UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Kristina KOŠMRLJ

UPORABA BIOTEHNOLOŠKIH PRISTOPOV ZA ŽLAHTNJENJE OLJNIH BUČ (*Cucurbita pepo* subsp. *pepo* var. *styriaca* Greb.)

DOKTORSKA DISERTACIJA

Ljubljana, 2015

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DOKTORSKA DISERTACIJA

APPLICATION OF BIOTECHNOLOGICAL APPROACHES FOR BREEDING OF OIL-SEED PUMPKINS (*Cucurbita pepo* subsp. *pepo* var. *styriaca* Greb.)

DOCTORAL DISSERTATION

Ljubljana, 2015

Doktorska disertacija je zaključek Interdisciplinarnega doktorskega študija Bioznanosti, znanstveno področje biotehnologije. Opravljena je bila na Katedri za biotehnologijo, genetiko, statistiko in žlahtnjenje rastlin Oddelka za agronomijo Biotehniške fakultete Univerze v Ljubljani. Del raziskav je bil opravljen na Katedri za botaniko Oddelka za biologijo Biotehniške fakultete Univerze v Ljubljani.

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SA BOHANEC, Borut (mentor)
KZ SI-1000 Ljubljana, Jamnikarjeva 101
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AI Kljub vedno večjemu zanimanju za pridelavo oljnih buč (*Cucurbita pepo* subsp. *pepo* var. *styriaca*) se le-tem posveča relativno malo pozornosti na področju žlahtnjenja in razvoju metod, ki bi omogočile hitrejše prilagajanje spreminjajočim se okoljskim razmeram. Za razvoj čistih linij za žlahtnjenje hibridov smo želeli optimizirati indukcijo haploidov in *in vitro* podvojevanje genoma za pridobivanje podvojenih haploidov. Preučili smo faktorje, ki vplivajo na uspešnost indukcije haploidov s pomočjo obsevanega peloda in kulturo nezrelih embrijev. Ugotovili smo, da je učinkovitost indukcije odvisna tako od akcesije donorja ženskih cvetov kot tudi donorja peloda, doze obsevanja in sezone. Kot optimalno smo določili obsevanje z 200 do 300 Gy in dosegli do 10 % haploidnih regenerantov v eni izmed testiranih akcesij. Pri analizi diploidnih regenerantov z mikrosatelitskimi markerji nismo potrdili spontanega podvojevanja genoma. Znano je, da so postopki indukcije bolj učinkoviti pri višjih dozah obsevanja, zato smo želeli razviti postopek, ki bi pelod delno zaščitil pred izgubo kalivosti pri daljšem času obsevanja. Ugotovili smo, da je z obsevanjem pri višji zračni vlažnosti možna uporaba obsevanja z rentgenskimi žarki do 600 Gy, medtem ko obsevanje pri sobni vlažnosti primerljivo kalivost omogoča pri dozi obsevanja 500 Gy. Za študij somaklonske variabilnosti smo optimizirali postopek adventivne regeneracije iz kotiledonov. S pretočno in slikovno citometrijo smo v različnih organih ter znotraj kotiledonskega tkiva določili razlike v stopnji endoreduplicirani. Bazalni del kotiledona, ki velja za najodzivnejšega za regeneracijo, je bil najmanj endoreduplicirani. Najvišjo regeneracijo smo dosegli na MS gojišču z dodatkom 1 mg/l BA, 0,25 mg/l PABA in 5 mg/l fuzarične kisline (FA). Presenetljivo je FA, dodana gojišču kot selekcijski agens za toleranco na glive rodu *Fusarium*, stimulirala regeneracijo in inducirala podvojevanje genoma *in vitro*, medtem ko podvojevanja genoma na gojiščih brez FA nismo detektirali. Kot nadgradnjo fenotipskemu ocenjevanju odpornosti na virus ZYMV smo želeli vzpostaviti metodo relativne kvantifikacije virusa ZYMV z metodo qPCR. Za normalizacijo podatkov sta v primeru okužbe z virusom ZYMV zadoščala referenčna gena UFP in EF-1A. Preliminarni rezultati nakazujejo, da tolerantna in občutljiva sorta oljnih buč akumulirata primerljive količine virusa ZYMV v rastlinah z izrazitimi bolezenskimi znamenji in se na okužbo odzivata z znižanjem ekspresije gena za katalazo 1.

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AU KOŠMRLJ, Kristina
AA BOHANEC, Borut (supervisor)
PP SI-1000 Ljubljana, Jamnikarjeva 101
PB University of Ljubljana, Biotechnical Faculty, Interdisciplinary Doctoral Programme in Biosciences, Scientific Field Biotechnology
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AB Despite traditional breeding improvements, little effort has been made in terms of developing styrian oil pumpkin (*Cucurbita pepo* subsp. *pepo* var. *styriaca*) germplasm-specific modern breeding methods that would enable accelerated delivery of cultivars adapted to changing environmental conditions. With the goal of developing pure lines suitable for hybrid breeding, we aimed to optimize the haploid induction protocol and subsequent genome doubling. Female parent and pollen donor, as well as irradiation dose and season, were found to affect the efficiency of haploid induction via pseudofertilization with X-ray irradiated pollen. A dose of 200 to 300 Gy was found optimal and resulted in up to 10 % of haploid regenerants in one of the tested female parents. Spontaneous genome doubling was not confirmed by microsatellite analysis of diploid regenerants. Similar studies have shown that haploid induction is more efficient if pollen is irradiated with higher doses. By irradiating at higher humidity we were able to conserve the germinability of styrian oil pumpkin pollen during prolonged irradiation. By using the modified irradiation method we expect a successful use of doses as high as 600 Gy, whereas only doses up to 500 Gy seem feasible with irradiation at room humidity. Finally, we aimed to optimize the adventitious regenerantion protocol, required for somaclonal variation studies. Major differences in the endoreduplication status of different organs suggested that the choice of explant tissue and its endopolyploidy might play an important role in its responsiveness for regeneration. The analysis of different cotiledonary sections by image cytometry revealed that the basal section, which has been reported as the most responsive, is less endoreduplicated than the central and distal parts of the cotyledon. MS medium supplemented with 1 mg/l BA, 0,25 mg/l PABA and 5 mg/l fusaric acid (FA) was found optimal. FA, which was added to the media as a possible selective agent for *Fusarium* tolerance, was found to stimulate regeneration and induce genome doubling. To support phenotypic virus resistance screening, we also aimed to develop a qPCR-based relative quantification method for ZYMV. Under conditions of ZYMV infection two reference genes (UFP and EF-1A) were found sufficient for data normalization. Our preliminary results show comparable quantities of ZYMV detected in symptomatic plants of both, the tolerant and the susceptible cultivar. Moreover, a downregulation of the catalase 1 gene was observed in the same samples.

KAZALO VSEBINE

str.

Ključna dokumentacijska informacija III
Key words documentation IV
Kazalo vsebine V
Kazalo znanstvenih del VII
Kazalo preglednic VIII
Kazalo slik IX
Kazalo prilog X
Okrajšave in simboli XI
Slovarček XIII

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE 1
2 ZNANSTVENA DELA 7
2.1 OBJAVLJENA ZNANSTVENA DELA 7
2.1.1 Partenogenetska indukcija haploidov pri golosemenskih bučah s pomočjo z rentgenskimi žarki obsevanega peloda 7
2.1.2 Kalivost peloda oljnih buč pri višjih dozah obsevanja: optimizacija *in vitro* postopka kalitve in obsevanja 15
2.1.3 Adventivna regeneracija pri oljnih bučah, vpliv endoreduplikacije na sposobnost regeneracije in podvojevanje genoma na gojiščih z dodatkom fuzarične kisline 23
2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO 32
2.2.1 Izbor stabilno izraženih referenčnih genov in kvantifikacija virusa ZYMV v okuženih listih oljnih buč s qPCR 32
2.2.1.1 Priprava rastlinskega materiala, mehanska inokulacija rastlin z virusom ZYMV in priprava cDNA vzorcev za qPCR analizo 32
2.2.1.2 qPCR in analiza pridobljenih rezultatov pomnoževanja 33
2.2.1.3 Rezultati preliminarnega poskusa mehanske inokulacije z virusom ZYMV 35
2.2.1.4 Rezultati validacije referenčnih genov 36
2.2.1.5 Preliminarni poskus primerjave ekspresije gena CAT1 v občutljivi in tolerantni sorti oljnih buč 38
2.2.1.6 Preliminarni poskus primerjave relativne količine virusa ZYMV v občutljivi in tolerantni sorti oljnih buč 39
3 RAZPRAVA IN SKLEPI 41

3.1 INDUKCIJA HAPLOIDOV Z OPRAŠEVANJEM Z OBSEVANIM PELODOM 41
3.2 KALITEV PELODA PRI VIŠJIH DOZAH OBSEVANJA IN POTENCIALNA UPORABA V OPTIMIZACIJI POSTOPKA INDUKCIJE HAPLOIDOV 43
3.3 ENDOPOLIPLOIDIJA IN NJEN VPLIV NA ADVENTIVNO REGENERACIJO 44
3.4 ADVENTIVNA REGENERACIJA IN UPORABA SOMAKLONSKE VARIABILNOSTI V ŽLAHTNJITELJSKE NAMENE 45
3.5 IZBOR STABILNO IZRAŽENIH REFERENČNIH GENOV OB OKUŽBI RASTLIN Z VIRUSOM ZYMV 46
3.6 EKSPRESIJA GENA CAT1 IN PRISOTNOST VIRUSA ZYMV V OKUŽENIH RASTLINAH 47
4 POVZETEK (SUMMARY) 50
4.1 POVZETEK 50
4.2 SUMMARY 52
5 VIRI 55
ZAHVALA
PRILOGE

KAZALO ZNANSTVENIH DEL

str.

Košmrlj K., Murovec J., Bohanec B. 2013. Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray-irradiated pollen. Journal of the American Society for Horticultural Science, 138: 310-316 8

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KAZALO PREGLEDNIC

str.

Pregl. 1: Izbor referenčnih in tarčnih genov s pripadajočimi sekvencami začetnih oligonukleotidov, uporabljenih v qPCR analizi. 34

Pregl. 2: Učinkovitosti pomnoževanja amplikonov in Ct vrednosti referenčnih genov, pridobljene z analizo qPCR. 37

Pregl. 3: Razpored referenčnih genov s programom RefFinder (Xie in sod., 2011), pri čemer stabilno izražene referenčne gene najdemo na vrhu posameznih lestvic, medtem ko so manj stabilni na dnu. 37

KAZALO SLIK

str.

Sl. 1: Mehanska inokulacija oljnih buč z virusom ZYMV. Za inokulacijo smo uporabili homogenizirano listno tkivo oljnih buč, ki so kazale tipična bolezenska znamenja okužbe z virusom ZYMV (A). Na inokuliranih rastlinah so se prva bolezenska znamenja pojavila približno 1 teden po inokulaciji na mladih listih (B). Vzorčenje za ekstrakcijo RNA smo izvedli 21 dni po inokulaciji. Inokulirane rastline so razvile izrazita bolezenska znamenja (C) ali šibka oziroma le-ta niso bila izražena (D). Kontrolne rastline niso kazale bolezenskih znamenj (E). 36

Sl. 2: Relativna ekspresija gena CAT1 v mladih listih z virusom ZYMV okuženih oljnih buč sort 'GL Opal' (tolerantna na virus ZYMV) in 'Gleisdorfer Ölkürbis' (občutljiva na virus ZYMV), podana kot $\log_2$ večkratnik razlike glede na ekspresijo pri kontroli iste sorte. Negativne vrednosti predstavljajo znižanje ravni izražanja gena CAT1, medtem ko pozitivne vrednosti predstavljajo zvišanje ravni izražanja glede na dano kontrolo. Standardna napaka je izračunana na podlagi bioloških ponovitev. Skupina »ZYMV skupno« je izračunana na osnovi vseh rastlin, okuženih z virusom ZYMV, in se naprej deli na skupini »ZYMV simptomatski« (rastline z izrazitimi bolezenskimi znamenji) ter »ZYMV asimptomatski« (rastline s šibkimi oziroma neizrazitimi bolezenskimi znamenji). 39

Sl. 3: Relativna kvantifikacija virusa ZYMV v mladih listih z virusom ZYMV okuženih oljnih buč sort 'GL Opal' (tolerantna na virus ZYMV) in 'Gleisdorfer Ölkürbis' (občutljiva na virus ZYMV), podana kot $\log_2$ večkratnik glede na vzorec z najnižjo detektirano količino virusa ZYMV. Standardna napaka je izračunana na podlagi bioloških ponovitev. Skupina »ZYMV skupno« je izračunana na osnovi vseh rastlin, okuženih z virusom ZYMV, in se naprej deli na skupini »ZYMV simptomatski« (rastline z izrazitimi bolezenskimi znamenji) ter »ZYMV asimptomatski« (rastline s šibkimi oziroma neizrazitimi bolezenskimi znamenji). 40

KAZALO PRILOG

Priloga A: Dovoljenje založnika revije Journal of the American Society for Horticultural Science, American Society for Horticultural Science, za objavo članka v tiskani in elektronski obliki doktorske disertacije

Priloga B: Dovoljenje založnika revije Turkish Journal of Biology, TÜBİTAK, za objavo članka v tiskani in elektronski obliki doktorske disertacije

Priloga C: Dovoljenje založnika revije Plant Growth Regulation, Springer Science + Business Media, za objavo članka v tiskani in elektronski obliki doktorske disertacije

OKRAJŠAVE IN SIMBOLI

2iP	2-izopentenil adenin
ACT	aktin (angl. actin)
angl.	angleško
BA	6-bezil amino purin
BYDV	virus rumene pritlikavosti ječmena (angl. Barley yellow dwarf virus)
CAC	srednja podenota adaptorskega kompleksa klatrina (angl. clathrin adaptor complexes medium subunit)
CAT1	katalaza 1 (angl. catalase 1)
cDNA	komplementarna deoksiribonukleinska kislina
CMV	virus mozaika kumar (angl. Cucumber mosaic virus)
COX	citokrom oksidaza (angl. cytochrome oxidase)
Ct	pražni cikel (angl. threshold cycle)
CUC18S	18S ribosomalna RNA (angl. 18S ribosomal RNA)
CVYV	virus rumenenja žil kumare (angl. Cucumber vein yellowing virus)
CYSDV	virus rumenenja in zakrnelosti buče (angl. Cucurbit yellow stunting disorder virus)
DH	podvojeni haploid (angl. doubled haploid)
dH_2O	deionizirana voda
DNA	deoksiribonukleinska kislina
EF-1A	elongacijski faktor-1α (angl. elongation factor-1α)
ELISA	encimskoimunski test (angl. enzyme-linked immunosorbent assay)
FA	fuzarična kislina (angl. fusaric acid)
FAO	Svetovna organizacija za hrano in kmetijstvo (angl. Food and Agriculture Organization of the United Nations)
Gy	Gray (enota za absorbirano dozo sevanja)
HELI	DEAD-box RNA helikazi podoben protein (angl. DEAD-box RNA helicase-like protein)
HH	obsevanje pri višji zračni vlažnosti (angl. high humidity)
IAA	indol-3-ocetna kislina
IAEA	Mednarodna agencija za jedrsko energijo (angl. International Atomic Energy Agency)
iPA	2-izopentil adenozin
PABA	para-aminobenzoična kislina
PCR	verižna reakcija s polimerazo (angl. polymerase chain reaction)
PP2A	regulatorna podenota A proteinske fosfataze 2A (angl. protein phosphatase 2A regulatory subunit A)
PRSV	virus obročkaste pegavosti papaje (angl. Papaya ringspot virus)

qPCR	kvantitativna verižna reakcija s polimerazo (angl. quantitative polymerase chain reaction)
RH	obsevanje pri sobni vlažnosti (angl. room humidity)
RNA	ribonukleinska kislina
RPL36Aa	60S ribosomalni protein L36a/L44 (angl. 60S ribosomal protein L36a/L44)
RT-PCR	obratna transkripcija in verižna reakcija s polimerazo (angl. reverse transcription polymerase chain reaction)
SqMV	virus mozaika buč (angl. Squash mosaic virus)
TNV	virus nekroze tobaka (angl. Tobacco necrosis virus)
TUA	α-tubulin
UFP	ubikvitin fuzijski protein (angl. ubiquitin fusion protein)
WMV	virus mozaika lubenic (angl. Watermelon mosaic virus)
ZEA	zeatin
ZYMV	virus rumenega mozaika bučk (angl. Zucchini yellow mosaic virus)

SLOVARČEK

celična diferenciacija	spremembe, ki so vključene v progresivno diverzifikacijo strukture in funkcije celic
endopoliploidija	obstoj celic z različno vsebnostjo jedrne DNA v istem organizmu
haploid	organizem, ki ima v somatskih celicah gametsko število kromosomov
hibrid	križanec dveh genetsko različnih osebkov (širše); v žlahtnjenju rastlin križanec dveh genetsko izenačenih, vendar različnih, starševskih linij (t.i. čistih linij)
lokus	mesto gena na kromosomu, določeno glede na relativni vrstni red z drugimi geni
občutljivost	nezmožnost rastline, da prepreči škodljiv vpliv patogenega organizma
odpornost / rezistenca	zmožnost rastline, da prepreči škodljiv vpliv patogenega organizma
patogeni organizem	organizem, zmožen povzročitve bolezenskih znamenj
populacijska sorta	sorta, ki je nastala z masovno selekcijo
somaklonska variabilnost	variabilnost, ki nastane zaradi gojenja v *in vitro* pogojih in se kaže tako na genotipu kot tudi fenotipu
toleranca	zmožnost rastline, da prestane okužbo s patogenim organizmom z nezmanjšano rastjo oziroma brez bistvene izgube pridelka

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

Buče sodijo med najstarejše kmetijske rastline. Rod *Cucurbita* izvira iz Amerike, kjer najdemo tudi največjo genetsko raznolikost (Ivančič, 2002). Prvi pisni vir uporabe buč z namenom pridobivanja bučnega olja sega v zgodnje 18. stoletje. Najden je bil na avstrijskem Štajerskem, vendar takrat verjetno še niso bili v uporabi golosemenski tipi oljnih buč (*Cucurbita pepo* subsp. *pepo* var. *styriaca* Greb.), saj naj bi se ti kot posledica spontane mutacije pojavili šele v drugi polovici 19. stoletja. Zaradi močno zreducirane semenske ovojnice je bilo pridobivanje olja olajšano, to pa je posledično povzročilo hitro širjenje pridelave v celotni regiji (Lelley in sod., 2009). V Evropi se tradicionalno pridelujejo v Avstriji, v zahodnem delu Madžarske in severozahodu Hrvaške ter na področju severovzhodne Slovenije. Na svetu se pridela približno 200000 ton oljnih buč na 600000 hektarjih pridelovalne površine letno, od česar se jih približno 10 % porabi za pridelavo olja, 80 % za pekarsko industrijo in 10 % v druge namene. Svetovno gledano je največja pridelovalka oljnih buč Kitajska s 120000 ton pridelka (Kranjec, 2014).

Po letu 2010, ko smo v Sloveniji dosegli maksimalno površino pri pridelavi oljnih buč doslej (6141 ha), se je le-ta znižala na 3433 ha v letu 2013 (Podatkovni portal SI-STAT, 2013). Manjši trend upada je bil sicer opazen tudi v sosednji Avstriji, vendar naj bi po prvih ocenah leta 2014 pridelovali oljne buče na skupno 20536 ha, kar je za 14,8 % več kot leta 2013 (Statistik Austria, 2014). Manjša nihanja v pridelovalnih površinah je sicer mogoče pripisati različnih razlogom. Na avstrijskem Štajerskem kot razlog navajajo predvsem pozno setev zaradi vremenskih razmer v aprilu 2013 (Landwirtschaftskammer Steiermark, 2013). Kljub kratkoročnemu upadu pridelovalnih površin pa zanimanje za pridelavo ostaja razmeroma visoko. Leta 2013 je bila cena na kilogram bučnih semen pri nas v povprečju 2,8 eur (Podatkovni portal SI-STAT, 2013), medtem ko so bile odkupne cene na avstrijskem Štajerskem višje (med 3,5 in 3,8 eur; Landwirtschaftskammer Steiermark, 2013). Na Sortni listi poljščin, zelenjadnic, sadnih rastlin in trte za leto 2014 (Ministrstvo za kmetijstvo in okolje, 2014) najdemo 2 populacijski sorti, in sicer 'Slovensko golico' (Semenarna Ljubljana d.d., Slovenija) in 'Gleisdorfer Ölkürbis' (Saatzucht Gleisdorf Ges.mbH, Avstrija). V zadnjih letih pa se na trgu pojavljajo tudi hibridi, ki jih odlikuje predvsem boljša odpornost na bolezni, izenačenost ter večji hektarski donos. Med najbolj pomembnimi podatki za pridelovalce je gotovo hektarski donos, ki je bil v zadnjih letih nizek in je leta 2013 v Sloveniji znašal le 0,5 tone na hektar (Podatkovni portal SI-STAT, 2013), kar je relativno nizko celo ob setvi populacijskih sort. V idealnih pogojih bi ob uporabi visoko donosnih hibridov lahko dosegli pridelek do 1,07 ton na hektar (Neubauer in sod., 2010). Skupaj z načeloma nižjo porabo fitofarmacevtskih sredstev, večji pridelek gotovo upraviči višjo nabavno ceno hibridnega semena.

Žlahtnjenje hibridov predpostavlja razvoj starševskih linij oziroma t.i. čistih linij. Ustrezna izbira kombinacije starševskih linij zagotavlja križancu boljše lastnosti zaradi pojava imenovanega hibridni vigor. Vzgoja čistih linij s tradicionalnimi postopki povratnih križanj za doseganje homozigotnosti traja več let, medtem ko se je temu možno izogniti z razvojem tehnologije za indukcijo haploidov in kasnejšega podvojevanja genoma za pridobivanje podvojenih haploidov (angl. doubled haploid, DH), s čimer znižamo stroške razvoja novih sort, saj popolno homozigotnost na vseh lokusih dosežemo že v eni sami

generaciji (Bohanec, 2004). V osnovi obstajata 2 različna načina indukcije haploidov, in sicer androgenetska in ginogenetska pot. V rodu *Cucurbita* sta znani 2 poročili o uspešni indukciji po androgenetski poti (Metwally in sod., 1998a; Mohamed in Refaei, 2004). Nasprotno pa je znanih več poskusov indukcije haploidov po ginogenetski poti, in sicer s kulturo neoprašenih ovul (Dumas de Vaulx in Chambonnet, 1986; Metwally in sod., 1998b; Shalaby, 2007), medvrstnimi križanji (Dumas de Vaulx, 1979) in opraševanjem z obsevanim pelodom (Kurtar in sod., 2002, 2009; Kurtar in Balkaya, 2010; Baktemur in sod., 2014).

Sodeč po številu objav uspešne indukcije haploidov s pseudofertilizacijo za indukcijo haploidov v rodu *Cucurbita* (Kurtar in sod., 2002, 2009; Kurtar in Balkaya, 2010; Baktemur in sod., 2014) in drugih predstavnikih družine Cucurbitaceae (Sauton in Dumas de Vaulx, 1987; Sauton, 1988; Gürsöz in sod., 1991; Cuny in sod., 1993; Katoh in sod., 1993; Sari in sod., 1994; Przyborowski in Niemirowicz-Szczytt, 1994; Caglar in Abak, 1996; Faris in sod., 1999; Lotfi in sod., 2003; Claveria in sod., 2005; Lim in Earle, 2008; Gonzalo in sod., 2011; Godbole in Murthy, 2012) smo predpostavljali, da bo izbrani partenogenetski postopek primerljivo uspešen tudi pri oljnih bučah. Z namenom optimizacije postopka smo v doktorski nalogi želeli ovrednotiti vpliv akcesije donorja peloda, donorja ženskih cvetov in prejete doze obsevanja rentgenskih žarkov. Rentgenski žarki so sicer redkeje uporabljen vir sevanja za indukcijo haploidov (Katoh in sod., 1993; Sato in sod., 2000; Yahata in sod., 2010), vendar vedno večja restrikcija uporabe gama žarkov upravičuje in spodbuja uporabo rentgenskih žarkov (FAO/IAEA, 2013). Mehanizem same indukcije haploidov sicer ostaja isti. Raziskovalci si niso enotni, kako pravzaprav sevanje stimulira partenogenetski razvoj haploidnega embrija, vendar sta se uveljavili dve hipotezi. Prva govori o stimulaciji razvoja embrija že s samo kalitvijo peloda po brazdi brez dejanske oploditve jajčnih celic (Murovec in Bohanec, 2011), medtem ko druga hipoteza fenomen pojasnjuje z eliminacijo »očetovih« kromosomov v prvih delitvah novonastalega embrija (Murovec in Bohanec, 2013). Poleg časovne prednosti, ki jo ponuja indukcija haploidov, ni zanemarljiva niti prednost eliminacije zarodkov s slabšim vigorjem zaradi t.i. inbriding depresije, ki povzroča težave pri vzgoji čistih linij tudi v družini Cucurbitaceae (Stephenson in sod., 2001; Cardoso, 2004).

Poleg več dejavnikov, ki vplivajo na uspešnost indukcije haploidov (sezona, uporabljene akcesije, tip sevanja, stopnja razvoja embrija ob reševanju itd.), je gotovo vredno več pozornosti posvetiti sami dozi obsevanja, saj se le-ta večkrat navaja kot najpomembnejši dejavnik uspeha. Več avtorjev (Sauton in Dumas de Vaulx, 1987; Chalak in Legave, 1997; Grouh in sod., 2011; Baktemur in sod., 2014) poroča o boljši učinkovitosti oziroma višji frekvenci haploidov pri višjih dozah obsevanja. Pelod rodu *Cucurbita* zaradi svojih neugodnih lastnosti (Nepi in Pacini, 1993) že v naravnih pogojih nekaj ur po odprtju moških cvetov skoraj popolnoma izgubi viabilnost, kar otežuje uporabo višjih doz obsevanja, saj mora pelod preživeti daljše čase obsevanja. Zaradi svoje izredne velikosti (100 – 200 μm) naj bi bil pelod tudi bolj radiosenzitiven, torej bolj občutljiv na poškodbe zaradi sevanja (Brewbaker in Emery, 1962). Za namene vrednotenja viabilnosti oziroma kalivosti peloda po obsevanju smo v sklopu doktorske naloge želeli optimizirati postopek *in vitro* kalitve in obsevanja samega. Metoda obsevanja, ki bi zagotovila primerno kalivost tudi po daljšem obsevanju, bi namreč omogočala uporabo višjih doz rentgenskih žarkov.

Primerno optimiziran postopek obsevanja bi tako omogočil poskus nadaljnje optimizacije indukcije haploidov.

