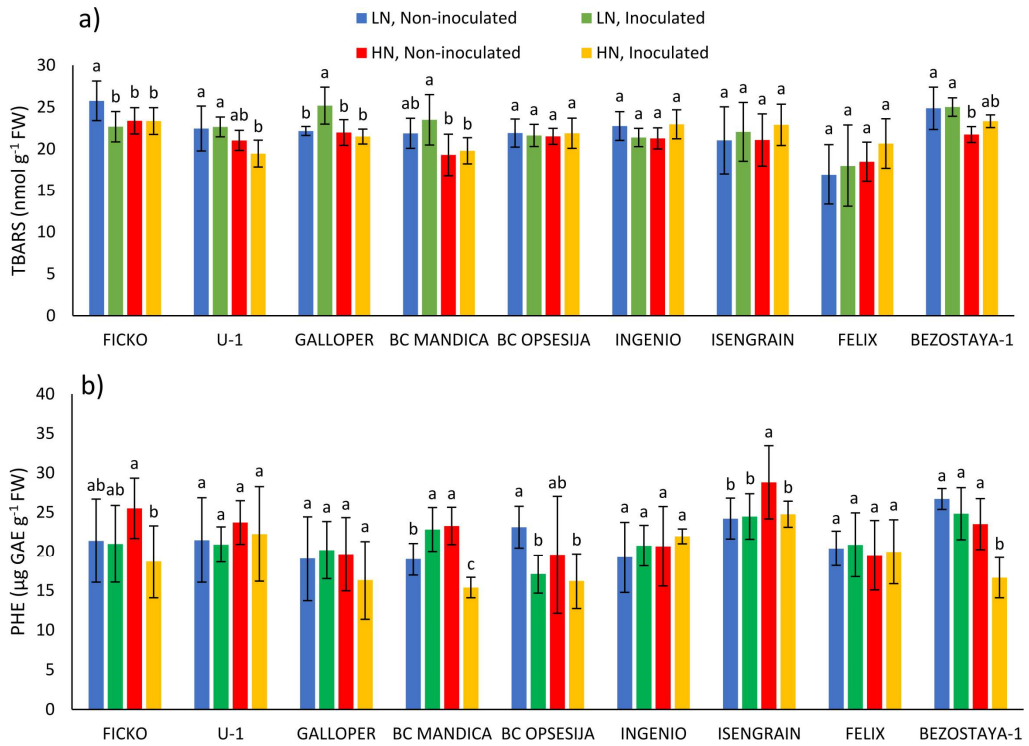


**Figure 1.** TBARS content (**a**) and soluble phenolic (PHE) content (**b**) in spikes of nine wheat varieties under different nitrogen (LN—low nitrogen; HN—high nitrogen) and *Fusarium* (non-inoculated and inoculated) treatments. Values are means of six replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different treatments in each variety separately.

The soluble phenolic content was most significantly affected by *Fusarium* treatment and wheat variety (Table 1). *Fusarium* treatment caused an increase in soluble phenolic content in BC Mandica variety at low nitrogen level, while in the BC Opsesija variety this content was decreased (Figure 1b). In conditions of high nitrogen level, *Fusarium* treatment caused a decrease in phenolic content in the Ficko, BC Mandica, Isengrain and, Bezostaya-1 varieties. In most non-inoculated varieties, low nitrogen level decreased phenolic content, although a significant decrease was only found in the BC Mandica and Isengrain varieties.

The CAT activity was significantly influenced by all three main factors, wheat variety ($p \leq 0.001$), nitrogen ($p \leq 0.01$), and *Fusarium* treatment ($p \leq 0.05$; Table 1). On average, a low nitrogen level caused a decrease of 5.46% in CAT activity compared to a high nitrogen level. Observing the changes in CAT activity in each variety separately, in the U-1, BC Mandica, Isengrain, Felix, and Bezostaya-1 varieties, at low nitrogen level, *Fusarium* treatment caused an increase in CAT activity (Figure 2a). In conditions of high nitrogen level, *Fusarium* treatment caused a decrease in CAT activity in the Isengrain variety. In most non-inoculated varieties, low nitrogen level decreased CAT activity, although a significant decrease was found in the BC Mandica and Isengrain varieties.

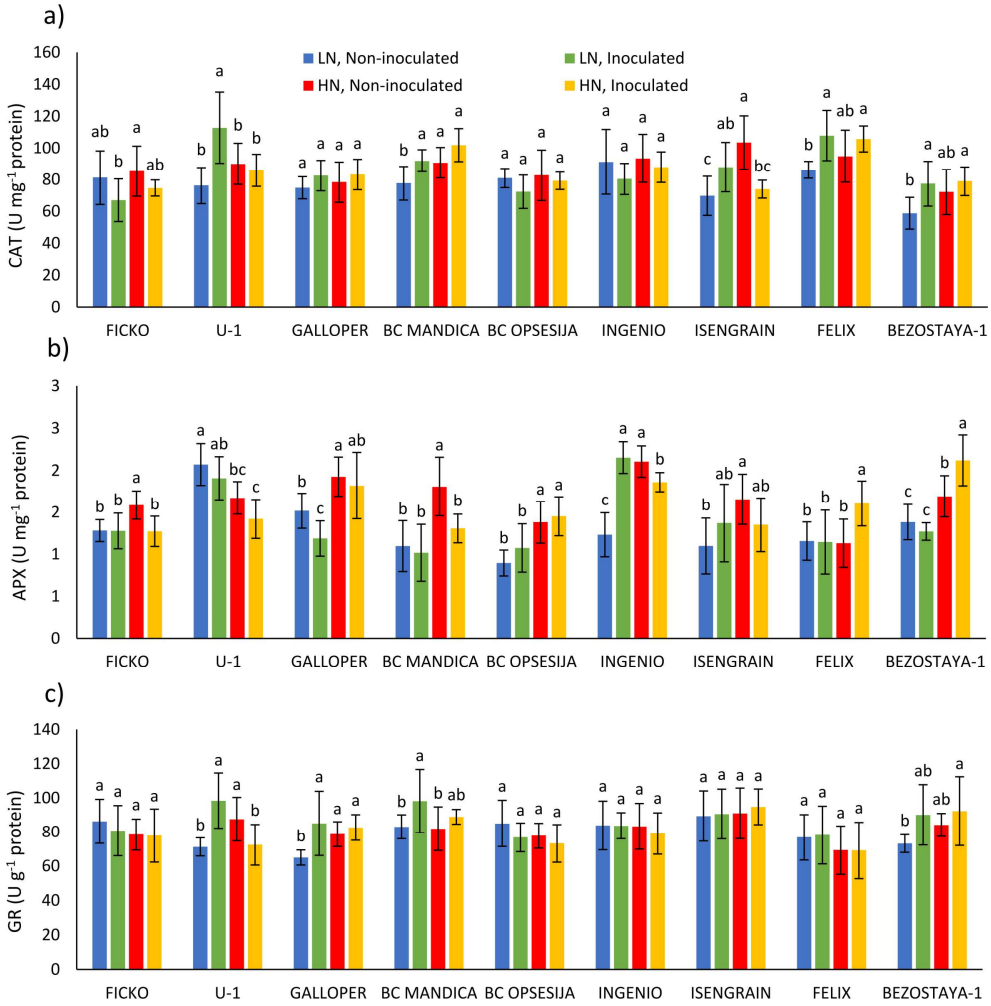


**Figure 2.** Antioxidant enzyme activity: catalase (CAT; (**a**)), ascorbate peroxidase (APX; (**b**)), and glutathione reductase (GR; (**c**)) in spikes of nine wheat varieties under different nitrogen (LN—low nitrogen; HN—high nitrogen) and *Fusarium* (non-inoculated and inoculated) treatments. Values are means of six replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different treatments in each variety separately.

The APX activity was most significantly influenced by nitrogen treatment and wheat variety (Table 1). On average, a low nitrogen level caused a decrease of 17.26% in APX activity compared to a high nitrogen level. Observing the changes in APX activity in each variety separately, in the Galloper variety at low nitrogen level, *Fusarium* treatment caused a decrease in APX activity, while in the Ingenio APX activity was increased (Figure 2b). In conditions of high nitrogen level, *Fusarium* treatment caused a decrease in APX activity in Ficko, BC Mandica, and Ingenio varieties, while in Felix and Bezostaya-1 varieties, APX activity was increased. In most non-inoculated varieties (Ficko, Galloper, BC Mandica, BC Opsesija, Ingenio, Isengrain, and Bezostaya-1), low nitrogen level significantly decreased APX activity, compared to high nitrogen level.

The GR activity was most significantly influenced by wheat variety ($p \leq 0.001$), and to a lesser extent, *Fusarium* treatment ($p \leq 0.05$; Table 1). In U-1, Galloper and BC Mandica varieties, at low nitrogen, *Fusarium* treatment caused an increase in GR activity, while, at high nitrogen conditions, GR activity was decreased only in U-1 variety (Figure 2c). Compared to high nitrogen level, low nitrogen level significantly decreased GR activity in non-inoculated U-1 and Galloper varieties.

Three-way ANOVA revealed a wide variety, nitrogen and *Fusarium* treatment effects for tested chloroplast pigments (Table 3). Wheat variety and nitrogen treatment significantly affected all tested chloroplast pigments in wheat spikes. *Fusarium* treatment significantly affected almost all tested chloroplast pigments, except the Chl a/Chl b ratio.

**Table 3.** Three-way ANOVA for different chloroplast pigments under different nitrogen and *Fusarium* treatment in spikes of nine winter wheat varieties.

| | | MS | | | | |
|---|---|---|---|---|---|---|
| **Source of Variation** | **df** | **Chl a** | **Chl b** | **Chl a+b** | **Car** | **Chl a/Chl b** |
| VARIETY (V) | 8 | 0.0128 *** | 0.0015 *** | 0.0228 *** | 0.0008 *** | 0.0745 ** |
| N LEVEL (N) | 1 | 0.0363 *** | 0.0058 *** | 0.0712 *** | 0.0019 *** | 0.1192 * |
| FUSARIUM (F) | 1 | 0.0204 *** | 0.0033 *** | 0.0402 *** | 0.0014 *** | 0.0592 ns |
| V × N | 8 | 0.0011 ns | 0.0002 ns | 0.0022 ns | 0.0001 ns | 0.0217 ns |
| V × F | 8 | 0.0025 * | 0.0004 * | 0.0050 * | 0.0002 ns | 0.0258 ns |
| N × F | 1 | 0.0028 ns | 0.0003 ns | 0.0049 ns | 0.0002 ns | 0.0059 ns |
| V × N × F | 8 | 0.0049 *** | 0.0008 *** | 0.0097 *** | 0.0003 *** | 0.0229 ns |

ns—not significant, *, ** and ***—significant at the level of probability $p \leq 0.05$, 0.01, and 0.001, respectively. Df, degrees of freedom; MS, mean sum of squares; Chl a, chlorophyll a; Chl b, chlorophyll b; Chl a+b, total chlorophyll; Car, carotenoids; Chl a/Chl b, chlorophyll a/b ratio.

The strongest effect on chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl a+b), carotenoids (Car) content, and chlorophyll a/b ratio in wheat spikes was found in the nitrogen treatment (Table 3). On average, a low nitrogen level decreased Chl a, Chl b, Chl a+b, and Car content in wheat spikes, while chlorophyll a/b ratio was increased, compared to high nitrogen level. Wheat variety also had a strong effect on chloroplast pigment content in wheat spikes. On average for all varieties, Ingenio and Isengrain varieties showed the highest Chl a, Chl b, Chl a+b, and Car content in wheat spikes, while the lowest content of chloroplast pigments was in Bezostaya-1 variety (data not shown).

Observing the changes in chloroplast pigment content in each variety separately, *Fusarium* treatment, at both nitrogen levels, caused a decrease in Chl a, Chl b, and Car content in BC Mandica and Isengrain varieties (Figure 3a–c). In conditions of low nitrogen level, most non-inoculated varieties showed decrease in the content of chloroplast pigments compared to high nitrogen level. However, a significant decrease was found in Ingenio, Isengrain, and Bezostaya-1 varieties for Chl a, Chl b, and Car content, and for Chl a in the Galloper variety.

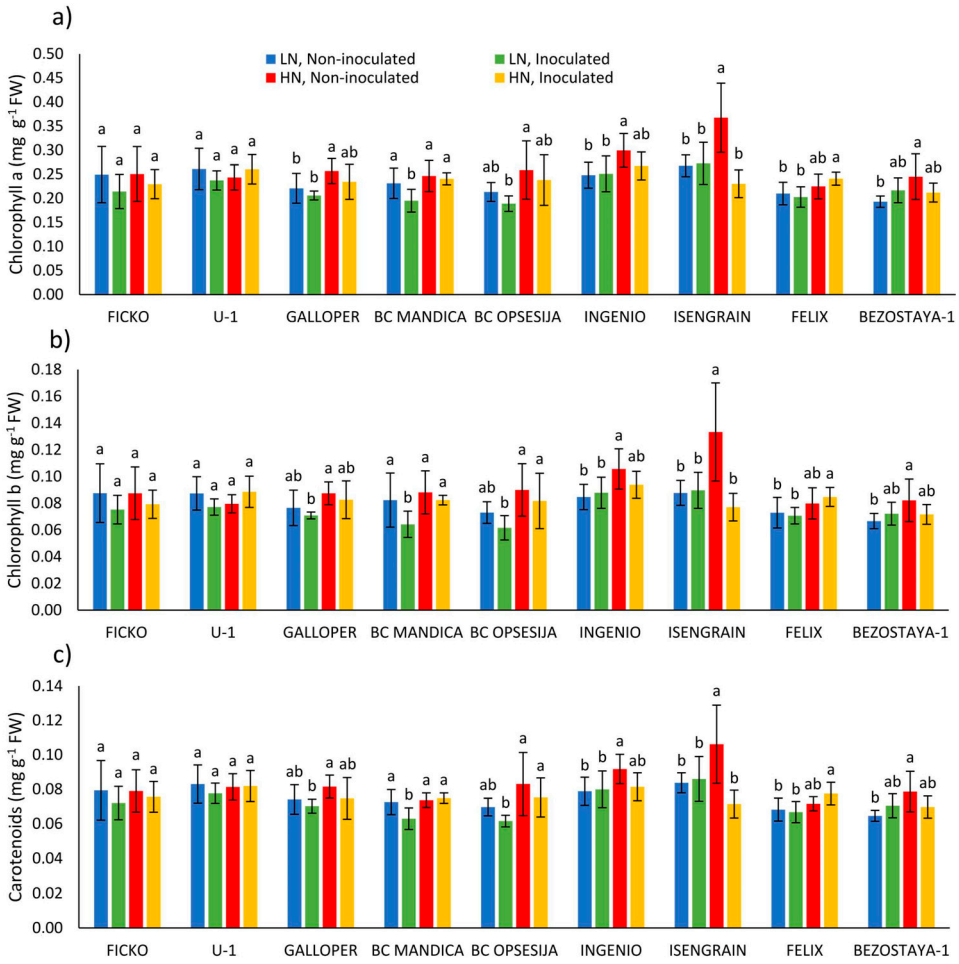


**Figure 3.** Chlorophyll a (**a**), chlorophyll b (**b**), and carotenoids (**c**) content in spikes of nine wheat varieties under different nitrogen (LN—low nitrogen; HN—high nitrogen) and *Fusarium* (non-inoculated and inoculated) treatments. Values are means of six replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different treatment in each variety separately.

## 3. Discussion

Both inadequate nitrogen fertilization and pathogen attacks can cause abiotic and biotic stress conditions in wheat production. To date, a scarce amount of studies are available on the influence of both FHB and different nitrogen fertilization on the oxidative and antioxidant response of wheat. In the present study, we examined the effect of nitrogen and *Fusarium* treatment on the occurrence of FHB and biochemical changes in spikes of nine wheat varieties in field conditions. In general, wheat variety, nitrogen, and *Fusarium* treatment had an impact on all tested parameters of oxidative stress and antioxidative response.

Considering different experimental conditions, various reports on the effect of nitrogen fertilization on FHB are available in the literature. According to some studies, high nitrogen levels increase disease incidence and intensity [16–18], whereas others reported restriction impact of nitrogen on *Fusarium* infection [19–21]. In the present study, in, Ficko, Galloper, and Felix varieties high nitrogen levels caused an increase in visual symptoms, compared to low nitrogen levels. Contrarily, in U-1 variety high nitrogen level caused a decrease in visual symptoms, suggesting a variety-specific response. However, to determine a more accurate effect of different nitrogen levels on FHB incidence and intensity, it is necessary to include testing at different nitrogen levels and more extensive mycological, and mycotoxin analyses.

As an indicator of oxidative stress, lipid peroxidation can be a useful tool to evaluate cultivars' susceptibility to FHB [14]. However, in our study, we did not find any significant changes in the levels of lipid peroxidation caused by nitrogen or *Fusarium* treatment. Under the nitrogen deficiency and *Fusarium* treatment, increased lipid peroxidation was recorded only in Galloper variety, which could be explained by varietal susceptibility to *Fusarium*. On the other hand, in conditions of high nitrogen level, *Fusarium* treatment did not cause any significant changes in lipid peroxidation level. This could be explained by different plant responses to individual stress compared to a combination of stresses [22–24].

In conditions of low nitrogen level, *Fusarium* treatment considerably increased activities of some measured antioxidative enzymes (CAT, APX or GR) in most varieties. The exception was the Galloper variety, the only variety with increased lipid peroxidation level, where APX activity was decreased, indicating the importance of this enzyme in the defense response. Induction of enzymatic defense mechanisms is connected with FHB-resistance, wherein more tolerant varieties activate the antioxidative enzymes faster and earlier in the infection process [25,26]. On the other hand, in conditions of high nitrogen levels, antioxidative enzyme activities were decreased in most infected wheat varieties. Although there were no changes in TBARS content, decreased activities of antioxidative enzymes could increase the incidence and intensity of FHB in conditions of high nitrogen levels. Different Fusarium impacts on antioxidative response in conditions of high and low nitrogen level could be due to different ROS formation and scavenging in conditions of single stress (*Fusarium* treatment) and combination of stresses (low nitrogen level and *Fusarium* treatment).

Polyphenols content, such as phenolic acids and flavonoids, increased under abiotic or biotic stress conditions, helping the plant to cope with environmental constraints [27,28]. In the present study, *Fusarium* treatment of wheat at low nitrogen level increased the phenolic content in BC Mandica variety, while in BC Opsesija variety phenolic content decreased in response to *Fusarium* treatment. Increased content of phenolics in BC Mandica variety could be a part of its defense response that could contribute against pathogen attack and spread. In conditions of high nitrogen level, *Fusarium* treatment of wheat decreased the phenolic content in Ficko, BC Mandica, Isengrain, and Bezostaya-1 varieties. When exposed to pathogen infection, plants often suffer significant chloroplast pigment loss [29]. In the present study, *Fusarium* treatment caused a decrease in all chloroplast pigments in BC Mandica and Isengrain varieties, in conditions of low and high nitrogen levels, respectively.

In this study, the most noticeable impact on the measured biochemical parameters in wheat spikes had a low nitrogen level itself. Various abiotic stress conditions lead to the overproduction of ROS and imbalanced ROS detoxification, which lead to lipid peroxidation [8,30]. The present study provided similar results, in which low nitrogen level as an abiotic stress factor caused an increase in lipid peroxidation in most varieties. Although a significant increase in lipid peroxidation was found only in non-inoculated Ficko and Bezostaya-1 varieties, which also showed reduced APX activities, indicating the importance of this enzyme in scavenging ROS. Moreover, in most varieties, low nitrogen level caused a decrease in antioxidant enzyme activities. Decreased activities of CAT, APX, and GR have also been demonstrated in other N-deficient plants [31,32]. The reduction in antioxidant enzymes activity could be connected with lower amino acid and protein synthesis in nitrogen deficiency conditions, compared to high nitrogen level conditions. In the present study, low nitrogen level caused a decrease in the soluble phenolic content in two varieties (BC Mandica and Isengrain). This finding is in contrast to other studies, in which the accumulation of phenolic components in plant tissues was enhanced under limited nitrogen conditions, due to increased C:N ratio within plants [33–35]. Although results were not shown in this study, phenolic contents were increased in flag leaves of most varieties, suggesting different tissue responses to nitrogen deficiency. Furthermore, low nitrogen level caused the reduction in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids content in wheat spikes in non-inoculated plants. Our findings are in accordance with previous studies in which decreased content of photosynthetic pigments

under nitrogen-deficient conditions were found [3,36]. Low nitrogen level induces inhibition of photosynthesis and reduces photosynthetic capacity, which consequently inhibits plant growth and development [37,38].

The obtained results provided a better understanding on the biochemical aspects as a part of wheat defense mechanisms against necrotrophic fungi *F. culmorum*, under different nitrogen treatments. To our knowledge, this is the first report on the oxidative/antioxidative response of wheat to combined stress caused by *Fusarium* attack and different nitrogen levels. This research can serve as an additional tool in assessing wheat tolerance to various environmental stress conditions.