Za pridobivanje DH je potrebno podvojevanje genoma pridobljenih haploidov. Splošno je največ v uporabi tretiranje z antimitotskimi sredstvi kot so kolhicin, orizalin, amiprofos metil itd. Poleg razmeroma nizke uspešnosti podvojevanja so omenjena sredstva tudi zdravju škodljiva. Na voljo so predvsem rezultati raziskav v rodu *Cucumis* (Lotfi in sod., 2003; Claveria in sod., 2005; Lim in Earle, 2008), ki prav tako kot rod *Cucurbita* (in s tem oljne buče) spada v družino Cucurbitaceae. Uspešnost podvojevanja s kolhicinom ne presega 30 %, pri čemer so zelo pogosti miksoploidni regeneranti, katerih je po podatkih raziskav na melonah (*Cucumis melo*; Lotfi in sod., 2003) do 64 %. Podobno je znano tudi pri drugih rastlinskih vrstah. V izogib prisotnosti miksoploidov in nizki stopnji podvojevanja so pri hmelju (*Humulus lupulus*) Škof in sod. (2007) razvili postopek podvojevanja genoma s pomočjo adventivne regeneracije na gojiščih s povišano vsebnostjo citokininov, ki bi lahko tudi v našem primeru služil kot alternativna pot podvojevanju z antimitotskimi sredstvi.

Na voljo je več študij adventivne regeneracije v rodu *Cucurbita* (Ananthakrishnan in sod., 2003; Lee in sod., 2003; Kathiravan in sod., 2006; Zhang in sod., 2008; Kim in sod., 2010). Avtorji navajajo, da je regeneracija najbolj uspešna na Murashige in Skoog (1962) gojišču z dodatkom 6-bezil amino purina (BA) ali kombinacije zeatina (ZEA) in indol-3-ocetne kisline (IAA). Poleg sestave gojišča na učinkovitost regeneracije vplivajo tudi drugi dejavniki: genotip, starost rastlinic ob odvzemu izsečkov, tip izsečkov in endogena vsebnost hormonov. Pri kumarah (*Cucumis sativus*) je bilo dokazano, da je regeneracija odvisna tudi od frekvence celic z diploidnimi jedri (2C) v tkivu kotiledonskih izsečkov (Colijn-Hooymans in sod., 1994). Znano je, da je *C. pepo* ena izmed rastlinskih vrst, ki kaže visoko stopnjo endoredupliciranosti v različnih tkivih (Barow in Meister, 2003). Stopnja endoredupliciranosti oziroma endopoliploidija je pri rastlinah sicer znan in razširjen pojav, ki ga večinoma povezujemo s sposobnostjo za hitro rast in specializacijo celic (Barow, 2006). Na osnovi teh informacij je možno sklepati, da endopoliploidija vpliva tudi na sposobnost regeneracije pri oljnih bučah, zato smo ovrednotili stopnjo endoredupliciranosti različnih rastlinskih organov in sekcij kotiledona s pomočjo pretočne in slikovne citometrije. Z namenom razvoja postopka adventivne regeneracije za podvojevanje genoma smo preverili uspešnost regeneracije izsečkov bazalnega dela kotiledonov na različnih gojiščih in regenerantom določili ploidnost s pretočno citometrijo.

Poleg žlahtnjenja za večji pridelek, večjo vsebnost olja in grmičasto obliko rasti je velikega pomena tudi žlahtnjenje za odpornost na bolezni. Izpade pridelka pri oljnih bučah povzročajo številne mikoze, viroze in bakterioze. Pridelovalci buč se srečujejo z vrsto bolezni med katerimi so pomembnejše 4 viroze, in sicer rumeni mozaik bučk (Zucchini yellow mosaic virus; ZYMV), mozaik lubenic (Watermelon mosaic virus; WMV), obročkasta pegavost papaje (Papaya ringspot virus; PRSV) ter kumarni mozaik (Cucumber mosaic virus; CMV). Prvi trije virusi spadajo v družino Potyviridae, medtem ko zadnji spada v družino virusov Bromoviridae (Gaba in sod., 2004). Poleg viroz se pojavlja še vrsta mikoz in bakterioz, in sicer pepelovka bučnic (*Podosphaera fusca* in *Golovinomyces orontii*), kumarna plesen (*Pseudoperonospora cubensis*), fuzarijska uvelost bučnic (*Fusarium oxysporum*), verticilijska uvelost bučnic (*Verticillium albo-atrum* in *V. dahliae*),

črna stebelna gniloba bučnic (*Didymella bryoniae*; Celar, 2000), črna pegavost bučnic (*Alternaria cucumerina*) ter bakterijski ožig bučnic, ki ga povzroča *Pseudomonas syringae* pv. *lachrymans* (Zitter in sod., 1996).

Zaradi razmeroma majhnega območja pridelave oljnih buč, omejenega zanimanja za žlahtnjenje in selekcije zelo specifičnih lastnosti je zmanjšana genetska raznolikost. Zato je za žlahtniteljske namene potrebno razširiti genski nabor. Za nekatere izmed naštetih bolezni so na voljo viri odpornosti oziroma tolerance v divjih sorodnikih in požlahtnjenih sortah (Zitter in sod., 1996; Paplomatas in sod., 2000; Cohen in sod., 2003, 2007; Pachner in Lelley, 2004; Paris in Brown, 2005; Huss in Pucher, 2007; Pachner in Lelley, 2009; Lebeda in Cohen, 2011), ki jih je mogoče uporabiti kot izhodiščne genotipe za nadaljnjo žlahtnjenje odpornejših oljnih buč. Medvstna križanja so sicer zahtevna in velikokrat neuspešna, vendar vseeno mogoča (Lelley in sod., 2009).

Biotehnologija nam, poleg tradicionalnega križanja, ponuja tudi druge načine izboljšanja lastnosti kmetijskih rastlin. Znano je, da z gojenjem v *in vitro* pogojih in z vegetativnim razmnoževanjem prihaja do somaklonske variabilnosti. To lastnost je mogoče uporabiti tudi v žlahtnjiteljske namene, saj lahko celično ali tkivno kulturo izpostavimo tudi dodatnemu selekcijskemu pritisku, npr. filtratu gliv, ki pri patogenezi izločajo toksine. Tako je mogoče izselekcionirati regenerante, ki so rezistentni oziroma tolerantni na izbrano glivo. O uporabnosti te metode poročajo Thakur in sod. (2002) ter Saxena in sod. (2008). V omenjenih raziskavah so avtorji uporabili filtrate gliv *Fusarium oxysporum* f. sp. *dianthi* in *Alternaria alternata*, katerih bližnji sorodniki so znani tudi kot povzročitelji bolezni pri bučah, tako je podobne uspehe v smislu rezistence oziroma tolerance proti izbranim patogenim organizmom mogoče pričakovati tudi v naših raziskavah. Postopek adventivne regeneracije, ki se v prvi vrsti uporablja za hitrejšo mikropropagacijo rastlin, se lahko prilagodi in uporablja tudi za študijo izzvane variabilnosti in selekcijo somaklonov. Fuzarijsko uvelost pri bučah povzroča gliva *Fusarium oxysporum*, značilna za lubenice (*Citrullus lanatus*; Zitter in sod., 1996). Bolezenska znamenja so izrazitejša v toplem vremenu (podnevi), pri višji zračni vlagi (ponoči) pa si rastlina ponovno opomore. Najprej opazimo venenje listov in kloroze, kasneje pa bolezen privede do propada rastline (Celar, 2000). Za omejevanje širjenja se priporoča predvsem kolobarjenje in fumigacija, vendar je tu problematična predvsem hitra rekolonizacija prsti z glivami. Fuzarična kislina (FA), gostiteljsko neselektiven toksin gliv rodu *Fusarium*, se uporablja za vzpostavitev selekcijskega pritiska v tkivnih kulturah za indukcijo na *Fusarium* odpornih oziroma tolerantnih mutantov (Švabova in Lebeda, 2005). Glede na to, da so bili toksini rodu *Fusarium* dokazani tudi v semenih buč (Schollenberger in sod., 2005), bi pridobljeni genotipi lahko služili tudi kot žlahtnjiteljske linije za znižanje vsebnosti teh toksinov v novih sortah, kar je izrednega pomena, saj se oljne buče uporabljajo tako za prehrano kot tudi krmo. Posebno zanimiva bi bila aplikacija selekcijskega pritiska direktno na haploidnih izsečkih hkrati s podvojevanjem genoma s pomočjo adventivne regeneracije. V doktorski nalogi smo želeli ovrednotiti učinek dodatka FA gojišču za adventivno regeneracijo na regeneracijo izsečkov in tako proučiti morebitno uporabnost FA v postopku *in vitro* selekcije.

Virus ZYMV se je prvič pojavil leta 1973 v Severni Italiji (Lisa in sod., 1981). Povzroča različna bolezenska znamenja, ki so odvisna od občutljivosti gostitelja, seva, časa okužbe

ter okolja. Bolezenska znamenja so rumenenje, temno zelene nabrekline na listni površini, pokončna rast, mozaik vzdolž žil, nazobčani listi, manj ženskih cvetov, deformirani plodovi itd. Prenaša se na nepersistenten način z rastlinskimi ušmi (*Aphis gossypii*, *Myzus persicae* itd.) in mehansko, zelo redko pa s semenom. Spada v rod Potyvirus. Njegov genom je sestavljen iz enoverižne RNA s približno 9600 nukleotidi (Desbiez in Lecoq, 1997). Kot vse druge viruse, ki se prenašajo z rastlinskimi ušmi, tudi širjenje ZYMV težko nadziramo z uporabo insekticidov in mineralnih olj, zato je najbolj obetavna uporaba odpornih sort. Znana sta znana dva vira rezistence, in sicer pri vrstah *Cucurbita ecuadorensis* in *C. moschata* (Zitter in sod., 1996). Izbor primernih izhodiščnih linij za žlahtnjenje navadno naredimo s fenotipsko oceno bolezenskih znamenj po umetni inokulaciji s patogenim organizmom in testom poljske odpornosti, vendar pri tem težko natančno ločimo med dejanskimi odpornostnimi razredi. Poleg tega ocene niso standardizirane, kar otežuje primerjavo med ocenjevalci in žlahtniteljskimi inštitucijami.

Potreba po standardizaciji in razvoju metod za natančnejšo oceno odpornosti je prisotna tudi pri drugih rastlinskih vrstah in njihovih patogenih organizmih. Tako kot virus ZYMV, tudi virus rumenenja žil kumare (Cucumber vein yellowing virus; CVYV) spada v družino Potyviridae in povzroča precejšne izpade pridelka pri kumarah in drugih vrstah družine Cucurbitaceae. Pico in sod. (2003) so s pomočjo hibridizacijskih tehnik v delno odpornih akcesijah kumar detektirali manj virusa CVYV kot v občutljivih. Za namene natančnejšega razločevanja med delno odpornimi in občutljivimi sortami kumar na virus CVYV so Pico in sod. (2005) razvili metodo absolutne kvantifikacije virusa CVYV s kvantitativno verižno reakcijo s polimerazo (qPCR). Poročajo o manjši koncentraciji virusnih delcev na rastlini v primeru odpornih genotipov v primerjavi z občutljivimi. Do podobnega zaključka so prišli tudi Gil-Salas in sod. (2009), ki so preverjali koncentracijo virusa CVYV v različnih sortah kumar na trgu po mehanski inokulaciji. Slednji so se v nasprotju s Pico in sod. (2005), ki so za kvantifikacijo uporabili SYBR Green tehnologijo, poslužili kvantifikacije s TaqMan tehnologijo in metodo priporočajo kot podporo pri žlahtnjenju za odpornost na CVYV. Poleg natančnejše razporeditve v odpornostne razrede, lahko rezultati služijo tudi za pojasnjevanje mehanizmov odpornosti ter morebitne kasnejše genetske študije. Kljub temu, da je virus ZYMV eden pomembnejših virusov pri pridelavi buč, je podobnih študij razmeroma malo. Metoda je bila prirejena in uporabljena za razlikovanje izvora omenjenega patogenega organizma v Avstraliji (Coutts in sod., 2011), pri kateri so izolate virusa razlikovali na osnovi polimorfizmov v sekvenci za plaščni protein virusa. Izvedena je bila tudi študija sinergističnih učinkov mešane okužbe virusa rumenenja in zakrnelosti buče (Cucurbit yellow stunting disorder virus; CYSDV) in virusa CVYV z virusom ZYMV (Gil-Salas in sod., 2011). Nedavno pa so s pomočjo qPCR ovrednotili tudi koncentracijo virusa ZYMV v odpornih akcesijah različnih vrst Cucurbitaceae (Svoboda in sod., 2013) ter metodo uporabili pri detekciji prenosa virusa ZYMV s semenom (Simmons in sod., 2013). Medtem ko so se Coutts in sod. (2011) ter Svoboda in sod. (2013) poslužili t.i. absolutne kvantifikacije, so Gil-Salas in sod. (2011) virusne delce kvantificirali relativno. V nasprotju z Gil-Salas in sod. (2011), ki so pri kvantifikaciji uporabili le en referenčni gen, je znano, da izbira lahko močno vpliva na dobljene rezultate in zaključke, zato je potrebno predhodno validirati stabilnost izražanja referenčnih genov pri različnih stresnih dejavnikih (Udvardi in sod., 2008). Kot izhodišče za relativno kvantifikacijo z metodo qPCR je potrebno izbrati najprimernejše referenčne gene za dano vrsto, včasih celo sorto, in eksperimentalne pogoje, ki so relevantni za raziskavo. Obrero in sod. (2011) so za

vrsto *C. pepo* objavili študijo validacije 13 referenčnih genov za različna tkiva, razvojne stopnje cveta in plodu ter stresne pogoje, med katerimi pa ne najdemo okužbe z virusi (npr. ZYMV) in drugimi patogenimi organizmi. Izbor primernih referenčnih genov bi pomembno pripomogel tako k kvantifikacijskim kot tudi ekspresijskim študijam, s katerimi bi lahko podrobneje opisali odziv rastline na virusno okužbo. Za družino Cucurbitaceae so na voljo rezultati podobnih študij odziva na biotski stres, vendar po pregledu literature ugotavljamo, da nobena ne vključuje okužbe z virusi (Kong in sod., 2014a, 2014b; Sestili in sod., 2014). Izbrane referenčne gene, ki jih odlikuje stabilno izražanje tudi v pogojih okužbe z virusom ZYMV, smo v okviru doktorske disertacije želeli uporabiti za normalizacijo podatkov pri ovrednotenju in določitvi relativne količine virusa ZYMV ter spremembe ekspresije gena za katalazo 1 (CAT1), ki naj bi bila vključen v odgovor rastline na biotski stres.

Glede na predstavljeno problematiko in področje doktorske disertacije smo si zastavili naslednje raziskovalne cilje:

- z metodo opraševanja z obsevanim pelodom in kulturo nezrelih embrijev pridobiti haploidne regenerante ter metodo optimizirati in primerjati uspešnost med različnimi genotipi golosemenskih buč,
- določiti optimalno dozo obsevanja z rentgenskimi žarki in preučiti možnost uporabe višjih doz za indukcijo haploidov,
- optimizirati postopek podvojevanja genoma s pomočjo adventivne regeneracije ter uspešnost primerjati z antimitotskimi sredstvi,
- testirati učinek FA na stopnjo regeneracije izsečkov in tako proučiti morebitno uporabnost FA v študijah izzvane variabilnosti,
- izbrati najprimernejše referenčne gene za normalizacijo relativne kvantifikacije virusa ZYMV in ekspresije gena CAT1 z metodo qPCR po umetni inokulaciji z virusom ZYMV.

Glavne predpostavke so bile sledeče:

- indukcija haploidov po partenogenetski poti bo uspešna, pri čemer bodo razlike v učinkovitosti vidne pri uporabi različnih akcesij donorjev ženskih cvetov, peloda in prejetih dozah obsevanja,
- višja zračna vlažnost med obsevanjem pelod zaščiti pred izgubo kalivosti pri daljših časih obsevanja,
- adventivna regeneracija poganjkov na gojiščih s povišano vsebnostjo citokininov vodi v podvojevanje genoma,
- stopnja endoredupliciranosti kotiledonskega izsečka vpliva na sposobnost adventivne regeneracije,
- FA kot dodatek gojiščem za regeneracijo je mogoče uporabiti za indukcijo tolerantih mutantov in v selekcijske namene za toleranco na glive rodu *Fusarium*,
- na osnovi relativne koncentracije virusa ZYMV v listih bo mogoče ločiti med tolerantnim in občutljivim genotipom oljnih buč, pri čemer bodo rastline občutljive na virus ZYMV predvidoma akumulirale več virusnih delcev.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Partenogenetska indukcija haploidov pri golosemenskih bučah s pomočjo z rentgenskimi žarki obsevanega peloda

Haploid Induction in Hull-less Seed Pumpkin through Parthenogenesis Induced by X-ray-irradiated Pollen

Kristina Košmrlj, Jana Murovec, Borut Bohanec

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Zanimanje za pridelavo oljnih buč (*Cucurbita pepo* subsp. *pepo* var. *styriaca* Greb.) narašča kot posledica vedno večjega povpraševanja po bučnem olju in uporabi semen v druge namene. Na trgu se v zadnjih letih pojavljajo hibridi, vendar do sedaj ni bila vzpostavljena metoda za indukcijo haploidov. Naše raziskave so bile usmerjene v razvoj postopka za indukcijo haploidov po partenogenetski poti, ki temelji na opraševanju s pelodom, ki je bil obsevan z rentgenskimi žarki (0, 50, 100, 150, 200, 300 in 350 Gy). Ugotovili smo, da se uspešnost oploditve in posledično tvorba plodov zmanjša pri 200 Gy, medtem ko je bilo zmanjšanje tvorbe embrijev opazno že pri 100 Gy. Med več testiranimi akcesijami smo opazili razlike v partenogenetski odzivnosti. Najbolj učinkovita je bila indukcija haploidov pri 'Turkey #2' (10,0 %), 'Gleisdorfer Ölkürbis' (4,4 %), in 'Naked Seed' (3,9 %), medtem ko sta bili akcesiji 'GL Opal' in 'White Acorn' učinkoviti kot donor peloda. S pomočjo pretočne citometrije smo ploidnost določili skupno 3830 domnevno partenogenetskim embrijem. Potrdili smo haploide, diploide, triploide in, zanimivo, tudi tetraploide, ki so se bolj pogosto pojavljali pri višjih prejetih dozah obsevanja. Spontanega podvojevanja genoma, ki bi ga zaznali kot homozigotnost na vseh lokusih, na testiranih diploidih nismo potrdili z mikrosatelitskimi markerji. Naša raziskava predstavlja prvi uspel poskus indukcije haploidov pri oljnih bučah. Poleg tega pa smo potrdili tudi uporabnost rentgenskih žarkov kot alternativo gama žarkom.

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Haploid Induction in Hull-less Seed Pumpkin through Parthenogenesis Induced by X-ray-irradiated Pollen

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Additional index words. *Cucurbita pepo* ssp. *pepo* var. *styriaca*, irradiation dose, female parent, pollen donor, flow cytometry, SSR markers

Abstract. **Production of hull-less seeds of styrian oil pumpkin (*Cucurbita pepo* ssp. *pepo* var. *styriaca*) is increasing as a result of demand for aromatic seed oil and for other uses. Hybrid cultivars have recently been released but a method for haploid induction has not been established. We focused on the development of a parthenogenetic haploid induction protocol based on pollination with pollen irradiated with X-ray radiation at 0, 50, 100, 150, 200, 300, and 350 Gy. Fruit set largely decreased at 200 Gy, whereas a decline in embryo formation was observed at 100 Gy. Various accessions were tested as the female parent or as the pollen donor and large differences were found. The best parthenogenetic response was found in 'Turkey #2' (10.0%), 'Gleisdorfer Ölkürbis' (4.4%), and 'Naked Seed' (3.9%), whereas 'GL Opal' and 'White Acorn' were efficient as pollen donors. The ploidy level of 3830 putative parthenogenetic embryos was determined using flow cytometry. Four ploidy levels (n, 2n, 3n, and 4n) were found with the majority being diploid. Interestingly, a significant proportion was determined to be tetraploid and this was clearly correlated with increased radiation delivered to pollen grains. Using selected simple sequence repeat markers on diploid embryos, no spontaneous chromosome doubling could be confirmed. In this study, haploid induction in styrian oil pumpkin was elaborated for the first time. We also showed that X-ray pollen irradiation provides an alternative to gamma radiation treatment, yielding a sufficient percentage of haploid plantlets.**

As a result of ever greater interest in the hull-less seeds of styrian oil pumpkin for oil production or roasted snacks in central Europe and elsewhere, new biotechnological methods are needed to accelerate breeding programs and offer producers modern cultivars with outstanding characteristics such as high yield, disease resistance, bush growth habit, and uniformity. The most recently released cultivars are hybrids, so the development of an efficient haploid induction protocol for creating homozygous lines is highly desirable. Inbreeding depression is well known in cucurbits (Cardoso, 2004; Stephenson et al., 2001). In addition, selection on the haploid level and the regeneration process per se might also exclude embryos possessing deleterious mutations.

Reliable protocols for haploid induction based on in situ parthenogenetic haploid induction by irradiated pollen have been published for some species of Cucurbitaceae. Obtaining haploids by in vitro rescue of parthenogenetic melon (*Cucumis melo*) embryos induced by pollination with irradiated pollen (Sauton and Dumas De Vaulx, 1987) was the first reported success in cucurbit crops. Three studies have been published on the genus *Cucurbita*. The authors used gamma ray pollen irradiation followed by in vitro culture of immature embryos (Kurtar and Balkaya, 2010; Kurtar et al., 2002, 2009). In these studies, season, maternal genotype, irradiation dose, embryo stage, and embryo type (necrotic vs. normal) are reported to be the main factors that affect haploid induction. Tested doses ranged between 25 and 400 Gy with the optimum found to be below 100 Gy. Confirmation of the ploidy level of regenerants was well described by chromosome counting, stomata and chloroplast observations, and morphological observations of plants. An irradiated pollen approach has also been found to be successful in related species such as melon (Godbole and Murthy, 2012; Gonzalo et al., 2011; Sauton and Dumas De Vaulx, 1987), cucumber [*Cucumis sativus* (Claveria et al., 2005; Przyborowski and Niemirowicz-Szczytt, 1994)], and watermelon [*Citrullus lanatus* (Sari et al., 1994)]. The gamma ray doses used in the related species were generally higher (200 to 500 Gy). In addition to gamma rays, ultraviolet irradiation and X-rays have also been used for in situ haploid induction. X-rays have proved to be efficient in melon (Katoh et al., 1993), carnation [*Dianthus caryophyllus* (Sato et al., 2000)], and pummelo [*Citrus maxima* (Yahata et al., 2010)].

The present study was designed to measure the effect of X-ray pollen irradiation on fruit set and embryo formation, and both irradiation dose and parental genotype on haploid embryo induction. Large-scale flow cytometric determination of ploidy level and an optimized protocol for simple sequence repeat (SSR) marker analysis were used to carefully examine the ploidy and homozygosity of obtained embryos.

Materials and Methods

Plant material and pollination with irradiated pollen. A total of 13 accessions (Table 1) were used in experiments conducted in 2011 and 2012. Plants were grown in spring and summer in greenhouse and open-field conditions managed using standard agronomic practices. Male and female flowers were isolated 1 d before opening as shown in Figure 1A to avoid undesirable crosses. The next morning anthers were collected,

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Table 1. List of *Cucurbita* accessions used as female parent and/or pollen donor in haploid induction experiments in 2011 and 2012.

Species	Accession no.	Accession name	Accession type	Provider[y]
C. pepo	/	Gleisdorfer Ölkürbis[z]	Cultivar	Saatzucht Gleisdorf, Austria
	/	GL Opal[z]	Hybrid	Saatzucht Gleisdorf, Austria
	/	GL Maximal[z]	Hybrid	Saatzucht Gleisdorf, Austria
	/	Gleisdorfer Diamant[z]	Hybrid	Saatzucht Gleisdorf, Austria
	/	Beppo[z]	Hybrid	Saatbau Linz, Austria
	/	Elite F1	Hybrid	Semenarna Ljubljana, Slovenia
	/	Slovenska golica[z]	Cultivar	Semenarna Ljubljana, Slovenia
	/	Rumena golica[z]	Breeding line	A. Ivančič, UniMB, Slovenia
	PI 267664	Yellow Long[z]	Uncertain	USDA
	PI 420329	Turkey #2[z]	Landrace	USDA
	PI 615102	Naked Seed[z]	Cultivar	USDA
	PI 615111	White Acorn	Cultivar	USDA
C. moschata	/	Muscade de Provence	Cultivar	Semenarna Ljubljana, Slovenia

[z]Hull-less accession.
[y]UniMB = University of Maribor, Faculty of Agriculture and Life Sciences; USDA = U.S. Department of Agriculture.

placed in petri dishes, and irradiated at different doses using an X-ray unit (RX-650; Faxitron Bioptics, Tucson, AZ). Female flowers were pollinated immediately after irradiation (from 0600 to 1030 HR) and re-isolated.

IN VITRO EMBRYO CULTURE. Immature fruit were harvested ≈4 weeks after pollination and cleaned under tap water. Seeds were extracted, surface-sterilized for 20 min using dichloroisocyanuric acid sodium salt (Acros Organics, Geel, Belgium) in a 2% solution (w/v) with Tween 20 (Sigma-Aldrich, St. Louis, MO) added as a surfactant, washed with sterilized water over a sterile stainless steel mesh, and opened aseptically in a laminar flow hood. The excised embryos were cultured on solid E20A medium (Sauton and Dumas De Vaulx, 1987) in 100-mm square petri dishes with 25 compartments (Sterilin, Newport, U.K.) at 23 °C with a 16-h photoperiod (Fig. 1B).

DETERMINATION OF PLOIDY LEVEL AND HOMOZYGOSITY TESTING. Ploidy level was determined by flow cytometry using 4',6'-diamidino-2-phenylindole (Partec, Görlitz, Germany) staining according to Bohanec (2003). Measurements were done on a CyFlow space flow cytometer (Partec) using a linear scale with a diploid *C. pepo* plant positioned at channel 200 as an external standard.

Total genomic DNA was extracted from fresh leaves of the parental plants and cotyledonary or leaf tissue of the cultured embryos using a modified cetyl trimethylammonium bromide extraction method (Kump and Javornik, 1996). Extracted DNA was resuspended in Tris-EDTA, DNA concentration was quantified by fluorimetry (Hoefer DyNA Quant 200; GE Healthcare, Little Chalfont, U.K.), and a dilution at a concentration of 5 ng·µL^{-1} was used as a template for polymerase chain reaction (PCR) amplification. Published SSR markers (Gong et al., 2008) were used to test possible homozygosity caused by spontaneous chromosome doubling. The total volume of the PCR mixture was 15 µL with 25 ng of genomic DNA, 0.5 U Taq DNA polymerase (Promega, Madison, WI), 0.2 mM of each dNTP (Sigma-Aldrich), 1× PCR buffer, 2 mM $MgCl_2$ (Promega), 0.2 µM of each primer, and 0.25 µM dye-labeled primer (6-FAM, VIC, NED, or PET; Applied Biosystems, Foster City, CA). Forward primers were designed with an M13 tail sequence added to their 5′ end (5′-TGTAAAACGACGGCCAGT-3′). Amplification of SSRs with touchdown PCR was performed according to Formisano et al. (2012). PCR products were analyzed by capillary electrophoresis (3130xl Genetic Analyzer; Applied Biosystems) with GeneScan™ 600 LIZ® (Applied Biosystems) as an internal size standard. The allele sizes were analyzed with Peak Scanner software (Version 1.0; Applied Biosystems).

Results

EFFECT OF DIFFERENT IRRADIATION DOSES ON FRUIT SET, MEAN EMBRYO NUMBER, AND HAPLOID EMBRYO INDUCTION. Optimization of a haploid induction protocol was tested in 2 consecutive years (2011 and 2012). Data were recorded for fruit set, mean embryo number per 100 seeds, and ploidy level of regenerated embryos. To test the effect of the irradiation dose on fruit set and embryo formation, 'Gleisdorfer Ölkürbis' was pollinated with pollen of 'GL Opal' irradiated at 0, 50, 100, 150, 200, 300, and 350 Gy (Table 2). In a control treatment (pollination with non-irradiated pollen), fruit set and mean number of embryos per 100 seeds were 55.6% and 73.4, respectively. A gradual decrease of both parameters was observed at increased irradiation doses. At the highest irradiation dose (350 Gy), fruit set and mean number of embryos was 25.0% and 18.8, respectively. In the next year (2012), the response to the most effective doses in terms of haploid induction (100, 200, and 300 Gy) was repeated. The ploidy level of in vitro-grown plantlets obtained in 2011 and 2012 is shown in Table 3. The results showed that the ploidy level of plantlets was haploid, diploid, triploid, or tetraploid. In both years, the highest proportion of haploids (4.4% in 2011, 1.1% in 2012) was found at 200 Gy, although the success of haploid induction was lower in 2012. Of 1397 plantlets analyzed, 12 were haploid, 1376 diploid, one triploid, and eight were tetraploid. Figures 2A and 2B show typical histograms of diploid and haploid embryos, respectively, whereas an example of a haploid sample revealing extensive endoreduplication is given in Figure 2C.

EFFECT OF FEMALE PARENT ON HAPLOID EMBRYO INDUCTION. Seven accessions ('Beppo', 'Rumena golica', 'Slovenska golica', 'Turkey #2', 'Naked Seed', 'Gleisdorfer Diamant', and 'GL Maximal') were used to evaluate whether the genetic constitution of the female parent affects the success of haploid embryo induction. Pollen of 'GL Opal' was irradiated at 200 and 300 Gy and used for pollination. Fruit set and embryo formation were observed in all tested accessions. In 2011, haploid embryos were

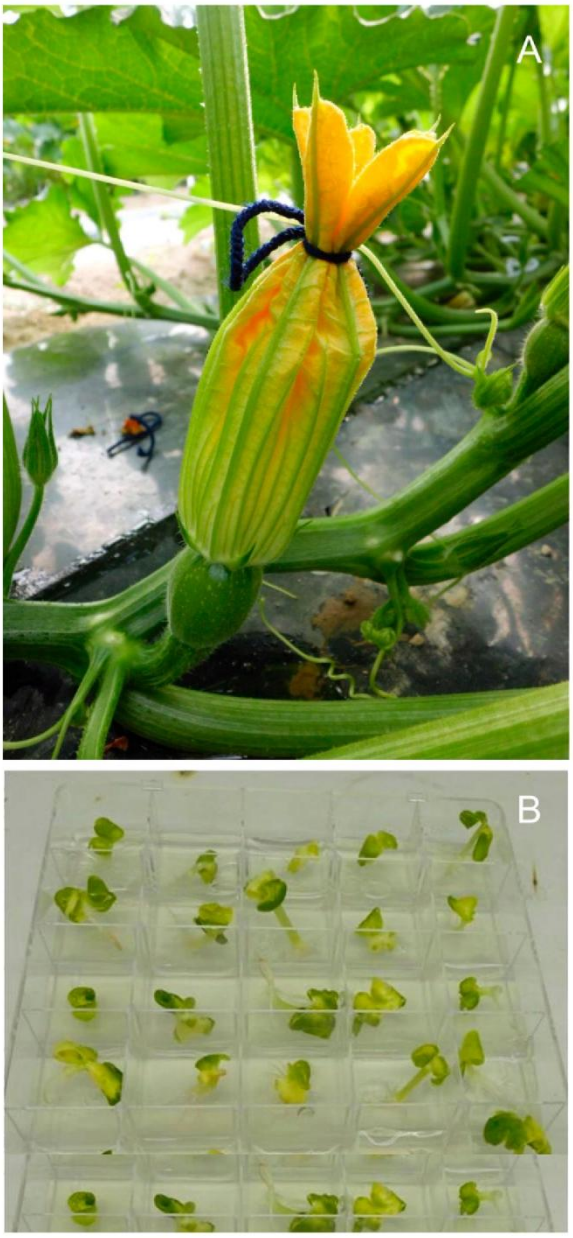

Fig. 1. Haploid induction in hull-less pumpkin. (**A**) Isolation of female flowers to avoid cross-pollination. (**B**) In vitro culture of rescued embryos on solid E20A medium (Sauton and Dumas De Vaulx, 1987).

obtained in all tested accessions except 'Slovenska golica', in which one treatment was lost as a result of contamination. The observed percentages of haploid embryos ranged from 0% in 'Rumena golica' and 'Naked Seed' to 10% in 'Turkey #2' for pollen irradiated at 200 Gy and from 0% in 'Slovenska golica' to 3.9% in 'Naked Seed' for pollen irradiated at 300 Gy (Table 4). In 2012, haploids were obtained only from 'GL Maximal', whereas 'Gleisdorfer Diamant' was not responsive in this year. Ploidy level was determined for a total of 1586 in vitro-grown plantlets. The plantlets were predominantly diploid (1549), 15 were haploid, one triploid, and 21 tetraploid. Generally, more tetraploids were found at higher irradiation doses.

Effect of pollen donor on haploid embryo induction. In 2012, pollen from five accessions ('Yellow Long', 'White Acorn', 'Elite F1', 'GL Maximal', and 'Muscade de Provence') were irradiated at 200 and 300 Gy and used for pollination of 'GL Opal' to evaluate the effect of the pollen donor on haploid embryo induction. One haploid was obtained when pollination was carried out with 'White Acorn' pollen irradiated at 300 Gy. No fruit set was observed after pollination with pollen of 'Yellow Long' irradiated at 300 Gy and 'Muscade de Provence' (cultivar of *Cucurbita moschata*) at 200 and 300 Gy (Table 5). Ploidy level was determined for a total of 847 samples, of which one was haploid, 818 diploid, and 28 tetraploid.

Homozygosity testing of diploid plantlets. For reliable testing of homo/heterozygosity, 23 published SSR markers (data not shown) were tested and six (CMTp80, CMTp88, CMTp125, CMTp142, CMTp235, and CMTp245) amplifying multiple discrete alleles were chosen for further analysis. Molecular analysis was performed on 253 diploids obtained from fruit of 'Gleisdorfer Ölkürbis', 'Beppo', and 'Turkey #2' pollinated with irradiated 'GL Opal' pollen, in which haploids were found. A list of observed SSR allele lengths at the given loci is given in Table 6. For each cross combination, an appropriate primer set was chosen based on the allelic constitution of the female parent and pollen donor. Each embryo was analyzed at least at two loci at which the pollen donor plant had at least one allele different from the female parent. To distinguish between diploids of zygotic origin and spontaneously doubled haploids (DHs), their allelic constitution was compared with the allelic constitution of parental plants; 245 (96.8%) samples showed heterozygosity at the first two loci tested, whereas eight were homozygous at both tested loci. Heterozygosity was confirmed for seven by testing an additional locus, thereby allowing 99.6% of tested samples to be confirmed as heterozygous. A single individual tested at four loci could not be determined as heterozygous but its homozygosity and thereby spontaneous chromosome doubling could also not be confirmed as a result of a lack of a completely distinct allele pattern of the parental plants. In four samples determined as heterozygous, the absence of the paternal allele was detected in one locus.

Discussion

In 'Gleisdorfer Ölkürbis', fruit set was observed at all tested irradiation doses. No fruit set was observed when 'GL Opal' was pollinated with 'Yellow Long' pollen irradiated at 300 Gy or after pollination with 'Muscade de Provence' (*C. moschata*). Although interspecific hybridization between *C. pepo* × *C. moschata* has been previously reported (Šiško et al., 2003), interspecific pollination with irradiated pollen did not induce fruit formation. For all other combinations, at least some pollinations resulted in fruit formation. Although increased X-ray doses affected the formation of embryos, some were formed even at the highest radiation treatment. These findings are in contrast with published results in *C. pepo* (Kurtar et al., 2002), in which fruit set was achieved at gamma ray doses up to 400 Gy, but no embryos were formed at doses higher than 50 Gy. However, a general decrease of both parameters was

Table 2. Effect of irradiation dose on fruit set and mean number of embryos per 100 seeds using hull-less pumpkin accessions 'GL Opal' as a pollen donor and 'Gleisdorfer Ölkürbis' as a female parent, tested in 2011.

Irradiation dose (Gy)	Flowers pollinated (no.)	Fruit set (%)	Fruit used for MNE[z] determination (no.)	MNE [mean ± SD (embryos/100 seeds)]
0	9	55.6	5	73.4 ± 23.8
50	9	55.6	5	64.1 ± 5.7
100	11	45.5	5	30.7 ± 7.3
150	10	60.0	5	31.2 ± 14.6
200	15	20.0	1	27.4[y]
300	9	33.3	3	14.8 ± 2.9
350	8	25.0	1	18.8[y]

[z]MNE = mean number of embryos per 100 seeds.
[y]SD could not be determined.

Table 3. Effect of irradiation dose on haploid induction using hull-less pumpkin accessions 'GL Opal' as a pollen donor and 'Gleisdorfer Ölkürbis' as a female parent.

Yr	Irradiation dose (Gy)	Embryos with ploidy determined (no.)	Ploidy level (%)			
			n	2n	3n	4n
2011	50	251	0.4	99.6	0	0
	100	208	1.0	99.0	0	0
	150	357	0	99.4	0.3	0.3
	200	113	4.4	95.6	0	0
	300	85	2.4	97.6	0	0
	350	53	0	98.1	0	1.9
2012	100	37	0	100	0	0
	200	175	1.1	97.1	0	1.7
	300	118	0	97.5	0	2.5

also observed at higher doses of radiation. A different situation was reported in X-ray treatment of watermelon pollen (Sugiyama and Morishita, 2000), in which fruit set was unaffected at 800 Gy, but the number of normal seeds was significantly reduced.

In 2011, most accessions tested as female parents responded positively to haploid induction by induced parthenogenesis, whereas in 2012, haploid induction rates were generally lower. A comparison is given in the case of 'Gleisdorfer Ölkürbis' and 'Gleisdorfer Diamant', which were used as female parents in both years. Lower or no response to the haploid induction treatments was observed in 2012. This variation between years can probably be attributed to weather conditions. According to data provided by the Ministry of the Environment and Spatial Planning, Environmental Agency of the Republic of Slovenia, days with maximum daily temperature of at least 30 °C occurred more often in 2012. To the best of our knowledge, there are no published studies comparing haploid induction protocol efficiencies in *Cucurbita* species for 2 consecutive years but differences in haploid induction have been found even within one growing season (Kurtar and Balkaya, 2010; Kurtar et al., 2009).

Within Cucurbitaceae, studies using both gamma (Godbole and Murthy, 2012; Gonzalo et al., 2011; Sauton and Dumas De Vaulx, 1987) and X-ray (Katoh et al., 1993) pollen irradiation are available only for melon, in which applied X-ray doses (1000 Gy) were at least twice as high as gamma ray irradiation (up to 500 Gy). In the genus *Cucurbita*, only gamma rays have

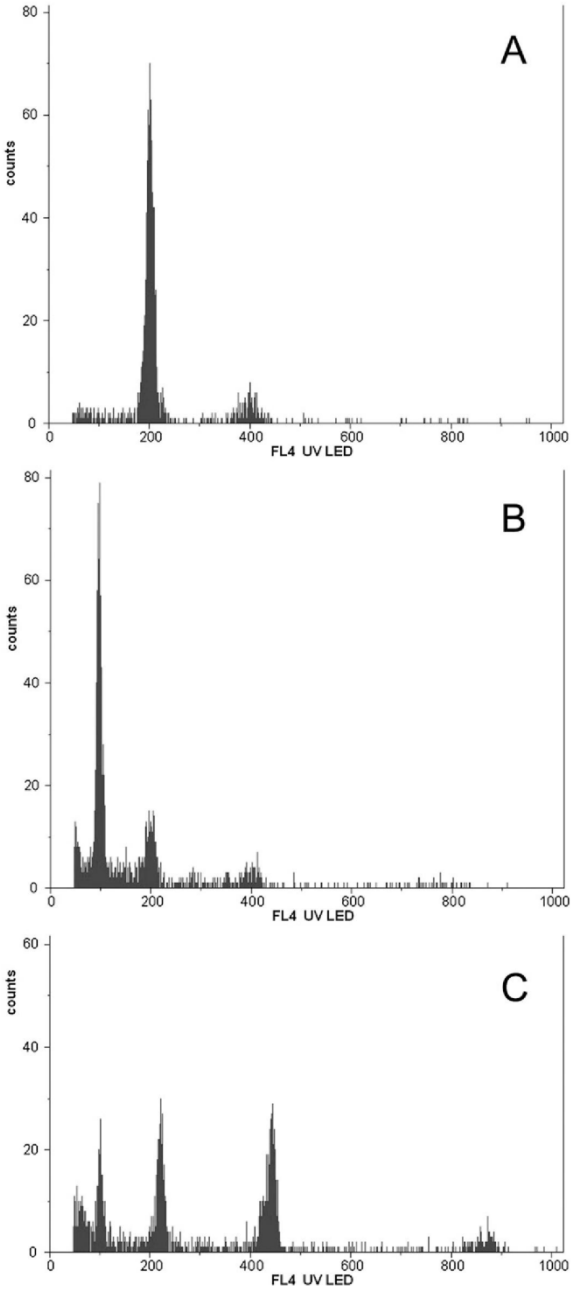

Fig. 2. Flow cytometric ploidy determination of regenerated pumpkin embryos. 4′,6′-diamidino-2-phenylindole fluorescence of nuclei (x-axis) vs. number of nuclei counted (y-axis) in (**A**) diploid, (**B**) haploid, and (**C**) haploid showing extensive endoreduplication.

so far been used and haploids were obtained only up to 100 Gy (Kurtar and Balkaya, 2010; Kurtar et al., 2002, 2009). These reports might be comparable to our results, in which different X-ray doses led to successful haploid embryo induction with 200 Gy being the most effective for 'Gleisdorfer Ölkürbis',

Table 4. Comparison of haploid induction efficiencies when using different hull-less pumpkin accessions as a female parent and 'GL Opal' as a pollen donor.

Yr	Female parent	Irradiation dose (Gy)	Embryos with ploidy determined (no.)	Ploidy level (%)			
				n	2n	3n	4n
2011	Beppo	200	114	0.9	99.1	0	0
		300	80	2.5	95	1.3	1.3
	Gleisdorfer Diamant	200	124	2.4	96.8	0	0.8
		300	84	1.2	98.8	0	0
	Naked Seed	200	58	0	100	0	0
		300	102	3.9	91.2	0	4.9
	Rumena golica	200	234	0	100	0	0
		300	96	1.0	97.9	0	1.0
	Slovenska golica	200	0[z]	/	/	/	/
		300	12	0	100	0	0
	Turkey #2	200	10	10.0	90.0	0	0
		300	72	1.4	98.6	0	0
2012	Gleisdorfer Diamant	200	202	0	98.0	0	2.0
		300	40	0	92.5	0	7.5
	GL Maximal	200	207	0	99.0	0	1.0
		300	151	0.7	96.7	0	2.6

[z]Lost as a result of contamination during in vitro culture.

Table 5. Comparison of haploid induction efficiencies when using different *Cucurbita* accessions as a pollen donor and 'GL Opal' as a female parent.

Pollen donor	Irradiation dose (Gy)	Embryos with ploidy determined (no.)	Ploidy level (%)			
			n	2n	3n	4n
Yellow Long	200	139	0	97.8	0	2.2
	300	0[z]	/	/	/	/
GL Maximal	200	161	0	98.1	0	1.9
	300	106	0	97.2	0	2.8
White Acorn	200	163	0	94.5	0	5.5
	300	61	1.6	86.9	0	11.5
Muscade de Provence	200	0[z]	/	/	/	/
	300	0[z]	/	/	/	/
Elite F1	200	94	0	100	0	0
	300	123	0	97.6	0	2.4

[z]No fruit set.

'Gleisdorfer Diamant', and 'Turkey #2', whereas other accessions ('Beppo', 'Naked Seed', 'Rumena golica', and 'GL Maximal') exhibited a better response at 300 Gy. The irradiation doses are much lower than the doses used in melon but, as summarized by Kurtar and Balkaya (2010), *Cucurbita* pollen tends to rapid viability loss as a result of dehydration and radiosensitivity during irradiation. This could be attributed to its extraordinary size (180 to 200 μm) and morphology [12 operculated pores (Nepi and Pacini, 1993)], which is not the case in other genera of this family. These characteristics might also limit the success of haploid induction.

When studying the effect of a female parent on haploid embryo induction, differences between accessions were observed. The highest percentages of haploid embryos were found in 'Turkey #2' (200 Gy) followed by 'Gleisdorfer Ölkürbis' (200 Gy) and 'Naked Seed' (300 Gy), with 10%, 4.4%, and 3.9%, respectively, although the small sample number might have led to an overestimation of the haploid percentage in 'Turkey #2'. One cultivar, Slovenska golica, remained unresponsive as a female parent to haploid induction; however, the number of measured embryos was the lowest among all tested accessions. Similar to our results obtained in hull-less pumpkin, Kurtar and Balkaya (2010) were able to obtain haploids only from certain accessions of *Cucurbita maxima*, whereas in cucumber (Claveria et al., 2005; Przyborowski and Niemirowicz-Szczytt, 1994), no significant differences among tested accessions were reported.

The effect of pollen donor on parthenogenesis has been recently studied in persian walnut [*Juglans regia* (Grouh et al., 2011)], in which one donor was found to be effective in all tested female parents, whereas the other only in some of them. A more detailed study was carried out in kiwifruit [*Actinidia deliciosa* (Pandey et al., 1990)], in which all pollen donors were found to be effective but with differences in the haploid induction efficiencies. In our experiments, only one cultivar (White Acorn) effectively induced haploids when 'GL Opal' was used as the female parent. However, 'GL Opal' used as a pollen donor was effective in all tested accessions, except 'Slovenska golica'.

Our study provides the first large-scale analysis of ploidy in putative parthenogenetic regenerants in *C. pepo*. It should be noted that, although flow cytometric determination of ploidy is undoubtedly a superior technique for ploidy analysis, it is to some extent problematic in Cucurbitaceae. As shown previously in watermelon (Sari et al., 1994) and cucumber (Gilissen et al., 1993), young tissues often exhibit endoreduplication. These additional peaks (as shown in Fig. 2C) might partly obscure the presence of the first, often smaller, G1 peak representing ploidy level. We detected four ploidy levels in this study. Although the majority of the 3830 samples tested were diploid, a significant proportion was found to be tetraploid. Determination of a relatively large proportion of tetraploids was unexpected but clearly correlated with increased dose delivered to pollen grains. Searching published data, we were unable to find similar reports of induced tetraploidy in haploid induction attempts through pseudofertilization. The mechanism of the formation of tetraploids is unclear and needs further analysis; so far we can only speculate that a zygote formed by a normal egg cell and a damaged pollen nucleus is somehow directed into early genome duplications.

Table 6. Observed allele lengths of chosen polymorphic simple sequence repeat loci in bps in tested parental hull-less pumpkin plants given by accession.

	Accession			
Locus[z]	Gleisdorfer Ölkürbis	Turkey #2	Beppo	GL Opal
CMTp80	168, 170, 172	168	170, 172	168, 170, 172
CMTp88	182, 192	182, 192	182, 192	182, 192
CMTp125	118, 128, 130	130	118, 130	128, 130
CMTp142	161, 173, 209	165	173, 183	165, 173, 183, 209
CMTp235	146, 161, 170, 173	161, 170	167, 170	164, 167, 173
CMTp245	134, 149	149	134, 149	134, 149

[z]Gong et al. (2008).

Finding an appropriate polymorphic codominant SSR marker specific for an accession suitable for homozygosity testing was a difficult task because of the high genetic similarity within the gene pool of hull-less pumpkin, which has previously been reported (Gong et al., 2012), and the low level of uniformity within accessions used, so a marker for individual plants and pollination combinations was required. Spontaneous chromosome doubling is generally regarded as a rare event in gynogenic haploid induction (Bohanec, 2009) and our decision to perform molecular analysis on a significant number of diploid plantlets was based on the complete absence of homozygosity studies during haploid (and DH) induction in *Cucurbita* sp. However, homozygosity studies of induced parthenogenetic embryos have been reported in other species of the Cucurbitaceae family. In cucumber, the authors found no spontaneously DH plants using SSR markers (Claveria et al., 2005), whereas in melon, Gonzalo et al. (2011) used SSRs and restriction fragment length polymorphism markers and found that two of 141 in vitro-rescued parthenogenetic embryos were diploid and homozygous for all the molecular markers tested. Our finding that none of the 253 tested diploid embryos could be confirmed as DH therefore contradicts the speculation of Kurtar and Balkaya (2010) that spontaneous diploidization occurs among parthenogenetic embryos of *C. maxima*. Our results show that the use of SSR markers is efficient, because the majority (96.8%) of putatively parthenogenetic embryos were discriminated by the first two loci tested, whereas three were sufficient for the determination of heterozygosity of 99.6% of embryos. An interesting observation was that the paternal SSR amplicon at one of the tested markers was missing from four heterozygous plantlets, which could be the result of mutation or chromosome deletion after irradiation or segregation of a null allele at a paralogous locus.

In the presented study, a method for haploid induction in hull-less pumpkin was elaborated for the first time. We showed that, using selected polymorphic markers, homozygosity can be efficiently tested and our results suggest that there is no further need for the testing of diploid embryos because of their zygotic origin. Rigid security regulations for gamma ray facilities limit future use of a gamma source of radiation and an increased interest in X-ray treatments is anticipated (Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, 2013). Also for this reason, the elaborated method might have broad applicability.

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2.1.2 Kalivost peloda oljnih buč pri višjih dozah obsevanja: Optimizacija *in vitro* postopka kalitve in obsevanja

Styrian oil pumpkin pollen germinability at higher irradiation doses: optimization of the *in vitro* germination protocol and irradiation procedure

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Postopki za indukcijo haploidov v družini Cucurbitaceae temeljijo na opraševanju z obsevanim pelodom, vendar so učinkovitosti nizke in večina pridobljenih embrijev je diploidnih. Že v naravnih pogojih je dolgoživost peloda vrste *Cucurbita pepo* kratka, po obsevanju pa se kalivost še dodatno zmanjša. Študija je bila usmerjena v optimizacijo gojišča za *in vitro* kalitev peloda oljnih buč (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.), ki bi omogočal zadovoljivo natančno določitev kalivosti. Ovrednotili smo vpliv pH in dodatka saharoze, manitola ter polietilenglikola Brewbaker in Kwack gojišču na kalivost peloda. 12,5 % (w/v) saharoze in pH 9 smo določili kot optimalno, medtem ko drugi testirani dodatki in koncentracije niso bile učinkovite. Preučili smo tudi vpliv zračne vlažnosti med obsevanjem (od 0 do 700 Gy) na kalivost peloda. Kalivost peloda po obsevanju pri sobni vlažnosti (angl. room humidity, RH) je bila nižja kot kalivost po obsevanju pri višji vlažnosti (angl. high humidity, HH). Meritve premera peloda so pokazale visoko variabilnost v velikosti znotraj celotne populacije peloda (premeri od 79,2 do 196,5 μm) in dve subpopulaciji. Kaleč pelod, obsevan v pogojih višje vlažnosti, je bil večji v primerjavi s kalečim pelodom, obsevanim pri sobni vlažnosti, in neobsevano kontrolo.

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Styrian oil pumpkin pollen germinability at higher irradiation doses: optimization of the in vitro germination protocol and irradiation procedure

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Abstract: Protocols for haploid induction in cucurbits are based on pollination with irradiated pollen, but the induction frequency is low and the majority of obtained embryos are zygotic. The longevity of *Cucurbita pepo* L. pollen is short even under natural conditions; following irradiation, germinability is decreased even further. This study was initiated to develop an optimal in vitro germination medium for styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) pollen, which would enable accurate germination testing. Different pH values and the addition of sucrose, mannitol, and polyethylene glycol to the Brewbaker and Kwack germination medium were tested. The optimum medium condition was pH 9 and 12.5% (w/v) sucrose, while other tested components were not efficient. Using the optimized medium, X-ray–irradiated (100–700 Gy) pollen germinability was assessed under 2 air humidity conditions. Germinability of pollen irradiated at room humidity (RH) was generally lower than that of pollen irradiated at high humidity (HH). A major variability in pollen size (diameters ranged from 79.2 to 196.5 µm) and 2 subgroups were found in the pollen population. Following irradiation, HH conditions allowed germination of larger pollen grains than those of the nonirradiated control and RH.

Key words: *Cucurbita pepo* subsp. *pepo* var. *styriaca*, media composition, X-ray irradiation, pollen diameter, haploid induction

1. Introduction

An efficient method for haploid induction is highly desirable for hybrid breeding protocols. As reviewed by Murovec and Bohanec (2011), doubled haploid technology not only helps to overcome inbreeding depression in the production of homozygous lines, but also allows the fixation of mutations and screening in the first generation after the mutagenic treatment. Pollen irradiation is applied in both haploid induction and mutation breeding. The determination of an appropriate irradiation dose delivered to pollen grains that still allows pollen tube growth and induces embryo development is therefore essential.

In *Cucurbita* spp., efficient haploid induction protocols are based on the application of irradiated pollen to the stigma, triggering parthenogenesis by pseudofertilization. For this approach, mature pollen grains are exposed to various doses of gamma ray [squash (*Cucurbita pepo* L.; Kurtar et al., 2002), pumpkin (*Cucurbita moschata* Duchesne ex. Poir.; Kurtar et al., 2009), and winter squash (*Cucurbita maxima* Duchesne ex Lam.; Kurtar and Balkaya, 2010)] or X-ray irradiation [styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.; Košmrlj et al., 2013)]. The success of haploid induction has been very limited, with the majority of rescued embryos being a result of fertilization and therefore of zygotic origin. In contrast to other plant genera, in which irradiation doses for triggering haploid induction frequently exceed 900 Gy, this is not the case in *C. pepo*. In this species, the authors generally succeeded with doses of up to 100 Gy for gamma ray and from 200 to 300 Gy for X-ray, while irradiation of pollen with higher doses resulted in minimal or no embryo formation. An exception in the family Cucurbitaceae so far is muskmelon (*Cucumis melo* L.), in which higher doses (>300 Gy; gamma ray) were efficient and resulted exclusively in haploids (Sauton and Dumas De Vaulx, 1987).