## 4. Materials and Methods

### *4.1. Field Trial*

Field trial with nine winter wheat varieties of different origin (Table 4) was planted in 2017/18 season at location Osijek. The experiment was set-up in a split-split-plot-factorial design in three replicates with nitrogen fertilization levels as main plots, nine wheat cultivars as sub-plots, and *Fusarium* infection was applied at sub-sub-plot level.

**Table 4.** Winter wheat varieties and their origin.

| Variety | Country | Breeding Institution | Year of Release |
|---|---|---|---|
| BC Mandica | Croatia | Bc Institut d.d. Zagreb | 2015 |
| BC Opsesija | Croatia | Bc Institut d.d. Zagreb | 2016 |
| Bezostaya-1 | Russia | Krasnodar Lukyanenko Research Institute | 1959 |
| Felix | Croatia | Agricultural Institute Osijek | 2007 |
| Ficko | Croatia | Agricultural Institute Osijek | 2007 |
| Galloper | Croatia | Agricultural Institute Osijek | 2014 |
| Ingenio | France | CC Benoist SA | 2010 |
| Isengrain | France | Florimond Desprez Veuve et Fils (FR) | 1997 |
| U-1 | Croatia | Agricultural Institute Osijek | 1936 |

The soil type was eutric cambisol and the experimental plot size was 7.56 $m^2$. Basic fertilization of 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$ and 120 kg $K_2O$ $ha^{-1}$ was applied by adding 100 kg $ha^{-1}$ of urea (46% N) and 400 kg $ha^{-1}$ NPK (7:20:30). The nitrogen treatment comprised of two nitrogen fertilization levels, 0 kg N $ha^{-1}$ (LN − low nitrogen) and 100 kg N $ha^{-1}$ (HN—high nitrogen), applied as top-dressings of 50 kg N $ha^{-1}$ at tillering (Zadok's scale 23–25) and stem extension (Zadok's scale 33–35) growth stages (Table 5). All other cultural practices including the application of herbicides, insecticides, and fungicides to control major weeds, insects and foliar diseases were typical for commercial wheat production in Croatia.

**Table 5.** Soil nitrogen (N) content (kg $ha^{-1}$) in Osijek in 2017/18 year.

| Location | Soil Type | Season | Residual Soil N (kg N $ha^{-1}$) | Basic N Fertilization (kg N $ha^{-1}$) | N Top-Dressing (kg N $ha^{-1}$) | | Total N (kg N $ha^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | LN | HN | LN | HN |
| Osijek | Eutric cambisol | 2017/18 | 20 | 74 | 0 | 50 + 50 | 94 | 194 |

### *4.2. Inoculum Production, Inoculation Procedure, and Disease Evaluation*

Isolate *F. culmorum* obtained from the fungal culture collection of Faculty of Agrobiotechnical Sciences Osijek (Croatia) was used for inoculum production, according to the method of Snijders and Eeuwijk [39]. A mixture of wheat and oat grains (3:1, v/v) was soaked in water overnight. The following day, excess water was decanted and seeds were autoclaved and inoculated with *F. culmorum* isolate. Inoculated grains were incubated for three weeks at 25 °C, protected from sunlight. Macroconidia were washed

off the colonized grains, and conidial suspension was diluted to a final concentration of $1 \times 10^6$ conidia $mL^{-1}$. Only the first $m^2$ of each subplot (variety) was inoculated, while the rest of the subplot was left to natural infection. Spray inoculations were carried out using a motor-driven backpack-sprayer in the late afternoon. Inoculations were performed individually on each subplot when 50% of the plants had reached anthesis (Zadok's scale 65) and repeated two days later. To maintain moisture for optimal infection conditions, plants were sprayed with water several times during the day. Visual scoring of the overall percentage of inoculated spikes showing FHB symptoms was performed 18 days after inoculation. The percentage of diseased spikes in each plot was determined on a linear scale (0–100%) [40].

### *4.3. Sample Preparation and Measurements*

Wheat spikes for measuring biomarkers of oxidative stress and antioxidant response were sampled 7 days after inoculation. Collected samples were immediately frozen in liquid nitrogen before being stored at −80 °C prior to analysis. Wheat spikes were ground using a TissueLyser (Qiagen Retsch GmbH, Hannover, Germany) for 1 min at 30 Hz. A fine powder obtained was weighed into microtubes for further analysis.

### *4.4. Determination of Lipid Peroxidation Level*

The level of lipid peroxidation in wheat spikes was determined by measuring the concentration of reactive substances of thiobarbituric acid (TBARS), mainly malondialdehyde (MDA), according to the method of Verma and Dubey [41]. About 150 mg of wheat spikes tissue was homogenized on ice with 1 mL of 0.1% (w/v) solution of trichloroacetic acid (TCA) and centrifuged at 6000× *g* for 5 min. To an aliquot (0.5 mL) of the supernatant, 1 mL of 0.5% thiobarbituric acid in 20% TCA was added, and the mixture was incubated in a water bath at 95 °C for 30 min. The produced red pigment was measured spectrophotometrically at 532 nm and 600 nm. The absorbance at 600 nm is deducted from the absorbance at 532 nm due to the correction for a non-specific reaction. Results were expressed in nmol of TBARS per gram of fresh weight (nmol TBARS $g^{-1}$ FW).

### *4.5. Determination of the Soluble Phenolic Content*

Powdered wheat tissue was homogenized on ice with 1 mL of 80% ethanol (1:10, w/v) and phenolic compounds were extracted for 24 h at −20 °C. After extraction, samples were centrifuged at 21,000× *g* at 4 °C for 15 min and supernatant was used for further measurements. Soluble phenolic content was determined by the Folin–Ciocalteu method [42]. The reaction mixture contained 20 µL of sample, 1.58 mL of $H_2O$, 100 µL of Folin–Ciocalteu reagent, and 300 µL of the saturated $Na_2CO_3$ solution. The reaction mixture was incubated in a water bath at 37 °C for 60 min, after which the absorbance was measured at 765 nm. Soluble phenolic content was calculated from a standard curve using gallic acid as a standard and expressed as µg gallic acid equivalents (GAE) per $g^{-1}$ fresh weight (µg GAE $g^{-1}$ FW).

### *4.6. Enzyme Activities*

A fine powder obtained after grinding was homogenized on ice in cold 100 mM potassium phosphate buffer (1:5, w/v) containing 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.2% (w/v) polyvinylpyrrolidone (PVP), pH 7.0. The homogenized samples were then centrifuged at 21,000× *g* at 4 °C for 15 min, and the supernatants were used for the spectrophotometric determination of the activity of the enzymes catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). Additionally, protein concentration in the enzyme extracts was determined using bovine serum albumin as a protein standard [43].

Briefly, catalase (CAT, EC 1.11.1.6) activity was measured according to Aebi [44]. The reaction mixture (1.5 mL) consisted of enzyme extract (50 µL) and 0.036% in 50 mM potassium phosphate buffer (pH 7.0). The decrease in absorbance due to the oxidation of

$H_2O_2$ was monitored at 240 nm over 2 min. The CAT activity was calculated using a molar extinction coefficient ($\varepsilon$ = 0.04 mM/cm) and expressed in U $mg^{-1}$ protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by an adjusted method from Nakano and Asada [45]. The reaction mixture (1 mL) consisted of enzyme extract (50 µL), 0.5 mM ascorbic acid, 0.12 mM $H_2O_2$ and 0.1 mM EDTA in 50 mM potassium phosphate buffer (pH 7.0). The decrease in absorbance due to the oxidation of ascorbate was monitored at 290 nm every 15 s for 3 min. The APX activity was calculated using a molar extinction coefficient ($\varepsilon$ = 2.8 mM/cm) and expressed in U $mg^{-1}$ protein.

Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to Halliwell and Foyer [46]. The reaction mixture (1 mL) consisted of protein extract (50 µL), 1 mM oxidized glutathione (GSSG), 0.1 mM reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and 1 mM EDTA in 100 mM potassium phosphate buffer (pH 7.5). A decrease in absorbance due to the oxidation of NADPH was monitored at 340 nm every 15 s for 2 min. The GR activity was calculated using a molar extinction coefficient for NADPH ($\varepsilon$ = 6.220 mM/cm) and expressed in U $mg^{-1}$ protein.

### *4.7. Determination of Photosynthetic Pigment Concentration*

A fine powder obtained after grinding (about 100 mg) was homogenized on ice with the cold absolute acetone and reextracted until plant tissue was completely colorless. The samples were centrifuged at 21,000× *g* at 4 °C for 15 min, and the supernatants were used for further measurements. The absorption of extracted photosynthetic pigments was measured at 470, 645 and, 662 nm. Concentrations of photosynthetic pigments were calculated according to Lichtenthaler [47] and expressed as mg $g^{-1}$ fresh weight.

### *4.8. Statistical Analysis*

All data analyses were performed using the SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA) software. Assays were carried out in six replicates and their results were expressed as mean ± standard deviation (SD). Factorial analysis of variance (ANOVA) was performed, and statistically significant differences among the treatments in each variety separately were determined using the Fisher's LSD test ($p \leq 0.05$).

**Author Contributions:** Conceptualization, M.M., R.V., K.V., J.Ć., K.D. and D.N.; formal analysis, M.M., R.V. and D.N.; investigation, M.M., R.V., I.Š.Č., A.V., K.S., N.S. and D.N.; resources, R.V., K.V., I.Š.Č. and D.N.; writing—original draft preparation, M.M.; writing—review and editing, R.V., K.V., I.Š.Č., J.Ć., A.V. and D.N.; visualization, M.M., R.V., K.V., I.Š.Č. and D.N.; funding acquisition, D.N. All authors have read and agreed to the published version of the manuscript.

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**Naslova rada:** The effect of nitrogen fertilization and *Fusarium culmorum* inoculation on the biomarkers of oxidative stress in wheat flag leaves

**Autori:** Magdalena Matić, Rosemary Vuković, Karolina Vrandečić, Ivna Štolfa Čamagajevac, Ana Vuković, Jasenka Ćosić, Krešimir Dvojković i Dario Novoselović

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Utjecaj gnojidbe dušikom i inokulacije vrstom *Fusarium culmorum* na biomarkere oksidacijskoga stresa listova zastavičara pšenice

**Matić, M., Vuković R., Vrandečić, K., Štolfa Čamagajevac I., Vuković A., Ćosić, J., Dvojković, K., Novoselović, D.**

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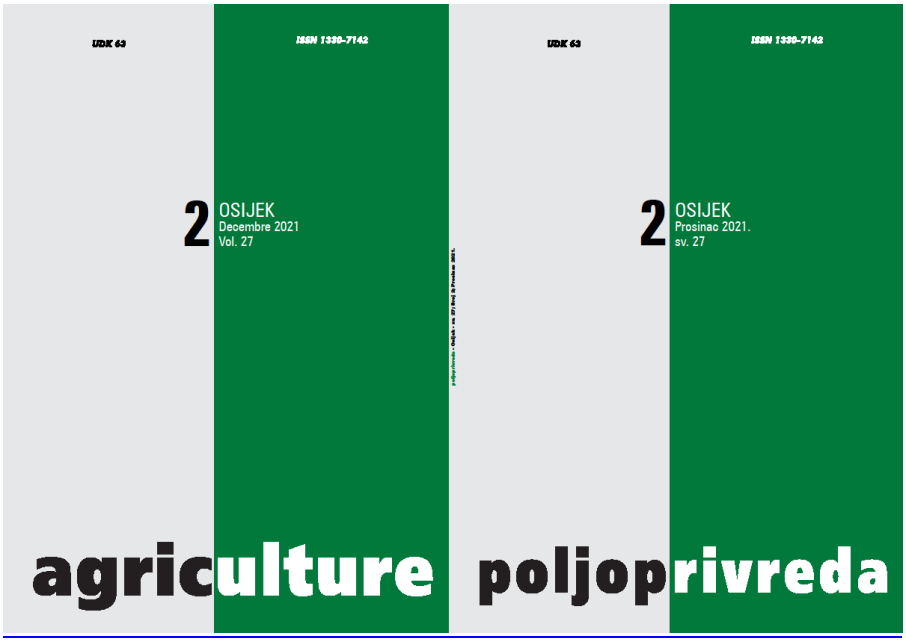


**Fakultet agrobiotehničkih znanosti Osijek, Poljoprivredni institut Osijek**

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# THE EFFECT OF NITROGEN FERTILIZATION AND *Fusarium culmorum* INOCULATION ON THE BIOMARKERS OF OXIDATIVE STRESS IN WHEAT FLAG LEAVES

***Matić, M.[(1)], Vuković R. [(2)], Vrandečić, K. [(1)], Štolfa Čamagajevac I. [(2)], Vuković A. [(2)], Ćosić, J. [(1)], Dvojković, K. [(3)], Novoselović, D. [(3)]***

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**SUMMARY**

***During cultivation, wheat is exposed to several abiotic and/or biotic stress conditions that may adversely impact the wheat yield and quality. The impact of abiotic stress caused by nitrogen deficiency and biotic stress caused by phytopathogenic fungus Fusarium culmorum on biomarkers of oxidative stress in the flag leaf of nine winter wheat varieties (*Ficko*, U-1, Galloper, BC Mandica, BC Opsesija, Ingenio, Isengrain, Felix, and Bezostaya-1*) was analyzed in this study. Hydrogen peroxide concentration and lipid peroxidation level were measured as indicators of oxidative stress, while the antioxidant response was determined by measuring the concentration of phenolic compounds and activities of antioxidant enzymes. Wheat variety and nitrogen treatment had a significant effect on all examined biomarkers of oxidative stress in the flag leaf, while the impact of Fusarium treatment was less pronounced. The most significant impact on the measured stress biomarkers had a low nitrogen level, which mainly increased hydrogen peroxide concentration and lipid peroxidation level and decreased activities of antioxidant enzymes in most varieties. The obtained results were discussed and compared with the previous study in which biochemical analyzes were performed on the wheat spike. There was no significant strong correlation between flag leaf and spike response in the measured parameters, which, in addition to the variety-specific response, also indicates a tissue-specific antioxidant response.***

***Keywords: wheat,* Fusarium culmorum*, nitrogen fertilization, oxidative stress, antioxidative response***

## INTRODUCTION

Wheat (*Triticum* spp.) is one of the most important cereal crops both globally and in Croatia. In the five-year period (2016-2020), the average wheat yield was 5.7 t $ha^{-1}$, which is why wheat is the grain with the highest yield in Croatia, after the corn (Statistical Yearbook of the Republic of Croatia, 2021). In field conditions, wheat is often exposed to several abiotic and/or biotic stress conditions that can significantly reduce yields.

As one of the major nutritional elements of wheat, nitrogen (N) is essential for achieving high yields and grain quality. In winter wheat, N fertilization can be considered as a critical agrotechnical measure because it is almost impossible to achieve high yields and grain quality without its adequate quantity and timely application

*(1) Magdalena Matić, MSc., Prof. Dr. Karolina Vrandečić, Prof. Dr. Jasenka Ćosić – Josip Juraj Strossmayer University of Osijek, Faculty of Agrobiotechnical Sciences Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia, (2) Assist. Prof. Rosemary Vuković (rosemary@biologija.unios.hr), Prof. Dr. Ivna Štolfa Čamagajevac, Ana Vuković, MSc. – Josip Juraj Strossmayer University of Osijek, Department of Biology, Cara Hadrijana 8/A, 31000 Osijek, Croatia, (3) Krešimir Dvojković, Ph. D., Dario Novoselović, Ph. D. – Agricultural Institute Osijek, Južno predgrađe 17, 31000 Osijek, Croatia*

(Vukadinović and Lončarić, 1999). Adequate fertilization impacts increasing photosynthetic activity and grain protein content, resulting in higher quality and higher yields (Hawkesford, 2014). Insufficient fertilization can reduce protein synthesis resulting in lower yields and lower market price, while excessive fertilization can cause lodging and increase wheat susceptibility to various pathogens (Wagan et al., 2003). Inadequate N fertilization can also affect the incidence of Fusarium Head Blight (FHB), which can also cause significant economic losses in wheat production. FHB is a devastating disease of wheat caused by phytopathogenic fungi in the genus *Fusarium*, of which *Fusarium graminearum* Schwabe (*Gibberella zeae* Schwein. Petch.) and *Fusarium culmorum* (Wm. G. Sm.) Sacc. are the most common and most virulent in Croatia (Ćosić et al., 2004).

In the plant tissues exposed to various abiotic and/or biotic stress conditions, a rapid accumulation of reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$), superoxide radical ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$) can occur, whose excessive amount can cause lipid peroxidation (LPO), i.e. oxidative damage to the structural components of plants (Hasanuzzaman et al., 2020). ROS can have several roles in the plant organism. They can act as reactive agents that damage cells, as signaling molecules, and they have an important role in plant pathogen defense (Demidchik, 2015; Camejo et al., 2016). In order to regulate levels of ROS and maintain redox homeostasis, plants have developed complex enzymatic and non-enzymatic antioxidant mechanisms (Apel and Hirt, 2004). The enzymatic antioxidants are enzymes such as catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), while the non-enzymatic antioxidants are biomolecules such as glutathione, phenols (PHE), carotenoids (Car), and others.

This study aimed to determine the effect of two different nitrogen levels and *F. culmorum* inoculation on the biomarkers of oxidative stress ($H_2O_2$, LPO, antioxidant enzymes activities, PHE, and Car) in flag leaves of nine wheat varieties. In order to establish the existence of a correlation between the antioxidant response of flag leaves and spikes, the obtained results were discussed and compared with the previous results of biochemical analyses performed under the same experimental conditions in wheat spikes (Matić et al., 2021).

## MATERIAL AND METHODS

### Field Trial

A field experiment with nine winter wheat varieties of different origins was conducted in 2017/2018 at the Agricultural Institute Osijek (45°32'N, 18°44'E). The experiment was set up according to the split-split plot design in three replicates. Three research factors included nine winter wheat varieties, two different nitrogen fertilization levels, and *F. culmorum* inoculation. The winter wheat varieties included in the study were *Ficko*, *U-1*, *Galloper*, *BC Mandica*, *BC Opsesija*, *Ingenio*, *Isengrain*, *Felix*, and *Bezostaya-1*. Basic fertilization of 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$, and 120 kg $K_2O$ $ha^{-1}$ was applied by adding 100 kg $ha^{-1}$ of urea (46% N) and 400 kg $ha^{-1}$ NPK (7:20:30). The nitrogen supplementation included two different nitrogen levels: (i) without supplementation 0 kg N $ha^{-1}$ (low nitrogen level) and (ii) 100 kg N $ha^{-1}$ (high nitrogen level), which was applied as top-dressings of 50 kg N $ha^{-1}$ at tillering (Zadok's scale 23 – 25) and 50 kg N $ha^{-1}$ at stem extension (Zadok's scale 33 – 35) growth stages. All cultural practices typical for commercial wheat production in Croatia (including herbicides, insecticides, and fungicides to control major weeds, insects, and foliar diseases) were used.

The method of Snijders and Van Eeuwijk (1991) was used for inoculum production. A mixture of wheat and oat grains (3:1) was left in water overnight, after which it was autoclaved and inoculated with spores of the fungus *F. culmorum* obtained from the fungal culture collection of Faculty of Agrobiotechnical Sciences Osijek, Department of Phytopathology. Inoculated grains were incubated for three weeks at 25 °C, protected from sunlight. After incubation, *F. culmorum* spores were washed off the colonized grains with sterile $H_2O$ and filtered. The final concentration of spores in the inoculum was adjusted to $1 \times 10^6$ spores $mL^{-1}$. Spray inoculations using a motor-driven backpack-sprayer were performed primarily on wheat spikes and only on the first $m^2$ of each plot (150 mL of suspension $m^{-2}$). The rest of the plants on the plot are left to natural infection. Inoculations were performed individually on each plot when 50% of the plants per plot had reached anthesis (Zadoks scale 65) and were repeated after 48 h.

### Determination of biomarkers of oxidative stress in flag leaves

For measuring biomarkers of oxidative stress (oxidative/antioxidant status), wheat flag leaves were sampled seven days after inoculation. The collected samples were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. The samples were ground into a fine powder in liquid nitrogen with a pestle and mortar. Biomarkers of oxidative stress were determined spectrophotometrically, using a UV-VIS spectrophotometer Perkin Elmer Lambda 25 (PerkinElmer, Waltham, USA). The oxidative status of wheat flag leaves was determined by measuring $H_2O_2$ concentration and LPO level. The $H_2O_2$ concentration was determined according to Mukherjee and Choudhuri (1983), where a standard curve with $H_2O_2$ was used for the calculation. Results were expressed in nmol of $H_2O_2$ per milligram of fresh weight (nmol $H_2O_2$ $mg^{-1}$ FW). The level of LPO levels was determined by measuring the concentration of reactive substances of thiobarbituric acid (TBARS), mainly malondialdehyde

(MDA), by the method of Verma and Dubey (2003). The amount of TBARS was calculated based on the standard curve with 1,1,3,3-tetramethoxypropane. Results were expressed in nmol of TBARS per gram of fresh weight (nmol TBARS $g^{-1}$ FW).