Only a few species of Cucurbitaceae have been described to have partially dehydrated pollen [watermelon (*Citrullus lanatus* (Thunb.) Matsumura & Nakai), cucumber (*Cucumis sativus* L.), red hailstone (*Thladiantha dubia* Bunge), bryony (*Bryonia dioica* Jacq.), Vietnamese gourd (*Luffa aegyptiaca* Mill.), and stuffing cucumber (*Momordica pedata* L.)] by Nepi et al. (2001) and Franchi et al. (2002), while others belong to the group of species with partially hydrated pollen, which typically results in greater vulnerability to water loss. Moreover, several other unfavorable properties of *C. pepo* pollen were identified by the same authors, causing rapid viability loss in a few

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hours after anthesis. Published attempts (Kurtar, 2009) at in vitro germination of pumpkin and winter squash pollen showed that the germinability of nonirradiated pollen is low (18.3% and 22.6%, respectively). Because of their extraordinary size (100–200 µm), the pollen grains of *C. pepo* may suffer from even greater radiosensitivity (Brewbaker and Emery, 1962).

There is no universal test to assess pollen viability, so the use of multiple tests is crucial for an accurate prediction of germinability and fertilization ability (Dafni and Firmage, 2000). For subsequent use in breeding and research of styrian oil pumpkin, optimization of the irradiation and germination protocols is highly relevant. Our goal was to optimize the composition of the in vitro pollen germination medium so as to establish an optimal viability assay. An optimized protocol was then used for the study of pollen germinability following irradiation under 2 air humidity conditions. Additionally, the pollen diameters were measured to reveal differences in the diameter in relation to germination and treatment.

2. Materials and methods

2.1. Plant material

Pollen of the hybrid styrian oil pumpkin variety GL Opal (Saatzucht Gleisdorf, Austria) was used in this study. Plants were grown in the greenhouse of the University of Ljubljana, Biotechnical Faculty, Slovenia, using standard agronomic practices. The germination tests were conducted with pollen from anthers collected in the morning (from 0600 to 0830 hours) on the day of anthesis in June and July.

2.2. In vitro pollen germination and medium optimization

The pollen germinability was assessed with hanging drop culture in liquid Brewbaker and Kwack (BK) (1963) medium. A pollen mixture from at least 2 plants was prepared and sprinkled on 25-µL drops on the petri dish cover. The cover was then inverted over the bottom half of the petri dish containing approximately 15 mL of water and sealed with Parafilm to prevent moisture loss. Each petri dish contained 3 drops of germination medium. The pollen was allowed to germinate at 23 °C in the dark for 2 to 3 h. In order to determine the optimum medium composition for pollen germination, various parameters were tested, including variations in pH values, sucrose, polyethylene glycol 4000 (PEG 4000; Duchefa Biochemie, the Netherlands), and D-Mannitol (Duchefa Biochemie) concentrations.

With the optimized medium, the germinability of pollen irradiated at different doses using an X-ray unit (RX-650; Faxitron Bioptics, USA) was evaluated. To test whether air humidity during irradiation affects germination, 2 different air humidity conditions, room humidity (RH) and high humidity (HH; achieved by placing the petri dish containing pollen in a larger petri dish filled with water), were studied.

2.3. Germination assessment

Direct microscopy (Nikon Eclipse 80i; Nikon Corporation, Japan) was used to count the number of germinated pollen grains. Pollen was accepted as germinated when the pollen tube length was at least equal to or greater than the grain diameter. The germination of pollen grains was counted on 3 different optical fields at 40× magnification in each drop. The mean value of at least 3 petri dishes (each with 3 drops) per treatment was calculated and considered to be the germination rate. In total, the germinability of at least 300 pollen grains per treatment was assessed. The diameter of pollen grains was measured using Lucia Cytogenetics 2 software (version 2.3; Laboratory Imaging, Czech Republic).

2.4. Statistical analysis

ANOVA for 2-factor experiments was performed on the irradiated pollen germination data to evaluate the impact of X-ray irradiation at 9 doses (0, 100, 200, 300, 350, 400, 500, 600, and 700 Gy) and 2 air humidity conditions (RH and HH). Duncan's multiple range test was used for the comparison of averages of germination rates between treatments at a 0.05 significance level. The empirical distribution of pollen diameter was first tested for normality with the Shapiro–Wilk normality test. The shapes of the kernel density estimation of pollen diameter distributions for different treatments were compared using the Kolmogorov–Smirnov test and the permutation test. Statistical analysis was performed using the statistical software R (http://www.R-project.org/).

3. Results

3.1. Optimization of germination media

To determine the effect of pH on germinability, BK medium with the pH value adjusted to 5, 6, 7, 8, 9, 10, and 11 was used. The highest germination rate was found in the range between pH 8 and pH 10, with the optimum being at pH 9 (Figure 1A). Using BK medium at pH 9, the influence of 3 other medium components was tested. Sucrose concentrations of 10%, 12.5%, 15%, 17.5%, and 20% (w/v) were tested. Lower concentrations were found to be superior to higher, with the optimum at 12.5% (Figure 1B). BK medium containing 10% sucrose at pH 9 was supplemented with either mannitol or PEG 4000, at concentrations of 0%, 2.5%, 5%, and 10% (w/v), and investigated. The addition of mannitol resulted in a sharp decrease in germinability, between 0% and 2.5%, while higher concentrations totally inhibited germination (Figure 1C). The addition of PEG 4000 gradually decreased germination and caused complete inhibition at the highest tested concentration (Figure 1D).

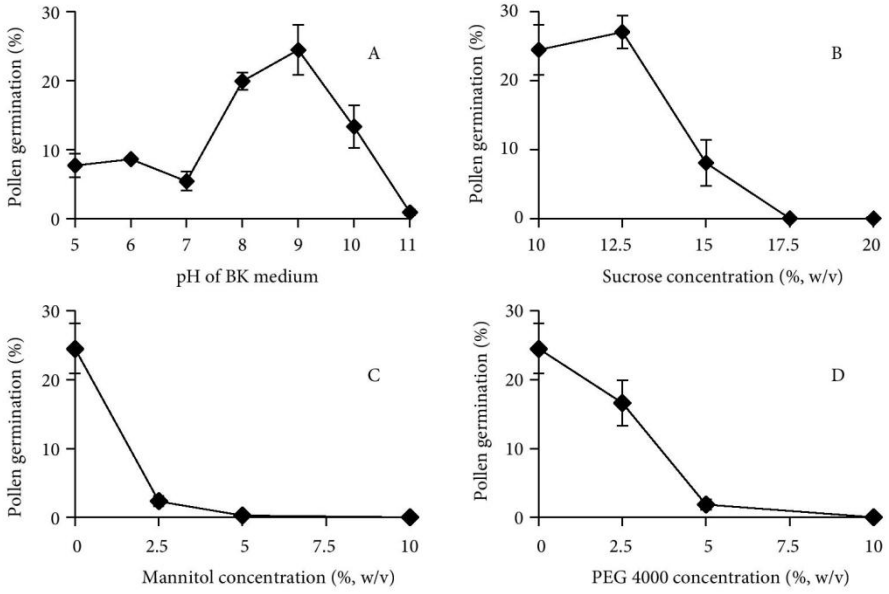

Figure 1. The effect of pH (A) and the addition of sucrose (B), mannitol (C), and PEG 4000 (D) in various concentrations to the Brewbaker and Kwack (1963) medium on the germinability of styrian oil pumpkin pollen. In variants B, C, and D, the pH was adjusted to 9. In C and D, the medium contained 10% (w/v) sucrose. Error bars indicate ±standard error.

Occasionally, polysiphony as well as pollen tube branching was observed. Figure 2 shows a typical example of such a pollen grain with 2 tubes emerging from a single grain.

3.2. Effect of different irradiation doses on germinability

The effect of the X-ray irradiation dose on pollen germination was tested at 2 air humidity conditions (RH and HH) using the previously optimized BK medium (12.5% sucrose and pH 9). Pollen of GL Opal was irradiated at 100, 200, 300, 350, 400, 500, 600, and 700 Gy under both conditions and was compared to a nonirradiated control (0 Gy). Figure 3 shows the germination rate against the irradiation dose for both humidity conditions (RH and HH). The nonparametric regression LOESS function was used for locally weighted scatter plot smoothing of the data. It is evident that the variability of the data depends on the irradiation dose and is much higher for the nonirradiated control (0 Gy) than for high doses. The data are portions of germinated pollen, so an arcsine transformation was used before ANOVA was performed. In the control treatment, germination reached 41.5 ± 1.0%. ANOVA showed that even at the lowest irradiation dose (100 Gy), pollen germination significantly decreased to 12.5% and 13.9% at RH and HH, respectively. Irrespective of treatment, an exponential decline in germinability was observed with increasing irradiation doses. The interaction between irradiation dose and treatment was statistically significant, showing a larger decline in germinability in the RH treatment than in the HH treatment at doses of ≥350 Gy, indicating less pronounced irradiation effects at higher doses at HH (Table 1).

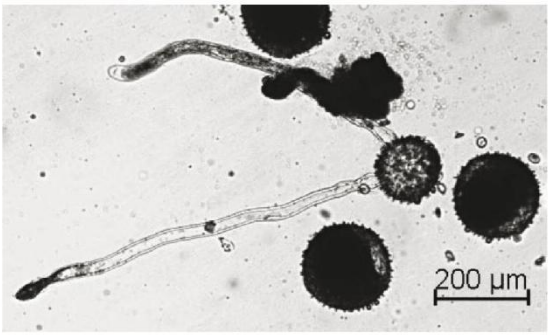

Figure 2. Germinating styrian oil pumpkin pollen grain emitting 2 pollen tubes.

3.3. Pollen diameter

The diameters in the total pollen population obtained from 366 measured pollen grains (germinating and nongerminating) in the germination medium ranged from 79.2 to 196.5 µm, with a median of 134.9 µm. The kernel density estimate of the empirical distribution of diameters revealed the existence of 2 size subgroups within the GL Opal pollen population, with the center of the second

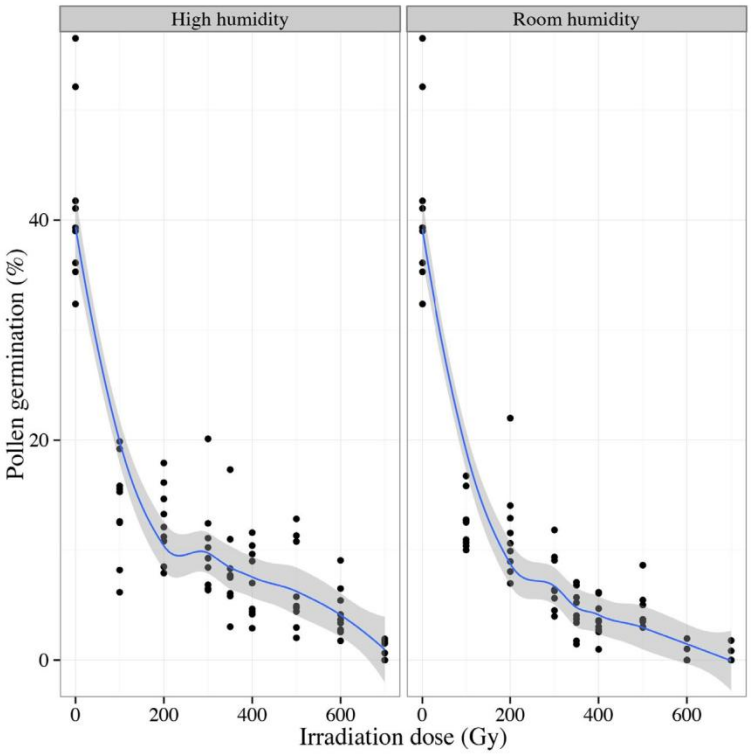

Figure 3. Comparison of the dependence of the germinability of styrian oil pumpkin pollen irradiated under high and room humidity conditions with a LOESS curve.

group around 182 µm. Germinated pollen grains were of similar diameters in the control treatment (118.8–186.6 µm) and RH treatment (107.1–184.8 µm), while pollen grains of a wider range were able to germinate following HH treatment (86.9–190.7 µm). However, germinating pollen with a diameter of greater than 170 µm was rare in all groups. A comparison of the kernel density estimation of the grain diameter distribution is shown in Figure 4. Based on the Shapiro–Wilk normality test, it can be concluded that none of the pollen diameter distributions are derived from a normally distributed population. The permutation test on the probability densities and the 2-sample Kolmogorov–Smirnov test revealed different germination responses among treatments; P-values are given in Table 2. The diameters of germinating pollen from the nonirradiated and HH group were found to differ the most ($P < 0.0001$).

4. Discussion

In vitro pollen germination was extensively studied by Brewbaker and Kwack (1963) for a number of plant species, including genera of the family Cucurbitaceae (*Cucumis*, *Lagenaria*, and *Momordica*). Although semisolid modified BK medium was used to evaluate the effects of gamma irradiation on pollen germination in 2 species of *Cucurbita* (Kurtar, 2009), the author did not provide any information on the optimization of in vitro germination conditions. Our improvement of the in vitro germination medium resulted in a high germination rate (41.5%) and was useful for the assessment of the impact of irradiation on styrian oil pumpkin pollen. In contrast with studies using sitting drop culture and semisolid medium in related species (Brewbaker and Kwack, 1963; Kurtar, 2009; Zaman, 2009), we used liquid medium in hanging drop culture based on preliminary tests, in which solid medium and sitting drop culture consistently resulted in lower germinability (data not shown). While pH value was not found to be of major importance for cucumber pollen (Vižintin and Bohanec, 2004), it seems to be essential in the genus *Cucurbita*. As with our results, Zaman (2009) concluded that pH values from 8.5 to 9 are optimal for a range of cucurbit species, whereas the germination values given in this reference appear improbable. Moreover, an optimal osmoticum concentration is crucial for maximal germination in vitro. Authors reported beneficial effects of PEG (Rihova et al., 1996; Conner, 2011) and to some

Table 1. The effect of irradiation method and dose on the germination of X-ray irradiated styrian oil pumpkin pollen.

Irradiation treatment	Irradiation dose (Gy)	Pollen germination ± SE (%)	
Nonirradiated	0	41.5 ± 1.0	a*
Room humidity	100	12.5 ± 0.4	b
	200	11.7 ± 1.0	bc
	300	7.1 ± 0.4	def
	350	4.4 ± 0.3	fg
	400	3.7 ± 0.2	g
	500	4.3 ± 0.2	fg
	600	0.3 ± 0.2	h
	700	0.3 ± 0.2	h
High humidity	100	13.9 ± 0.7	b
	200	12.5 ± 0.3	b
	300	10.1 ± 1.2	bcd
	350	8.3 ± 0.8	cde
	400	7.1 ± 0.6	def
	500	6.6 ± 0.2	efg
	600	4.4 ± 0.8	fg
	700	0.8 ± 0.1	h

*: Values with identical letters indicate no significant difference according to Duncan's multiple range test ($P < 0.05$) on arcsine square root transformed data; SE: standard error.

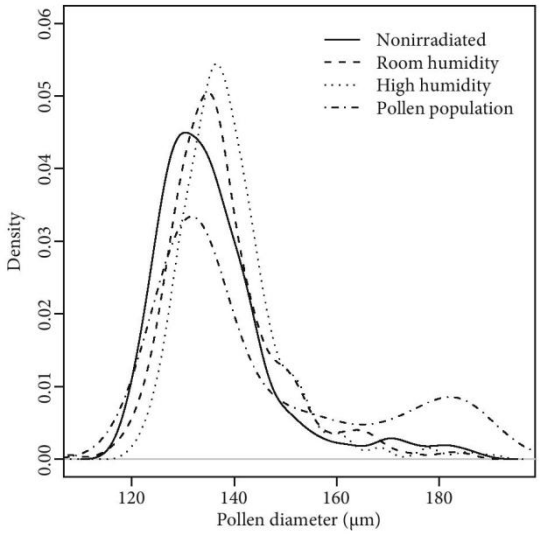

Figure 4. Comparison of the kernel density estimate for pollen diameter (µm) distributions in the GL Opal pollen population and 3 germinating pollen groups: nonirradiated, irradiated at room humidity, and high humidity.

extent also mannitol (Vasil, 1960; Rihova et al., 1996) as a substitute for sucrose or a supplement to sucrose-based medium. Based on our results, it can be concluded that the optimal sucrose concentration in liquid BK medium for styrian oil pumpkin is 12.5%, whereas the addition of PEG 4000 and mannitol is detrimental.

Several factors are known to impair *C. pepo* pollen performance in vitro and in vivo. Even under natural conditions without the application of stress, *C. pepo* pollen viability decreases rapidly. The same was shown for winter squash pollen (Agbagwa et al., 2007). The high water content (44.6%–50%) of *C. pepo* pollen at shedding (Nepi et al., 2001; Franchi et al., 2002) and high starch but low sucrose content (Speranza et al., 1997) are major causes for its vulnerability. Gay et al. (1987) observed no seed set when pollen water content fell below 24%, and, according to Brewbaker and Emery (1962), drier and larger pollen suffers from greater radiosensitivity. Due to its size and the aforementioned characteristics, a rapid decline in the germinability of *C. pepo* pollen is expected if the pollen is not somehow protected during the irradiation procedure. With these limitations in mind, it is essential to evaluate the effect of irradiation on germinability prior to haploid

Table 2. Test for equality of the diameter of germinating pollen distributions among different irradiation treatments.

Pairs of distributions of germinating pollen diameter		P-value	
		Two-sample Kolmogorov–Smirnov test	Permutation test
Nonirradiated	Room humidity	0.047	0.01
Nonirradiated	High humidity	<0.0001	<0.0001
Room humidity	High humidity	0.028	0.01

induction experiments. In our study, a significant drop in germinability was observed even at the lowest applied irradiation dose (100 Gy) in both tested methods (RH and HH) and a similar pattern was found in the fruit set and mean number of embryos per 100 seeds in a field trial using pollen irradiated under RH conditions (Košmrlj et al., 2013). Less severe but still pronounced damage by irradiation in terms of germinability has been reported in a number of species, including tomato (*Lycopersicon esculentum* L.; Akbudak and Seniz, 2009), walnut (*Juglans regia* L.; Grouh et al., 2011), *Cucumis* spp. (Denissen and Den Nijs, 1987; Cuny et al., 1993), and *Citrus* spp. (Kundu et al., 2014), as well as pumpkin and winter squash (Kurtar, 2009). Conversely, no significant decrease was observed in the germinability of pomelo pollen [*Citrus maxima* (Burm.) Merr.; Yahata et al., 2010]. Similar results were obtained by Sugiyama and Morishita (2000) in the fruit set of watermelon pollinated with soft X-ray–irradiated pollen, but the number of normal seeds still decreased with increased doses and reached 0 in 1 of the tested cultivars at 400 Gy. Assuming that fruit set follows the germinability pattern (Cuny et al., 1993), in spite of drawbacks such as low embryo number or lower fruit set, and based on our results obtained in hull-less pumpkin accessions (Košmrlj et al., 2013), in which fruits and embryos developed after pollination with pollen irradiated at 350 Gy RH, it can be concluded that when using irradiation at HH, embryos will be obtained with doses as high as 600 Gy, whereas only doses of up to 500 Gy are feasible with RH. An explanation of the observed differences between the 2 irradiation methods might be given by Nepi et al. (2010), who observed a significant decrease in the viability of *C. pepo* pollen at 30% relative humidity after 1 h, whereas this decrease occurred after 2 h if the pollen was exposed to 75% relative humidity. It therefore appears crucial to maintain high humidity during irradiation.

The kernel density estimate revealed the existence of 2 subgroups in the GL Opal pollen population. Generally, smaller pollen (near the median of the population) was more likely to germinate in vitro. However, when comparing the germinating pollen groups, a slight shift towards larger pollen grains was observed in the irradiated groups (RH and HH) and was more pronounced in the HH group. The finding of Brewbaker and Emery (1962) that larger pollen is less likely to germinate after irradiation thus might be true across species, but our results show a contrary situation within species. Similarly, Tejaswini (2002) found that in carnation (*Dianthus caryophyllus* L. and *D. chinensis* L.), in the presence of biological stress (culture filtrate of *Fusarium oxysporum* f. sp. *dianthi*), larger pollen grains exhibited higher germinability, suggesting an adaptation to environmental conditions. It can therefore be speculated that a certain diameter group of pollen might be favored in stress conditions and that a broad spectrum in pollen size serves as a survival strategy in an adverse environment.

It has been demonstrated that relatively more haploids (Chalak and Legave, 1997; Grouh et al., 2011), or even exclusively haploids (Sauton and Dumas de Vaulx, 1987), are obtained by pollination with pollen irradiated at higher doses, probably as a result of the so-called Hertwig effect, which has been shown to occur in animals (Hertwig, 1911) as well as in plants (Pandey and Phung, 1982). The haploid induction protocol for styrian oil pumpkin (Košmrlj et al., 2013) and squash (Kurtar et al., 2002), as well as for other *Cucurbita* spp. (Kurtar et al., 2009; Kurtar and Balkaya; 2010), resulted in a majority of zygotic diploids. Based on the assumption that more haploids are produced and are more likely to survive at higher irradiation doses, it is crucial to ensure pollen germinability after irradiation at higher doses in order to overcome the predominance of zygotic diploids. In conclusion, we confirmed that with a slight modification of the irradiation protocol (HH conditions), it was possible to conserve the germinability of irradiated pollen at doses as high as 600 Gy. Although not tested under field conditions, a higher frequency of haploids can now be expected, which is of great interest for breeding and research purposes. Moreover, the presented relationship between germinability and pollen diameter in *C. pepo* will allow for further studies of the application of stress and reproductive fitness under diverse environmental conditions.

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2.1.3 Adventivna regeneracija pri oljnih bučah, vpliv endoreduplikacije na sposobnost regeneracije in podvojevanje genoma na gojiščih z dodatkom fuzarične kisline

Adventitious regeneration in styrian oil pumpkin in relation to the endoreduplication pattern and induced tetraploidy on fusaric acid-supplemented media

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Plant Growth Regulation, doi: 10.1007/s10725-014-9961-5 (v tisku)

Za študij somaklonske variabilnosti oljnih buč je potrebna optimizacija postopka adventivne regeneracije. S pomočjo pretočne citometrije smo določili velike razlike v stopnji endoreduplicranosti različnih organov rastlin, to pa naj bi vplivalo tudi na sposobnost regeneracije *in vitro*. Minimalno stopnjo endoreduplicranosti smo določili v listih, medtem ko smo v drugih organih, predvsem hipokotilu in epikotilu, zaznali jedra s vsebnostjo DNA do 64C. Bazalni del kotiledona je bil večkrat potrjen kot najbolj odziven vir izsečkov za adventivno regeneracijo. S pomočjo slikovne citometrije smo le-tega določili kot najmanj endoreduplicranega z vrednostjo celičnega cikla 1,29 v primerjavi z 1,78 v centralnem oziroma 1,80 v distalnem delu kotiledona. Izsečke bazalnega dela kotiledona smo izpostavili različnim gojiščem in določili Murashige in Skoog (1962) gojišče z dodatkom 1 mg/l BA, 0,25 mg/l para-aminobenzoične kisline (PABA) in 5 mg/l FA kot optimalno (73,3 % regeneriranih izsečkov z 1,8 poganjkov na regeneriran izseček). FA je bila sicer dodana gojiščem kot potencialni selekcijski agens za študijo somaklonske variabilnosti za povišano toleranco na okužbo z glivami rodu *Fusarium*, vendar je, presenetljivo, z dodatkom v nizkih koncentracijah (5 mg/l) stimulirala regeneracijo in inducirala podvojevanje genoma v nekoliko višjih koncentracijah (10 in 20 mg/l).

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ORIGINAL PAPER

Adventitious regeneration in styrian oil pumpkin in relation to the endoreduplication pattern and induced tetraploidy on fusaric acid-supplemented media

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Abstract We attempted to improve the adventitious regeneration protocol in styrian oil pumpkin for subsequent use in somaclonal variation studies. The endoreduplication status of tissues might play an important role, so endopolyploidy was analyzed by flow cytometry in different organs and major differences were revealed. Endoreduplication was minimal in leaves but in other organs—particularly in the hypocotyl and epicotyl—levels up to 64C were found. The basal part of cotyledons has been reported to be the most responsive for adventitious regeneration and, using image cytometry, we identified it as the least endoreduplicated section, with a cycle value of 1.29 compared to 1.78 and 1.80 in the central and distal parts. Basal cotyledonary explants were subjected to various media combinations, revealing Murashige and Skoog (Physiol Plant 15:473–497, 1962, doi:10.1111/j.1399-3054.1962.tb08052.x) medium supplemented with 1 mg L^{-1} N^6-benzylaminopurine (BA), 0.25 mg L^{-1} *p*-aminobenzoic acid (PABA) and 5 mg L^{-1} fusaric acid (FA) to be optimal (73.3 % regenerated explants with 1.8 shoots per regenerating explants). FA was added to media as a possible selective agent for somaclonal variation inducing increased tolerance to *Fusarium* but was surprisingly found to stimulate regeneration at a low concentration (5 mg L^{-1}) and to induce genome doubling at medium concentrations (10 and 20 mg L^{-1}).

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Keywords *Cucurbita pepo* ssp. *pepo* var. *styriaca* · Cotyledon · Flow cytometry · Image cytometry · Somaclonal variation · Genome doubling

Introduction

Styrian oil pumpkin (*Cucurbita pepo* ssp. *pepo* var. *styriaca*) is a crop of ever greater interest in the agriculture of Central Europe and elsewhere. Despite traditional genetic improvements, little effort has been made in terms of the development of modern breeding techniques. An efficient protocol for adventitious shoot regeneration would offer a possibility of developing breeding strategies ranging from genetic transformation to somaclonal variation and in vitro selection for germplasm enhancement.

Adventitious regeneration has been studied for a range of *Cucurbita* sp., including *C. pepo* (Ananthakrishnan et al. 2003; Kathiravan et al. 2006), *C. maxima* (Lee et al. 2003), *C. moschata* (Zhang et al. 2008) and *C. ficifolia* (Kim et al. 2010). Authors have reported the most efficient shoot regeneration on Murashige and Skoog (MS) based medium (Murashige and Skoog 1962) supplemented with N^6-benzylaminopurine (BA) or zeatin (ZEA) in combination with indole-3-acetic acid (IAA). In addition to regeneration media, several other factors are known to affect the regeneration ability of *Cucurbita* sp., such as genotype (Kathiravan et al. 2006), explant age (Lee et al. 2003; Zhang et al. 2008), explant type (Ananthakrishnan et al. 2003; Lee et al. 2003; Kim et al. 2010) and endogenous hormonal contents (Zhang et al. 2008).

Endopolyploidy, the occurrence of different ploidy levels in adjacent cells within an organism, is a widespread phenomenon in seed plants. It is reported to be organ specific (Galbraith et al. 1991; Gilissen et al. 1993; Chen et al. 1994;

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Smulders et al. 1995; Barow and Meister 2003; Agullo-Anton et al. 2013) and, as reviewed by Barow (2006), appears to be crucial for fast growth and cell specialization. As determined by flow cytometry genome sizes of *Cucurbita* spp. vary (Šiško et al. 2003) but have an equal number of chromosomes, $2n = 40$ (Robinson and Decker-Walters 1997). *C. pepo* has been reported to be one of the plant species with extensive endopolyploidization (Barow and Meister 2003). It has been demonstrated in cucumber (*Cucumis sativus*; Colijn-Hooymans et al. 1994) that the regeneration of cotyledonary explants is correlated to the frequency of cells with 2C nuclei. Several factors have been studied in relation to regeneration ability but, to the best of our knowledge, no reports of the influence of endopolyploidy on regeneration in *Cucurbita* sp. have been published so far.