The antioxidant response was determined spectrophotometrically by measuring the activity of the antioxidant enzymes CAT, APX, and GR. CAT activity was determined following the method of Aebi (1984). The decrease in absorbance due to the degradation of $H_2O_2$ was measured at 240 nm. APX activity was determined according to Nakano and Asada (1981) with some modifications. The decrease in absorbance due to the oxidation of ascorbate was monitored at 290 nm. GR activity was determined by the method of Halliwell and Foyer (1978), and a decrease in absorbance due to the oxidation of NADPH was monitored at 340 nm. The specific activity of each enzyme was expressed as the amount ($\mu$mol) of degraded substrate per minute per milligram of protein, i.e., as the number of units per milligram of protein (U $mg^{-1}$ protein; U = $\mu$mol $min^{-1}$ protein). The concentration of soluble proteins was determined using the Bradford method (1976).

As non-enzymatic biomarkers of antioxidant status, soluble phenolic content and concentrations of photosynthetic pigments were determined. The soluble phenolic content was determined by the method of Folin-Ciocalteu (Folin and Ciocalteu, 1927). A standard curve, using gallic acid as a standard, was used for the calculation. The results were expressed as microgram gallic acid equivalents (GAE) per $g^{-1}$ fresh weight ($\mu$g GAE $g^{-1}$ FW). Photosynthetic pigments were extracted using ice-cold acetone, and the absorbance of extracted pigments was measured at 470, 645, and 662 nm. The extracted photosynthetic pigments' concentration was calculated according to the equations proposed by Lichtenthaler (Lichtenthaler, 1987) and expressed as mg $g^{-1}$ fresh weight.

### Statistical Analysis

The collected data were analyzed using the SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA) software. The effect of the examined factors (variety, nitrogen treatment, and *F. culmorum* inoculation) on the investigated traits was determined by a three-way analysis of variance (ANOVA) ($p \leq 0.05$, 0.01, and 0.001). Significant differences among the treatments in each variety separately were determined using the Fisher LSD test ($p \leq 0.05$).

## RESULTS AND DISCUSSION

Tolerance to stressful conditions can be increased by using various treatments to enhance the plant's defense response or by growing varieties more resistant to adverse environmental effects. This study analyzed the effect of abiotic stress caused by nitrogen deficiency and biotic stress caused by phytopathogenic fungus *F. culmorum* on the biomarkers of oxidative stress in flag leaves of nine winter wheat varieties. Wheat variety, nitrogen treatment, and inoculation with *F. culmorum* significantly impacted the examined biomarkers of oxidative stress in flag leaves. Furthermore, the results are discussed in the context of a previously published study where, under the same experimental conditions, the same analyzes were performed on the wheat spikes (Matić et al., 2021), which are most commonly used in biochemical analyzes of the FHB infection impact. Although FHB occurs primarily on wheat spikes, spike infection can cause changes in some metabolic pathways in other plant organs, e.g., changes in the rate of flag leaf photosynthesis (Yang et al., 2016; Španić et al., 2017a). In this study, we wanted to determine whether the spikes infection also reflects on the antioxidant response of flag leaves and whether there is a correlation in the antioxidant response between wheat flag leaves and spikes. Although a correlation between measured biochemical parameters could allow the use of flag leaves for a detailed analysis of oxidative stress caused by FHB, the results of this study did not show a significantly strong correlation between flag leaves and spikes in measured parameters (Pearson correlation test results not shown). The antioxidant response was both variety- and tissue-specific.

Three-way ANOVA revealed a significant variety effect on all examined biomarkers of oxidative stress in wheat flag leaves (Table 1). Under the same experimental conditions, an equally significant variety effect on biomarkers of oxidative stress was found in wheat spikes (Matić et al., 2021). Nitrogen treatment significantly affected the concentration of $H_2O_2$, PHE content, and the activity of antioxidant enzymes CAT, APX, and GR. Inoculation with *F. culmorum* isolate significantly affected the concentration of $H_2O_2$ and TBARS and PHE content ($p \leq 0.001$). In contrast to wheat flag leaves, *F. culmorum* inoculation of wheat spikes affected most of the measured biomarkers of oxidative stress (PHE, CAT, GR, Car). The interaction between the three main factors was significant for all examined biomarkers except for PHE content.

**Table 1. Three-way ANOVA of the influence of variety, nitrogen treatment, and *Fusarium culmorum* inoculation and their interactions on the examined biomarkers of oxidative stress in the flag leaf of wheat**

*Tablica 1. Trofaktorijalna analiza varijanca (ANOVA) utjecaja sorte, tretmana dušikom i inokulacije vrstom Fusarium culmorum i njihovih interakcija na ispitivane biomarkere oksidacijskoga stresa u listu zastavičaru pšenice*

| Mean sum of squares / *Srednja suma kvadrata* | | | | | | | |
|---|---|---|---|---|---|---|---|
| Source of Variation / *Izvor variranja* | Degrees of freedom / *Stupnjevi slobode* | HP | TBARS | PHE | CAT | APX | GR |
| Variety (V)/ *Sorta* | 8 | 95.36*** | 99.96*** | 2115.80*** | 1300.17* | 0.29*** | 787.75*** |
| Nitrogen treatment (N)/ *Tretman dušikom* | 1 | 50.63*** | 3.53 ns | 2199.62*** | 19041.20*** | 0.13* | 3764.30*** |
| Fusarium inoculation (F)/ *Fusarium inokulacija* | 1 | 47.00*** | 65.25*** | 422.01*** | 1491.89 ns | 0.04 ns | 421.11 ns |
| V×N | 8 | 11.85*** | 9.97*** | 40.39 ns | 1598.01** | 0.16*** | 124.06 ns |
| V×F | 8 | 8.87*** | 10.32*** | 77.31* | 1480.71** | 0.04 ns | 70.60 ns |
| N×F | 1 | 10.41** | 1.67 ns | 53.49 ns | 1306.70 ns | 0.07 ns | 2.11 ns |
| V×N×F | 8 | 5.11*** | 9.97*** | 48.36 ns | 1899.69*** | 0.10*** | 221.61* |

ns – not significant/nije statistički značajno; *, ** and *** – significant at the level of probability $p \leq 0.05$, 0.01, and 0.001/značajno na razini $p \leq 0,05$, 0,01 i 0,001. HP, hydrogen peroxide/vodik peroksid; TBARS, thiobarbituric acid reactive substances/reaktivne supstance tiobarbiturne kiseline; PHE, phenolics/fenoli; CAT, catalase/katalaza; APX, ascorbate peroxidase/askorbat-peroksidaza; GR, glutathione reductase/glutation-reduktaza.

In the *U-1*, *Galloper*, and *BC Opsesija* varieties, at low nitrogen level, *F. culmorum* inoculation caused an increase in $H_2O_2$ concentration, while at high nitrogen level, $H_2O_2$ concentration was increased in the *Galloper*, *BC Mandica*, *BC Opsesija*, *Felix*, and *Bezostaya-1* varieties (Figure 1a). In most non-inoculated varieties, low nitrogen level tended to increase $H_2O_2$ concentration, and a significant increase was found in the *Ficko*, *U-1*, *BC Opsesija*, *Ingenio*, and *Bezostaya-1* varieties. Since the lack of nitrogen supplementation in non-inoculated plants mainly caused an increase in TBARS content and a decrease in the activity of antioxidant enzymes, $H_2O_2$ could act as a promoter of oxidative stress. Mamenko (2018) also found an increase in the concentration of $H_2O_2$ in wheat leaves under an insufficient supply of soil nitrogen. However, the author also found an increase in the activity of antioxidant enzymes and concluded that $H_2O_2$ acted as a signaling molecule that affects the activation of wheat defense mechanisms under insufficient nitrogen supply.

Numerous studies have shown that different abiotic and/or biotic stress conditions can induce LPO, which can cause oxidative damage to the structural components of plants (Španić et al., 2017b; Hasanuzzaman et al., 2020). The occurrence of LPO caused by pathogen attack and/or insufficient nitrogen fertilization was also found in some varieties in this study. In the *Ficko*, *BC Opsesija*, and *Bezostaya-1* varieties at low nitrogen level, inoculation with *F. culmorum* caused an increase in TBARS content (Figure 1b). In contrast, the TBARS content was increased at high nitrogen level in the *Galloper*, *BC Opsesija*, and *Ingenio* varieties. On average, nitrogen treatment itself did not cause any significant changes in TBARS content in wheat flag leaves (Table 1). However, low nitrogen level caused an increase in TBARS content in non-inoculated varieties *BC Opsesija*, *Ingenio*, and *Isengrain*. Although the level of LPO is variety and tissue-specific, the increase in LPO is more pronounced in flag leaves than wheat spikes, i.e., it is present in a much larger number of varieties at different treatments (Matić et al., 2021).

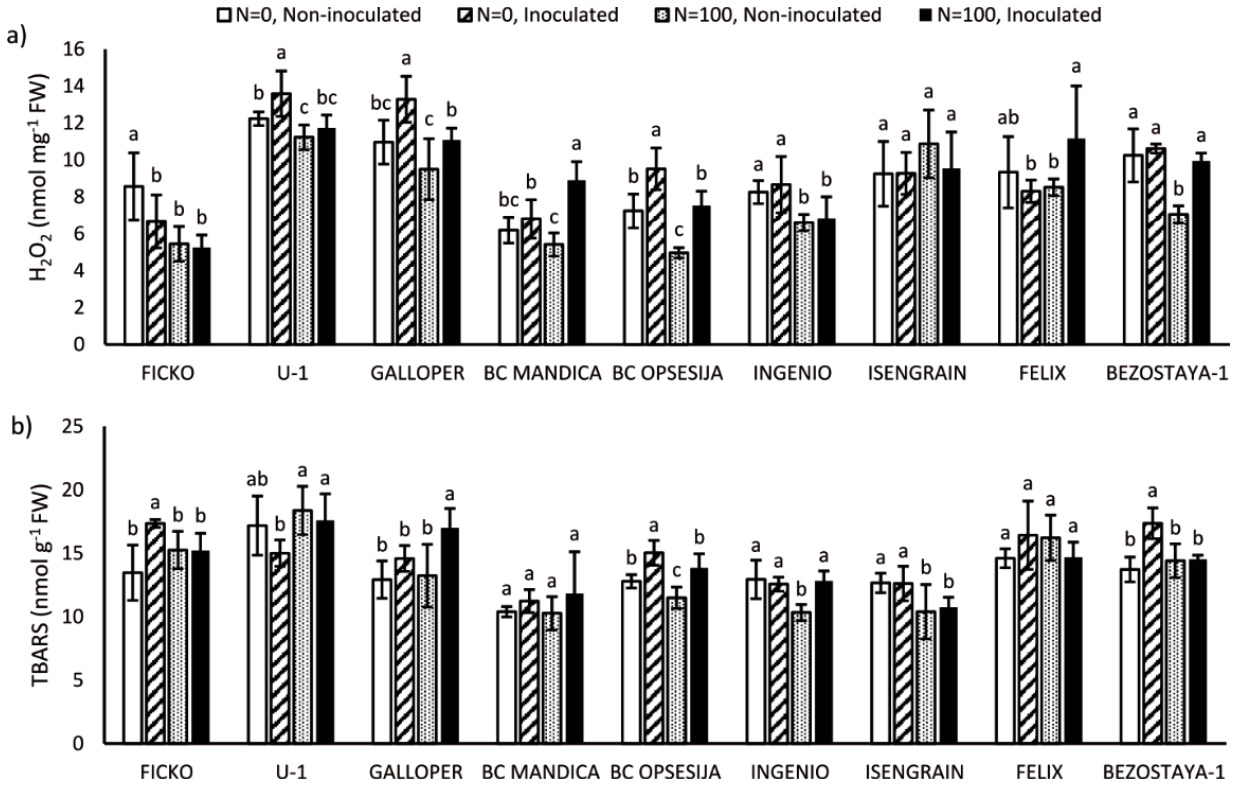


**Figure 1. Hydrogen peroxide ($H_2O_2$; a) and thiobarbituric acid reactive substances (TBARS; b) content in flag leaves of nine wheat varieties under different nitrogen (0 kg N ha$^{-1}$ and 100 kg N ha$^{-1}$) and *Fusarium culmorum* (non-inoculated and inoculated) treatments. Values are means of six replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different treatments in each variety separately.**

*Grafikon 1. Koncentracija vodika peroksida ($H_2O_2$; a) i količina reaktivnih supstanci tiobarbiturne kiseline (TBARS; b) listova zastavičara devet sorata pšenice pri različitim prihranama dušikom (0 kg N ha$^{-1}$ i 100 kg N ha$^{-1}$) i tretmanom* Fusarium culmorum *(neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinoga stupca označavaju statistički značajne razlike između tretmana u svakoj sorti pojedinačno, prema Fisherovu LSD testu ($p \leq 0,05$).*

The PHE content was significantly influenced by all three main factors, nitrogen treatment, variety, and *F. culmorum* inoculation ($p \leq 0.001$) (Table 1). The most significant effect had a nitrogen treatment itself, and on average, a low nitrogen level caused an increase of 8.82% in the PHE content compared to a high nitrogen level. Inoculation with *F. culmorum* isolate caused an increase in the PHE content in the *Galloper* and *BC Opsesija* varieties at low nitrogen level (Figure 2). The trend of increasing PHE content due to *F. culmorum* inoculation was also observed at a high nitrogen level, and a significant increase was found in the *BC Mandica*, *BC Opsesija*, and *Bezostaya-1* varieties. In most non-inoculated varieties, low nitrogen level tended to increase PHE content, although a significant increase was found in the *Ficko*, *Galloper*, *BC Mandica*, *BC Opsesija*, and *Bezostaya-1* varieties. The obtained results are in accordance with other studies in which it was found that under conditions of nitrogen deficiency, due to the increase of C:N ratio within plants, there is an increased formation and accumulation of phenolic compounds (Ibrahim et al., 2011; Munene et al., 2017; Deng et al., 2019). Phenolic compounds are the most significant and widespread plant secondary metabolites that play an important role in plant defense mechanisms under abiotic and/or biotic stress conditions (Kulbat, 2016; Sharma et al., 2019). Therefore, nitrogen fertilization is of great importance because it can affect primary and secondary plant metabolites (Chen et al., 2011). In the research of Matić et al. (2021), growing wheat at a low nitrogen level caused a decrease in the PHE content in wheat spikes of non-inoculated plants, suggesting a tissue-specific wheat response.

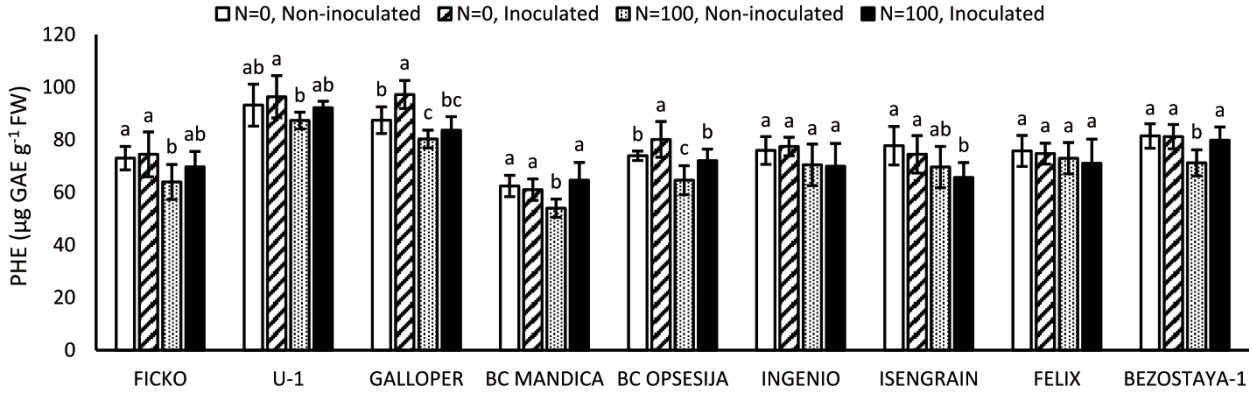


**Figure 2. Soluble phenolics (PHE) content in flag leaves of nine wheat varieties under different nitrogen (0 kg N ha$^{-1}$ and 100 kg N ha$^{-1}$) and *Fusarium culmorum* (non-inoculated and inoculated) treatments. Values are means of six replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different treatments in each variety separately.**

*Grafikon 2. Koncentracija topljivih fenola (PHE) listova zastavičara devet sorata pšenice pri različitim prihranama dušikom (0 kg N ha$^{-1}$ i 100 kg N ha$^{-1}$) i tretmanom vrstom* Fusarium culmorum *(neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinoga stupca označavaju statistički značajne razlike između tretmana u svakoj sorti pojedinačno, prema Fisherovu LSD testu ($p \leq 0,05$).*

Nitrogen treatment and wheat variety significantly affected the antioxidant enzymes CAT, APX, and GR (Table 1). On average, compared to high levels, a low nitrogen level caused a decrease in the activity of the antioxidant enzymes CAT and GR in wheat flag leaves. The decrease in antioxidant enzyme activity was 9.21% for CAT and 7.63% for GR, respectively. On average, *F. culmorum* inoculation did not significantly affect the activity of antioxidant enzymes in wheat flag leaves (Table 1). However, analysis of the effect of *F. culmorum* inoculation, in each variety separately, showed significant changes in enzymes activity, which were variety-specific (Figure 3). Inoculation with *F. culmorum* isolate caused an increase in CAT activity in the *Ficko* variety at low nitrogen level, while in the *BC Opsesija* and *Felix* varieties CAT activity was decreased (Figure 3a). In conditions of high nitrogen level, inoculation with *F. culmorum* isolate caused an increase in CAT activity in the *Bezostaya-1* variety and a decrease in CAT activity in the *Ingenio* variety. At low nitrogen level, inoculation with *F. culmorum* isolate caused an increase in APX activity in the *BC Opsesija* and *Isengrain* varieties, while in conditions of high nitrogen level, APX activity was increased in the *Ficko* variety (Figure 3b). Inoculation with *F. culmorum* isolate tended to decrease GR activity in almost all varieties at both nitrogen levels. However, a significant decrease in GR activity was found in the *Ingenio* variety at a low nitrogen level and the *Felix* variety at a high nitrogen level (Figure 3c). In addition to the fact that the response of antioxidant enzymes to *F. culmorum* inoculation depended on the variety, a comparison of the results of this analysis with previous analysis in wheat spikes (Matić et al., 2021) suggest a tissue-specific response. In the non-inoculated varieties *Ficko*, *U-1*, *Galloper*, and *Ingenio*, low nitrogen level decreased the CAT activity, while in the variety *Isengrain* decreased the APX activity. Also, compared to the high nitrogen level, the low nitrogen level caused a trend of decreasing GR activity in almost all non-inoculated varieties, although a significant decrease in GR activity was found only in the *Felix* variety. According to Matić et al. (2021), insufficient nitrogen supplementation caused a decrease in the activity of antioxidant enzymes in wheat spikes of most non-inoculated varieties. Decreased activity of antioxidant enzymes in conditions of nitrogen deficiency may be associated with reduced amino acids and protein synthesis in conditions of low nitrogen supply.