Adventitious regeneration is a helpful tool for micropropagation and has been used for many other purposes. Protocols can be adapted for in vitro screening and selection pressure for the development of stress tolerant plants. Fusaric acid (FA), a non-host selective *Fusarium* sp. toxin, is used for selection pressure in tissue culture in order to develop *Fusarium* resistant mutants (reviewed by Švabova and Lebeda 2005). *Fusarium* toxins have been reported to occur in pumpkin kernels (Schollenberger et al. 2005) and the obtained mutants can exhibit not only enhanced field resistance but also a lower toxin content, which is of major importance if crops are used for foodstuffs. Moreover, adventitious regeneration can be utilized for genome doubling as an alternative to treatments with toxic chemicals such as colchicine (Škof et al., 2007). Consequently, an optimized method would allow simultaneous screening, selection pressure towards *Fusarium* resistance, and diploidization, if applied on haploid embryos (Košmrlj et al. 2013), for the development of high-value doubled haploid lines for hybrid breeding.

In this study, we aimed to determine the extent of endoreduplication in different organs of styrian oil pumpkin plants and to analyze whether the site-specific endoreduplication pattern correlates with adventitious regeneration ability. In addition, we tested various culture media in order to optimize the adventitious regeneration protocol and examined whether and how fusaric acid could be utilized as a selective agent. Finally, regenerants were subjected to ploidy analysis.

Materials and methods

Flow cytometric analysis of different plant organs

Greenhouse-grown plants of the hybrid styrian oil pumpkin variety 'GL Opal' (Saatzucht Gleisdorf, Austria) were managed using standard agronomic practices. Samples of five biological replicates were collected from 1 month old seedlings from root, hypocotyl, cotyledon (distal part), epicotyl and first, second and third leaf. Samples were prepared using 4′,6′-diamidino-2-phenylindole (DAPI) staining (Bohanec 2003). The analysis was performed on a CyFlow space flow cytometer (Partec GmbH, Görlitz, Germany) using a logarithmic scale, with a 'GL Opal' leaf sample as external standard. A cycle value representing the average number of endocycles per nucleus (Barow and Meister 2003) was calculated from relative frequencies of nuclei of different endopolyploidy levels.

Seed sterilization and germination

Seeds of 'GL Opal' were surface-sterilized for 20 min using sodium dichloroisocyanurate (Sigma-Aldrich, St. Louis, MO) in a 4 % solution (w/v), with Tween 20 (Sigma-Aldrich) added as a surfactant, and washed with sterilized water over a sterile stainless steel mesh. Six seeds per tissue culture vessel were placed on MS medium including vitamins (Duchefa Biochemie B. V., Haarlem, The Netherlands) supplemented with 30 g/L sucrose and 8 g/L agar, with pH adjusted to 5.8 before autoclaving. Seeds were allowed to germinate for 7 days at 23 ± 1 °C and 16-h photoperiod.

Image cytometry of cotyledonary sections

Endopolyploidy in cotyledon cells was measured using DNA image cytometry (Vilhar et al. 2001). Cotyledons from 7 day old in vitro seedlings were dissected to basal, central and distal parts, fixed in ethanol:acetic acid (3:1) overnight at 4 °C and transferred to 96 % ethanol. The samples were stained by Feulgen reaction and nuclear DNA was measured following the protocol of Dolenc Koce et al. 2003. Briefly, samples were hydrolyzed in 5 M HCl for 1 h at 20 °C, stained with Feulgen reagent for 2 h and washed with several changes of SO_2-water. Squash preparations were prepared in 45 % acetic acid, dehydrated and mounted in DPX (Fisons Scientific Equipment, Loughborough, UK). The nuclear DNA amount was measured using the interphase-peak method (Vilhar et al. 2001), as the integrated optical density (IOD) of nuclei. IOD was measured in at least 1,000 nuclei per slide. The peaks in the IOD histograms represent ploidy levels, with the first peak representing 2C. Cycle values (Barow and Meister 2003) were calculated from the number of nuclei in different endopolyploidy classes.

Adventitious regeneration of cotyledonary explants

Cotyledonary explants were prepared from the basal third of cotyledons from 7-day old in vitro seedlings. The

Table 1 Composition of media used for adventitious regeneration of cotyledonary explants of the hybrid styrian oil pumpkin variety 'GL Opal'

Medium[a]	Concentration (mg L^{-1})					
	BA[d]	ZEA[e]	mT[f]	2iP[g]	PABA[h]	FA[i]
BA1[b]	1	–	–	–	–	–
BA12iP0.5[b]	1	–	–	0.5	–	–
BA1PABA0.25[c]	1	–	–	–	0.25	–
BA3[b]	3	–	–	–	–	–
BA5[b]	5	–	–	–	–	–
BA5PABA0.25[c]	5	–	–	–	0.25	–
MS0[b]	–	–	–	–	–	–
mT1[b]	–	–	1	–	–	–
mT3[b]	–	–	3	–	–	–
ZEA1[b]	–	1	–	–	–	–
ZEA3[b]	–	3	–	–	–	–
FA5[c]	1	–	–	–	0.25	5
FA10[c]	1	–	–	–	0.25	10
FA20[c]	1	–	–	–	0.25	20
FA30[c]	1	–	–	–	0.25	30

[a] All media contained 30 g/L sucrose, 8 g/L agar with pH adjusted to 5.8 before autoclaving

[b] MS medium including vitamins (Duchefa Biochemie B. V.)

[c] MS medium basal salt mixture (Duchefa Biochemie B. V.)

[d] BA: N^6-benzylaminopurine (Sigma-Aldrich)

[e] ZEA: Zeatin (Duchefa Biochemie B. V.)

[f] T: Meta-topolin (Duchefa Biochemie B. V.)

[g] 2iP: N^6-(2-isopentenyl)-adenine (Sigma-Aldrich)

[h] PABA: *p*-aminobenzoic acid (Sigma-Aldrich)

[i] FA: Fusaric acid (Sigma-Aldrich)

cotyledons were separated, resulting in two explants, and the apical bud was carefully removed. Three explants, with their abaxial part facing the medium, were placed on a 90 mm petri dish containing approx. 20 mL of regeneration medium (Table 1). Cultures were kept at 23 ± 1 °C and 16-h photoperiod for 3–4 weeks. Regenerated shoots were subcultured on MS medium containing 0.1 mg L^{-1} BA and kept under the aforementioned conditions for another 4 weeks before they were subjected to ploidy analysis.

Ploidy analysis of regenerated shoots

The ploidy level of regenerated shoots was determined using exclusively leaf tissue, by flow cytometry using DAPI staining (Bohanec 2003). Measurements were done on a CyFlow space flow cytometer (Partec GmbH) using a linear scale with a 'GL Opal' leaf sample as external standard.

Results

Endoreduplication is differently expressed in different plant organs

We examined the extent of endoreduplication in different organs of greenhouse-grown 'GL Opal' plants (Table 2). Major differences between organs were found, with the most endoreduplicated organ being the hypocotyl, followed by the epicotyl and cotyledon. Moreover, cotyledonary tissue showed the lowest frequency of 2C nuclei (7.8 %) among all tested organs. The least endoreduplicated cells were found in leaf tissues. In the youngest leaf (third leaf), the majority of cells contained 2C and 4C nuclei (95.4 %; 4C represents both mitotically active cells in the G2 phase and endoreduplicated nuclei with one completed endocycle). Older leaves (first and second) showed a greater extent of endoreduplication, which can be seen from a comparison of the cycle values (the higher the value, the greater the endoreduplication extent). Typical examples of the flow cytometric histograms of studied organs are shown in Fig. 1.

Endoreduplication is differently expressed within cotyledons

Our attempt to analyze cotyledonary tissue of 7-day old seedlings by flow cytometry failed, presumably due to the presence of starch and oil in partially etiolated pumpkin cotyledons, which interfered with DAPI staining. In order to measure the extent of endoreduplication in different sections of cotyledons, we used image cytometry as an alternative method. This method was found to be useful for the measurement of the amount of nuclear DNA in the studied cotyledonary tissues. The results of the analysis are shown in Table 3. The cotyledons were divided into three sections: basal, central and distal. The basal section was the least endoreduplicated (cycle value = 1.29) and contained a majority of putatively mitotically active nuclei (2C + 4C = 62.3 %). The proportion of endoreduplicated nuclei, 8C (27.5 %) and 16C (10.2 %), was the lowest among analyzed sections. Most cells in the central and distal part contained nuclei with 8C or higher DNA content. Figure 2a shows a microscopic image of nuclei varying in size and DNA content observed in cotyledonary samples. In Fig. 2b a typical example of peaks obtained by image analysis is shown.

Adventitious regeneration of cotyledonary explants: Fusaric acid promotes regeneration and induces genome doubling

Various media (Table 1) were tested for adventitious regeneration of cotyledonary explants. Shoots regenerated from the most basal part of the explants, as shown in

Table 2 Frequency of cells with different DNA contents and cycle values (average number of endocycles) in different organs of greenhouse-grown plants of the hybrid styrian oil pumpkin variety 'GL Opal' as analyzed by flow cytometry

SE standard error

Plant organ	Frequency of nuclei by DNA contents (%) ± SE						Cycle value ± SE
	2C	4C	8C	16C	32C	64C	
Root	18.9 ± 0.7	54.2 ± 1.1	23.9 ± 0.7	2.9 ± 0.4	0.1 ± 0.0	0.0 ± 0.0	1.11 ± 0.02
Hypocotyl	9.5 ± 0.8	39.9 ± 1.2	28.1 ± 0.8	14.8 ± 0.4	6.8 ± 0.3	1.0 ± 0.2	1.72 ± 0.01
Cotyledon	7.8 ± 0.6	54.7 ± 2.4	25.1 ± 1.7	11.0 ± 1.3	1.4 ± 0.6	0.0 ± 0.0	1.44 ± 0.05
Epicotyl	12.9 ± 1.1	38.9 ± 1.1	33.0 ± 0.5	13.0 ± 1.1	2.1 ± 0.3	0.1 ± 0.0	1.53 ± 0.04
First leaf	29.7 ± 2.7	63.3 ± 3.1	5.8 ± 0.9	1.0 ± 0.3	0.1 ± 0.1	0.0 ± 0.0	0.82 ± 0.04
Second leaf	35.6 ± 1.4	57.0 ± 1.3	6.0 ± 0.5	1.2 ± 0.2	0.2 ± 0.0	0.0 ± 0.0	0.79 ± 0.03
Third leaf	73.1 ± 2.3	22.3 ± 2.2	3.6 ± 0.5	0.7 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.33 ± 0.03

Fig. 3a. The percentage of explants with shoots ranged from 19.0 to 73.3 % on mT1 (meta-topolin) and FA5, respectively. The highest number of shoots per regenerating explant was found on FA10 (3.3), while mT3 was least effective, with 1.1 regenerating shoots per explant. Overall, mT was the least effective supplement, while N^6-(2-isopentenyl)-adenine (2iP), *p*-aminobenzoic acid (PABA) and FA in low concentration, promoted regeneration. The ploidy level of elongated shoots (Fig. 3b) was determined by the position of the first peak detected with flow cytometry. In total, 205 shoots were subjected to ploidy analysis and the majority was found to be diploid. Interestingly, FA not only promoted regeneration but also induced genome doubling, particularly at medium concentrations (10 and 20 mg L^{-1}). Overall, 6 samples were determined to be tetraploid. The results are shown in Table 4.

Discussion

Endopolyploidy is frequently observed in the plant kingdom and has been studied for a range of organs in various species, including *Arabidopsis thaliana* (Galbraith et al. 1991), cucumber (Gilissen et al. 1993), oilseed rape (*Brassica napus*; Chen et al. 1994), tomato (*Solanum lycopersicum*; Smulders et al. 1995) and carnation (*Dianthus caryophyllus*; Agullo-Anton et al. 2013), as well as many other seed plants (Barow and Meister 2003). Among others, the last named study analyzed organs of developed *C. pepo* plants and although this study concentrated on flower parts, it also included data for cotyledons and lower and upper leaves. Similarly to our results, a high level of endoreduplication was found in cotyledons. In developing seedlings, a comparable situation was found in cucumber plantlets (Gilissen et al. 1993; Colijn-Hooymans et al. 1994) and *A. thaliana* (Galbraith et al. 1991), in which cotyledons contained significantly fewer 2C cells than leaves. Although rather unusual in comparison to other studied seed plants, Barow and Meister (2003) reported that the lower (mature) leaf in *C. pepo* was less endoreduplicated than the upper (younger) leaf. Their finding cannot be confirmed by our data. As in our study, the hypocotyl was identified as the most endoreduplicated organ in oilseed rape (Chen et al. 1994).

Barow (2006) summarized that generally within a certain tissue of a given organ, a decreasing endopolyploidy gradient may occur from the tip to the base of an organ. In cucumber (Gilissen et al. 1993; Colijn-Hooymans et al. 1994), however, cotyledonary sections exhibited a different pattern. The frequency of 2C and 4C nuclei decreased towards the base or stayed constant but changed through developmental stages. Moreover, cotyledons of earlier developmental stages contained more 2C cells and exhibited a higher regeneration rate (Colijn-Hooymans et al. 1994). Several studies of adventitious regeneration in *Cucurbita* sp. (Ananthakrishnan et al. 2003; Lee et al. 2003; Kathiravan et al. 2006; Zhang et al. 2008; Kim et al. 2010) have reported that the highest regeneration frequency was achieved on the basal parts of the cotyledons, near the conjunction with the hypocotyl. Other tested tissues (distal parts of cotyledons, hypocotyl etc.) exhibited minimal or no regeneration response. Furthermore, the age of the cotyledonary explant has been listed as the most important factor for organogenesis in *Cucurbita* sp. (Lee et al. 2003; Zhang et al. 2008) but the endopolyploidy status of explants has not been investigated. While an endoreduplication pattern through developmental stages is yet to be determined, our results show that the basal section exhibited the lowest endoreduplication and the highest proportion of cells with 2C and 4C nuclei in comparison to other parts of the cotyledon. We therefore conclude that a lower endopolyploidy extent correlates with higher regeneration ability.

Cytokinins are reported to be required for the induction of shoots in *Cucurbita* sp. (Zhang et al. 2008; Kim et al. 2010), although regeneration was also observed on growth regulator-free media (MS0) in our study. Generally, the percentage of explants with shoots and no. of shoots per regenerating explant fell within the range of previously published studies in *C. pepo* (Ananthakrishnan et al. 2003; Kathiravan et al. 2006). Interestingly, fairly limited beneficial effects of the

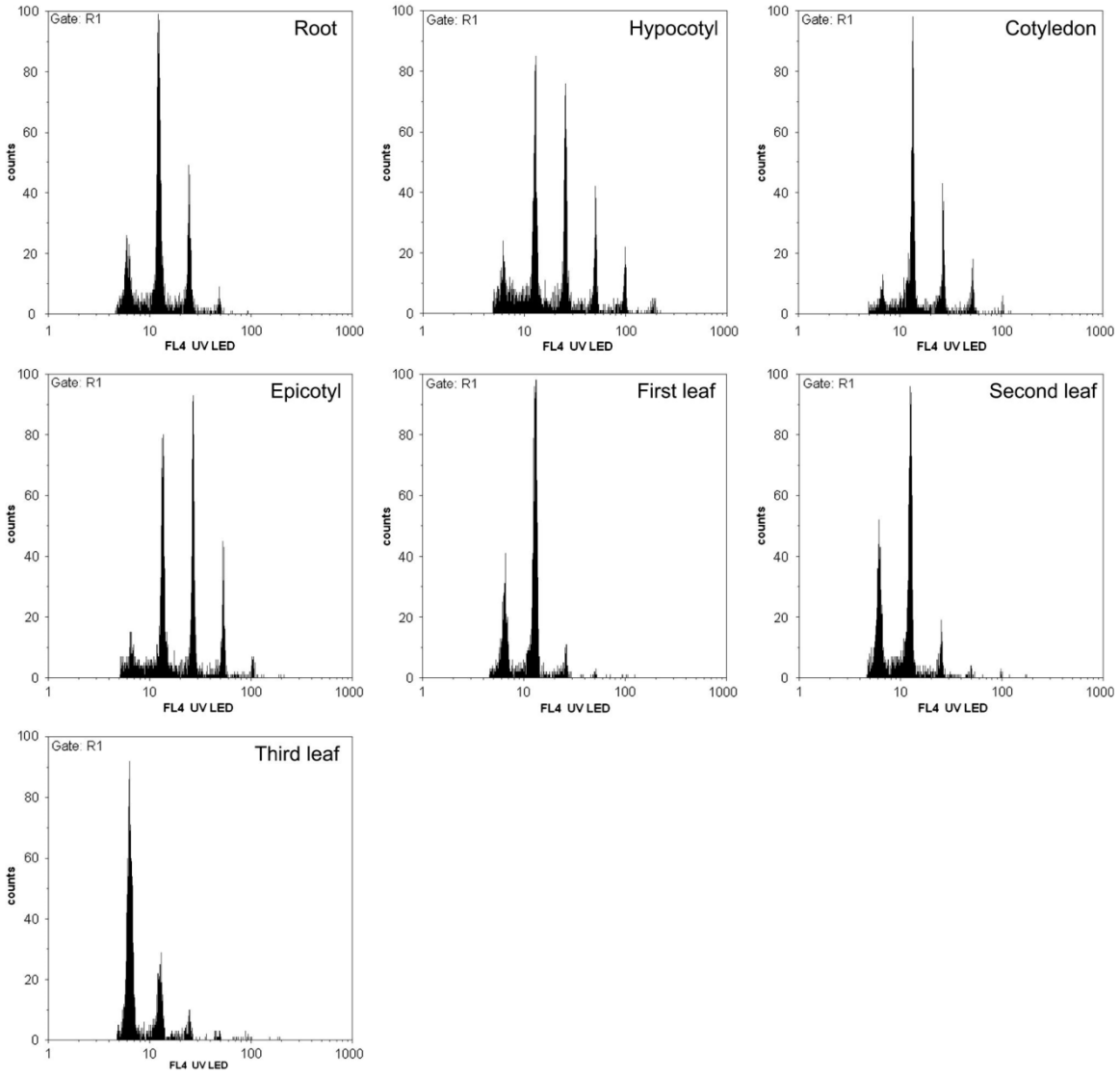

Fig. 1 Flow cytometric analysis of the endoreduplication pattern in organs of styrian oil pumpkin variety 'GL Opal'. 4′,6′-diamidino-2-phenylindole (DAPI) fluorescence of nuclei (x-axis) versus number of nuclei counted (y-axis) in root, hypocotyl, cotyledon, epicotyl, first, second and third leaf. The first peak corresponds to 2C nuclei

Table 3 Frequency of cells with different DNA contents and cycle values (average number of endocycles) in different cotyledonary sections of 7-day old in vitro seedlings of the hybrid styrian oil pumpkin variety 'GL Opal' as determined by image cytometry

Cotyledonary section	Frequency of nuclei by DNA contents (%) ± SE					Cycle value ± SE
	2C	4C	8C	16C	32C	
Basal	18.9 ± 5.5	43.4 ± 7.7	27.5 ± 9.1	10.2 ± 4.0	0.1 ± 0.1	1.29 ± 0.22
Central	7.4 ± 3.3	27.3 ± 6.7	45.2 ± 5.3	20.1 ± 6.0	0.0 ± 0.0	1.78 ± 0.17
Distal	8.3 ± 4.9	20.3 ± 4.0	54.2 ± 8.1	17.1 ± 2.7	0.1 ± 0.1	1.80 ± 0.12

SE standard error

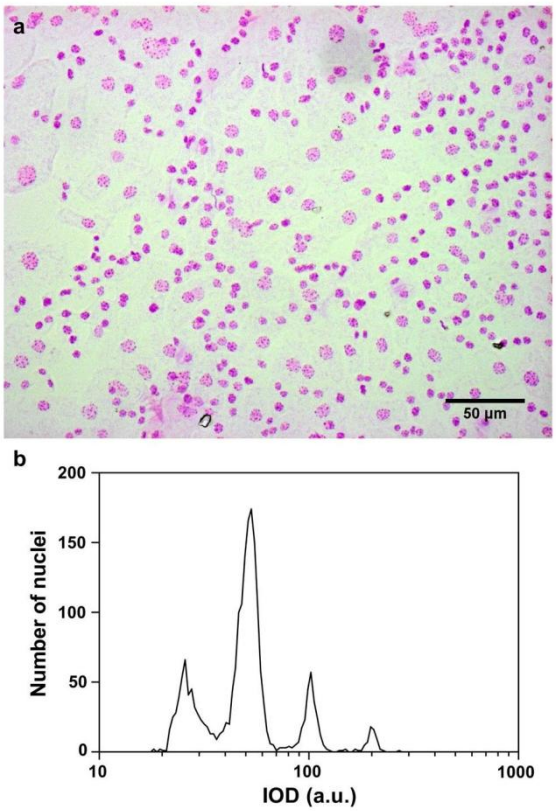

Fig. 2 Analysis of endopolyploidy in cotyledonary samples of styrian oil pumpkin. **a** Nuclei with different DNA contents in squash preparations, stained by Feulgen reaction. **b** An example of peaks obtained by measuring integrated optical density (IOD) of stained nuclei for analysis of nuclear DNA content. The first peak corresponds to 2C nuclei

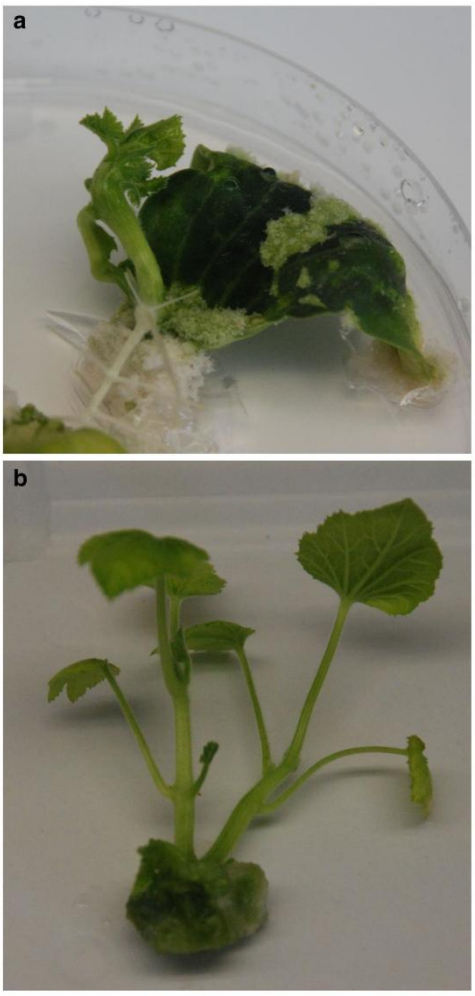

Fig. 3 Adventitious regeneration in the styrian oil pumpkin variety 'GL Opal'. Shoots regenerated at the most basal part of the cotyledonary explants (**a**) and were elongated (**b**) before leaf tissue was used for ploidy analysis

addition of cytokinins were observed, mT and ZEA even inhibited regeneration in comparison to growth regulator-free medium (MS0). We determined three supplements to the BA-based media to have a positive effect on regeneration: 2iP, PABA and FA. Adventitious regeneration has previously been shown to vary with endogenous N^6-(2-isopentenyl)-adenosine (iPA; a precursor of 2iP) content. Initial cotyledonary explants of *C. moschata* containing more iPA exhibited higher regeneration frequencies (Zhang et al. 2008) and our results show that exogenous 2iP has a similar effect. The addition of PABA resulted in increased regeneration frequencies when added to BA1 and BA5. PABA, most commonly added to tissue culture media as a vitamin source, is known to possess auxin-like properties (Pandey et al. 2010) and is structurally close to IAA. When added to a cytokinin-based medium, IAA is known to improve regeneration in Cucurbitaceae (Abrie and van Staden 2001; Kim et al. 2010; Ren et al. 2013). Surprisingly, FA added in low concentrations (5 mg L^{-1}) resulted in the highest regeneration frequency, while higher concentrations gave comparable regeneration rates to its basic media (BA1PABA0.25). For in vitro pressure and selection studies, the concentration of the selective agent (FA or fungal culture filtrate) should strongly limit survival and regeneration of explants. Furthermore, PABA, known to trigger systemic aquired resistance (Song et al. 2013), might alter the normal tissue response to FA and we therefore suggest the use of higher concentrations of FA or BA12iP0.5-based media for future somaclonal variation studies.

Table 4 Effect of various regeneration media on adventitious shoot regeneration, number of shoots per regenerating explants and ploidy of regenerated shoots in the hybrid styrian oil pumpkin variety 'GL Opal'

Regeneration medium	Explant with shoots ± SE (%)	No. of shoots per regenerating explant ± SE	No. of shoots with ploidy determined	Diploid regenerants (%)	Tetraploid regenerants (%)
BA1	38.9 ± 13.4 ab*	1.3 ± 0.1	8	100	0
BA12iP0.5	61.1 ± 13.4 ab	1.7 ± 0.2	19	100	0
BA1PABA0.25	50.0 ± 9.6 ab	2.0 ± 0.3	37	100	0
BA3	20.0 ± 13.3 a	2.0 ± 1.0	4	100	0
BA5	38.1 ± 4.8 ab	1.5 ± 0.2	12	100	0
BA5PABA0.25	50.0 ± 16.7 ab	1.8 ± 0.5	16	100	0
MS0	33.3 ± 13.6 ab	1.3 ± 0.2	12	100	0
mT1	19.0 ± 9.9 a	1.2 ± 0.2	4	100	0
mT3	20.8 ± 8.8 a	1.1 ± 0.1	5	100	0
ZEA1	33.3 ± 18.3 ab	2.7 ± 0.9	15	100	0
ZEA3	28.6 ± 13.5 ab	1.9 ± 0.7	11	100	0
FA5	73.3 ± 19.4 b	1.8 ± 0.1	20	100	0
FA10	50.0 ± 11.4 ab	3.3 ± 0.9	25	84	16
FA20	40.0 ± 19.4 ab	1.3 ± 0.3	9	78	22
FA30	33.3 ± 13.6 ab	2.3 ± 0.7	8	100	0

SE standard error

* Values with identical letters following the values indicate no significant difference according to Duncan's multiple range test ($P < 0.05$)

Somaclonal variation is defined as a genetic variation originating from tissue culture and although undesired in the case of true to type micropropagation, it can generate novel characteristics, which are the basis for genetic improvement in various species (reviewed by Bairu et al. 2011). Major drawbacks of traditional antimitotic treatments in Cucurbitaceae for doubled haploid production are mixoploid regenerants and relatively low genome doubling frequency (Lotfi et al. 2003; Claveria et al. 2005; Lim and Earle 2008). It has been shown in hop (*Humulus lupulus*; Škof et al. 2007) that this can be overcome with regeneration on media with a high cytokinin content. Polyploidization has been also observed in Cucurbitaceae following adventitious shoot regeneration (Colijn-Hooymans et al. 1994; Lee et al. 2003; Han et al. 2004; Ren et al. 2013), whereas in our study exclusively diploids regenerated on media without FA. Interestingly, tetraploids, determined as regenerants with a complete absence of diploid cells, were detected exclusively on media supplemented with FA (10 and 20 mg L^{-1}) and frequencies were comparable to those of antimitotic treatments in related species. Induced tetraploids may be produced as a result of the disruption of the microtubular mechanism or as a consequence of spliced chromosomes originating from endoreduplicated nuclei. Large proportions of endoreduplicated cells in cotyledonary tissues make this second assumption credible (Colijn-Hooymans et al. 1994). Although evidence is accumulating (reviewed by Barow and Jovtchev 2007) that chromosomes in endoreduplicated nuclei are bound together at least at centromeric regions, it is unclear whether or not such chromosomes can split to produce true polyploids. FA is associated with endogenous plant ethylene evolution (Wilson et al. 1978). Transient exposure to ethylene has been shown to increase cell DNA content up to eightfold and inhibit cytokinesis in cucumber hypocotyls (Dan et al. 2003). After removal of ethylene an exceptional burst in cell division was observed. Moreover, a companion study reported of prolonged competency of cells for division and alteration of cell fate (Kazama et al. 2004). Furthermore, using ethylene inhibitors during adventitious regeneration of bottle gourd (*Lagenaria siceraria*) cotyledons a decreasing frequency of tetraploid regenerants was observed (Han et al. 2004). These could not only explain the positive effect of FA on the regeneration frequency in our study but can also be associated with the regeneration of tetraploids. However, at this stage we can only speculate about the mode of action of FA and its involvement in polyploidization. So far, the only data indicating that FA can induce polyploidization was found in tomato (Nguyen et al. 1992). It seems that this study has never been repeated in other species but it should be noted that tomato is also known to exhibit major endoreduplication in various tissues (Smulders et al. 1995). Our finding can in any case be utilized as an alternative strategy for genome doubling in pumpkins so we are pursuing additional studies in this direction.

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Izbor stabilno izraženih referenčnih genov in kvantifikacija virusa ZYMV v okuženih listih oljnih buč s qPCR

Za natančno relativno kvantifikacijo virusa ZYMV v rastlinskem tkivu z metodo qPCR je potrebna validacija referenčnih genov pri pogojih okužbe z virusom ZYMV. V ta namen smo vzpostavili metodo mehanske inokulacije rastlin z virusom ZYMV, iz okuženih in kontrolnih (neokuženih) rastlin ekstrahirali celokupno RNA ter jo prepisali v cDNA. Ovrednotili smo primernost izbranih referenčnih genov, ki so jih Obrero in sod. (2011) objavili za vrsto *C. pepo*, Weller in sod. (2000) ter Gil-Salas in sod. (2009) pa za nekatere druge rastlinske vrste. Z izbranimi referenčnimi geni smo normalizirali podatke, ki smo jih dobili s pomnoževanjem virusa ZYMV s specifičnimi začetnimi oligonukleotidi (Svoboda in sod., 2013). Poleg tega smo ovrednotili tudi raven ekspresije gena za CAT1 (Obrero in sod., 2011), ki naj bi bil vključen v odziv rastline na stres.

2.2.1.1 Priprava rastlinskega materiala, mehanska inokulacija rastlin z virusom ZYMV in priprava cDNA vzorcev za qPCR analizo

Za poskus smo uporabili rastline sort 'GL Opal' (Saatzucht Gleisdorf Ges.mbH) in 'Gleisdorfer Ölkürbis'. Sorti se razlikujeta po stopnji odpornosti na virus ZYMV. 'GL Opal' velja za tolerantno sorto in je uvrščena v odpornostni razred 4, medtem ko 'Gleisdorfer Ölkürbis' spada med občutljive sorte z odpornostnim razredom 7 (lestvica od 0 do 9, pri čemer je 0 odporno; Sorteninformation Ölkürbis, 2014).

Semena smo posadili v lončke s prstjo ter jih skozi celotno obdobje gojili v rastni komori s 24°C in 16-urno fotoperiodo. Približno trikrat tedensko smo rastline zalivali z deionizirano vodo (dH_2O). Z namestitvijo rumenih lepljivih plošč v rastni komori smo spremljali morebiten pojav insektov, ki bi lahko služili kot prenašalci virusa. Rastline smo izolirali z vlaknasto kopreno, da bi dodatno onemogočili dostop insektom. Za okuževanje smo uporabili rastline, ki so že razvile prve prave liste (stare 11 dni za sorto 'GL Opal' in 26 dni za sorto 'Gleisdorfer Ölkürbis').

Za okuževanje z virusom ZYMV smo uporabili postopek mehanske inokulacije rastlin s karborundom. Kot vir virusa ZYMV smo uporabili homogenizirano listno tkivo oljnih buč, na katerih so bila vidna tipična bolezenska znamenja okužbe z virusom ZYMV (Slika 1A) in na katerih je bila z encimskoimunskim testom (ELISA) ter elektronsko mikroskopijo potrjena le prisotnost virusa ZYMV med testiranimi rastlinskimi virusi (CMV, PRSV, virus mozaika buč (Squash mosaic virus; SqMV), virus nekroze tobaka (Tobacco necrosis virus; TNV), WMV, ZYMV). Detekcija in potrjevanje prisotnosti virusa ZYMV je bila izvedena po standardnih postopkih preskusnega laboratorija Oddelka za biotehnologijo in sistemsko biologijo Nacionalnega inštituta za biologijo (Ljubljana, Slovenija). Vzorce listov smo od vzorčenja na poskusnem polju Fakultete za kmetijstvo in biosistemske vede (Univerza v Mariboru, Pivola, Slovenija; julij 2013) do okuževanja (oktober 2013) shranjevali pri -20°C. Sok za mehansko inokulacijo smo pripravili s homogenizacijo 1 g okuženega rastlinskega tkiva v 10 ml 0.01 M fosfatnega pufra s pH 7,2. Rastline smo 24 h

pred okuževanjem gojili ob šibki osvetlitvi. Kotiledone testnih rastlin smo posuli s karborundom (Carborundum Technical 0.037 mm; VWR International, Avstrija) ter jih premazali z homogenizatom okuženih listov. Nekaj minut po inokulaciji smo rastline sprali z dH_2O. Kontrolne rastline smo tretirali enako, le da smo namesto homogenizata listov na kotiledone nanesli le fosfatni pufer. Po okuževanju smo kontrolne in testne rastline gojili na ločenih policah in izolirane z vlaknasto kopreno 21 dni do odvzema vzorcev za ekstrakcijo RNA.

21 dni po mehanski inokulaciji smo iz kontrolnih in testnih rastlin izolirali RNA iz 100 do 150 mg tkiva mladih listov s Spectrum Plant Total RNA Extraction Kit (Sigma-Aldrich, ZDA) po navodilih proizvajalca. V izogib degradaciji RNA smo listno tkivo, ki je bilo odvzeto z rastline, nemudoma prenesli v terilnico in dodali raztopino Lysis solution. Vzorce za morebitno ponovitev poskusa in nadaljnje raziskave smo vzorčili ločeno. Rastlinsko tkivo smo najprej takoj zamrznili v tekočem dušiku in ga nato shranili pri temperaturi -80°C. Po ekstrakciji smo izmerili koncentracijo in čistost RNA (razmerje absorpcije pri 260 in 280 nm) s spektrofotometrom NanoVue (GE Healthcare, Združeno kraljestvo Velike Britanije in Severne Irske) ter jo hranili pri -80°C. Prepis 1000 ng RNA v cDNA smo izvedli s kitom High capacity cDNA Reverse Transcription Kit (Applied Biosystems, ZDA) po navodilih proizvajalca. Kit vsebuje naključno prilegajoče heksamerne začetne oligonukleotide in inhibitor RNaz. RT-PCR reakcija je potekala pri 25°C 10 min, 37°C 60 min, 85°C 5 s in 4°C ∞. Pridobljeno cDNA smo hranili pri -20°C.

2.2.1.2 qPCR in analiza pridobljenih rezultatov pomnoževanja

Iz literature smo izbrali 9 potencialnih referenčnih genov, ki so bili uporabljeni za ekspresijske in kvantifikacijske študije pri *C. pepo* ter drugih vrstah. Z uporabo stabilnih referenčnih genov smo želeli normalizirati podatke relativne kvantifikacije virusa ZYMV. V študijo ekspresijske analize smo vključili tudi gen, ki naj bi sodeloval pri odgovoru rastline na stres (CAT1). Podatki o izbranih tarčnih in referenčnih genih so zbrani v Preglednici 1.

Preglednica 1: Izbor referenčnih in tarčnih genov s pripadajočimi sekvencami začetnih oligonukleotidov, uporabljenih v qPCR analizi.
Table 1: List of reference and target genes and their primer sequences used in qPCR analysis.

Okrajšava	**Akcesijska številka v bazi GenBank**	**Ime gena**	**Sekvenci začetnih oligonukleotidov (forward/reverse; 5'-3')**
RPL36aA[a]	HM594174	60S ribosomal protein L36a/L44	GATAGTCTTGCTGCACAGGGAAA GGTCTGACCTCCATATCCTGATTG
ACT[a]	HM594170	Actin	CCTCTCAATCCCAAAGCTAACAG CGGCCTGGATAGCAACATACA
UFP[a]	CD726808	Ubiquitin fusion protein	CGGACCAGCAGAGGCTTATC GAGAGTTCGCCCATCCTCAA
PP2A[a]	HM594171	Protein phosphatase 2A regulatory subunit A	TGGTAGCATCCTTTCCCAATACA CATGCCCGTTCAGCTTTAGC
EF-1A[a]	HO702383	Elongation factor-1α	GCTTGGGTGCTCGACAAACT TCCACAGAGCAATGTCAATGG
CAC[a]	HM594173	Clathrin adaptor complexes medium subunit	GGACAAACAGAACCAACCATGA GGTTTCCTTTCCGTCACTGTAGA
HELI[a]	HM594176	DEAD-box RNA helicase-like protein	ACACTGGTCCCTCCCACACA GCGGGCACTTGGAGATTATC
CUC18S[b]	AF206894	18S ribosomal RNA	GGCGGATGTTGCTTTAAGGA GTGGTGCCCTTCCGTCAAT
COX[c]	/	Cytochrome oxidase	CGTCGCATTCCAGATTATCCA CAACTACGGATATATAAGAGCCAAAACTG
CAT1[a]	D55645	Catalase 1	GTCACCCATGAGATCCGCA CCAAGAGACCTATCCGCCTG
ZYMV[d]	DQ144054[e]	ZYMV coat protein	AAACCAATGGCTATCCAGATTTG CCATGAATTGTGCAGTTGCTTT

[a] Obrero in sod. (2011), določeno za vrsto *Cucurbita pepo*
[b] Gil-Salas in sod. (2009), določeno za vrsto *Cucumis sativus*
[c] Weller in sod. (2000), določeno za vrsto *Solanum tuberosum*
[d] Svoboda in sod. (2013), določeno za virus Zucchini yellow mosaic virus
[e] Začetni oligonukleotidi dizajnirani tudi na osnovi homologije z drugimi sekvencami virusa ZYMV (akcesijske številke DQ144061, DQ144055, DQ124244, DQ144062, DQ12445, DQ144063, DQ144056, DQ124246 v bazi GenBank)

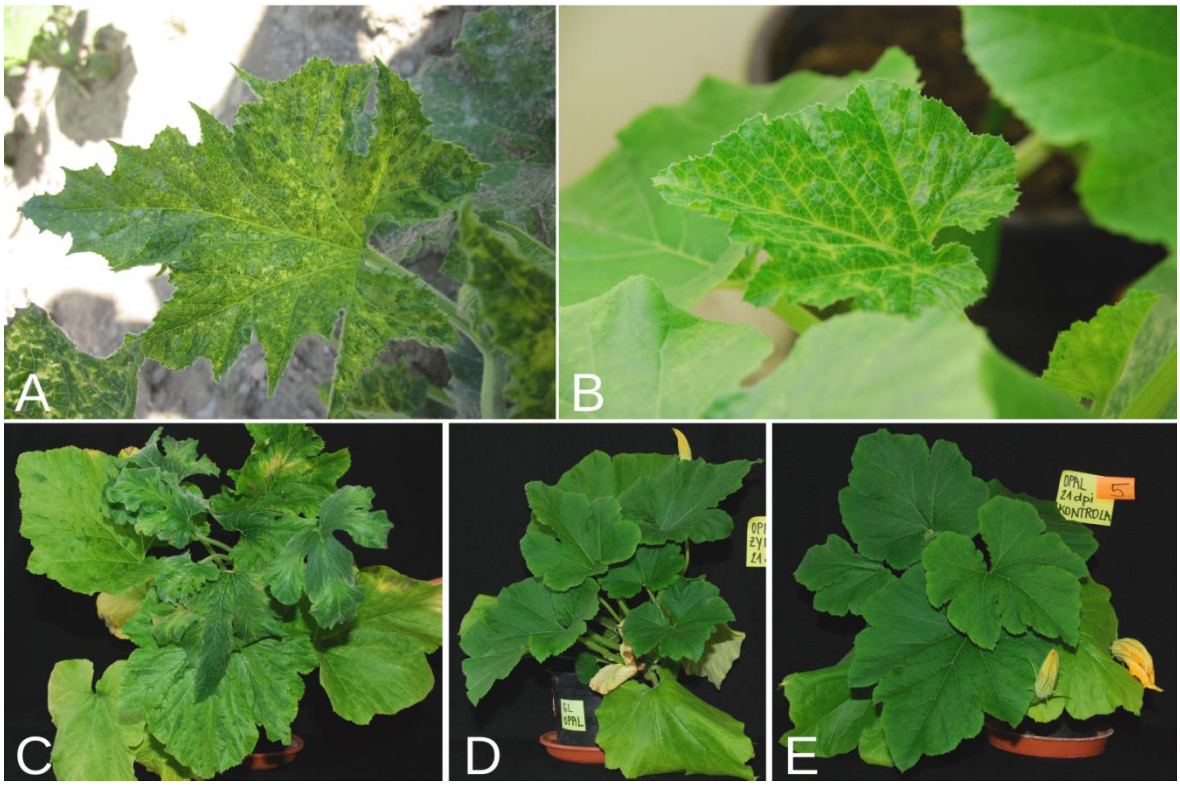
Analiza referenčnih in tarčnih genov je bila izvedena z uporabo Fast SYBR Green tehnologije na napravi ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Reakcija je potekala na optičnih ploščah za 96 PCR reakcij pri čemer je vsak vzorec vseboval naslednje komponente: 0,6 do 1,9 ng cDNA, 5 µl FastStart Universal SYBR Green Master (Rox) mešanice (Roche, Švica) in 0,6 µl (300 nM) vsakega začetnega oligonukleotida. Reakcija qPCR je potekala v naslednjih korakih: 10 min pri 95°C in 40 ciklov pri 95°C za 10 s ter 30 s pri 60°C. Program je vključeval tudi analizo disociacijske krivulje s temperaturnim gradientom od 60 do 95°C za preverjanje specifičnosti namnoževanja. Vsak vzorec smo na plošče nanesli v 3 tehničnih ponovitvah ter v poskus vsakič vključili vsaj 3 slepe vzorce, ki so namesto vzorca cDNA vsebovali sterilno dH_2O. Slednji so služili kot kontrola navzkrižne kontaminacije reakcijske mešanice in zaznavi

morebitne tvorbe dimerov začetnih oligonukleotidov. Pomnoževanje amplikona je vedno potekalo ob prisotnosti standardne krivulje iz ustreznih redčitev združenih vzorcev (opis v nadaljevanju) na isti plošči.

Učinkovitost pomnoževanja, ki je definirana z naklonom regresijske premice standardne krivulje, smo določili za vsak amplikon posebej, in sicer s pomočjo zaporednih štirikratnih redčitev združenih cDNA vzorcev v poskusu (12,5, 3,12, 0,8, 0,2, 0,05 ng itd.) ter izračuna v programu ABI 7500 SDS 2.0.4 (Applied Biosystems). Amplikon, katerega učinkovitost pomnoževanja ni bila v območju od 90 do 110 % za testirano redčitveno vrsto, je bil izključen iz nadaljnje analize. Analizo stabilnosti izražanja referenčnih genov smo izvedli s pomočjo programa RefFinder (Xie in sod., 2011), ki vključuje 4 algoritme (primerjalno delta Ct metodo (Silver in sod., 2006), BestKeeper (Pfaffl in sod., 2004), NormFinder (Andersen in sod., 2004) in geNorm (Vandesompele in sod., 2002)). Program izračuna tudi lestvico na osnovi vseh uporabljenih algoritmov (t.i. skupna razvrstitev). Število referenčnih genov potrebnih za normalizacijo podatkov ekspresije gena CAT1 in kvantifikacije virusa ZYMV smo določili z algoritmom geNorm na osnovi izračunane M vrednosti, ki opisuje povprečno stabilnost ekspresije gena. Nizka M vrednost ($\leq 0,5$) je indikator stabilne ekspresije danega gena (Hellemans in sod., 2007). Minimalno število referenčnih genov za normalizacijo se določi na osnovi parne variacije (V_n/V_{n+1}) med normalizacijskim faktorjem NF_n in normalizacijskim faktorjem NF_{n+1}. Parna variacija opiše efekt dodatnega referenčnega gena na normalizacijski faktor. Kombinacija referenčnih genov, katerih parna variacija je pod 0,15, je uporabna v kvantifikacijskih in ekspresijskih študijah (Vandesompele in sod., 2002). Razlike v ekspresiji gena CAT1 in relativno količino virusa ZYMV smo izračunali v programu Microsoft Excel (Microsoft, ZDA) po metodi delta-delta-Ct ($\Delta\Delta$Ct). Kot kalibrator smo v primeru CAT1 uporabili povprečje kontrolnih vzorcev dane sorte, medtem ko smo pri ZYMV za kalibracijo uporabili vzorec, pri katerem je bil določen najvišji povprečni pražni cikel (angl. threshold cycle; Ct vrednost).

2.2.1.3 Rezultati preliminarnega poskusa mehanske inokulacije z virusom ZYMV

Poskus mehanske inokulacije rastlin z virusom ZYMV je bil uspešen, saj smo na inokuliranih rastlinah zaznali bolezenska znamenja, značilna za virus ZYMV. Prva bolezenska znamenja so bila opazna približno 1 teden po inokulaciji (Slika 1B). Izmed 6 inokuliranih rastlin sorte 'GL Opal' so po 21 dneh 3 razvile izrazita bolezenska znamenja okužbe z virusom ZYMV. Podobno je bilo pri sorti 'Gleisdorfer Ölkürbis', kjer sta 2 izmed 4 razvili izrazita bolezenska znamenja. Rastline z izrazitimi bolezenskimi znamenji so v nadaljevanju poimenovane »simptomatske« (Slika 1C). Ostale inokulirane rastline so razvile šibka oziroma neizrazita znamenja (v nadaljevanju poimenovane »asimptomatske«; Slika 1D). Na kontrolnih rastlinah (4 sorte 'GL Opal' in 3 sorte 'Gleisdorfer Ölkürbis') bolezenskih znamenj nismo zaznali (Slika 1E). Obseg poskusa je zadoščal za izbor stabilnih referenčnih genov in preliminarno študijo kvantifikacije virusa ZYMV in ekspresijo gena CAT1. Vzorčenje za ekstrakcijo RNA smo izvedli 21 dni po inokulaciji. Iz zbranega rastlinskega materiala smo uspeli pridobiti RNA primerne čistosti za analizo z metodo qPCR. Koncentracije ekstrahirane RNA so bile v območju od 332 do 2435 ng/µl. Vrednosti A260/A280, ki opisujejo čistost RNA, so bile v območju od 1,8 do 2,2.



Slika 1: Mehanska inokulacija oljnih buč z virusom ZYMV. Za inokulacijo smo uporabili homogenizirano listno tkivo oljnih buč, ki so kazale tipična bolezenska znamenja okužbe z virusom ZYMV (A). Na inokuliranih rastlinah so se prva bolezenska znamenja pojavila približno 1 teden po inokulaciji na mladih listih (B). Vzorčenje za ekstrakcijo RNA smo izvedli 21 dni po inokulaciji. Inokulirane rastline so razvile izrazita bolezenska znamenja (C) ali šibka oziroma le-ta niso bila izražena (D). Kontrolne rastline niso kazale bolezenskih znamenj (E).

Figure 1: Mechanical inoculation of styrian oil pumpkin with ZYMV. For inoculation homogenized leaf tissue of plants showing typical disease symptoms was used (A). First mosaic symptoms were observed approximately 7 days post inoculation (dpi) on young leaves (B). At the time of sampling for RNA extraction (21 dpi) different stages of disease development were observed. In (C) a typical example of severe symptoms is shown, whereas in (D) a plant with mild or non-existent symptoms can be seen. The control plants remained symptomless (E).

2.2.1.4 Rezultati validacije referenčnih genov

Referenčni geni s primerno učinkovitostjo pomnoževanja (90 do 110 %) so bili izbrani za analizo stabilnosti izražanja. Iz nadaljnjih eksperimentov je bil tako izključen amplikon gena CUC18S, saj je bila učinkovitost pomnoževanja kljub visoki redčitvi vzorcev prenizka. Učinkovitosti pomnoževanja ostalih referenčnih genov in razponi Ct vrednosti so navedene v Preglednici 2. Vsi referenčni geni so se namnožili v amplikon z enojno disociacijsko krivuljo.

Preglednica 2: Učinkovitosti pomnoževanja amplikonov in Ct vrednosti referenčnih genov, pridobljene z analizo qPCR.
Table 2: Efficiencies and Ct values of the amplification of reference genes obtained by qPCR analysis.

Okrajšava	**Učinkovitost pomnoževanja (%)**	**Ct vrednosti**
RPL36aA	94,5	23,3 – 26,7
ACT	98,7	23,9 – 28,7
UFP	100,2	22,9 – 26,4
PP2A	100,2	25,1 – 28,4
EF-1A	99,7	21,0 – 24,8
CAC	92,2	27,8 – 30,6
HELI	94,1	29,3 – 31,1
COX	91,5	21,8 – 24,7

S programom RefFinder (Xie in sod., 2011) smo referenčne gene razvrstili po stabilnosti z različnimi algoritmi. Končna razporeditev genov je prikazana v Preglednici 3. Vsi uporabljeni algoritmi so pokazali, da sta COX in ACT najmanj stabilna gena. V skupni razvrstitvi so se kot najbolj stabilni izkazali UFP, PP2A, CAC in EF-1A. Splošno so končne razvrstitve referenčnih genov različnih algoritmov podobne, izstopajo le rezultati analize z algoritmom BestKeeper.

Preglednica 3: Razpored referenčnih genov s programom RefFinder (Xie in sod., 2011), pri čemer stabilno izražene referenčne gene najdemo na vrhu posameznih lestvic, medtem ko so manj stabilni na dnu.
Table 3: Final ranking of reference genes using RefFinder software (Xie et al., 2011). Stability decreases from top to bottom.

Delta Ct metoda		**BestKeeper**		**NormFinder**		**geNorm**		**Skupna razvrstitev**	
Okrajšava	**Povpr. Stdev[a]**	**Okrajšava**	**Stdev[b]**	**Okrajšava**	**Stabilnost**	**Okrajšava**	**M vrednost**	**Okrajšava**	**Geo. sred.[c]**
PP2A	0,53	HELI	0,35	PP2A	0,236	EF-1A	0,272	UFP	2,06
CAC	0,55	UFP	0,51	CAC	0,279	UFP	0,272	PP2A	2,21
UFP	0,56	RPL36aA	0,53	UFP	0,34	RPL36aA	0,326	CAC	2,99
EF-1A	0,57	CAC	0,55	RPL36aA	0,353	PP2A	0,435	EF-1A	3,16
RPL36aA	0,57	EF-1A	0,6	EF-1A	0,356	CAC	0,451	RPL36aA	3,66
HELI	0,71	PP2A	0,6	HELI	0,56	HELI	0,518	HELI	3,83
COX	0,78	COX	0,64	COX	0,68	COX	0,579	COX	7
ACT	0,8	ACT	0,92	ACT	0,694	ACT	0,634	ACT	8

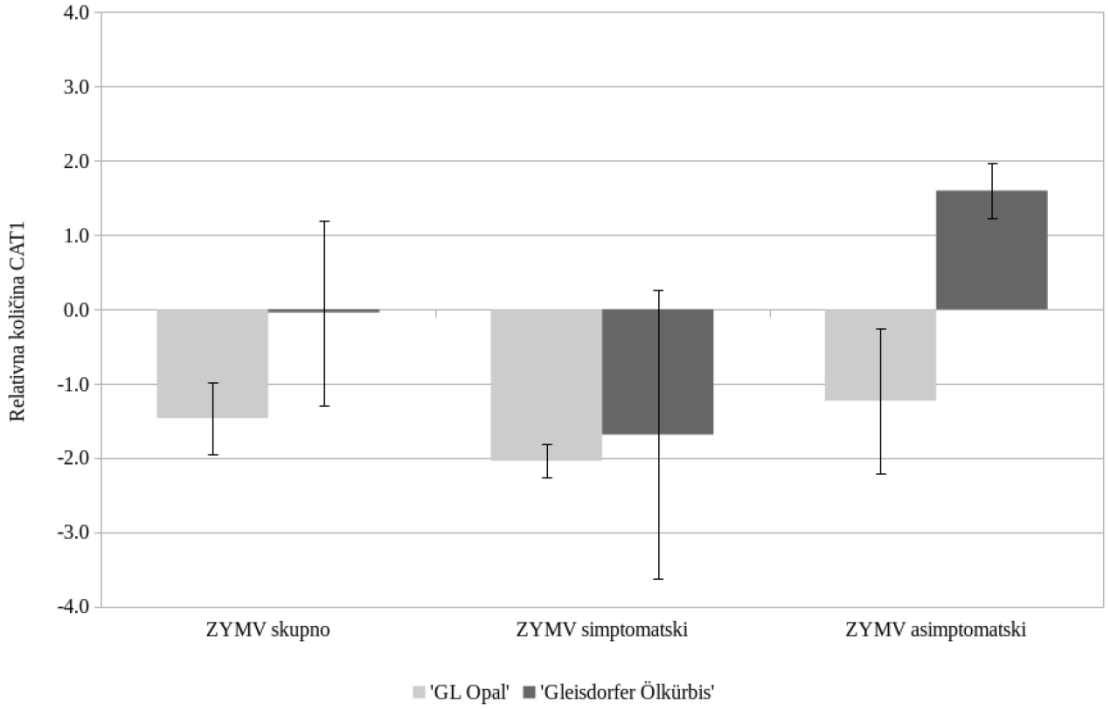
[a] Povprečje standardnih odklonov
[b] Standardni odklon
[c] Geometrična sredina je izračunana iz mest na lestvicah posameznih algoritmov

Za izbor in določitev optimalnega števila referenčnih genov za zanesljivo normalizacijo smo uporabili algoritem geNorm. Analiza parne variacije (V_n/V_{n+1}) je pokazala, da je optimalno število referenčnih genov v danih eksperimentalnih pogojih 2. Pri primerjavi normalizacijskih faktorjev 2 in 3 najbolj stabilnih referenčnih genov smo namreč določili

parno variacijo V < 0,15. Tako smo v nadaljnjih analizah ekspresije gena CAT1 in kvantifikacije virusa ZYMV kot normalizacijski faktor uporabili geometrično sredino referenčnih genov EF-1A in UFP.