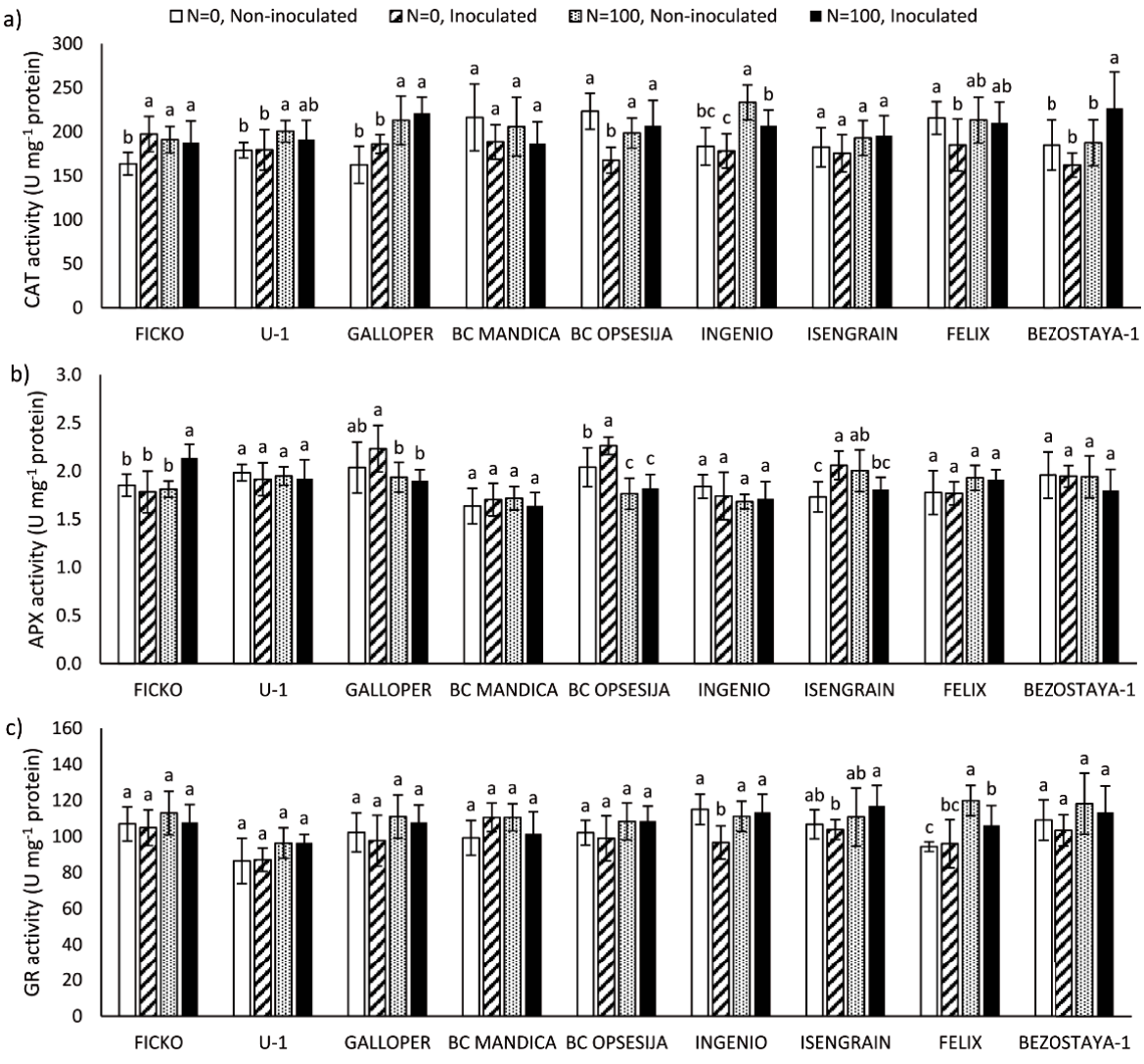


**Figure 3. Antioxidant enzyme activities: catalase (CAT; a), ascorbate peroxidase (APX; b), and glutathione reductase (GR; c) in flag leaves of nine wheat varieties under different nitrogen (0 kg N ha$^{-1}$ and 100 kg N ha$^{-1}$) and *Fusarium culmorum* (non-inoculated and inoculated) treatments. Values are means of six replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different treatments in each variety separately.**

*Grafikon 3. Aktivnosti antioksidacijskih ezima: katalaze (CAT; a), askorbat-peroksidaze (APX; b) i glutation-reduktaze (GR; c) listova zastavičara devet sorata pšenice pri različitim prihranama dušikom (0 kg N ha$^{-1}$ i 100 kg N ha$^{-1}$) i različitim tretmanom vrstom* Fusarium culmorum *(neinokulirane i inokulirane biljke). Rezultati su prikazani kao srednja vrijednost šest replika ± standardna devijacija. Različita slova iznad pojedinoga stupca označavaju statistički značajne razlike između tretmana u svakoj sorti pojedinačno, prema Fisherovu LSD testu ($p \leq 0,05$).*

Three-way ANOVA revealed a significant variety and nitrogen treatment effects on the content of photosynthetic pigments in wheat flag leaves ($p \leq 0.001$; Table 2). On the other hand, inoculation with *F. culmorum* isolate had no significant effect on the content of photosynthetic pigments in wheat flag leaves (Table 2), while its effect was significant in the wheat spikes (Matić et al., 2021). In their study of a large number of wheat genotypes, Molero and Reynolds (2020) observed a lack of correlation between flag leaves and spikes photosynthesis, suggesting their independence.

On average, a low nitrogen level decreased Chl a, Chl b, Chl a+b, and Car content in wheat flag leaves, compared to high nitrogen level. Decreased content of photosynthetic pigments under limited nitrogen conditions was also found in wheat spikes (Matić et al., 2021). As a macronutrient, nitrogen is an integral part of cells, proteins, nucleic acids, enzymes, and photosynthetic pigments of plants. Nitrogen deficiency during the growing season can cause a decrease in the content of photosynthetic pigments, which in turn can cause inhibition of photosynthesis and reduction of photosynthetic capacity (Boussadia et al., 2010; Prinsi et al., 2020). Reduced photosynthetic capacity, in turn, can negatively affect crop yield and quality.

**Table 2. Three-way ANOVA of the influence of variety, nitrogen treatment, and *Fusarium culmorum* inoculation and their interactions on the content of chloroplast pigments in the flag leaf of wheat**

*Tablica 2. Trofaktorijalna analiza varijanca (ANOVA) utjecaja sorte, tretmana dušikom i inokulacije izolatom* Fusarium culmorum *i njihovih interakcija na sadržaj kloroplastnih pigmenata u listu zastavičaru pšenice*

| Mean sum of squares / *Srednja suma kvadrata* | | | | | |
|---|---|---|---|---|---|
| Source of Variation / *Izvor variranja* | Degrees of freedom / *Stupnjevi slobode* | Chl a | Chl b | Chl a + b | Car |
| Variety (V)/ *Sorta* | 8 | 0.1836*** | 0.0297*** | 0.3547*** | 0.0090** |
| Nitrogen treatment (N) / *Tretman dušikom* | 1 | 1.7471*** | 0.3130*** | 3.5392*** | 0.0966*** |
| Fusarium inoculation (F) / *Fusarium inokulacija* | 1 | 0.0004 ns | 0.0012 ns | 0.0031 ns | 0.0013 ns |
| V×N | 8 | 0.0279 ns | 0.0053 ns | 0.0483 ns | 0.0025 ns |
| V×F | 8 | 0.0441 ns | 0.0088 ns | 0.0904 ns | 0.0040 ns |
| N×F | 1 | 0.0239 ns | 0.0063 ns | 0.0547 ns | 0.0035 ns |
| V×N×F | 8 | 0.0057 ns | 0.0046 ns | 0.0143 ns | 0.0009 ns |

ns – not significant/nije statistički značajno; *, ** and *** – significant at the level of probability $p \leq 0.05$, 0.01, and 0.001/značajno na razini $p \leq 0,05$, 0,01 i 0,001. Chl a, chlorophyll a/klorofil a; Chl b, chlorophyll b/klorofil b; Chl a+b, total chlorophyll/ukupni klorofil; Car, carotenoids/karotenoidi.

## CONCLUSION

Wheat variety and nitrogen treatment significantly affected all measured biomarkers of oxidative stress in the flag leaves, while the effect of *F. culmorum* treatment was less pronounced. The most significant effect on the measured biomarkers had a low nitrogen level itself, where a trend of increased $H_2O_2$ concentration and LPO level, and decreased activities of antioxidant enzymes was observed in most varieties. The obtained results were discussed and compared with the previous results of biochemical analyses performed under the same experimental conditions in wheat spikes. There was no significantly strong correlation between flag leaves and spikes in the measured parameters, which, in addition to the variety-specific response, also indicates a tissue-specific antioxidant response.

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# UTJECAJ GNOJIDBE DUŠIKOM I INOKULACIJE VRSTOM *Fusarium culmorum* NA BIOMARKERE OKSIDACIJSKOGA STRESA LISTOVA ZASTAVIČARA PŠENICE

***SAŽETAK***

***Pri uzgoju je pšenica izložena brojnim abiotičkim i/ili biotičkim stresnim čimbenicima koji mogu negativno djelovati na prinos i kvalitetu. U ovome je radu analiziran utjecaj abiotičkoga stresa uzrokovanog deficitom dušika i biotičkoga stresa izazvanog fitopatogenom gljivom* Fusarium culmorum *na biomarkere oksidacijskoga stresa u listu zastavičaru devet sorata ozime pšenice (*Ficko*,* U-1*,* Galloper*,* BC Mandica*,* BC Opsesija*,* Ingenio*,* Isengrain*,* Felix *i* Bezostaya-1*). Kao pokazatelji oksidacijskoga stresa mjereni su koncentracija vodikova peroksida i razina lipidne peroksidacije, dok je antioksidacijski odgovor određen mjerenjem koncentracije fenolnih spojeva i aktivnosti antioksidacijskih enzima. Sorta pšenice i tretman dušikom imali su značajan utjecaj na sve ispitivane biomarkere oksidacijskoga stresa u listu zastavičaru, dok je utjecaj tretmana gljivom Fusarium bio manje izražen. Najznačajniji utjecaj na mjerene pokazatelje stresa imao je uzgoj u uvjetima niske razine dušika, pri čemu je u većini sorata vidljiv trend povećanja koncentracije vodikova peroksida i razine lipidne peroksidacije te smanjenja aktivnosti antioksidacijskih enzima. Dobiveni su rezultati prokomentirani i uspoređeni s prijašnjim rezultatima biokemijskih analiza provedenih na klasu pšenice. Značajno jake korelacije između lista zastavičara i klasa u mjerenim parametrima nije bilo, što uz sortno-specifični odgovor upućuje i na tkivno-specifičan antioksidacijski odgovor.*

***Ključne riječi: pšenica,*** **Fusarium culmorum***, dušična gnojidba, oksidacijski stres, antioksidacijski odgovor***

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*Izvorni znanstveni rad broj 3 u obliku i izvornom jeziku na kojem je objavljen u znanstvenom časopisu*

**Naslova rada:** Defense Response to *Fusarium* Infection in Winter Wheat Varieties, Varying in FHB Susceptibility, Grown under Different Nitrogen Levels

**Autori:** Magdalena Matić, Rosemary Vuković, Karolina Vrandečić, Ivna Štolfa Čamagajevac, Jasenka Ćosić, Ana Vuković, Krešimir Dvojković i Dario Novoselović

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# Defense Response to *Fusarium* Infection in Winter Wheat Varieties, Varying in FHB Susceptibility, Grown under Different Nitrogen Levels

**Magdalena Matić [1], Rosemary Vuković [2,*], Karolina Vrandečić [1,3,*], Ivna Štolfa Čamagajevac [2], Jasenka Ćosić [1], Ana Vuković [2], Krešimir Dvojković [4] and Dario Novoselović [3,4]**

1 Faculty of Agrobiotechnical Sciences, Josip Juraj Strossmayer University of Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia; maticm@fazos.hr (M.M.); jcosic@fazos.hr (J.Ć.)
2 Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia; ivna@biologija.unios.hr (I.Š.Č.); ana.vukovic@biologija.unios.hr (A.V.)
3 Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), Svetošimunska Cesta 25, 10000 Zagreb, Croatia; darion@poljinos.hr
4 Department for Cereal Breeding and Genetics, Agricultural Institute Osijek, Južno Predgrađe 17, 31000 Osijek, Croatia; kresimir.dvojkovic@poljinos.hr
* Correspondence: rosemary@biologija.unios.hr (R.V.); kvrandecic@fazos.hr (K.V.)

**Abstract:** Fusarium head blight and inadequate nitrogen fertilization can cause numerous biochemical changes in wheat. The main aim of this study was to determine the effects of *Fusarium* inoculation and a broader range of different nitrogen fertilization on the defense response in the spikes of four wheat varieties, varying in FHB susceptibility. Total soluble phenolics content, activities of enzymes involved in phenol metabolism (PAL and PPO), and total antioxidant capacity were determined as indicators of defense response. In both growing seasons, *Fusarium* inoculation altered PHE content in wheat spikes, indicating involvement of PHE in the defense response to *Fusarium* attack. Increased PHE content in the partially resistant varieties (Apache and Graindor) indicates involvement of PHE in the defense response and better disease tolerance in the more resistant varieties. Breeding wheat varieties with enhanced PHE synthesis could be a promising strategy to control FHB. To the best of our knowledge, this is the first study that emphasizes the effects of *Fusarium* infection and a broader range of different nitrogen fertilization on PHE and enzymes involved in PHE metabolism. In addition, this is the first study using the FRAP method to determine the antioxidant capacity of wheat tissues under the influence of *Fusarium* infection and different nitrogen fertilization.

**Keywords:** winter wheat; FHB; nitrogen fertilization; phenols; defense response



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## 1. Introduction

Fusarium head blight (FHB) is one the most extensively studied fungal diseases of wheat and other small grain cereals because of its impact on yield and quality, but more importantly because of its potential to produce mycotoxins, that are harmful to humans and animals [1]. Yield losses in wheat production occur due to the sterility of infected florets or because the grains obtained from the infected spikes become small, shriveled, and light in test weight. Among the numerous *Fusarium* mycotoxins, deoxynivalenol (DON) and zearalenone (ZEA) are the most abundant and most studied mycotoxins [2]. The intensity and severity of FHB infection and mycotoxin contamination are strongly influenced by meteorological conditions during the growing season [3,4]. Therefore, the severity of the infection and mycotoxin concentrations vary considerably from year to year. Wheat is most susceptible to FHB infections at the flowering stage, and the overlap of the flowering stage with humid weather conditions favors the development of the disease and mycotoxin accumulation [5–7]. *Fusarium graminearum* and *Fusarium culmorum* are among the most common species causing FHB in Europe, but their prevalence may vary throughout the

growing season [8]. *F. graminearum* has been associated with warmer and humid conditions, while *F. culmorum* occurs in niches with cooler and humid conditions [9].

During the life cycle, plants produce many different secondary metabolites that play essential roles in plant growth, signal transduction, and response to stress conditions. The most abundant and important secondary metabolites are phenolic compounds. Phenolics are aromatic compounds with one or more hydroxyl groups attached to the aromatic benzene ring. Phenolics are classified, mainly according to the differences in their chemical structure, into flavonoids, phenolic acids, tannins, stilbenes, and lignans [10]. In plants, phenolics can be divided into two groups: the preformed phenolics, which are synthesized during normal development, and the induced phenolics, which are synthesized in response to various abiotic and biotic stress conditions [11]. Concerning the biotic stress, pathogen attack causes a multitude of biochemical changes in plants associated with stress signaling and activation of defense mechanisms, including various non-enzymatic components that include phenolic compounds, flavonoids, lignins, tannins, phytoalexins, and enzymes for phenol metabolism such as phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) and polyphenol oxidase (PPO; EC 1.14.18.1) [12]. Due to their chemical structure, phenolic compounds have strong antioxidant and antiradical activity expressed by various mechanisms [12–14]. Different assays can measure the antioxidant and antiradical activity of phenolic compounds, although ferric reducing antioxidant power assay (FRAP) has proven to be a useful method for screening the total antioxidant capacity (TAC) of different phenolic compounds [15].

The phenylpropanoid pathway provides precursors for a wide range of phenolic compounds and is an important regulation point between primary and secondary plant metabolism [16]. PAL is the primary enzyme in the phenylpropanoid pathway that catalyzes the deamination of L-phenylalanine to trans-cinnamic acid, thus providing precursors for the synthesis of several defense-related secondary compounds such as phenols, lignin, and salicylic acid [17]. The accumulation of phenolics in plant tissues is considered to be the first response to various internal and external factors such as trauma, wounding, drought, and pathogen attack [18].

The accumulated phenolics can be oxidized to antimicrobial quinones by the action of PPO, a copper-containing enzyme that catalyzes the oxidation of phenolics, using molecular oxygen as an electron acceptor. Plant PPOs are involved in numerous biological functions, including enzymatic browning of crops and their end products [19], a defense mechanism against plant pathogens, and detoxification of reactive oxygen species (ROS) [20–22]. Increased synthesis and activity of PPO can be used as a biochemical marker of the degree of resistance and/or susceptibility of plants to various negative abiotic and biotic stress conditions [19,20,23].

Nitrogen plays an important role in wheat production as a key nutrient for growth, development, high crop yields, and quality [24]. The role of nitrogen in plant growth and development is irreplaceable as it is a major component of plant cells, proteins, nucleic ac-ids, enzymes, and photosynthetic pigments. Nitrogen can alter a plant's biochemical de-fence response and increase susceptibility to various pathogens, although its role in host–pathogen interactions is still extremely complex [25]. There are conflicting results in the literature regarding the effect of N fertilization on FHB severity and incidence. Some studies found that an increase in nitrogen supply may lead to an increase in FHB occurrence and mycotoxin concentrations [26,27], while other studies reported that FHB was more severe under partial nitrogen deficiency condition [28]. The different response of the plant to different nitrogen fertilizations may be due to the use of different forms of nitrogen, and the effect of specific forms of nitrogen on disease severity depends on many factors and is not the same for all plant–pathogen interactions [29].

It is known that various abiotic and biotic stress conditions cause numerous biochemical changes in wheat. During pathogen attacks, plants employ various defense strategies to combat stress conditions. In our previous studies, we investigated the effects of *Fusarium* inoculation under two levels of nitrogen fertilization on antioxidant defense response, mea-

suring the activities of antioxidant enzymes (catalase, ascorbate peroxidase, glutathione reductase), PHE, and chloroplast pigments content [30,31]. In the present study, due to their importance in the defense response to various types of stress, PHE and enzymes related to phenolics metabolism (PAL and PPO), as well as total antioxidant capacity of wheat tissues, were investigated. In addition, the present study included a broader range of different nitrogen fertilization, and the experiment was conducted during two growing seasons to gain insight into the influence of climatic conditions. The aims of this study were: (i) to determine the effect of nitrogen fertilization and climate conditions on FHB severity, (ii) to determine and focus on the effects of *Fusarium* inoculation and a broader range of different nitrogen fertilization on PHE content and enzymes involved in phenol metabolism (PAL and PPO), (iii) to determine the variety-specific defense response, and (iv) to find a parameter that could serve as a good biomarker for breeding more resistant wheat varieties to the studied stress conditions. To the best of our knowledge, this is the first study that emphasizes the effects of *Fusarium* infection and a broader range of different nitrogen fertilization on PHE and enzymes involved in PHE metabolism. In addition, this is the first study using the FRAP method to determine the antioxidant capacity of wheat tissue under the influence of *Fusarium* infection and different nitrogen fertilization.

## 2. Materials and Methods

### *2.1. Field Trial*

A field trial with four winter wheat varieties was set up during two consecutive growing seasons, 2018/2019 and 2019/2020, in the experimental field of the Agricultural Institute Osijek (45°33′ N and 18°40′ E). Four winter wheat varieties were selected based on their varying levels of susceptibility to FHB: Srpanjka (very early maturity, short statured variety and moderately susceptible to *Fusarium* sp., the most widespread variety in Croatia until the year 2014), Sofru (early type maturity, high-yielding and susceptible to *Fusarium* sp., the second most widespread variety in Croatia today), Apache (medium type maturity, very adaptable and stable variety with excellent resistance to *Fusarium* sp.), and Graindor (medium to late type of maturity, high yielding and excellent resistance to *Fusarium* sp.). The experiment was set up in a split-split-plot factorial design in two replicates with nitrogen fertilization levels as main plots and four wheat varieties as sub-plots, and *Fusarium* infection was applied at a sub-sub-plots level. Basic fertilization of 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$, and 120 kg $K_2O$ $ha^{-1}$ was the same for all plots and was applied by adding 100 kg $ha^{-1}$ of urea (46% N; 100% of amide-N form) and, before the planting, adding 400 kg $ha^{-1}$ NPK (7:20:30; 8.5% of ammonium-N and 6.5% of nitrate-N forms). Nitrogen treatments consisted of a different level of nitrogen supplementation applied manually as a top-dressing with KAN fertilizers (27% N) during tillering (Zadok's scale 23–25) and stem extension (Zadok's scale 33–35) growth stages at rates of 0, 35, 70, and 140 kg N $ha^{-1}$ per treatment (Table 1). KAN fertilizers contain two forms of nitrogen (13.5% of ammonium-N and 13.5% of nitrate-N). Within each subplot, 50 wheat spikes were randomly selected for inoculation and 50 spikes were left to natural infection. Inoculated and non-inoculated wheat spikes were spatially separated within the subplot. Thus, a total of 32 subplots and 64 sampling sites was established. The soil type was Eutric Cambisol and the size of the experimental plot was 7.56 $m^2$. The previous crop for both growing seasons was soybean, and the cereals were grown in the experimental field every second year. All other cultural practices, including the use of herbicides, insecticides, and fungicides to control major weeds, insects, and foliar diseases, were typical for commercial wheat production in Croatia.

**Table 1.** Soil nitrogen (N) content (kg $ha^{-1}$) in Osijek in 2018/2019 and 2019/2020 year.

| Location | Soil Type | Season | Residual Soil N (kg N $ha^{-1}$) | Basic N Fertilization (kg N $ha^{-1}$) | N Top-Dressing (kg N $ha^{-1}$) | Total N (kg N $ha^{-1}$) |
|---|---|---|---|---|---|---|
| Osijek | Eutric Cambisol | 2018/2019 and 2019/2020 | 20 | 74 | 0 | 94 |
| | | | | | 35 | 129 |
| | | | | | 70 | 164 |
| | | | | | 140 | 234 |

### *2.2. Inoculum Production, Inoculation Procedure, and FHB Evaluation*

A conidial suspension of two species of the genus *Fusarium*, *F. graminearum* (IFA 65) and *F. culmorum*, was used for wheat infection. The slightly modified method of Snijders and Van Eeuwijk was used to prepare the inoculum [32]. A mixture of wheat and oats (2:1, *v*/*v*) was soaked in water overnight, and the next day the excess water was decanted and the mixture was autoclaved. After autoclaving at 120 °C for 20 min, the mixture was inoculated with *F. graminearum* conidia (IFA 65). The inoculated grains were incubated at 25 °C for two weeks and then in the refrigerator (4 °C) for three weeks to promote conidia formation. After incubation, conidia were washed with sterile $H_2O$ and counted under a microscope using a Bürker–Türk chamber. The same procedure was repeated to obtain a conidial suspension of *F. culmorum*. The conidia concentration in the suspension used for inoculation was adjusted to $1 \times 10^6$ $mL^{-1}$.

For each variety, 50 wheat spikes were randomly selected for inoculation and 50 spikes were left to natural infection. Hand sprayer inoculation with *Fusarium* suspension was performed individually on each variety when 50% of the plants per plot had reached anthesis (Zadok's scale 65). Inoculations were performed in the morning and were repeated after 48 h. To maintain optimal humidity for infection, spikes were covered with plastic bags for 48 h. FHB severity (percentage of infected spikelets per spike) was assessed using a linear scale (0–100%) on days 10, 14, 18, 22, 26, and 30 after the inoculation. The area under the disease progress curve (AUDPC) for FHB severity, expressed as general resistance, was calculated.

### *2.3. Sample Preparation and Measurements*

Wheat spikes for measuring the soluble phenolic content, total antioxidant capacity (TAC), and the activities of the enzymes involved in phenol metabolism (PAL and PPO) were sampled 7 days after inoculation. Eight biological replicates were taken, and each replicate consisted of four wheat spikes. The collected samples were immediately frozen in liquid nitrogen and stored at −80 °C. The wheat spikes were ground using a TissueLyser (Qiagen Retsch GmbH, Hannover, Germany) for 1 min at 30 Hz. The obtained fine powder was weighed into microtubes for further analysis.

### *2.4. Determination of the Soluble Phenolic Content*

Powdered wheat tissue was homogenized on ice with 1 mL of 80% ethanol (1:10, *w*/*v*), and phenolic compounds were extracted in a water bath at 80 °C for 30 min. After extraction, the samples were centrifuged at 21,000× *g* at 4 °C for 10 min, and the supernatant was used for further measurements. The soluble phenolic content was determined by the Folin–Ciocalteu method [33]. The reaction mixture contained 20 µL of the sample, 1.58 mL of $H_2O$, 100 µL of Folin–Ciocalteu reagent, and 300 µL of the saturated $Na_2CO_3$ solution. The reaction mixture was incubated in a water bath at 37 °C for 60 min, after which the absorbance was measured at 765 nm. Soluble phenolic content was calculated from a standard curve using gallic acid as a standard and expressed as microgram gallic acid equivalents (GAE) per gram of fresh weight (µg GAE $g^{-1}$ FW).

### 2.5. Determination of Total Antioxidant Capacity (TAC)

The TAC of wheat spikes tissue was determined using the ferric reducing antioxidant power (FRAP) assay according to Benzie and Straint, modified for microplate assay [34]. A FRAP assay was performed in 96-well plates with the FRAP reagent comprising 300 mM of sodium acetate buffer solution, pH 3.6, 10 mM TPZT in 40 mM HCl, and 20 mM $FeCl_3$ hexahydrate. Each sample was tested in triplicate. Absorbance was recorded using a Tecan Spark microplate reader at 593 nm after 3.5 min incubation at room temperature. Antioxidant capacity was calculated from a standard curve using 10 mM Trolox as a standard. Results were expressed as the FRAP value in milligram equivalents of Trolox per milligram of fresh weight (mg Trolox $mg^{-1}$ FW).

### 2.6. Determination of PPO and PAL Activities

Protein extracts for enzyme activity determination were prepared by homogenizing wheat spike tissue powder with different extraction buffers (1:5, *w*/*v*). For PPO extraction, 100 mM potassium phosphate buffer, pH 7.0 (1:5, *w*/*v*), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2% (*w*/*v*) polyvinylpyrrolidone was used, while 150 mM Tris–HCl buffer, pH 8.5, containing 0.2% (*w*/*v*) polyvinylpyrrolidone was used for PAL extraction. The homogenized samples were then centrifuged at 21,000× *g* at 4 °C for 15 min, and the supernatants were used for the spectrophotometric determination of the enzymes PPO and PAL.

Protein content in the tissue extracts was determined by the method of Bradford [35]. The assay was performed in 96-well plates, where 5 µL of diluted protein extracts was added to 250 µL of Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 5 min at 25 °C with shaking at 550 rpm on a PST-100 HL Thermo-Shaker (Biosan, Riga, Latvia), absorbance was recorded using a Spark multimode microplate reader with SparkControl software (Tecan, Männedorf, Switzerland). The protein concentration was calculated from a standard curve using bovine serum albumin as a protein standard. The obtained protein concentrations were used for calculations of the specific enzyme activities.

The microplate assay for PPO activity was based on the coupling reaction between the benzoquinone derivative, generated during the PPO-catalyzed oxidation of L-3,4-dihydroxyphenylalanine, and L-ascorbic acid to obtain dehydro-ascorbic acid [36]. The reaction mixture consisted of 0.17 mM L-3,4-dihydroxyphenylalanine, 0.07 mM L-ascorbic acid, 0.002 mM EDTA, and protein extract in 50 mM potassium phosphate buffer, pH 6.5, in a final volume of 0.3 mL. Spectrophotometric rate determination was performed on a Spark multimode microplate reader with SparkControl software (Tecan, Männedorf, Switzerland). The decrease in absorbance was monitored at 265 nm every 15 s for 3 min at room temperature. Each sample was measured in triplicate. The PPO activity was calculated using a molar extinction coefficient ($\varepsilon$ = 2.5 mM $cm^{-1}$) and expressed in U $mg^{-1}$ protein.

PAL activity was determined by the modified method based on Havir and Hanson [37]. The PAL assay mixture comprised 2 mM L-phenylalanine as a substrate and protein extract in 150 mM Tris–HCl buffer, pH 8.5, in a final volume of 1.5 mL. The protein sample was added after a 10 min pre-incubation period at 40 °C. The increase in absorbance due to the production of trans-cinnamic acid was monitored at 270 nm over 5 min every 30 s at 40 °C, using a UV-VIS spectrophotometer Perkin Elmer Lambda 25 equipped with PTP Peltier system and UV WinLab software package (PerkinElmer, Waltham, MA, USA). The PAL activity was calculated using a molar extinction coefficient ($\varepsilon$ = 19.73 mM $cm^{-1}$) and expressed in U $mg^{-1}$ protein.

### 2.7. Determination of DON and ZEA

Non-inoculated and inoculated wheat spikes from the 2019/2020 growing season were manually harvested. Obtained grains per replication were pooled, ground, and used for the determination of DON and ZEA. Mycotoxin analyses were performed in the ac-credited laboratory (Eurofins Croatiakontrola, Zagreb, Croatia). DON and ZEA were extracted

from the grain samples with deionized water, and the obtained extracts were purified using immunoaffinity columns DONStar® IAC (Romer Labs Diagnostic GmbH, Tulln, Austria). The mycotoxin concentrations were determined by high-performance liquid chromatography coupled with a detection diode array (HPLC-DAD). The chromatographic separation has been performed on an Agilent Zorbax C18 column (150 mm × 4.6 mm i.d., 5 µm).

### *2.8. Statistical Analysis*

All data analyses were performed using the SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA) software. Biochemical assays were carried out in eight replicates and their results were expressed as mean ± standard deviation (SD). Factorial analysis of variance (ANOVA) was performed, and statistically significant differences among the treatments in each variety were separately determined using the Fisher's LSD test ($p \leq 0.05$). For correlation analyses, Pearson's coefficient was used.

## 3. Results and Discussion

FHB severity, expressed as general resistance, varied under the effect of variety, nitrogen treatment, and year (Figure 1). Variety-specific differences in FHB severity in both growing seasons were clearly evident (Figure 1). The average AUDPC for disease severity per variety (regardless of N level) in 2018/2019 ranged from 257 (Sofru) to 136 (Srpanjka), 31 (Graindor), and 30 (Apache). An almost similar trend was present in 2019/2020, where the average AUDPC for disease severity per variety (regardless of N level) ranged from 155 (Sofru) to 64 (Srpanjka), 6 (Apache), and 5 (Graindor). Non-inoculated wheat varieties did not show any FHB symptoms. Therefore, scoring for general FHB resistance was not made for the non-inoculated wheat varieties. In the present study, the Sofru variety showed higher AUDPC values for general FHB resistance in both growing seasons, indicating higher FHB susceptibility. The higher AUDPC values for general FHB resistance in the Sofru variety could be related to the presence of awns, since the Sofru variety was the only variety with awns used in this study. However, this cannot be proven with certainty, as Mesterhazy reported that wheat varieties with awns were more susceptible to FHB when tested under the natural epidemic conditions in the field, although this trait had no effect on FHB severity in conditions of artificial inoculations [38]. Compared to the Sofru variety, lower average AUDPC values for general FHB resistance were observed in the Srpanjka variety in both growing seasons. Therefore, the Srpanjka variety was classified as moderately susceptible to FHB. In the present study, Apache and Graindor varieties were classified as partially resistant to FHB due to lower average AUDPC values for general FHB resistance in both growing seasons. Our results are in accordance with previous studies that evaluated FHB resistance in the same varieties and in which varieties' resistance to FHB was well characterized [8,39].

Meteorological data for the Osijek area were obtained from the Croatian Meteorological and Hydrological Service (DHMZ). The annual precipitation during the 2018/2019 and 2019/2020 growing seasons was 531.3 and 408.6 mm, and annual average temperatures were 10.9 and 11.1 °C, respectively (Figure S1 in Supplementary Materials). In May, during the pre-anthesis and anthesis stage, total precipitation was 150.8 mm in 2019 and 53.3 mm in 2020, while the mean daily temperature was 14.0 °C in 2019 and 15.3 °C in 2020. In June, during grain development, total precipitation was 112.8 mm in 2019 and 73.5 mm in 2020, while the mean daily temperature was 23.1 °C in 2019 and 20.2 °C in 2020.

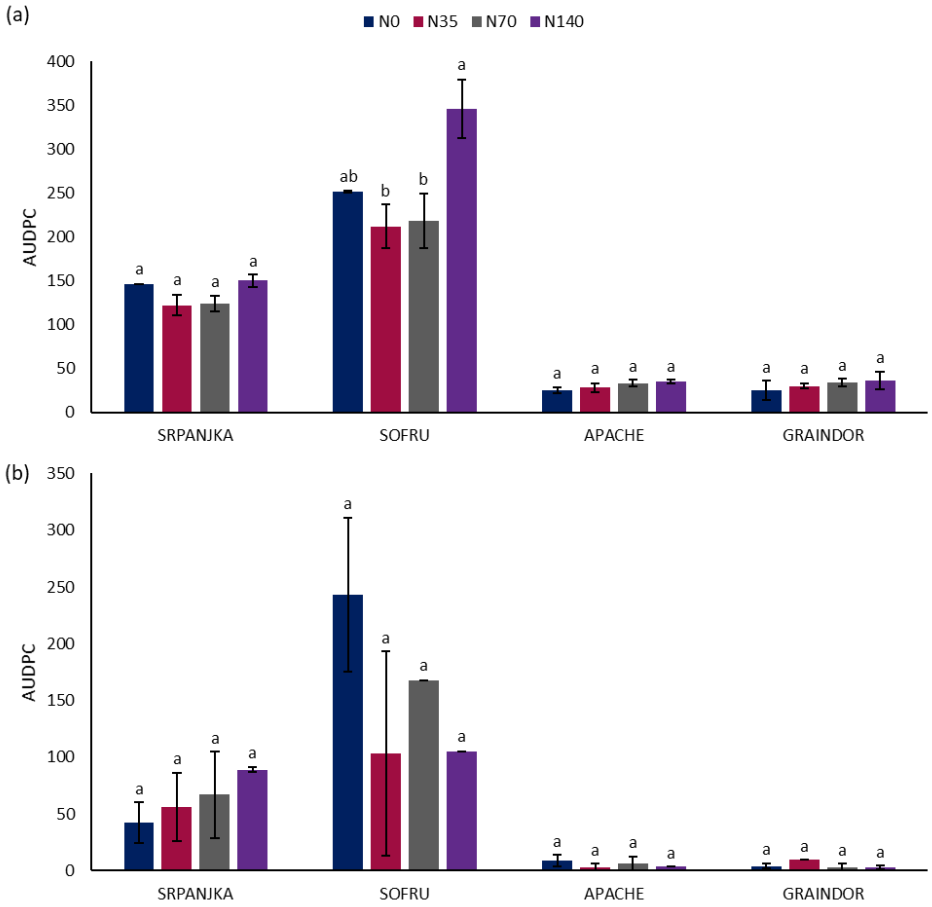


**Figure 1.** The area under the disease progress curve (AUDPC) for general FHB resistance in 2018/2019 (**a**) and 2019/2020 (**b**). Values are means of two replicates ± standard error (SE). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among different nitrogen levels in each variety separately.

In general, wheat is most susceptible to FHB at the anthesis stage, while warm and moist conditions during this period promote disease development [3]. Differences in the FHB severity can be observed between two growing seasons in which different climatic conditions prevailed (Figure 1). Overall, for all varieties and all nitrogen levels, higher AUDPC values were found in the 2018/2019 growing season compared to 2019/2020. Higher levels of FHB disease in the 2018/2019 growing season may be due to the much heavier rainfall and associated higher humidity in May 2019. In May, during the pre-anthesis and anthesis stage, total precipitation was almost three times higher in 2019 than in 2020 (Figure S1 in Supplementary Materials). Krnjaja et al. [40] reported similar results and pointed out that climatic conditions, especially intense precipitation during anthesis, cause a higher FHB index and a higher occurrence of FHB-causing species. In 2018/2019, higher AUDPC values were recorded in the inoculated wheat varieties grown under higher nitrogen supplementation level (N140). Higher nitrogen fertilization can increase FHB intensity by changing crop characteristics, especially by increasing crop density and altering the microclimate of the canopy [26]. A denser canopy has a more humid microclimate that favors disease development. However, in both growing seasons, application of different nitrogen fertilization levels did not significantly affect FHB severity. In our previous study, in some varieties (Ficko, Galloper, and Felix), high nitrogen resulted in an increase in visual FHB symptoms, whereas in variety U-1, high nitrogen resulted in a decrease in visual FHB symptoms [30], suggesting a variety-specific response to nitrogen fertilization. The literature contains numerous and sometimes conflicting data on the effects of nitrogen on infection and disease development in various plant–pathogen interactions [25,29]. However,

the contrasting results may not be surprising, because the effect of nitrogen in different plant–pathogen interactions is extremely complex and depends on the type of host plant and pathogen, as well as on the different amount and form of nitrogen. Different forms of nitrogen (ammonium vs. nitrate) appear to have different effects on plant disease resistance, at least in part, by utilizing different assimilation and metabolic pathways [25]. In the present study, both ammonium and nitrate forms of nitrogen were used, which are part of the standard agronomic practice in applying mineral fertilizers in wheat production in Croatia, so it was not possible to determine how each form of nitrogen affects the FHB's severity. Although numerous and conflicting studies are available for wheat, several studies reported that different levels of nitrogen fertilization have no significant effect on FHB and mycotoxin concentrations and that adaptation of nitrogen fertilization represents no relevant tool in managing FHB in practical wheat cultivation [26,40].

The determination of DON and ZEA concentrations was measured only in the growing seasons 2019/2020. It was additionally done to test the impact of the three main factors (wheat variety, *Fusarium*, and nitrogen treatment) on the mycotoxin's concentrations. The concentrations of mycotoxins in the grains of the non-inoculated wheat varieties were be-low the detection limit of 100 μg $kg^{-1}$ for DON and 8 μg $kg^{-1}$ for ZEA. Therefore, only the concentrations of DON and ZEA in the grains of the inoculated wheat varieties were shown (Table 2). Regardless of the treatment, high concentrations of DON were found in all inoculated wheat samples. More precisely, the levels of DON exceeded the European limit for unprocessed wheat of 1250 μg $kg^{-1}$ as set by the European Commission [41]. On average, the Sofru variety had the highest DON concentration (1680.40 ± 157.10), followed by Srpanjka (1668.03 ± 130.71), Apache (1667.03 ± 97.28), and Graindor (1532.05 ± 232.35) varieties. These values of DON concentration are in accordance with the disease severity. On average, the highest DON concentration was recorded in the inoculated wheat varieties that had grown at N0 (1724.48 ± 37.27), which was in accordance with the highest disease severity detected at N0 in season 2019/2020.

**Table 2.** The concentrations of mycotoxins deoxynivalenol (DON) and zearalenone (ZEA) in the grains of inoculated wheat varieties in the growing seasons 2019/2020.

| *Fusarium* Inoculated Wheat Varieties | Nitrogen Treatment | DON Concentration (μg $kg^{-1}$) | ZEA Concentration (μg $kg^{-1}$) |
|---|---|---|---|
| Srpanjka | N0 | 1691.30 | <8 |
| | N35 | 1504.80 | <8 |
| | N70 | 1822.50 | 16.30 |
| | N140 | 1653.50 | 19.90 |
| Sofru | N0 | 1769.90 | <8 |
| | N35 | 1623.10 | 49.80 |
| | N70 | 1487.90 | 17.30 |
| | N140 | 1940.70 | <8 |
| Apache | N0 | 1739.90 | <8 |
| | N35 | 1761.60 | <8 |
| | N70 | 1590.90 | <8 |
| | N140 | 1575.70 | <8 |
| Graindor | N0 | 1696.80 | <8 |
| | N35 | 1702.00 | <8 |
| | N70 | 1206.70 | <8 |
| | N140 | 1522.70 | <8 |

As for ZEA, mycotoxin concentrations in all samples of the inoculated wheat varieties did not exceed the tolerance limit (100 μg $kg^{-1}$) set by the European Commission [41]. In fact, the concentrations of ZEA were below the detection limit in almost all samples of the inoculated wheat varieties. On average, the highest concentrations of ZEA were detected

in Srpanjka and Sofru varieties, which was in accordance with the highest disease severity in these varieties.

Analysis of variance for variety, nitrogen, and *Fusarium* treatment effects on the measured parameters in the 2018/2019 and 2019/2019 growing seasons is shown in Tables 3 and 4. In the 2018/2019 growing season (Table 3), wheat variety significantly affected all tested biochemical parameters ($p \leq 0.001$), while *Fusarium* treatment affected the PHE content ($p \leq 0.001$) and TAC ($p \leq 0.01$). Nitrogen treatment only significantly affected PPO activity ($p \leq 0.05$). Variety × nitrogen treatment interaction was significant for PHE content, PAL, and PPO activity, while variety × *Fusarium* treatment interaction was significant for all tested parameters. Nitrogen × *Fusarium* treatment interaction was significant only for PHE content. Three-factor interaction between the variety, nitrogen, and *Fusarium* treatment was significant for PHE content and PPO activity.

**Table 3.** Analysis of variance (three-way ANOVA) for measured biochemical parameters under different nitrogen and *Fusarium* treatments in spikes of four different winter wheat varieties in the 2018/2019 growing season.

| | | MS | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **df** | **PHE** | **TAC** | **PAL** | **PPO** |
| VARIETY (V) | 3 | 5.31 *** | 36.95 *** | 75.43 *** | 354,684.34 *** |
| N LEVEL (N) | 3 | 0.01 ns | 0.04 ns | 0.64 ns | 2586.77 * |
| FUSARIUM (F) | 1 | 14.08 *** | 1.66 ** | 0.27 ns | 688.89 ns |
| V × N | 9 | 0.15 ** | 0.21 ns | 2.67 *** | 2957.68 *** |
| V × F | 3 | 2.01 *** | 2.62 *** | 3.05 ** | 15,940.55 *** |
| N × F | 3 | 0.30 *** | 0.19 ns | 0.02 ns | 1789.18 ns |
| V × N × F | 9 | 0.31 *** | 0.11 ns | 1.56 ns | 1941.68 * |

ns—not significant, *, **, and ***—significant at the level of probability $p \leq 0.05$, 0.01, and 0.001, respectively. Df, degrees of freedom; MS, mean sum of squares; PHE, phenolics; TAC, total antioxidant capacity; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase.