2.2.1.5 Preliminarni poskus primerjave ekspresije gena CAT1 v občutljivi in tolerantni sorti oljnih buč

Učinkovitost pomnoževanja amplikona gena CAT1 z metodo qPCR je bila 91,3 %. Analiza disociacijske krivulje je pokazala le en produkt pomnoževanja. Ct vrednosti kontrolnih vzorcev sorte 'GL Opal' so bile v razponu od 22,0 do 24,8, medtem ko je bil razpon pri simptomatskih vzorcih 23,8 do 24,6 in pri asimptomatskih od 21,9 do 25,0. Pri sorti 'Gleisdorfer Ölkürbis' so bile Ct vrednosti kontrolnih vzorcev v razponu od 22,6 do 23,2, medtem ko je bil razpon pri simptomatskih vzorcih 22,3 do 24,3 in pri asimptomatskih od 21,1 do 21,7. Rezultati preliminarnega poskusa študije ekspresije tarčnega gena CAT1 nakazujejo razliko v ravni izražanja v na virus ZYMV občutljivi ('Gleisdorfer Ölkürbis') in tolerantni ('GL Opal') sorti oljnih buč (Slika 2), vendar je bilo število bioloških ponovitev prenizko za relevantne zaključke. V okuženih rastlinah (simptomatske in asimptomatske rastline) 'Gleisdorfer Ölkürbis' je raven ekspresije praktično enaka kot v kontrolnih rastlinah, medtem ko je v 'GL Opal' ekspresija nižja (Slika 2, ZYMV skupno). V simptomatskih rastlinah je v obeh sortah vidna nižja ekspresija kot v kontrolnih rastlinah (Slika 2, ZYMV simptomatski), medtem ko so večje razlike med sortama vidne v asimptomatskih rastlinah. Tu je pri 'Gleisdorfer Ölkürbis' opazno povečanje ekspresije CAT1, medtem ko pri 'GL Opal' ni opaznejših razlik v primerjavi s simptomatskimi rastlinami (Slika 2, ZYMV asimptomatski).



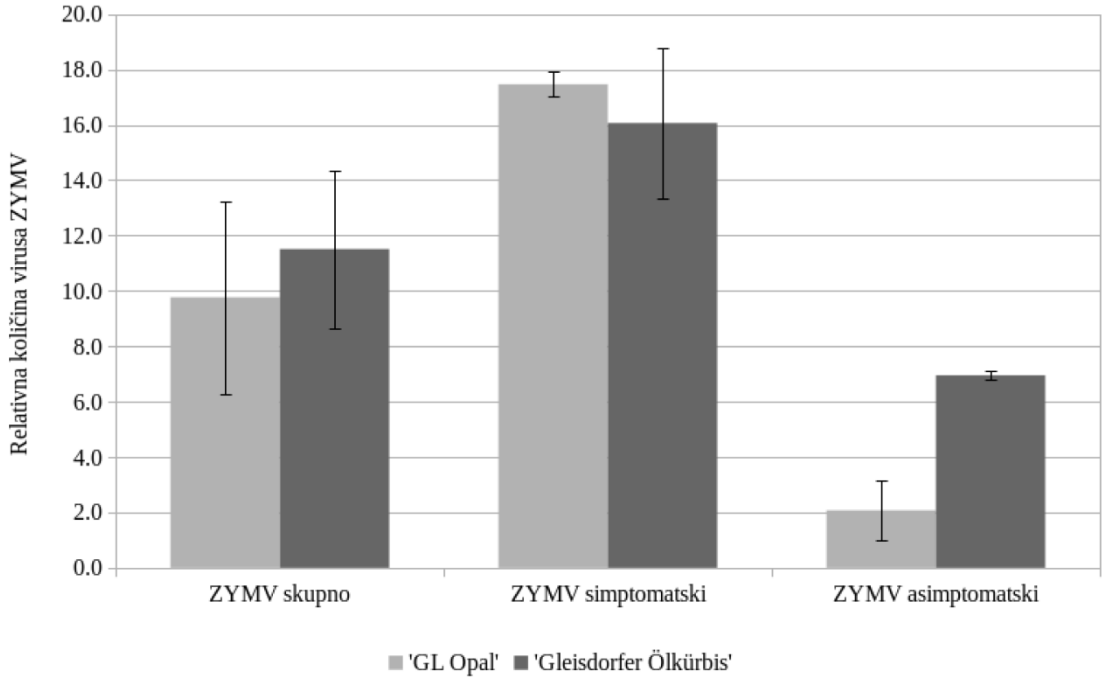
Slika 2: Relativna ekspresija gena CAT1 v mladih listih z virusom ZYMV okuženih oljnih buč sort 'GL Opal' (tolerantna na virus ZYMV) in 'Gleisdorfer Ölkürbis' (občutljiva na virus ZYMV), podana kot $\log_2$ večkratnik razlike glede na ekspresijo pri kontroli iste sorte. Negativne vrednosti predstavljajo znižanje ravni izražanja gena CAT1, medtem ko pozitivne vrednosti predstavljajo zvišanje ravni izražanja glede na dano kontrolo. Standardna napaka je izračunana na podlagi bioloških ponovitev. Skupina »ZYMV skupno« je izračunana na osnovi vseh rastlin, okuženih z virusom ZYMV, in se naprej deli na skupini »ZYMV simptomatski« (rastline z izrazitimi bolezenskimi znamenji) ter »ZYMV asimptomatski« (rastline s šibkimi oziroma neizrazitimi bolezenskimi znamenji).

Figure 2: Relative expression of CAT1 gene in young leaves of ZYMV-inoculated styrian oil pumpkin cultivars 'GL Opal' (ZYMV tolerant) and 'Gleisdorfer Ölkürbis' (ZYMV susceptible). The expression level is expressed as a Log_2 fold-difference and calibrated with the respective control treatment. Negative Log_2 values indicate downregulation, whereas positive values indicate upregulation of CAT1 gene in comparison to the respective control treatment. Error bars indicate the standard error of biological replicates. The group »ZYMV skupno« includes all plants inoculated with ZYMV and is further divided into »ZYMV simptomatski« (plants with severe symptoms) and »ZYMV asimptomatski« (plants with mild or non-existent symptoms).

2.2.1.6 Preliminarni poskus primerjave relativne količine virusa ZYMV v občutljivi in tolerantni sorti oljnih buč

Učinkovitost pomnoževanja amplikona ZYMV z metodo qPCR je bila 91,4 %. Analiza disociacijske krivulje je pokazala le en produkt pomnoževanja. Ct vrednosti simptomatskih vzorcev sorte 'GL Opal' so bile v razponu od 16,1 do 17,8, medtem ko je bil razpon pri asimptomatskih vzorcih 31,1 do 34,5. Pri sorti 'Gleisdorfer Ölkürbis' so bile Ct vrednosti simptomatskih vzorcev v razponu od 15,7 do 19,2, medtem ko je bil razpon pri asimptomatskih vzorcih 27,8 do 28,1. Vrednosti so bile kalibrirane na asimptomatski vzorec sorte 'GL Opal', v kateremu je bila določena najnižja količina virusa ZYMV (najvišja Ct vrednost). Rezultati preliminarne študije primerjave relativne količine virusa

ZYMV nakazujejo manjše razlike med na virus ZYMV občutljivo ('Gleisdorfer Ölkürbis') in tolerantno ('GL Opal') sorto oljnih buč (Slika 3), vendar je bilo število rastlin, vključenih v poskus, prenizko za relevantne zaključke. V okuženih rastlinah (simptomatske in asimptomatske rastline) 'Gleisdorfer Ölkürbis' je bilo prisotnega 1,2-krat več virusa ZYMV kot v 'GL Opal' (Slika 3, ZYMV skupno). Iz primerjave simptomatskih in asimptomatskih rastlin lahko povzamemo, da simptomatske rastline vsebujejo več virusa ZYMV (v tolerantni sorti 'GL Opal' 8,4-krat več; v občutljivi sorti 'Gleisdorfer Ölkürbis' 2,3-krat več). Največje razlike med sortama so bile detektirane v skupini asimptomatskih rastlin (Slika 3, ZYMV asimptomatski), pri čemer so asimptomatske rastline tolerantne sorte 'GL Opal' akumulirale 3,3-krat manj virusa kot rastline občutljive sorte 'Gleisdorfer Ölkürbis'.



Slika 3: Relativna kvantifikacija virusa ZYMV v mladih listih z virusom ZYMV okuženih oljnih buč sort 'GL Opal' (tolerantna na virus ZYMV) in 'Gleisdorfer Ölkürbis' (občutljiva na virus ZYMV), podana kot $\log_2$ večkratnik glede na vzorec z najnižjo detektirano količino virusa ZYMV. Standardna napaka je izračunana na podlagi bioloških ponovitev. Skupina »ZYMV skupno« je izračunana na osnovi vseh rastlin, okuženih z virusom ZYMV, in se naprej deli na skupini »ZYMV simptomatski« (rastline z izrazitimi bolezenskimi znamenji) ter »ZYMV asimptomatski« (rastline s šibkimi oziroma neizrazitimi bolezenskimi znamenji).
Figure 3: Relative quantification of ZYMV in young leaves of ZYMV-inoculated styrian oil pumpkin cultivars 'GL Opal' (ZYMV tolerant) and 'Gleisdorfer Ölkürbis' (ZYMV susceptible). The results are expressed as a Log_2 fold-difference and calibrated with the sample with the lowest relative ZYMV quantity. Error bars indicate the standard error of biological replicates. The group »ZYMV skupno« includes all plants inoculated with ZYMV and is further divided into »ZYMV simptomatski« (plants with severe symptoms) and »ZYMV asimptomatski« (plants with mild or non-existent symptoms).

3 RAZPRAVA IN SKLEPI

3.1 INDUKCIJA HAPLOIDOV Z OPRAŠEVANJEM Z OBSEVANIM PELODOM

Iz študij indukcije haploidov s pseudofertilizacijo pri vrsti *C. pepo* (Kurtar in sod., 2002; Baktemur in sod., 2014) je znano, da je razvoj plodu možen pri opraševanju s pelodom, ki je bil obsevan z visokimi dozami gama žarkov (do 400 Gy). V nasprotju z Baktemur in sod. (2014) pa Kurtar in sod. (2002) poročajo, da ni prišlo do razvoja embrijev pri dozah višjih od 50 Gy. V naši študiji je bil uspešen tako razvoj plodu kot tudi embrijev pri dozah do vključno 350 Gy za vse donorje peloda in ženskih cvetov z dvema izjemama. Opraševanje s pelodom sort *C. pepo* 'Yellow Long' (300 Gy; U.S. Department of Agriculture, ZDA) in *C. moschata* 'Muscade de Provence' (200 in 300 Gy; Semenarna Ljubljana d.d.) ni vodilo do razvoja plodov. Kljub temu, da so medvrstna križanja s *C. moschata* mogoča (Šiško in sod., 2003), obsevanje peloda verjetno dodatno zmanjša sposobnost oploditve. Podobno kot obsevanje peloda z gama žarki, je tudi obsevanje z rentgenskimi žarki vodilo v zmanjšanje števila embrijev z naraščanjem doze.

Učinkovitost postopka indukcije haploidov je odvisna od več dejavnikov. Avtorji poročajo o različni uspešnosti znotraj rastne sezone buč (Kurtar in Balkaya, 2010; Kurtar in sod., 2009). Iz naših rezultatov lahko povzamemo podobno. V letu 2011 smo haploide uspeli pridobiti z večino testiranih donorjev ženskih cvetov, medtem, ko je bila uspešnost v letu 2012 nižja. To je razvidno predvsem iz direktne primerjave odstotka haploidnih regenerantov sort 'Gleisdorfer Ölkürbis' in 'Gleisdorfer Diamant' (Saatzucht Gleisdorf Ges.mbH), ki sta bili vključeni v testiranje v obeh letih. Pri primerjavi učinkovitosti gama in rentgenskih žarkov lahko na primeru melone ugotovimo, da so optimalne doze rentgenskih žarkov za indukcijo haploidov (1000 Gy; Katoh in sod., 1993) vsaj dvakrat višje kot optimalne doze gama žarkov (do 500 Gy; Sauton in Dumas De Vaulx, 1987; Gonzalo in sod., 2011; Godbole in Murthy, 2012). Če primerjamo naše rezultate (optimalna doza med 200 in 300 Gy) s prvimi objavljenimi v rodu *Cucurbita* (do 100 Gy; Kurtar in sod., 2002, 2009; Kurtar in Balkaya, 2010), lahko sicer zaključimo podobno, vendar novejše raziskave kažejo, da so učinkovite tudi višje doze gama žarkov (do 300 Gy; Baktemur in sod., 2014). Za natančno primerjavo bi sicer bila potrebna podrobnejša študija, saj poleg vrste sevanja in doze na učinkovitost vpliva tudi vir sevanja in hitrost doze, zato so rezultati med seboj težje primerljivi. Možnosti aplikacije višjih doz rentgenskih žarkov pri oljnih bučah so predstavljene v Poglavju 2.1.2. Podobno kot Kurtar in Balkaya (2010) v *C. maxima* smo ugotovili, da je indukcija haploidov odvisna tudi od genetskega ozadja oziroma akcesije donorja ženskih cvetov. Ravno nasprotno pa se je izkazalo pri kumarah, kjer tako Claveria in sod. (2005) kot tudi Przyborowski in Niemirowicz-Szczytt (1994) niso ugotovili statistično značilnih razlik med uporabljenimi akcesijami. V naši raziskavi je bila indukcija najbolj uspešna pri 'Turkey #2' (U.S. Department of Agriculture), kjer smo z opraševanjem s pelodom 'GL Opal' (200 Gy), pridobili kar 10 % haploidnih regenerantov. Izmed vseh testiranih donorjev ženskih cvetov indukcija haploidov ni bila uspešna le pri sorti 'Slovenska golica', vendar je bilo število regenerantov z določeno ploidnostjo v tem primeru najnižje, saj smo del embrijev izgubili zaradi kontaminacije v tkivni kulturi. Medtem ko lahko iz uporabe različnih donorjev ženskih cvetov sklepamo, da je metoda uspešna v širšem naboru akcesij, se je pri testiranju donorjev peloda izkazalo ravno nasprotno. Izmed šestih testiranih, je bila indukcija

uspešna le z dvema ('GL Opal' in 'White Acorn' (U.S. Department of Agriculture)). Gotovo je možno, da je sorta 'GL Opal', s katero smo testirali vse donorje peloda razen 'GL Opal', slabše odzivna, vendar tudi drugi avtorji poročajo o različnih učinkovitostih donorjev peloda pri indukciji haploidov v drugih rastlinskih vrstah (Pandey in sod., 1990; Grouh in sod., 2011).

Skupno smo ploidnost določili 3830 regenerantom, ki smo jih pridobili s kulturo nezrelih embrijev po opraševanju z obsevanim pelodom. Večina regenerantov je bila diploidnih, določili pa smo tudi haploidne, triploidne in tetraploidne regenerante. Kljub temu, da je pretočna citometrija zagotovo najbolj primerna tehnika za določanje ploidnosti večjega števila vzorcev, je v družini Cucurbitaceae nekoliko problematična. Tako pri lubenicah (Sari in sod., 1994) kot tudi pri kumarah (Gilissen in sod.,1993) so mlada tkiva velikokrat močno endoreduplicirana. To smo dokazali tudi pri oljnih bučah (Poglavje 2.1.3). Posledično je interpretacija histograma, ki ga dobimo kot rezultat analize s pretočno citometrijo, lahko otežena, saj je prvi vrh, ki kot G1 faza celičnega cikla določa ploidnost, v nekaterih primerih nizek in ga lahko napačno razložimo kot ozadje. Tetraploidni regeneranti bi sicer lahko bili posledica napačne interpretacije histograma, vendar so se le-ti bolj pogosto pojavljali po opraševanju s pelodom, ki je bil obsevan z višjimi dozami rentgenskih žarkov. Po našem vedenju podobnih poročil o pojavu tetraploidov ni, zato lahko brez nadaljnjih raziskav sicer zgolj špekuliramo, kako pride do nastanka le-teh. Ena izmed možnih razlag je, da je zigota, ki je nastala kot posledica oprašitve s poškodovanim pelodom, v zgodnjem razvoju usmerjena v podvojevanje genoma. Če sklepamo, da je napačna interpretacija enako verjetna ne glede na ploidnost, to hipotezo potrjuje tudi dejstvo, da pri analizi diploidov z mikrosatelitskimi markerji nismo potrdili homozigotov. Napačno določen diploid (dejanski haploid) bi bil namreč navidezno homozigoten.

Pri ginogenetskih postopkih, med katere spada tudi pseudofertilizacija z obsevanim pelodom, je spontano podvojevanje genoma redko (Bohanec, 2009). Medtem ko pri kumarah (Claveria in sod., 2005) niso potrdili spontanega podvojevanja, so pri melonah (Gonzalo in sod., 2011) izmed 141 testiranih regenerantov potrdili 2 homozigota, ki sta nastala kot spontana DH pri indukciji z obsevanim pelodom. V rodu *Cucurbita* podobnih študij homozigotnosti za potrjevanje spontanega podvojevanja genoma ni. Zaradi ozkega genskega nabora oljnih buč (Gong in sod., 2012) in nizke izenačenosti znotraj sort, je bila izbira polimorfnih mikrosatelitskih markerjev (Gong in sod., 2008), primernih za analizo, težavna, saj smo morali le-te izbrati za vsako kombinacijo donorskih rastlin posebej. Skupno smo testirali 253 diploidnih regenerantov na štirih mikrosatelitskih lokusih. V nasprotju z domnevo Kurtar in Balkaya (2010), da je nizko število haploidov lahko posledica spontanega podvojevanja pri *C. maxima*, nismo potrdili nobenega DH z gotovostjo. Z našimi rezultati pa smo vseeno dokazali, da so mikrosatelitski markerji učinkoviti za preverjanje homozigotnosti, saj smo heterozigotnost večine (96,8 %) testiranih regenerantov določili že s prvima dvema testiranima lokusoma. S tremi je bilo mogoče potrditi heterozigotnost 99,6 % regenerantov. V štirih vzorcih enega izmed očetovih alelov v sicer heterozigotnem regenerantu pa verjetno nismo potrdili zaradi mutacije ali segregacije ničtega alela.

3.2 KALITEV PELODA PRI VIŠJIH DOZAH OBSEVANJA IN POTENCIALNA UPORABA V OPTIMIZACIJI POSTOPKA INDUKCIJE HAPLOIDOV

Ne glede na dejanski mehanizem indukcije haploidov s pseudofertilizacijo z obsevanim pelodom, ta metoda temelji na uporabi viabilnega in kalečega peloda. V več rastlinskih vrstah je bilo dokazano, da z višanjem doze obsevanja peloda, izboljšamo izplen haploidov (Sauton in Dumas de Vaulx, 1987; Chalak in Legave, 1997; Grouh in sod., 2011; Baktemur in sod., 2014) zaradi tako imenovanega Hertwigovega efekta (Hertwig, 1911), ki govori o razvoju haploidnih embrijev in boljšem preživetju le-teh po obsevanju spermalnih celic z višjimi dozami. Za poskus nadaljnje optimizacije indukcije haploidov pri oljnih bučah je zato pomembno razviti postopek obsevanja, ki bi omogočal kalitev tudi po višjih dozah obsevanja, saj bi tako teoretično lahko zmanjšali število diploidnih embrijev v prid haploidnih.

Ena izmed zanesljivejših metod za preverjanje viabilnosti peloda po obsevanju je *in vitro* kalitev. V ta namen smo optimizirali sestavo gojišča za *in vitro* kalitev peloda oljnih buč, s katerim smo dosegli 41,5 % kalečega peloda v kontrolnem vzorcu, kar zadostuje za zanesljivo oceno kalivosti. V nasprotju s študijami kalitve peloda sorodnih vrst (Brewbaker in Kwack, 1963; Kurtar, 2009; Zaman, 2009) smo uporabili tekoče gojišče in kulturo visečih kapljic, saj so rezultati preliminarnega testiranja pokazali, da je odstotek kalitve na trdnem in poltrdnem gojišču nižji. Kot najpomembnejši dejavnik se je izkazala pH vrednost gojišča. Kot optimalno smo določili pH vrednost 9, kar je v skladu s študijo vpliva pH na kalivost peloda drugih predstavnikov družine Cucurbitaceae (Zaman, 2009). Za kalitev je pomembna tudi optimalna koncentracija ozmotika. V literaturi navajajo pozitivne učinke uporabe polietilenglikola (Rihova in sod., 1996; Conner, 2011) in delno manitola (Vasil, 1960; Rihova in sod., 1996) kot alternativa saharozi ali dodatek gojiščem s saharozo, vendar tega z našimi rezultati ne moremo potrditi. Kot optimalno se je izkazalo Brewbaker in Kwack (1963) gojišče z 12,5 % (w/v) saharoze in pH 9.

Za uspešno opraševanje oljnih buč s pelodom, ki je bil obsevan z višjimi dozami, je potrebno zagotoviti ohranitev vlažnosti peloda. Ta je pri bučah ob odprtju cvetov zelo visoka v primerjavi z drugimi vrstami (od 44,6 do 50 % (Nepi in sod., 2001; Franchi in sod., 2002)). Padec vlažnosti pod 24 % pa že vodi v izgubo sposobnosti uspešne oploditve (Gay in sod., 1987). Poleg tega naj bi bil pelod buč tudi bolj občutljiv na sevanje zaradi svoje velikosti (Brewbaker in Emery, 1962). V izogib hitri izgubi sposobnosti kalitve zaradi zgoraj opisanih in drugih lastnosti (vsebnost saharoze itd.) je potrebno med samim postopkom obsevanja pelod zaščititi pred izgubo vlažnosti. V naših poskusih smo že ob aplikaciji najnižje doze (100 Gy) opazili statistično značilno izgubo kalivosti ne glede na metodo obsevanja (RH in HH). Podoben padec uspešnosti opraševanja je bil opazen tudi v poskusu opraševanja za indukcijo haploidov (Poglavje 2.1.1). V nasprotju z našimi rezultati je bil v sorodnih vrstah, kot so na primer melone (Cuny in sod., 1993) in buče (Kurtar, 2009), učinek obsevanja manj drastičen.

Ob predpostavki, da razvoj plodov sledi vzorcu kalivosti peloda (Cuny in sod., 1993) in na osnovi naših rezultatov indukcije haploidov (Poglavje 2.1.1), kjer smo uspeli pridobiti embrije tudi po obsevanju s 350 Gy (RH), lahko sklepamo, da je z obsevanjem v razmerah HH embrije možno pridobiti z dozami do 600 Gy, medtem ko z obsevanjem v RH

razmerah to pričakujemo do 500 Gy. Razlog za razliko med metodama je mogoče povzeti iz publikacije Nepi in sod. (2010), v kateri poročajo o statistično značilnem upadu kalivosti peloda po 1-urni inkubaciji peloda na 30 % zračni vlažnosti, medtem ko je bil upad opazen šele po 2 urah na 75 % zračni vlažnosti. Na osnovi navedenih rezultatov je zato vzdrževanje vlažnosti med obsevanjem izrednega pomena. Nadaljnje izboljšanje bi bilo mogoče pričakovati z nadgradnjo hladilnega sistema v napravi za obsevanje z rentgenskimi žarki.

Zanimivi so tudi rezultati meritev premera peloda. V populaciji peloda 'GL Opal' smo določili dve velikostni podpopulaciji. Splošno gledano je manjši pelod (okoli mediane populacije) bolj kalil. Pri primerjavi premerov kalečega peloda pa smo opazili rahel zamik proti večjemu pelodu v skupinah, ki so bile obsevane (RH in HH), pri čemer je bil le-ta bolj opazen v skupini HH. Naši rezultati tako nasprotujejo rezultatom Brewbaker in Emery (1962), ki sta prišla do sklepa, da je večji pelod bolj radiosenzitiven. Slednje verjetno drži pri primerjavi različnih rastlinskih vrst, vendar je odziv znotraj vrste drugačen. Podobno navaja tudi Tejaswini (2002) pri nageljnih (*Dianthus* spp.) ob prisotnosti biotskega stresa. Favoriziranje kalitve peloda določene velikosti ob stresu znotraj vrste tako podpira teorijo, da širok spekter velikosti omogoča odziv in prilagoditev na stresne dejavnike v okolju.

3.3 ENDOPOLIPLOIDIJA IN NJEN VPLIV NA ADVENTIVNO REGENERACIJO

O endopoliploidiji poroča več avtorjev pri različnih vrstah rastlinskega kraljestva. Barow in Meister (2003) sta med drugim v svojo študijo vključila tudi različne organe odraslih rastlin vrste *C. pepo*. V skladu z našimi rezultati sta določila visoko stopnjo endoredupliciranosti v kotiledonih. Do podobnega zaključka so prišli tudi pri analizi mladih rastlin kumar (Gilissen in sod., 1993) in navadnega repnjakovca (*Arabidopsis thaliana;* Galbraith in sod., 1991). V obeh primerih so kotiledoni vsebovali statistično značilno manj 2C jeder kot listi. Naše analize kažejo, da so jedra v celicah mladih listov manj endoredupliciranа kot v starejših listih. O obratni situaciji pa poročata Barow in Meister (2003), kar najverjetneje lahko pripišemo razlikam v analiziranih akcesijah. Tako kot pri analizi oljne ogrščice (*Brassica napus*; Chen in sod., 1994) smo tudi mi določili najvišjo stopnjo endoredupliciranosti v hipokotilu.

Splošno velja, da stopnja endoredupliciranosti pada od distalnega proti bazalnemu delu rastlinskih organov (Barow, 2006), vendar so pri kotiledonih kumar dokazali ravno nasprotno (Gilissen in sod., 1993; Colijn-Hooymans in sod., 1994). Frekvenca 2C in 4C jeder je proti bazalnemu delu upadala oziroma ostala enaka, spremembe pa so predvsem zaznavne skozi različne razvojne stopnje embrija oziroma rastlinice. Colijn-Hooymans in sod. (1994) so poleg tega dokazali tudi, da je učinkovitost regeneracije *in vitro* povezana s frekvenco 2C jeder v tkivu izsečka. Zaključili so, da več kot je v kotiledonskem tkivu prisotnih celic z 2C jedri, višji je odstotek regeneracije. Več avtorjev (Ananthakrishnan in sod., 2003; Lee in sod., 2003; Kathiravan in sod., 2006; Zhang in sod., 2008; Kim in sod., 2010) poroča o najboljši adventivni regeneraciji v rodu *Cucurbita* iz bazalnega dela kotiledona, medtem ko je bila regeneracija iz drugih tkiv (distalni del kotiledona, hipokotil itd.) minimalna oziroma do regeneracije ni prišlo. Kot pomemben faktor za uspeh Lee in sod. (2003) ter Zhang in sod. (2008) navajajo tudi starost rastlinic, iz katerih pridobivamo

izsečke. S pomočjo slikovne citometrije (Vilhar in sod., 2001) smo analizirali različne sekcije kotiledonov (bazalno, centralno in distalno) in ugotovili, da največ 2C in 4C jeder vsebuje ravno bazalni del. Kljub temu, da endopoliploidije nismo analizirali skozi različne razvojne stopnje rastlinic, naši rezultati nakazujejo, da na uspeh adventivne regeneracije tudi pri vrsti *C. pepo* vpliva stopnja endoredupliciranosti kotiledonskega izsečka.