**Table 4.** Analysis of variance (three-way ANOVA) for measured biochemical parameters under different nitrogen and *Fusarium* treatments in spikes of four different winter wheat varieties in the 2019/2020 growing season.

| | | MS | | | |
|---|---|---|---|---|---|
| **Source of Variation** | **df** | **PHE** | **TAC** | **PAL** | **PPO** |
| VARIETY (V) | 3 | 4.46 *** | 15.53 *** | 26.35 *** | 238,480.99 *** |
| N LEVEL (N) | 3 | 0.17 ** | 0.12 ns | 7.88 *** | 13,153.02 *** |
| FUSARIUM (F) | 1 | 0.11 ns | 6.70 *** | 13.50 ** | 34,039.71 *** |
| V × N | 9 | 0.09 ** | 0.23 * | 1.89 ns | 8876.24 *** |
| V × F | 3 | 1.05 *** | 0.66 *** | 237.92 *** | 56,574.48 *** |
| N × F | 3 | 0.40 *** | 1.99 *** | 4.86 * | 4996.04 ns |
| V × N × F | 9 | 0.15 *** | 0.27 * | 14.94 *** | 11,892.92 *** |

ns—not significant, *, **, and ***—significant at the level of probability $p \leq 0.05$, 0.01, and 0.001, respectively. Df, degrees of freedom; MS, mean sum of squares; PHE, phenolics; TAC, total antioxidant capacity; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase.

In the 2019/2020 growing season (Table 4), wheat variety significantly affected all tested biochemical parameters ($p \leq 0.001$). Nitrogen treatment had a significant effect on PHE content ($p \leq 0.01$), PAL, and PPO activity ($p \leq 0.001$), while *Fusarium* treatment affected TAC, PPO activity ($p \leq 0.001$), and PAL activity ($p \leq 0.01$). Variety × nitrogen treatment interaction was significant for PHE content, TAC, and PPO activity, while variety × *Fusarium* treatment interaction was significant for all tested parameters ($p \leq 0.001$). Nitro-gen × *Fusarium* treatment interaction was significant for PHE content, TAC, and PAL. Three-factor interaction between the variety, nitrogen, and *Fusarium* treatment was significant for all tested parameters.

In 2018/2019, the PHE content was most significantly affected by *Fusarium* treatment and wheat variety (Table 3). A trend of increased PHE content in the inoculated plants, compared to non-inoculated at almost all N levels, was found in the Sofru, Apache, and Graindor varieties (Figure 2a). A significant increase in PHE content due to *Fusarium* infection in the Sofru variety ranged from 46% at N140 to 71% at N35, in the Apache variety from 44% at N140 to 67% at N0, and in the Graindor variety from 26% at N70 to 39% at N140. In the Srpanjka variety, *Fusarium* infection caused a significant increase in PHE content only at N140.

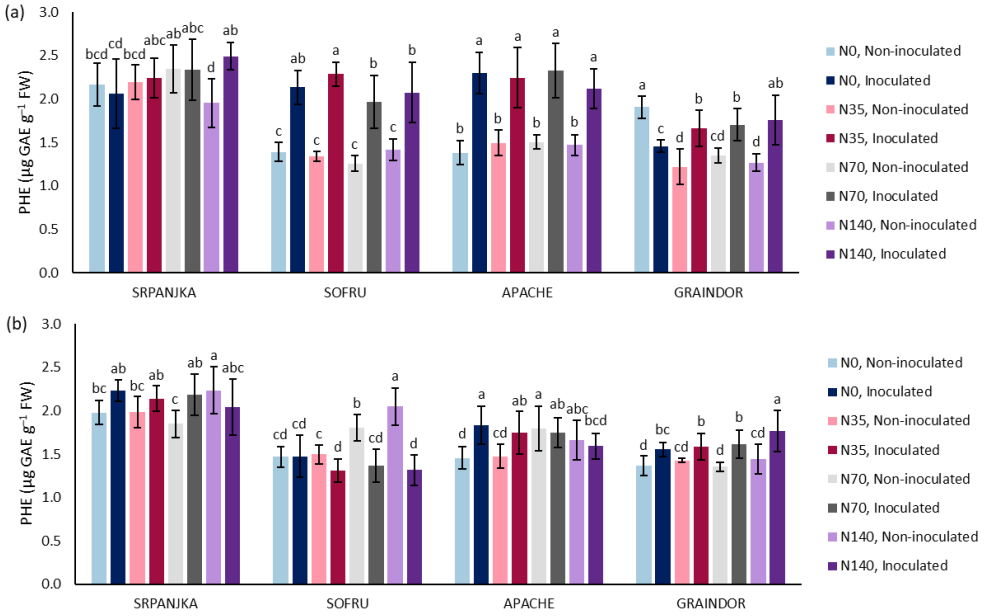


**Figure 2.** Soluble phenolic (PHE) content in spikes of four wheat varieties under different *Fusarium* (non-inoculated and inoculated) and nitrogen treatments (0, 35, 70, and 140 kg N $ha^{-1}$) in 2018/2019 (**a**) and 2019/2020 (**b**). Values are means of eight replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among treatments in each variety separately.

In 2019/2020, the PHE content was significantly affected by wheat variety and nitrogen treatment, although some significant differences have also been found for the effect of *Fusarium* treatment (Table 4). In the Srpanjka variety, *Fusarium* infection caused a significant increase in PHE content only at N70 (Figure 2b). In the Apache variety, *Fusarium* infection caused a significant increase in PHE content by 25% and 19% at N0 and N35, respectively. A trend of increased PHE content in the inoculated plants, compared to non-inoculated at all N levels, was found in the Graindor variety, where the increase in PHE content ranged from 10% at N35 to 23% at N140. Unlike in the previously mentioned varieties, in the Sofru variety, *Fusarium* infection caused a significant decrease in PHE content by 13%, 24%, and 36% at N35, N70, and N140, respectively. In the present study, the effect of nitrogen on PHE content was much more pronounced in 2019/2020. When examining the differences between N0 and N140 and between non-inoculated and inoculated plants, N0 caused a decrease in PHE content in the Srpanjka, Sofru, and Apache varieties in 2019/2020. Similarly, in our previous study, low nitrogen level caused a decrease in PHE content in two varieties (BC Mandica and Isengrain) [30]. All this indicates the great influence of climatic conditions and the variety itself on PHE content.

In both growing seasons, *Fusarium* inoculation altered the PHE content in wheat spikes, indicating the involvement of PHE in the defense response to *Fusarium* attack. Many studies confirm the involvement of PHE in the protective mechanisms against FHB, where increased phenolic synthesis in plant tissue indicates better adaptability and tolerance [11,42,43]. As reported by Chowdhary et al. [44], the plant defense mechanism against pathogens occurs

in two stages: in the first response, there is a rapid accumulation of phenols at the infection site, which slows down the growth of the pathogen, and in the second response, plants biosynthesize specific stress-related substances (simple phenols, phenolic phytoalexins, hydroxycinnamic acids, etc.) that restrict the pathogen at the infected site.

In the present study, during both growing seasons, *Fusarium* infection led to an increase in PHE content in the partially resistant varieties (Apache and Graindor), indicating the involvement of PHE in the defense response and better disease tolerance in the more resistant varieties. These results are in accordance with the results reported by Gunnaiah and Kushalappa [42]. Gunnaiah and Kushalappa [42] found that in the resistant wheat cultivar Sumai-3, the resistance was due to the accumulation of metabolites belonging to the phenylpropanoid pathway that reduced pathogen advance by increasing host cell wall thickening and also reduced pathogen growth by antifungal and/or antioxidant properties, which in turn reduced subsequent mycotoxin biosynthesis. On the other hand, the PHE content in the Sofru variety differs between the growing seasons. In the 2018/2019 growing season, the PHE content was increased in the inoculated wheat spikes, while in 2019/2020, the PHE content was decreased at almost all N levels in the inoculated compared to the non-inoculated plants. In both growing seasons, the Sofru variety showed the most pronounced FHB symptoms compared to the other inoculated varieties. Therefore, PHE failed to prevent the spread of the pathogen and the development of the disease in the Sofru variety.

Comparing the two growing seasons, more pronounced changes in PAL activity were observed in 2019/2020. Due to favorable climatic conditions (higher total rainfall) during 2018/2019, a high level of FHB pressure disabled the differentiation of varieties depending on differences in PAL activity. In 2018/2019, the PAL activity was significantly affected only by wheat variety, although some significant differences have also been found for the effect of *Fusarium* treatment (Table 3). Those differences were found in the Graindor variety, where *Fusarium* infection caused a significant increase in the PAL activity by 34% and 29% at N70 and N140, respectively (Figure 3a).

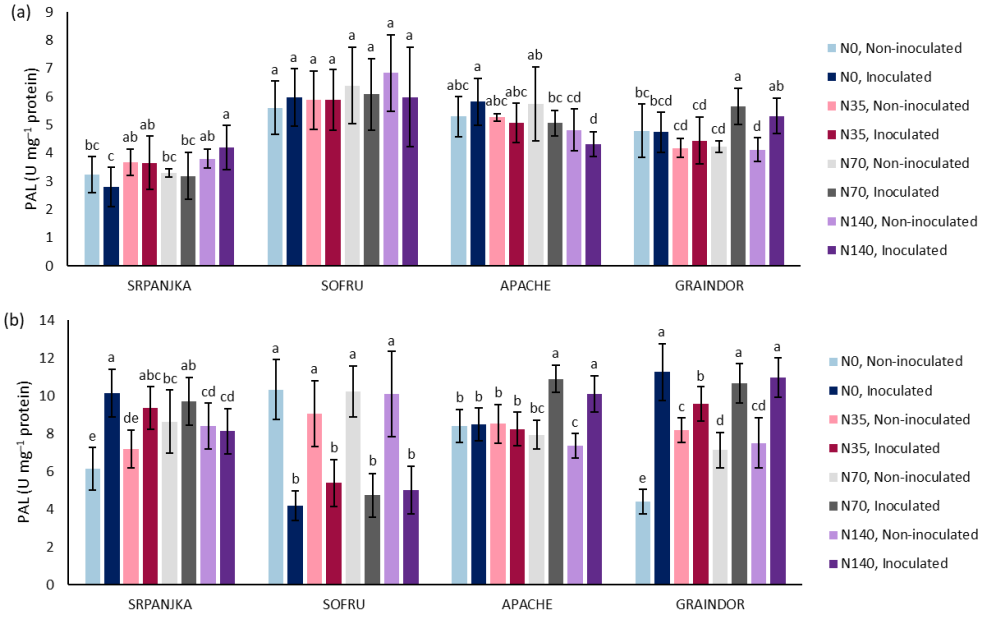


**Figure 3.** Phenylalanine ammonia-lyase (PAL) activity in spikes of four wheat varieties under different *Fusarium* (non-inoculated and inoculated) and nitrogen treatments (0, 35, 70, and 140 kg N ha$^{-1}$) in 2018/2019 (**a**) and 2019/2020 (**b**). Values are means of eight replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among treatments in each variety separately.

In 2019/2020, the PAL activity was significantly affected by all three main factors: wheat variety, *Fusarium* treatment, and nitrogen treatment (Table 4). In the Srpanjka variety, *Fusarium* infection caused a significant increase in PAL activity by 65% and 31% at N0 and N35, respectively (Figure 3b). In the Apache variety, *Fusarium* infection caused a significant increase in PAL activity by 37% at N70 and N140. Increased PAL activities in the inoculated plants compared to non-inoculated plants at all N levels were found in the Graindor variety (Figure 3b), where the increase in PAL activity ranged between 17% at N35 and 156% at N0. In the Graindor variety, classified as a partially resistant variety, increased PAL activity and less symptoms of the FHB disease may indicate the importance of PAL in the defense response. On the contrary, Sofru variety showed a significant decrease in PAL activity in the inoculated plants at all N levels, ranging from 41% (N35) to 60% (N0). In 2019/2020, the Sofru variety exhibited the most pronounced symptoms of FHB infection, and a severe infection could cause inhibition of PAL activity. Although a different pathogen was used, Riaz et al. reported that PAL was present in both the resistant and susceptible wheat varieties, but the PAL activity was more pronounced in the varieties that were more resistant to *Puccinia triticina* infection [45].

PAL is the main enzyme in the metabolism of phenylpropanoids and is involved in the synthesis of several secondary metabolites, including phenols (coumarins, flavonoids, lignins), phenolic derivatives, and lignin [12]. Consequently, inhibition of PAL activity could lead to decreased synthesis of molecules involved in the defense response and enhanced susceptibility to pathogens [16]. Considering the stated theoretical knowledge about the mechanism of PAL action, PAL and PHE content should be positively correlated. However, a weak negative correlation between PHE and PAL was found in 2018/2019 (Table 5), while in 2019/2020, a weak positive correlation was found (Table 6).

**Table 5.** Pearson correlation coefficients (r) and corresponding significance levels between the measured biochemical parameters in 2018/2019.

| | PHE | TAC | PAL | PPO |
|---|---|---|---|---|
| PHE | 1 | | | |
| TAC | 0.53 *** | 1 | | |
| PAL | −0.21 *** | −0.44 *** | 1 | |
| PPO | −0.48 *** | −0.53 *** | 0.17 ** | 1 |

**, and ***—significant at the level of probability $p \leq 0.05$, 0.01, and 0.001, respectively. PHE, phenolics; TAC, total antioxidant capacity; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase.

**Table 6.** Pearson correlation coefficients (r) and corresponding significance levels between the measured biochemical parameters in 2019/2020.

| | PHE | TAC | PAL | PPO |
|---|---|---|---|---|
| PHE | 1 | | | |
| TAC | 0.57 *** | 1 | | |
| PAL | 0.33 *** | 0.16 * | 1 | |
| PPO | −0.44 *** | −0.20 ** | −0.30 *** | 1 |

*, **, and ***—significant at the level of probability $p \leq 0.05$, 0.01, and 0.001, respectively. PHE, phenolics; TAC, total antioxidant capacity; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase.

When the correlations between PHE content and PAL activity were observed in each variety separately, positive correlation was found in Sofru variety (r = 0.40, $p \leq 0.01$) in 2018/2019. In 2019/2020, a positive correlation was found between PHE content and PAL activity in Sofru (r = 0.57, $p \leq 0.001$) and Graindor (r = 0.63, $p \leq 0.001$) varieties. In 2019/2020, *Fusarium* infection caused a decrease in PAL activity in the Sofru variety, which was associated with lower PHE content. It is supposed that severe FHB infection in the Sofru variety caused inhibition of PAL activity, resulting in reduced PHE synthesis. During both growing seasons, positive correlations between PHE content and PAL activity were found in the Graindor variety. The increase in PAL activity has consequently led to

increased PHE synthesis. Thus, both PAL and PHE, as products of its activity, contribute to greater FHB tolerance in more resistant varieties. The Srpanjka variety, which was classified as moderately susceptible to FHB, did not show significant changes in either PHE content nor in PAL activity.

In 2018/2019, the PPO activity was significantly influenced by wheat variety and nitrogen treatment, although some significant differences have also been found for the effect of *Fusarium* treatment (Table 3). Observing the changes in PPO activity in each variety separately, in the Srpanjka variety, *Fusarium* treatment caused an increase in PPO activity at all N levels, ranging from 53% at N70 to 140% at N0 (Figure 4a). In the Graindor variety, *Fusarium* infection caused a significant increase in PPO activity only at N0. Alternatively, decreased PPO activity was found in the Apache variety, where *Fusarium* infection caused a significant decrease of 42%, 31%, and 21% at N0, N35, and N70, respectively.

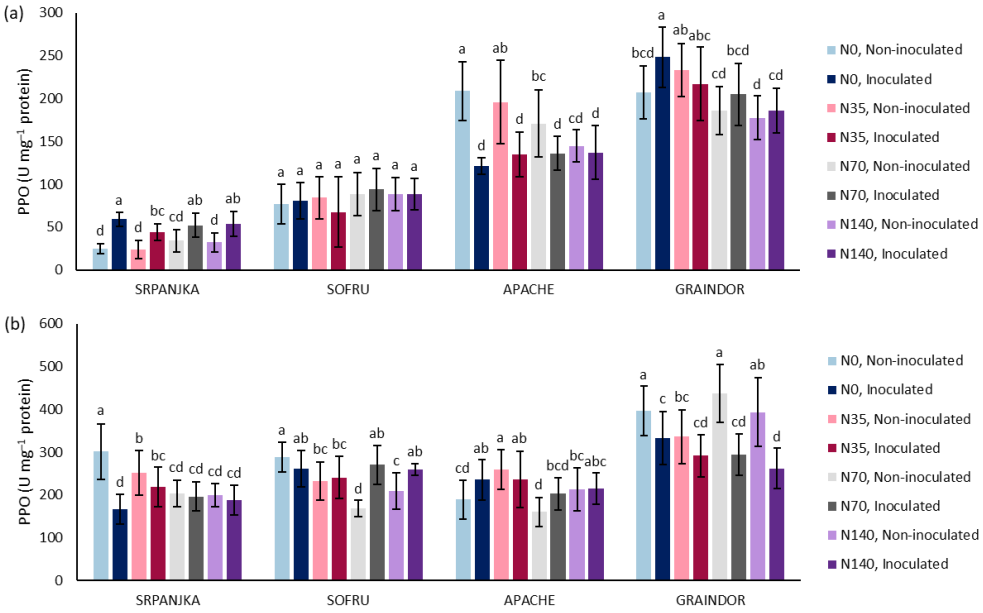


**Figure 4.** Polyphenol oxidase (PPO) activity in spikes of four wheat varieties under different *Fusarium* (non-inoculated and inoculated) and nitrogen treatments (0, 35, 70, and 140 kg N ha$^{-1}$) in 2018/2019 (**a**) and 2019/2020 (**b**). Values are means of eight replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among treatments in each variety separately.

In 2019/2020, the PPO activity was significantly influenced by all three main factors: wheat variety, *Fusarium* treatment, and nitrogen treatment (Table 4). *Fusarium* infection tended to decrease PPO activity in the Srpanjka variety, although a significant decrease was found only at N0. A trend of decreased PPO activity was also found in the Graindor variety, where *Fusarium* infection caused a significant decrease by 16%, 33%, and 33% at N0, N70, and N140, respectively. Unlike in the previously mentioned varieties, in the Sofru and Apache varieties, *Fusarium* infection caused a significant increase in the PPO activity. More precisely, in the Sofru variety, *Fusarium* infection caused a significant increase in the PPO activity by 60% at N70 and 24% at N140, while in the Apache variety, increase in the PPO activity was detected only at N0.

To date, the role of PPO in plant defense against pathogens has been recognized, although the exact information about the actual mechanism is still unknown. The proposed mechanisms of action: (1) direct toxicity of quinones, (2) reduced bioavailability and alkylation of cellular proteins to the pathogen, (3) cross-linking of quinones with protein or other phenolics, forming physical barriers, and (4) production of ROS, which are known to play an important role in defense signaling [19].

In 2018/2019, the Graindor variety had the highest PPO activity at all N levels, while the PPO activity was lowest in the Srpanjka variety (four to five times lower depending on N level). Similar results were reported in other studies, where PPO activity was higher in the spikes of wheat varieties resistant to *F. graminearum* than in the more susceptible varieties [21,22]. Pathogen-induced high PPO activity is often associated with increased resistance to pathogens, highlighting the role of PPO in plant defense against pathogens [20,46,47].

A moderate negative correlation was found between PHE content and PPO activity in both growing seasons (Tables 5 and 6). Since PPO catalyzes the oxidation of phenolic compounds to highly reactive quinones under different stress conditions, this could explain the negative correlation between these two variables.

In 2018/2019, TAC was significantly affected by wheat variety and *Fusarium* treatment (Table 3). A significant increase in TAC due to *Fusarium* infection was found in the inoculated plants of the Apache and Graindor varieties at all N levels (Figure 5a). In the Apache variety, a significant increase in TAC ranged from 9% at N35 and N70 to 11% at N0, while in the Graindor variety, it ranged from 8% at N140 to 10% at N35 and N70. Unlike Graindor and Apache, in the Srpanjka variety, *Fusarium* infection caused a significant decrease in TAC at N140, while no significant changes were observed in the Sofru variety (Figure 5a).

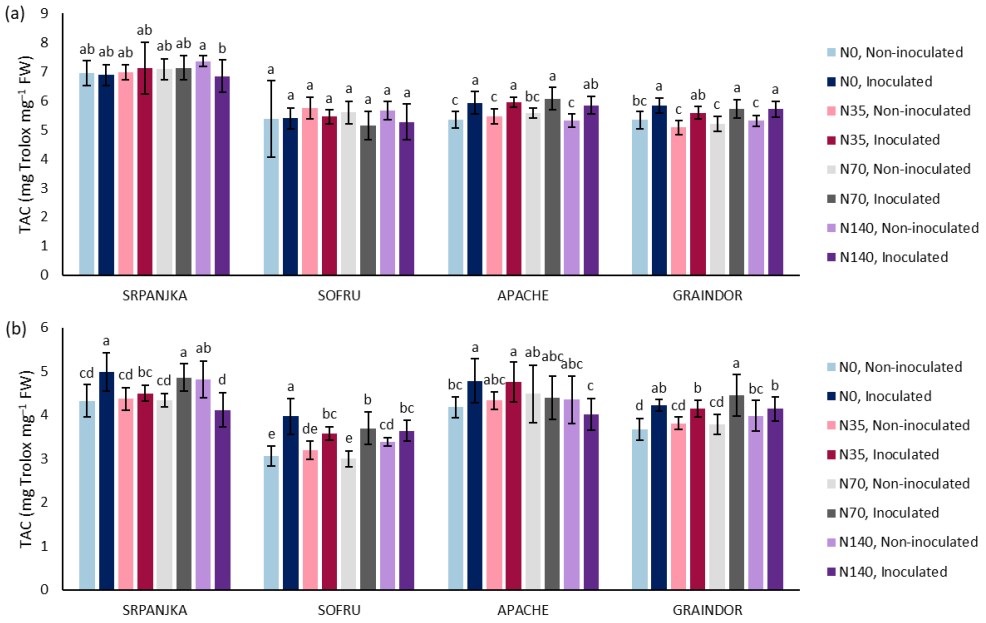


**Figure 5.** Total antioxidant capacity (TAC) in spikes of four wheat varieties under different *Fusarium* (non-inoculated and inoculated) and nitrogen treatments (0, 35, 70, and 140 kg N ha$^{-1}$) in 2018/2019 (**a**) and 2019/2020 (**b**). Values are means of eight replicates ± standard deviation (SD). Different letters above the bars indicate significant differences according to Fisher's LSD test ($p \leq 0.05$) among treatments in each variety separately.

In 2019/2020, TAC was significantly affected by wheat variety and *Fusarium* treatment (Table 4). A trend of increased TAC in the inoculated plants compared to non-inoculated plants at almost all N levels was found in the Sofru and Graindor varieties (Figure 5b). In the Sofru variety, a significant increase in TAC ranged from 12% (N35) to 30% (N0), while in the Graindor variety, it ranged from 8% at N35 to 18% at N70. In the Apache variety, *Fusarium* infection caused a significant increase in TAC by 14% only at N0. In the Srpanjka variety, *Fusarium* infection caused a significant increase in TAC by 15% (N0) and 12% (N70) and a decrease by 14% at N140.

A moderate positive correlation was found between PHE content and TAC in both growing seasons (Tables 5 and 6). The significant correlations between PHE content and TAC indicate a high contribution of PHE to the antioxidant capacity of winter wheat

subjected to different environmental stress conditions. This result is consistent with the previous study by Atanasova-Penichon et al. [48], in which the phenolics were highlighted as the main contributors to the total antioxidant capacity of cereal grains.

## 4. Conclusions

In the present study, we found a variety-specific response of winter wheat to FHB during cultivation at different nitrogen fertilization levels. The Sofru variety showed higher AUDPC values for general FHB resistance in both growing seasons, indicating higher FHB susceptibility. Lower average AUDPC values for general FHB resistance were observed in the Srpanjka variety in both growing seasons. Therefore, the Srpanjka variety was classified as moderately susceptible to FHB. The Apache and Graindor varieties were classified as partially resistant varieties, due to lower average AUDPC values for general FHB resistance in both growing seasons. No significant differences were found for the effect of nitrogen on FHB severity. The FHB severity was more affected by the prevailing climatic conditions during growing seasons, especially heavy precipitation during anthesis.

In both growing seasons, *Fusarium* inoculation altered the PHE content in wheat spikes, indicating the involvement of PHE in the defense response to *Fusarium* attack. Increased PHE content in the partially resistant varieties (Apache and Graindor) indicates the involvement of PHE in the defense response and better disease tolerance in the more resistant varieties. In addition, positive correlations between PHE content and PAL activity were reported in the Graindor variety. Thus, both PAL and PHE contribute to greater FHB tolerance in more resistant varieties. Significant correlations were also found between PHE content and TAC, indicating a high contribution of PHE to the antioxidant capacity of winter wheat subjected to different environmental stress conditions. In summary, breeding wheat varieties with enhanced PHE synthesis could be a promising strategy for controlling FHB.

Due to the still unclear and complex role of nitrogen in wheat infection with FHB, it would be interesting for future research to obtain information on how the timing of application and different forms of nitrogen may affect and modulate the plant's immune response to FHB. In addition, future research should include more parameters, such as measurements of different types of phenolic compounds (phenolic acids, anthocyanins, flavonoids) or different phytohormones (salicylic acid, jasmonic acid, abscisic acid, and indole acetic acid), which are important compounds in plant defense mechanisms against various abiotic and biotic stress conditions.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12081746/s1, Figure S1: Climate diagrams for two wheat growing seasons, 2018/2019 (a) and 2019/2020 (b), in Osijek, Croatia.

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**Naslov izvornog znanstvenog rada broj 1:** Oxidative Status and Antioxidative Response to *Fusarium* Attack and Different Nitrogen Levels in Winter Wheat Varieties

**Prošireni sažetak:**

Abiotički i biotički stresni čimbenici, kao što su nedostatak mineralnog gnojiva (osobito dušika) i napad patogena (*Fusarium culmorum*), na globalnoj razini mogu imati značajan negativan utjecaj na prinos zrna i kvalitetu pšenice. Cilj je ovog preliminarnog istraživanja utvrditi utjecaj *Fusarium* inokulacije i dvije različite razine prihrane dušikom na oksidacijski status i antioksidacijski odgovor u devet sorti ozime pšenice. Poljski pokus postavljen je tijekom vegetacijske godine 2017./2018. na poljima Poljoprivrednog instituta Osijek. Pokus je postavljen prema *split-split plot* dizajnu kao kompletni randomizirani blok u tri ponavljanja, kao višefaktorijalni pokus s tri glavna faktora: sorta, gnojidba dušikom i infekcija vrstama roda *Fusarium* (prirodna i umjetna infekcija). Istraživanje je provedeno na devet sorti ozime pšenice različitog podrijetla (BC Mandica, BC Opsesija, Bezostaya-1, Felix, Ficko, Galloper, Ingenio, Isengrain i U-1). Osnovna je gnojidba bila jednaka za sve parcele i iznosila je 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$ i 120 kg $K_2O$ $ha^{-1}$ te je primijenjena dodatkom 100 kg $ha^{-1}$ Uree i 400 kg $ha^{-1}$ NPK. Tretman dušikom uključivao je dvije različite prihrane dušikom, bez prihrane 0 kg N $ha^{-1}$ (niska razina dušika) i 100 kg N $ha^{-1}$ (visoka razina dušika), koja je primijenjena u dva obroka po 50 kg N $ha^{-1}$ u fazi vlatanja (Zadoksova skala 23 – 25) te u fazi izduživanja stabljike (Zadoksova skala 33 – 35). Za inokulaciju pšenice korištena je konidijska suspenzija vrste *F. culmorum* koncentracije $1\times10^6$ $mL^{-1}$. Umjetna inokulacija ručnom prskalicom vršena je primarno na klas pšenice te samo na prvom $m^2$ svake parcele (150 mL suspenzije $m^{-2}$). Ostatak biljaka na parceli prepušten je prirodnoj infekciji. Inokulacija je izvršena pojedinačno na svakoj parceli kada se 50 % biljaka po parceli nalazilo u fenofazi cvatnje (Zadoksova skala 65) te je ponovljena nakon 48 sati. Za održavanje vlage za optimalne uvjete infekcije pšenica je nekoliko puta tijekom dana prskana vodom. Vizualna procjena simptoma FHB-a provedena je 18 dana nakon inokulacija, a postotak zaraženih klasova procijenjen je prema linearnoj skali (0 – 100 %) prema EPPO standardu. Kao pokazatelj oksidacijskog stresa mjerena je razina lipidne peroksidacije (LPO). Antioksidacijski odgovor određen je mjerenjem aktivnosti antioksidacijskih enzima: katalaze (CAT), askorbat-peroksidaze (APX), glutation-reduktaze (GR) te mjerenjem koncentracije fenola (PHE) i sadržaja fotosintetskih pigmenata. Sva tri faktora istraživanja (sorta pšenice, tretman dušikom i *Fusarium* tretman) imala su utjecaj na vizualne simptome FHB-a te na ispitivane pokazatelje oksidacijskog stresa i antioksidacijskog odgovora. U ovom je istraživanju visoka razina dušika, u usporedbi s niskom razinom dušika,

uzrokovala povećanje simptoma FHB-a u sortama Ficko, Galloper i Felix. Suprotno tomu, visoka je razina dušika u sorti U-1 uzrokovala smanjenje vizualnih simptoma FHB-a, što ukazuje na sortno-specifični odgovor pšenice. U uvjetima niske razine dušika, *Fusarium* tretman utjecao je na značajno povećanje aktivnosti nekih mjerenih antioksidacijskih enzima (CAT, APX ili GR) u većine sorti. Iznimka je pronađena u sorti Galloper, jedinoj sorti s povećanom razinom LPO, koja ima smanjenu aktivnost APX-a, što ukazuje na važnost ovog enzima u obrambenom odgovoru te sorte. S druge strana, u uvjetima visoke razine dušika aktivnosti antioksidacijskih enzima bile su smanjenje u većine inokuliranih sorti pšenice. U ovom je istraživanju, *Fusarium* tretman pri niskoj razini dušika uzrokovao povećanje koncentracije PHE u sorti BC Mandica, dok je u sorti BC Opsesija koncentracija PHE bila smanjena. U uvjetima visoke razine dušika, *Fusarium* tretman utjecao je na smanjenje koncentracije PHE u sortama Ficko, BC Mandica, Isengrain i Bezostaya-1. U uvjetima niske i visoke razine dušika, *Fusarium* tretman je uzrokovao smanjenje sadržaja mjerenih fotosintetskih pigmenata u sortama BC Mandica i Isengrain.

U ovom je istraživanju najznačajniji utjecaj na pokazatelje oksidacijskog stresa i antioksidacijskog odgovora imala niska razina dušika. Naime, niska je razina dušika, kao abiotički stresni čimbenik, uzrokovala povećanje LPO u većini neinokuliranih sorti. Ipak, značajno je povećanje LPO pronađeno samo u neinokuliranim sortama Ficko i Bezostaya-1 koje su također imale i smanjenu aktivnost APX-a, što ukazuje na važnost ovog enzima u uklanjanju reaktivnih kisikovih jedinki. Nadalje, niska je razina dušika većinom djelovala na smanjenje aktivnosti antioksidacijskih enzima te na smanjenje sadržaja fotosintetskih pigmenata u neinokuliranim biljaka. Također, uzgoj pšenice pri niskoj razini dušika uzrokovao je smanjenje koncentracije topljivih PHE u dvije sorte (BC Mandica i Isengrain). Dobiveni su rezultati pružili uvid o oksidacijskom i antioksidacijskom odgovoru pšenice na kombinaciju utjecaja infekcije vrstama roda *Fusarium* i različite gnojidbe dušikom. Rezultati ovog istraživanja mogu poslužiti kao dodatni alat u procjeni tolerancije pšenice na stresne uvjete okoliša.

**Ključne riječi:** *Triticum aestivum*, *Fusarium*, dušik, oksidacijski stres, antioksidacijski sustav

**Naslov izvornog znanstvenog rada broj 2:** The Effect of Nitrogen Fertilization and *Fusarium culmorum* Inoculation on the Biomarkers of Oxidative Stress in Wheat Flag Leaves

**Prošireni sažetak:**

Pri uzgoju je pšenica izložena brojnim abiotičkim i/ili biotičkim stresnim čimbenicima koji mogu negativno djelovati na njezin prinos i kvalitetu. U ovom je radu analiziran utjecaj abiotičkog stresa uzrokovanog deficitom dušika i biotičkog stresa izazvanog fitopatogenom gljivom *Fusarium culmorum* na biomarkere oksidacijskog stresa u listu zastavičaru devet sorti ozime pšenice (Ficko, U-1, Galloper, BC Mandica, BC Opsesija, Ingenio, Isengrain, Felix i Bezostaya-1). Kao pokazatelji oksidacijskog stresa mjereni su koncentracija vodikova peroksida ($H_2O_2$) i razina lipidne peroksidacije (LPO), dok je antioksidacijski odgovor određen mjerenjem koncentracije ukupnih topljivih fenola (PHE), sadržaja fotosintetskih pigmenata i mjerenjem aktivnosti antioksidacijskih enzima: katalaze (CAT), askorbat-peroksidaze (APX) i glutation-reduktaze (GR). Cilj je ovog istraživanja bio utvrditi utjecaj dvije različite prihrane dušikom i inokulacije patogenim izolatom *F. culmorum* na biomarkere oksidacijskog stresa ($H_2O_2$, LPO, antioksidacijske enzime, PHE i fotosintetske pigmente) u listovima zastavičarima devet sorti pšenice. Kako bi ustanovili postojanje povezanosti antioksidacijskog odgovora lista zastavičara i klasa, pri istim eksperimentalnim uvjetima, dobiveni su rezultati prokomentirani i uspoređeni s prijašnjim rezultatima biokemijskih analiza provedenih na klasu pšenice (Matić i sur., 2021a). U ovom smo istraživanju željeli utvrditi odražava li se zaraza u klasu i na antioksidacijski odgovor lista zastavičara, te postoji li povezanost u antioksidacijskom odgovoru između lista zastavičara i klasa.

Poljski pokus s devet sorti ozime pšenice različitog podrijetla proveden je tijekom vegetacijske godine 2017./2018. na Poljoprivrednom institutu Osijek. Pokus je postavljen prema *split-split plot* dizajnu u tri ponavljanja. Tri faktora istraživanja uključivala su devet sorti ozime pšenice, dvije različite razine prihrane dušikom i inokulaciju izolatom *F. culmorum*. Osnovna je gnojidba bila jednaka za sve parcele i iznosila je 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$ i 120 kg $K_2O$ $ha^{-1}$ te je primijenjena dodatkom 100 kg $ha^{-1}$ Uree (46 % N) i 400 kg $ha^{-1}$ NPK (7 : 20 : 30). Prihrana dušikom uključivala je dvije različite količine dušika, bez prihrane 0 kg N $ha^{-1}$ (niska razina dušika) i 100 kg N $ha^{-1}$ (visoka razina dušika), koja je primijenjena u dva obroka po 50 kg N $ha^{-1}$ u fazi vlatanja (Zadoksova skala 23 – 25) te u fazi izduživanja stabljike (Zadoksova skala 33 – 35). Za umjetnu inokulaciju pšenice korištena je konidijska suspenzija vrste *F. culmorum* konačne koncentracije spora $1 \times 10^6$ spora $mL^{-1}$. Umjetna inokulacija ručnom

prskalicom vršena je primarno na klas pšenice te samo na prvom $m^2$ svake parcele (150 mL suspenzije $m^{-2}$). Ostatak biljaka na parceli prepušten je prirodnoj infekciji. Inokulacija je izvršena pojedinačno na svakoj parceli kada se 50 % biljaka po parceli nalazilo u fenofazi cvatnje (Zadoksova skala 65) te je ponovljena nakon 48 sati.

Sorta pšenice i tretman dušikom imali su značajan utjecaj na sve ispitivane biomarkere oksidacijskog stresa u listu zastavičaru, dok je utjecaj *Fusarium* tretmana bio manje izražen. U sortama Ficko, BC Opsesija i Bezostaya-1 pri niskoj razini dušika inokulacija izolatom *F. culmorum* uzrokovala je povećanje LPO, dok je u uvjetima visoke razine dušika LPO bila povećana u sortama Galloper, BC Opsesija i Ingenio. Nedostatak prihrane dušikom u neinokuliranih biljaka uzrokovao je povećanje količine TBARS-a u sortama BC Opsesija, Ingenio i Isengrain. Utvrđeno je kako je razina LPO sortno i tkivno-specifična, a u listu zastavičaru izraženiji je porast LPO, tj. prisutan je u znatno većem broju sorti pri različitim tretmanima, u odnosu na klas. U sortama U-1, Galloper i BC Opsesija (pri niskoj razini dušika) inokulacija patogenom *F. culmorum* uzrokovala je povećanje koncentracije $H_2O_2$, dok je u uvjetima visoke razine dušika koncentracije $H_2O_2$ bila povećana u sortama Galloper, BC Mandica, BC Opsesija, Felix i Bezostaya-1. Nedostatak prihrane dušikom u neinokuliranih biljaka uzrokovao je trend povećanja koncentracije $H_2O_2$ u gotovo svim sortama, a značajan rast utvrđen je u sortama Ficko, U-1, BC Opsesija, Ingenio i Bezostaya-1. Budući da je nedostatak prihrane dušikom u neinokuliranih biljaka većinom uzrokovao povećanje razine LPO te smanjenje aktivnosti antioksidacijskih enzima, možemo zaključiti kako je u ovom slučaju $H_2O_2$ djelovao kao promotor oksidacijskog stresa. Inokulacija izolatom *F. culmorum* uzrokovala je porast koncentracije PHE u sortama Galloper i BC Opsesija pri niskoj razini dušika. Trend povećanja koncentracije PHE uslijed inokulacije izolatom *F. culmorum* uočen je i pri visokoj razini dušika, a značajan rast utvrđen je u sortama BC Mandica, BC Opsesija i Bezostaya-1. U većine neinokuliranih sorti, niska je razina dušika uzrokovala trend povećanja koncentracije PHE, a statistički značajan porast zabilježen je u sortama Ficko, Galloper, BC Mandica, BC Opsesija i Bezostaya-1. U prosjeku je niska razina dušika, u usporedbi s visokom razinom, uzrokovala smanjenje sadržaja fotosintetskih pigmenata.

U prosjeku, inokulacija izolatom *F. culmorum* nije značajno utjecala na aktivnost antioksidacijskih enzima listova zastavičara pšenice. Ipak, analizom utjecaja inokulacije izolatom *F. culmorum* svake sorte pojedinačno, vidljive su značajne promjene u aktivnosti enzima koje su sortno-specifične. Inokulacija izolatom *F. culmorum* je u uvjetima niske razine dušika, uzrokovala povećanje aktivnosti enzima CAT u sorti Ficko, dok je u sortama BC

Opsesija i Felix uzrokovala smanjenje aktivnosti enzima CAT. Pri visokoj razini dušika, inokulacija izolatom *F. culmorum* uzrokovala je povećanje aktivnosti enzima CAT u sorti Bezostaya-1, a smanjenje aktivnosti u sorti Ingenio. U uvjetima niske razine dušika, inokulacija izolatom *F. culmorum* uzrokovala je povećanje aktivnosti APX-a u sortama BC Opsesija i Isengrain, dok je u uvjetima visoke razine dušika aktivnost APX-a bila povećana u sorti Ficko. Inokulacija izolatom *F. culmorum* uzrokovala je trend smanjenja aktivnosti GR-a u gotovo svim sortama pri obje razine dušika. Međutim, značajno smanjenje aktivnosti enzima GR utvrđeno je u sorti Ingenio pri niskoj razini dušika te u sorti Felix pri visokoj razini dušika. Osim što je odgovor antioksidacijskih enzima na inokulaciju izolatom *F. culmorum* ovisio o sorti, usporedbom rezultata analize lista s prijašnjom analizom klasa (Matić i sur., 2021a) uočava se i tkivno-specifični odgovor. U neinokuliranim sortama Ficko, U-1, Galloper i Ingenio, niska razina dušika utjecala je na smanjenje aktivnosti enzima CAT, dok je u sorti Isengrain utjecala na smanjenje aktivnosti enzima APX. Također, u odnosu na visoku razinu dušika, niska je razina dušika uzrokovala trend smanjenja aktivnosti GR-a u gotovo svih neinokuliranih sorti, iako je statistički značajan pad aktivnosti utvrđen samo u sorti Felix.

Najznačajniji utjecaj na mjerene pokazatelje stresa imao je uzgoj u uvjetima niske razine dušika, pri čemu je u većini sorti vidljiv trend povećanja koncentracije vodikovog peroksida i razine lipidne peroksidacije te smanjenja aktivnosti antioksidacijskih enzima. Dobiveni su rezultati prokomentirani i uspoređeni s prijašnjim rezultatima biokemijskih analiza provedenih na klasu pšenice. Značajno jake korelacije između lista zastavičara i klasa u mjerenim parametrima nije bilo, što upućuje na tkivno-specifični antioksidacijski odgovor.

**Ključne riječi:** pšenica, *Fusarium culmorum*, dušična gnojidba, oksidacijski stres, antioksidacijski odgovor

**Naslov izvornog znanstvenog rada broj 3:** Defense Response to Fusarium Infection in Winter Wheat Varieties, Varying in FHB Susceptibility, Grown under Different Nitrogen Levels

**Prošireni sažetak:**

Fuzarijska palež klasa (engl. *Fusarium head blight*, FHB) i neadekvatna gnojidba dušikom mogu uzrokovati brojne biokemijske promjene u tkivu pšenice. Ciljevi ovog istraživanja bili su: (i) utvrditi utjecaj gnojidbe dušikom i klimatskih prilika na ozbiljnost FHB-a, (ii) odrediti utjecaj *Fusarium* inokulacije i šireg raspona različitih razina gnojidbe dušikom na koncentraciju ukupnih topljivih fenola (PHE), aktivnost enzima uključenih u metabolizam fenola (PAL i PPO) i ukupni antioksidacijski kapacitet (engl. *total antioxidant capacity*, TAC), (iii) odrediti sortno-specifični obrambeni odgovor i (iv) pronaći parametar koji bi mogao poslužiti kao dobar biomarker za daljnje oplemenjivanje i uzgoj otpornijih sorti pšenice na proučavane stresne uvjete.

U svrhu istraživanja postavljeni su poljski pokusi tijekom dvije uzastopne vegetacijske godine (2018./2019. i 2019./2020.) na Poljoprivrednom institutu Osijek. Pokusi su postavljeni prema *split-split plot* dizajnu kao kompletni randomizirani blokovi u dva ponavljanja, kao višefaktorijalni pokusi s tri glavna faktora: sorta, gnojidba dušikom i infekcija vrstama roda *Fusarium* (prirodna i umjetna infekcija). U istraživanju su korištene četiri sorte ozime pšenice koje su odabrane na temelju različite razine osjetljivosti na FHB: Srpanjka, Sofru, Apache i Graindor. Srpanjka je vrlo rana sorta, izuzetno niske stabljike, srednje osjetljiva na *Fusarium* sp. te najrasprostranjenija sorta u Hrvatskoj do 2014. godine. Sofru je rana, visokoprinosna sorta, osjetljiva na *Fusarium* sp., druga najraširenija sorta u Hrvatskoj danas. Apache je srednje kasna, vrlo prilagodljiva i stabilna sorta s izvrsnom otpornošću na *Fusarium* sp. Graindor je srednje kasna do kasna sorta, visokoprinosna sorta s odličnom otpornošću na *Fusarium* sp.

Osnovna gnojidba od 74 kg N $ha^{-1}$, 80 kg $P_2O_5$ $ha^{-1}$ i 120 kg $K_2O$ $ha^{-1}$ primijenjena je dodatkom 100 kg $ha^{-1}$ Uree (udio dušika od 46 %, pri čemu je 100 % dušika u amidnom obliku) i 400 kg ha-1 NPK (7 : 20 : 30; 8,5 % dušika u amonijskom i 6,5 % u nitratnom obliku) i bila je jednaka za sve parcele. Tretman dušikom uključivao je različite prihrane dušikom koja je primijenjena ručno dodatkom KAN gnojiva (udio dušika od 27 %) u fazi vlatanja (Zadoksova skala 23 – 25) te fazi izduživanje stabljike (Zadoksova skala 33 – 35) u razinama od 0, 35, 70 i 140 kg N $ha^{-1}$ po tretmanu. Unutar svake parcelice nasumično je odabrano 50 klasova pšenice za *Fusarium* inokulaciju, a 50 klasova prepušteno je prirodnoj infekciji. Za proizvodnju

inokuluma korištena je modificirana metoda po Snijders i Van Eeuwijk (1991.). Inokulum za infekciju pšenice sastojao se od suspenzije konidija dviju vrsta roda *Fusarium*, *F. graminearum* (IFA 65) i *F. culmorum*. Konačna koncentracija spora u inokulumu podešena je na $1 \times 10^6$ spora $mL^{-1}$. *Fusarium* inokulacija vršena je ručnom prskalicom na svakoj sorti pojedinačno kada se 50 % biljaka po parcelici nalazilo u fenofazi cvatnje (Zadoksova skala 65). Inokulacije su obavljene u prijepodnevnim satima te su ponovljene nakon 48 sati. Kako bi se održala optimalna vlažnost za infekciju, klasovi su tijekom 48 sati bili prekriveni polietilenskim vrećicama. Ozbiljnost FHB-a (točnije postotak zaraženih klasića po klasu) procijenjena je pomoću linearne skale (0 – 100 %) 10., 14., 18., 22., 26. i 30. dana nakon inokulacije. Na temelju dobivenih postotaka zaraze izračunato je područje unutar progresivne krivulje bolesti (engl. *area under the disease progress curve*, AUDPC), kao integrirana jedinica za ukupni intenzitet bolesti, odnosno ukupnu otpornost na napad patogena. U ovom se istraživanju mogu uočiti razlike u ozbiljnosti FHB-a između dvije vegetacijske godine tijekom kojih su prevladavale različite klimatske prilike. U prosjeku, za sve sorte i sve razine dušika, veće vrijednosti AUDPC-a utvrđene su u vegetacijskoj godini 2018./2019. u usporedbi s 2019./2020. Navedeno može biti posljedica obilnijih oborina i povezane veće vlažnosti zraka tijekom 2018./2019. U obje vegetacijske godine primjena različitih razina gnojidbe dušikom nije značajno utjecala na ozbiljnost FHB-a. Tijekom obje vegetacijske godine, infekcija vrstama roda *Fusarium* izazvala je povećanje koncentracije PHE u djelomično otpornim sortama (Apache i Graindor), što ukazuje na uključenost PHE u obrambene reakcije i bolju toleranciju na bolest kod otpornijih sorti. Oplemenjivanje sorti pšenice koje su nositelj poželjnog svojstva (pojačana sinteza PHE) mogla bi biti jedna od strategija u kontroli FHB-a.

**Ključne riječi:** ozima pšenica, FHB, gnojidba dušikom, fenoli, obrambeni odgovor

## SAŽETAK

Pojava fuzarijske paleži klasa (engl. *Fusarium head blight*, FHB) i neadekvatna gnojidba dušikom, kao abiotički i biotički stresni čimbenici, mogu uzrokovati brojne fiziološke i biokemijske promjene u tkivu pšenice. Budući da je pšenica jedna od najvažnijih žitarica na svijetu zbog nezamjenjivog značaja u prehrani ljudi, od velikog je značaja razumjeti načine na koje pšenica reagira na stresne čimbenike okoliša. Ciljevi ovog istraživanja bili su utvrditi utjecaj različite gnojidbe dušikom na pojavnost i intenzitet FHB-a; odrediti utjecaj inokulacije vrstama roda *Fusarium* i različite gnojidbe dušikom na fiziološki odgovor, tj. oksidacijski i antioksidacijski status lista zastavičara i klasa različitih sorti pšenice; utvrditi odražava li se infekcija vrstama roda *Fusarium* u klasu na antioksidacijski odgovor lista zastavičara te postoji li povezanost fiziološkog odgovora lista zastavičara i klasa. Također, cilj nam je bio pronaći fiziološki parametar koji bi mogao poslužiti kao dobar biomarker za daljnje oplemenjivanje i uzgoj otpornijih sorti pšenice na proučavane stresne uvjete. Poljski su pokusi provedeni tijekom tri vegetacijske godine (2017./2018., 2018./2019. i 2019./2020.), kao višefaktorijalni pokusi s tri glavna faktora: sorta, gnojidba dušikom i infekcija vrstama roda *Fusarium*. U pokusu tijekom 2017./2018. vegetacijske godine korišteno je devet sorti ozime pšenice (BC Mandica, BC Opsesija, Bezostaya-1, Felix, Ficko, Galloper, Ingenio, Isengrain i U-1). Tretman dušikom uključivao je dvije različite prihrane dušikom, bez prihrane 0 kg N $ha^{-1}$ (niska razina dušika) i 100 kg N $ha^{-1}$ (visoka razina dušika). Tijekom 2017./2018. vegetacijske godine uzorkovani su listovi zastavičari i klasovi navedenih sorata. Tijekom 2017./2018. fokus je bio na istraživanju povezanosti oksidacijskog statusa i antioksidacijskog odgovora lista zastavičara i klasa. Ovisno o tome je li se radilo o listu zastavičaru ili klasu pšenice, mjereni su sljedeći pokazatelji oksidacijskog statusa i antioksidacijskog odgovora: koncentracija vodikova peroksida ($H_2O_2$), razina lipidne peroksidacije (LPO), koncentracija ukupnih topljivih fenola (PHE), sadržaj fotosintetskih pigmenata i aktivnosti antioksidacijskih enzima (katalaze, askorbat-peroksidaze i glutation-reduktaze). Sva tri faktora istraživanja imala su utjecaj na vizualne simptome FHB-a te na mjerene pokazatelje oksidacijskog stresa i antioksidacijskog odgovora. U ovom je dijelu istraživanju najznačajniji utjecaj na pokazatelje oksidacijskog stresa i antioksidacijskog odgovora imala niska razina dušika. Naime, niska je razina dušik, kao abiotički stresni faktor, uzrokovala povećanje koncentracije $H_2O_2$ (u listu) i povećanje razine LPO (u listu i klasu) u većini neinokuliranih sorti. Nadalje, niska je razina dušika većinom djelovala na smanjenje aktivnosti antioksidacijskih enzima (u listu i klasu). Iako bi korelacija u mjerenim biokemijskim parametrima omogućila upotrebu lista zastavičara za detaljniju analizu

biomarkera oksidacijskog stresa uzrokovanog FHB-om, rezultati nisu pokazali značajno jaku korelaciju između lista zastavičara i klasa u mjerenim parametrima. Na temelju dobivenih rezultata može se zaključiti kako je antioksidacijski odgovor bio tkivno-specifičan.

U pokusima tijekom 2018./2019. i 2019./2020. fokus je bio na istraživanju biokemijskih mehanizama otpornosti koji uključuju PHE i enzime metabolizma PHE: fenilalanin-amonij-lijazu (PAL) i polifenol-oksidazu (PPO). U istraživanju su korištene četiri sorte ozime pšenice koje su odabrane na temelju različite razine osjetljivosti na FHB: Srpanjka, Sofru, Apache i Graindor. U ovom je dijelu istraživanja raspon razina gnojidbe dušikom bio veći te je primijenjen u razinama od 0, 35, 70 i 140 kg N $ha^{-1}$ po tretmanu. U ovom se istraživanju mogu uočiti razlike u ozbiljnosti FHB-a između dvije vegetacijske godine tijekom kojih su prevladavale različite klimatske prilike. U prosjeku, za sve sorte i sve razine dušika, veći ukupni intenzitet bolesti utvrđen je u vegetacijskoj godini 2018./2019. u usporedbi s 2019./2020. Navedeno može biti posljedica obilnijih oborina i povezane veće vlažnosti zraka tijekom 2018./2019. U obje vegetacijske godine primjena različitih razina gnojidbe dušikom nije značajno utjecala na ozbiljnost FHB-a. Tijekom obje vegetacijske godine, infekcija vrstama roda *Fusarium* izazvala je povećanje koncentracije PHE u djelomično otpornim sortama (Apache i Graindor), što ukazuje na uključenost PHE u obrambene reakcije i bolju toleranciju na bolest kod otpornijih sorti. Oplemenjivanje sorti pšenice koje su nositelj poželjnog svojstva (pojačana sinteza PHE) mogla bi biti jedna od strategija u kontroli FHB-a. Zaključno, dobiveni su rezultati pružili uvid o oksidacijskom i antioksidacijskom odgovoru pšenice na kombinaciju utjecaja infekcije vrstama roda *Fusarium* i različite gnojidbe dušikom. Rezultati ovog istraživanja mogu poslužiti kao dodatni alat u procjeni tolerancije pšenice na stresne uvjete okoliša.

**SUMMARY**

Fusarium head blight (FHB) and inadequate nitrogen fertilization, as abiotic and biotic stress factors, can cause numerous physiological and biochemical changes in wheat tissue. Since wheat is one of the most important cereals in the world due to its irreplaceable importance in human nutrition, it is of great importance to understand the way it responds to environmental stressors. The aims of this research were: (i) to determine the effect of different nitrogen fertilization levels on the occurrence and intensity of FHB, (ii) to determine the effect of *Fusarium* inoculation and different nitrogen fertilization levels on the physiological response, i.e. oxidative and antioxidative status in flag leaves and spikes of winter wheat, (iii) to determine whether the spikes infection also reflects on the antioxidant response of flag leaves and whether there is a correlation in the antioxidant response between wheat flag leaves and spikes. Also, our aim was to find a physiological parameter that could serve as a good biomarker for further breeding and breeding of wheat varieties more resistant to the studied stress conditions. The field trials were set up during three growing seasons (2017/2018, 2018/2019, and 2019/2020) as multifactorial trials with three main factors: variety, nitrogen fertilization level and *Fusarium* infection. During 2017/2018 growing season, nine winter wheat varieties (BC Mandica, BC Opsesija, Bezostaya-1, Felix, Ficko, Galloper, Ingenio, Isengrain and U-1) were used. The nitrogen treatment included two different nitrogen top-dressings, without top-dressing 0 kg N $ha^{-1}$ (low nitrogen level) and 100 kg N $ha^{-1}$ (high nitrogen level). During 2017/2018 growing season, flag leaves and spikes of the mentioned varieties were sampled. During 2017/2018 the focus was on determining the relationship between oxidative status and antioxidative response in flag leaves and spikes. Depending on whether it was a flag leaf or a wheat spike, the following parameters of oxidative status and antioxidative response were measured: concentration of hydrogen peroxide ($H_2O_2$), level of lipid peroxidation (LPO), concentration of total soluble phenolics (PHE), content of photosynthetic pigments and activities of antioxidant enzymes (catalase, ascorbate-peroxidase and glutathione-reductase). All three research factors had an impact on visual FHB symptoms and measured indicators of oxidative stress and antioxidative response. In this part of the research, the most significant effect on the indicators of oxidative stress and antioxidative response had a low nitrogen level. Namely, a low nitrogen level, as an abiotic stress factor, caused an increase in concentration of $H_2O_2$ (in leaves) and an increase in the level of LPO (in the leaves and spikes) in most non-inoculated wheat varieties. Furthermore, a low nitrogen level mostly decreased activities of antioxidant enzymes (in leaves and spikes). Although a

correlation between measured biochemical parameters could allow the use of flag leaves for a detailed analysis of oxidative stress caused by FHB, the results of this study did not show a significantly strong correlation between flag leaves and spikes in measured parameters. More precisely, the antioxidant response was both variety- and tissue-specific.

During 2018/2019 and 2019/2020 the focus was on studying biochemical resistance mechanisms involving PHE and enzymes related to PHE metabolism: phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO). Four winter wheat varieties were used, selected based on their varying levels of susceptibility to FHB: Srpanjka, Sofru, Apache, and Graindor. In this part of the research, the range of nitrogen fertilization levels was broader and top-dressing was applied at rates of 0, 35, 70, and 140 kg N $ha^{-1}$ per treatment. In this research, differences in the FHB severity can be clearly observed between two growing seasons in which different climatic conditions prevailed. On average, for all varieties and all nitrogen levels, higher overall intensity of the disease was found in the 2018/2019 growing season compared to 2019/2020. This may be due to the more abundant precipitation and associated higher humidity during 2018/2019. In both growing seasons, application of different nitrogen fertilization levels did not significantly affect FHB severity. In both growing seasons, infection with *Fusarium* species caused an increase in PHE concentration in the partially resistant varieties (Apache and Graindor), indicating involvement of PHE in defense response and better disease tolerance in more resistant varieties. Breeding wheat varieties with desirable trait (enhanced PHE synthesis) could be one of the promising strategies to control FHB. In conclusion, the obtained results provided insight into wheat's oxidative and antioxidative response to the combination of *Fusarium* infection and different nitrogen fertilization levels. The results of this research can serve as an additional tool in assessing wheat tolerance to environmental stress conditions.

## ŽIVOTOPIS

Magdalena Matić rođena je 30. rujna 1993. godine u Vinkovcima. Osnovnu školu i Opću Gimnaziju završila je u Županji. Godine 2012. upisuje preddiplomski sveučilišni studij Biologija na Odjelu za biologiju Sveučilišta J. J. Strossmayera u Osijeku. Akademski naziv sveučilišne prvostupnice biologije stekla je 2015. godine i upisala diplomski sveučilišni studij Biologija, smjer znanstveni, također na Odjelu za biologiju u Osijeku. U svibnju 2018. godine obranila je diplomski rad pod nazivom *Učinak različitih oblika selena na oksidacijski i antioksidacijski odgovor klijanaca pšenice (Triticum aestivum L.)* i time stekla akademski naziv magistre biologije (mag. biol.). Na Filozofskome fakultetu u Osijeku 2018. godine završava program Pedagoško-psihološko-didaktičko-metodičke izobrazbe. Od listopada 2018. godine zaposlena je na Fakultetu agrobiotehničkih znanosti Osijek, kao asistentica na projektu Hrvatske zaklade za znanost pod nazivom *Genetsko poboljšanje i optimizacija potencijala rodnosti pšenice*. Iste godine na Fakultetu agrobiotehničkih znanosti Osijek upisuje Poslijediplomski doktorski studij Poljoprivrednih znanosti, smjer: zaštita bilja. Kao autor ili koautor objavila je šest znanstvenih radova u časopisima indeksiranim u Web of Science bazi te četiri rada koji su zastupljeni u drugim bibliografskim bazama. Sudjelovala je na nekoliko domaćih i međunarodnih znanstvenih skupova. Tijekom poslijediplomskog doktorskog studija boravila je na stručnom usavršavanju, u trajanju od dva mjeseca, na IFA-Tulln, Odjel za biotehnologiju u biljnoj proizvodnji, Sveučilišta za prirodne resurse i primijenjene bioznanosti u Beču – BOKU (Austrija) u sklopu Erasmus+ programa. Kao suradnik je bila uključena u rad istraživačkog projekta *Genetsko poboljšanje i optimizacija potencijala rodnosti pšenice* Hrvatske zaklade za znanost, voditelja dr. sc. Darija Novoselovića i bilateralnog projekta (Hrvatska-Srbija) *Nove tekuće formulacije u zaštiti od uzročnika bolesti pšenice u području panonskog bazena*. Također je sudjelovala u radu Erasmus+ projekta *Harmonizacija i inovacije na doktorskim studijskim programima u biljnom zdravstvu i održivoj poljoprivredi (HarISA)*, u sklopu kojega je boravila na stručnom usavršavanju na Poljoprivrednom fakultetu Univerziteta u Beogradu. Članica je Hrvatskoga društva biljne zaštite (HDBZ-a) i Hrvatskoga društva za biljnu biologiju (HDBB-a).

## CURRICULUM VITAE

Magdalena Matić was born on September 30, 1993, in Vinkovci. She completed elementary and secondary school in Županja. In 2012, she enrolled in the Undergraduate University Study Programme in Biology at the Department of Biology of J. J. Strossmayer University in Osijek. She obtained the university bachelor's degree in biology in 2015 and enrolled in the Graduate University Study Programme in Biology, also at the Department of Biology in Osijek. In May 2018, she defended her master's thesis entitled *The Effect of Different Forms of Selenium on the Oxidative Stress and Antioxidative Response in Wheat Seedlings (Triticum aestivum L.)* and thus obtained the academic title of Master of Biology. In 2018, she completed the Pedagogical-Psychological-Didactic-Methodical Education at the Faculty of Humanities and Social Sciences in Osijek. Since October 2018, she has been working at the Faculty of Agrobiotechnical Sciences Osijek, as an assistant in the project of the Croatian Science Foundation entitled *Genetic Improvement and Optimization of Wheat Yield Potential*. In the same year, she enrolled in the Postgraduate University Study Programme in Agricultural Sciences at the Faculty of Agrobiotechnical Sciences in Osijek, course of studies in Plant Protection. As an author or co-author, she published six scientific papers in journals indexed in the Web of Science database and four papers categorized in other bibliographic databases. She participated in several national and international scientific meetings. During her postgraduate university studies, she participated in a two-month professional training at IFA-Tulln, Institute of Biotechnology in Plant Production, University of Natural Resources and Life Sciences in Vienna – BOKA (Austria), as part of the Erasmus+ programme. As a collaborator, she was involved in the work of the research project *Genetic Improvement and Optimization of Wheat Yield Potential* of the Croatian Science Foundation led by Dario Novoselović, PhD, and the bilateral project (Bilateral Cooperation Serbia – Croatia) *New Liquid-based Formulations in Protection against Causes of Wheat Diseases in the Pannonian Basin Region*. She also participated in the work of the Erasmus+ project *Harmonization and Innovation in PhD Study Programs for Plant Health in Sustainable Agriculture (HarISA)*, in whose part she attended a professional training at the Faculty of Agriculture, University of Belgrade. She is a member of the Croatian Plant Protection Society and the Croatian Society of Plant Biology.