3.4 ADVENTIVNA REGENERACIJA IN UPORABA SOMAKLONSKE VARIABILNOSTI V ŽLAHTNJITELJSKE NAMENE

Odstotek regeneracije v naši študiji se je gibal v rangu drugih študij pri vrsti *C. pepo* (Ananthakrishnan in sod., 2003; Kathiravan in sod., 2006). Medtem ko Zhang in sod. (2008) ter Kim in sod. (2010) poročajo, da so citokinini nujno potrebni za adventivni razvoj poganjkov v rodu *Cucurbita*, smo v naši raziskavi uspešno regenerirali poganjke tudi na brezhormonskem gojišču. Naši rezultati pravzaprav kažejo na omejen pozitiven učinek dodatka citokininov v regeneracijsko gojišče. Meta-topolin in zeatin (ZEA) sta celo zavrla regeneracijo v primerjavi z brezhormonskim gojiščem. Najbolj pozitivno so na regeneracijo kot dodatek gojišču z BA vplivali 2-izopentenil adenin (2iP), PABA in FA. Zhang in sod. (2008) so pri študiji adventivne regeneracije pri vrsti *C. moschata* dokazali, da so kotiledonski izsečki, ki vsebujejo več endogenega 2-izopentil adenozina (iPA), bolj odzivni. Slednje bi lahko razložilo učinek hormona 2iP, saj je iPA njegov prekurzor. PABA, ki jo gojiščem ponavadi dodajamo kot vitamin, je znana tudi po avksinom-podobnih učinkih (Pandey in sod., 2010) in je strukturno podobna IAA. O boljši regeneraciji v družini Cucurbitaceae na gojiščih s citokinini in dodatkom IAA pa poroča več avtorjev (Abrie in van Staden, 2001; Kim in sod., 2010; Ren in sod., 2013). Presenetljivo je bila najboljša regeneracija izsečkov določena na gojišču z dodatkom FA v nizki koncentraciji (5 mg/l), medtem ko smo na gojiščih z višjimi koncentracijami dosegali primerljive vrednosti regeneracije kot v ustreznem kontrolnem gojišču z dodatkom BA in PABA. Za uporabo v *in vitro* selekcijskih študijah naj bi selekcijski agens (FA ali filtrat kulture gliv) močno omejil preživetje in regeneracijo izsečkov. Tako lahko zaključimo, da je bila koncentracija dodane FA najverjetneje prenizka, da bi testirana gojišča lahko uporabili tudi v te namene. Gojišča s FA so vsebovala tudi PABA, ki je bila povezana z indukcijo sistemsko pridobljene odpornosti v rastlinah (Song in sod., 2013). PABA je tako lahko spremenila odziv rastline na glivni toksin. Za prihodnje raziskave oziroma aplikacije FA v selekcijske namene zato priporočamo višje koncentracije FA in/ali gojišča z dodatkom BA in 2iP.

Somaklonska variabilnost je definirana kot fenotipska ali genotipska variabilnost, ki je izzvana z gojenjem *in vitro*. V postopkih mikropropagacije je večinoma nezaželena, lahko pa privede do zanimivih somaklonov, ki jih uporabimo v žlahtnjenju (Bairu in sod., 2011). Škof in sod. (2007) so adventivno regeneracijo na gojiščih s povišano vsebnostjo citokininov uspešno uporabili za podvojevanje genoma brez neželenih miksoploidov, ki so poleg razmeroma nizke učinkovitosti podvojevanja (Lotfi in sod., 2003; Claveria in sod., 2005; Lim in Earle, 2008) tudi ena izmed slabih strani uporabe antimitotskih sredstev pri družini Cucurbitaceae. Poliploidi so bili večkrat detektirani tudi med adventivnimi regeneranti družine Cucurbitaceae (Colijn-Hooymans in sod., 1994; Lee in sod., 2003; Han in sod., 2004; Ren in sod., 2013), medtem ko naši rezultati kažejo, da se na gojiščih brez

dodatka FA pri oljnih bučah regenerirajo zgolj diploidi. Tetraploide, torej podvojene diploide, smo detektirali izključno na gojiščih z dodano FA v srednjih koncentracijah (10 in 20 mg/l). Ti so se pojavljali v odstotkih, ki so primerljivi uporabi antimitotskih sredstev. Tetraploidi so v tem primeru lahko rezultat motnje delovanja delitvenega vretena pri celični delitvi ali vsaj teoretično posledica razcepitve kromosomov endoredupliciranih jeder. Colijn-Hooymans in sod. (1994) so zaradi stopnje endoredupliciranosti izsečkov kot razlog navedli slednje. O podvojevanju genoma ob prisotnosti FA poročajo tudi pri paradižniku (*Solanum lycopersicum*; Nguyen in sod., 1992), pri katerem so tudi dokazali visoko stopnjo endopoliploidije v več tkivih (Smulders in sod., 1995). FA naj bi v tkivu povzročala nastajanje endogenega etilena (Wilson in sod., 1978). Etilen pa lahko povežemo z več procesi, ki bi lahko privedli do regeneracije tetraploidov iz sicer diploidnih izsečkov. Tekom prehodne izpostavljenosti hipokotilnega tkiva etilenu so pri kumarah opazili kopičenje DNA v jedrih brez vmesne citokineze (Dan in sod., 2003). Po prenehanju izpostavljenosti so se celice ponovno pričele deliti. Vzporedna študija (Kazama in sod., 2004) govori o podaljšani kompetenci celic za delitev in druge procese vključene v celično diferenciacijo. Z uporabo inhibitorjev etilena pri adventivni regeneraciji kotiledonov vodnjače (*Lagenaria siceraria*) pa so opazili manjšo pogostost tetraploidnih regenerantov (Han in sod., 2004). Poleg regeneracije tetraploidov lahko z zgoraj opisanimi pojavi delno razložimo tudi višji odstotek regeneracije na gojiščih s FA. Zagotovo pa predstavljena teorija potrebuje natančnejšo analizo in poskuse, ki pa niso bili del pričujočega doktorskega dela.

3.5 IZBOR STABILNO IZRAŽENIH REFERENČNIH GENOV OB OKUŽBI RASTLIN Z VIRUSOM ZYMV

Za normalizacijo podatkov pri analizi izražanja genov, ki sodelujejo pri odzivu rastline na okužbo z virusom ZYMV, in kvantifikacijo virusa ZYMV je potreben izbor stabilno izraženih referenčnih genov. Za validacijo stabilnosti izražanja genov smo uporabili 4 algoritme (Delta Ct metoda, NormFinder, geNorm, BestKeeper), ki jih vključuje program RefFinder (Xie in sod., 2011). Rezultati razvrstitve genov po stabilnosti izražanja z Delta Ct metodo, algoritmom NormFinder in geNorm so podobni, medtem ko rezultati z algoritmom BestKeeper nekoliko odstopajo od ostalih, saj le-ta uvršča gen HELI na prvo mesto po stabilnosti, medtem ko ga ostali uvrščajo na 6. mesto. Na osnovi analize z algoritmom geNorm so bile referenčnim genom EF-1A, UFP, RPL36aA, PP2A in CAC pripisane M vrednosti $\leq$ 0,5, kar jih uvršča med stabilno izražene referenčne gene (Hellemans in sod., 2007). Kot najmanj stabilna sta se izkazala v vseh primerih gena COX in ACT. Manjše razlike v končni razvrstitvi referenčnih genov med uporabljenimi algoritmi so bile opažene tudi v drugih raziskavah stabilnosti izražanja referenčnih genov tako ob abiotskem kot tudi biotskem stresu pri predstavnikih družine Cucurbitaceae (Obrero in sod, 2011; Kong in sod., 2014a, 2014b; Sestili in sod., 2014). Ker smo se pri naši raziskavi odločili, da v nadaljnjih analizah sledimo rezultatom algoritma geNorm kot največkrat uporabljenega v literaturi, v nadaljevanju primerjamo zgolj rezultate pridobljene s tem algoritmom. V raziskavi, ki so jo izvedli Kong in sod. (2014a), sta se pri lubenici, okuženi z glivo *Fusarium oxysporum* f.sp. *niveum* in bakterijo *Acidovorax avenae* subsp. c*itruli*, med stabilne referenčne gene uvrstila CAC in EF-1A. Gen ACT je bil tudi v tem primeru uvrščen med nestabilne. V nasprotju z našimi rezultati se je med nestabilne uvrstil

tudi gen PP2A. Zanimivi so tudi rezultati analize ekspresije referenčnih genov pri meloni (Kong in sod., 2014b), kjer so ravno tako okuževali z glivo *Fusarium oxysporum* f.sp. *niveum* in bakterijo *Acidovorax avenae* subsp. c*itruli* ter določili stabilno izražanje gena ACT in gena za α-tubulin (TUA), medtem ko so se geni EF-1A, RPL36aA ter PP2A uvrstili med nestabilne. Pri okužbi melone z glivo *Fusarium oxysporum* f. sp. *melonis* pa so Sestili in sod. (2014) določili najstabilnejše izražanje gena za ribosomalni protein L2 in ACT, za primerno normalizacijo pa je bil potreben še gen za ciklofilin. Ne glede na podobne oziroma enake stresne pogoje filogenetsko izredno sorodnih rastlinskih vrst, se rezultati razlikujejo. Do zaključka, da je izbor referenčnih genov različen ne glede na enake stresne pogoje (okužba z virusom rumene pritlikavosti ječmena (Barley yellow dwarf virus; BYDV)) sta prišla tudi Jarošova in Kundu (2010) pri primerjavi stabilnosti izražanja referenčnih genov različnih vrst žit. Zaključimo lahko, da je potreben izbor referenčnih genov vsakič, ko spremenimo vrsto ne glede na podobnost eksperimentalnih pogojev.

Stabilno izražanje genov UFP in EF-1A so pri *C. pepo* določili tudi Obrero in sod. (2011), vendar njihovi eksperimentalni pogoji niso vključevali okužbe s patogenimi organizmi. Omenjena gena sta se kot najstabilnejša izkazala pri kombinaciji vseh vzorcev oziroma eksperimentalnih pogojev, ki so vključevali različne razvojne stopnje plodu in cveta, odziv na slano okolje, tretiranje s hormoni in temperaturni stres (10°C). Tudi v posameznih stresnih pogojih oziroma razvojni stopnji, so se geni EF-1A, UFP, RPL36aA, PP2A in CAC uvrščali razmeroma visoko po M vrednosti. Splošno sta se najslabše uvrstila gena 18S in TUA. Primerjava izbora znotraj iste vrste kaže, da je stabilnost referenčnih genov v različnih stresnih pogojih oziroma razvojnih stopnjah podobna, vendar ne nujno enaka.

Kot priporočajo Vandesompele in sod. (2002) ter Udvardi in sod. (2008) smo se odločili za normalizacijo z več kot enim referenčnim genom. Na osnovi parne variacije, ki naj bi bila manjša od 0,15, je algoritem geNorm določil, da je optimalno število uporabljenih referenčnih genov v našem primeru 2. Tako smo kot normalizacijski faktor za študijo izražanja gena CAT1 in kvantifikacijo virusa ZYMV uporabili geometrično sredino dveh najbolj stabilnih genov (EF-1A in UFP), ki sta se kot najprimernejša izkazala tudi na skupni lestvici raziskave Obrero in sod. (2011). Vendar pa omenjeni avtorji poročajo, da je zaradi raznolikosti analiziranih eksperimentalnih pogojev potrebnih več (4) referenčnih genov za normalizacijo, ki pa so se na lestvici algoritma geNorm tudi v pogojih okužbe z virusom ZYMV uvrstili najvišje (UFP, EF-1A, RPL36aA in PP2A).

3.6 EKSPRESIJA GENA CAT1 IN PRISOTNOST VIRUSA ZYMV V OKUŽENIH RASTLINAH

Rastline, ki so izpostavljene stresu, pospešeno proizvajajo reaktivne kisikove spojine (npr. vodikov peroksid), ki so za rastlino škodljive. Katalaze sodelujejo pri eliminaciji vodikovega peroksida, kar pa v primeru okužbe lahko vodi do oslabljenega odziva rastline na okužbo. Vodikov peroksid namreč v sklopu hipersenzitivnega odgovora omejuje širjenje patogenega organizma, odstranjevanje pa lahko vodi do znižane aktivacije s patogenezo povezanih genov. V odgovor je vključena tudi salicilna kislina, ki inhibira delovanje katalaze v primeru okužbe. Skupaj z vodikovim peroksidom okrepita odziv

rastline na okužbo in vodita v t.i. sistemsko pridobljeno odpornost (Van Breusegem in sod., 2001). Rezultati preliminarne študije ekspresije gena CAT1 v sistemsko okuženih mladih listih z virusom ZYMV okuženih rastlin oljnih buč nakazujejo znižanje ravni izražanja pri tolerantni sorti oljnih buč 'GL Opal' 21 dni po okužbi z virusom, medtem ko izražanje pri občutljivi sorti 'Gleisdorfer Ölkürbis' ostane praktično nespremenjeno v primerjavi s kontrolo (ZYMV skupno, Slika 2). V skupini simptomatskih rastlin obeh sort se nakazuje trend znižanja ravni ekspresije gena CAT1 v sistemsko okuženih mladih listih (ZYMV simptomatski, Slika 2). Do podobnega zaključka so prišli tudi Kundu in sod. (2013) pri primerjavi občutljive in odporne sorte vrste *Vigna mungo* in okužbi z virusom Mungbean Yellow Mosaic India Virus. Nasprotno pa Radwan in sod. (2006) navajajo, da je v na virus ZYMV izredno občutljivi sorti buč 'Eskandarani', okuženi z virusom ZYMV, prišlo do povišanja ravni aktivnosti katalaze v sistemsko okuženih mladih listih z izrazitimi bolezenskimi znamenji. V rastlinah, ki so bile tretirane s salicilno kislino, so bila bolezenska znamenja šibkejša in aktivnost katalaze nižja. Ravno tako je prišlo do povišanja aktivnosti katalaze v sistemsko okuženih starejših listih občutljive sorte oljnih buč 'Gleisdorfer Ölkürbis' (Riedle-Bauer, 2000). Natančna analiza in primerjava rezultatov je sicer otežena, saj so naši rezultati zgolj preliminarni, poleg tega pa primerjamo tudi različne parametre (ekspresija gena CAT1 in skupna aktivnost katalaz) v različnih časovnih točkah in tkivih. Pri analizi asimptomatskih rastlin smo pri občutljivi sorti 'Gleisdorfer Ölkürbis' določili višjo raven ekspresije gena CAT1 v primerjavi s kontrolo, medtem ko je bila ekspresija pri tolerantni sorti 'GL Opal' znižana (ZYMV asimptomatski, Slika 2). Pri tem je vredno rezultate primerjati s prisotnostjo virusa ZYMV (ZYMV asimptomatski, Slika 3). V mladih listih asimptomatskih rastlin občutljive sorte 'Gleisdorfer Ölkürbis' smo namreč detektirali večjo količino virusa ZYMV kot v asimptomatskih rastline tolerantne sorte 'GL Opal'. Kljub večji detektirani količini virusa ZYMV pa pri občutljivi sorti nismo zaznali znižanja ekspresije gena CAT1. Za relevantne zaključke je bilo število rastlin, vključenih v analizo, prenizko. Kljub temu pa preliminarni rezultati nakazujejo splošne trende, ki bi jih bilo zanimivo preveriti v prihodnje. Poleg večjega števila bioloških ponovitev je za jasne zaključke o odzivu rastline na okužbo z virusom ZYMV seveda potrebna tudi analiza izražanja več genov.

Rezultati preliminarnega poskusa kvantifikacije virusa ZYMV nakazujejo minimalne razlike v relativni količini med občutljivo ('Gleisdorfer Ölkürbis') in tolerantno sorto ('GL Opal') 21 dni po inokulaciji (ZYMV skupno in ZYMV simptomatski, Slika 3). Pojav asimptomatskih rastlin je sicer najverjetneje posledica razlik v uspešnosti mehanske inokulacije, vseeno pa s tem ne moremo razložiti tudi razlike v detektirani relativni količini virusa ZYMV med sortama (ZYMV asimptomatski, Slika 3); v rastlinah občutljive sorte 'Gleisdorfer Ölkürbis' je bilo detektiranega 3,3-krat več virusa ZYMV kot v rastlinah tolerantne sorte 'GL Opal'. Tako Svoboda in sod. (2013) kot tudi Lecoq in sod. (2004) navajajo, da količina virusa ZYMV na posameznih rastlinah bučk ni nujno povezana s stopnjo odpornosti, kar je v skladu z rezultati naše preliminarne študije, v kateri smo, razen v primeru asimptomatskih rastlin, detektirali primerljive količine virusa ZYMV. Kot navajajo Svoboda in sod. (2013) je količina virusa ZYMV odvisna tudi od časovne točke, v kateri virus detektiramo. Pri okuževanju bučk z virusom ZYMV so največje razlike med različnimi sortami opazili 28 dni po inokulaciji, medtem ko smo v našem poskusu vzorčili po 21 dneh, kar bi lahko bil vzrok neizrazitim razlikam v bolezenskih znamenjih kot tudi razmeroma majhnim razlikam v količini detektiranega virusa z metodo qPCR (ZYMV

skupno in ZYMV simptomatski, Slika 3). Svoboda in sod. (2013) so se poslužili pri primerjavi akcesij metode ELISA, medtem ko so za detekcijo virusa ZYMV v popolnoma odpornih akcesijah vrst družine Cucurbitaceae razvili metodo absolutne kvantifikacije ZYMV s qPCR in tehnologijo TaqMan. Kljub pomnoževanju do 50. cikla pa virusa niso detektirali. Iz navedenega je možno sklepati, da metoda lahko dopolni fenotipsko ocenjevanje odpornosti. Z ustrezno optimizacijo mehanske inokulacije in obširnejšo študijo akumulacije virusa ZYMV v večjem številu akcesij metoda gotovo lahko pomembno pripomore k standardizaciji in olajša primerljivost testiranj ter opisov različnih akcesij.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Zanimanje za pridelavo oljnih buč po svetu narašča, vseeno pa razvoj biotehnoloških pristopov za žlahtnjenje zaostaja za drugimi predstavniki vrste *C. pepo* in družine Cucurbitaceae. Hibride, ki prihajajo na trg in izpodrivajo starejše populacijske sorte, odlikuje predvsem višji pridelek in boljša odpornost na bolezni, pomembne pri pridelavi oljnih buč. Namen doktorske naloge je bil razvoj metod, ki bi jih lahko uporabili za učinkovitejše žlahtnjenje, in izvedba optimizacije le-teh z ozirom na specifične lastnosti akcesij oljnih oziroma golosemenskih buč.

Alternativa dolgotrajnemu postopku razvoja čistih linij z večkratnimi povratnimi križanji za kasnejše žlahtnjenje hibridov je indukcija haploidov, kateri sledi podvojevanje genoma in regeneracija DH. Glede na število uspešnih poskusov je v družini Cucurbitaceae obetavna indukcija z opraševanjem z obsevanim pelodom. Večina protokolov temelji na obsevanju z gama žarki, katerih uporaba pa je vedno bolj omejena. V ta namen smo v naših poskusih uporabili rentgenske žarke. Indukcija je bila uspešna v večini testiranih akcesij golosemenskih buč. Največ haploidnih regenerantov smo detektirali v akcesiji 'Turkey #2' (10,0 %), kateri sta sledili 'Gleisdorfer Ölkürbis' (4,4 %), in 'Naked Seed' (3,9 %). Izmed vseh testiranih akcesij indukcija ni bila uspešna zgolj pri sorti 'Slovenska golica'. Učinkovitost je bila odvisna tudi od uporabljenega donorja peloda. Haploide smo pridobili samo pri opraševanju s pelodom 'GL Opal' in 'White Acorn', medtem ko opraševanje z drugimi akcesijami ni bilo uspešno oziroma ni vodilo v regeneracijo haploidov. Indukcija haploidov je bila najbolj učinkovita pri uporabi višjih testiranih doz (200 in 300 Gy). Skupno smo ploidnost določili 3830 domnevno partenogenetskim regenerantom. Večina jih je bila diploidnih, določili pa smo tudi haploidne, triploidne in, zanimivo, tetraploidne regenerante, ki so se bolj pogosto pojavljali po opraševanju s pelodom obsevanim z višjimi dozami. 99,6 % diploidnih regenerantov, analiziranih z mikrosatelitskimi markerji, je bilo heterozigotnih, pri enem pa heterozigotnosti nismo mogli ovreči zaradi odsotnosti ustreznih polimorfnih markerjev. Tako lahko zaključimo, da so diploidi zigotskega izvora in je za pridobivanje DH za žlahtnjenje hibridov potrebno podvojevanje genoma. Naša raziskava predstavlja prvo obširnejšo študijo indukcije haploidov pri oljnih bučah. Zaradi vedno večjega zanimanja za uporabo rentgenskih žarkov so rezultati tudi širše uporabni, saj je bilo doslej objavljenih izredno malo študij z uporabo le-teh.

Dosežena učinkovitost indukcije haploidov je za komercialno uporabo prenizka, zato smo se odločili preučiti možnost uporabe višjih doz za obsevanje peloda, ki vsaj teoretično odpira možnost nadaljnje optimizacije postopka. Pelod vrste *C. pepo* je že v naravnih pogojih zaradi izgube vlažnosti podvržen izgubi kalivosti, zato je potrebna prilagoditev razmer med obsevanjem. Za učinkovito preverjanje kalivosti smo optimizirali gojišče za *in vitro* kalitev peloda. Tekoče Brewbaker in Kwack (1963) gojišče z 12,5 % (w/v) saharoze in pH 9 je bilo določeno kot optimalno za kalitev in določanje kalivosti peloda po obsevanju, medtem ko druge testirane pH vrednosti in koncentracije saharoze ter dodatek manitola oziroma polietilenglikola niso izboljšali odstotka kalitve. Kalivost peloda, obsevanega pri HH, je bila statistično značilno višja kot kalivost peloda, obsevanega pri RH. Obsevanje s 100 Gy (RH in HH pogoji) je povzročilo statistično značilen upad

kalivosti. Večje razlike med metodama obsevanja so bile vidne predvsem pri dozah višjih od 350 Gy. Na osnovi poskusov opraševanja, pri katerih smo pelod obsevali v RH razmerah, lahko sklepamo, da je z uporabo HH pogojev možno uporabiti doze do 600 Gy, medtem ko je z RH možno pričakovati indukcijo formacije embrijev le do 500 Gy. Z meritvami premera peloda smo potrdili prisotnost dveh subpopulacij peloda, pri čemer je v tekočem gojišču kalil predvsem pelod blizu mediane populacije. Kaleč obsevan pelod je bil ne glede na metodo obsevanja večji kot neobsevan kaleč pelod. Zamik proti večjemu pelodu je bil bolj opazen pri pelodu, ki je bil obsevan pri HH pogojih. Velika variabilnost v velikosti peloda znotraj populacije in pa favoriziranje kalitve peloda določene velikosti verjetno kaže na možnost oziroma sposobnost prilagoditve različnim okoljskim pogojem.

Na osnovi izkušenj, ki smo jih pridobili z določevanjem ploidnosti regenerantom pri indukciji haploidov, smo se odločili analizirati endopoliploidnost različnih organov rastlin oljnih buč. S pomočjo pretočne citometrije smo ugotovili, da je listno tkivno najmanj endoreduplicirano in vsebuje največ celic z 2C in 4C jedri, medtem ko smo v hipokotilu in epikotilu detektirali jedra do 64C. Med najbolj endorepliciranimi je bilo tudi kotiledonsko tkivo. Iz literature je znano, da je za adventivno regeneracijo najbolj odziven bazalni del kotiledona. S slikovno citometrijo smo določili ravno ta del kotiledona kot najmanj endoreduplicirani v primerjavi s centralnim in distalnim delom. Izmed testiranih gojišč se je kot najboljše v smislu najvišjega odstotka regenerantov izkazalo Murashige in Skoog (1962) gojišče z dodatkom BA, PABA in FA. Uporabljene koncentracije FA so bile za uporabo v selekcijske namene prenizke, saj bi pričakovali, da bo dodatek glivnega toksina (FA) močno omejil regeneracijo in omogočil le-to zgolj poganjkom, ki so se regenerirali kot posledica izzvane variabilnosti. Ker so gojišča s FA vsebovala tudi PABA, ki je bila povezana z indukcijo sistemsko pridobljene imunosti v rastlinah, predlagamo uporabo višjih koncentracij FA ali pa gojišča na osnovi BA in 2iP. Presenetljivo pa smo na gojiščih s FA v srednjih koncentracijah (10 in 20 mg/l) detektirali tetraploidne regenerante. Tako lahko zaključimo, da FA inducira podvojevanje genoma, saj na gojiščih brez dodatka FA, kljub razmeroma visokim koncentracijam dodanih citokininov, poliploidov nismo detektirali.

Med najpomembnejšimi patogenimi organizmi pri pridelavi oljnih buč je virus ZYMV. V doktorskem delu smo želeli vzpostaviti metodo kvantifikacije virusa ZYMV z metodo qPCR, ki bi jo lahko uporabili v podporo fenotipskemu ocenjevanju in razporejanju v odpornostne razrede. Uspešno smo vzpostavili metodo mehanske inokulacije z virusom ZYMV in tako pridobili rastlinski material za pripravo vzorcev za analizo qPCR. Za kvantifikacijo virusa ZYMV in študijo ekspresije gena CAT1, ki je vključen v odgovor rastline na stres, smo najprej analizirali stabilnost izražanja 9 referenčnih genov (RPL36aA, ACT, UFP, PP2A, EF-1A, CAC, HELI, CUC18S, COX) v pogojih okužbe z virusom ZYMV in uporabili najprimernejše za normalizacijo. Med stabilno izražene referenčne gene so se po analizi s 4 algoritmi (geNorm, NormFinder, BestKeeper, Delta Ct metoda), ki jih vključuje program RefFinder, uvrstili EF-1A, UFP, RPL36aA, PP2A in CAC. Gena ACT in COX sta se v vseh analizah izkazala kot najmanj stabilna. Za normalizacijo podatkov sta bila po rezultatih analize z algoritmom geNorm potrebna le gena EF-1A in UFP. Ker je bilo število vzorcev prenizko za relevantne zaključke, lahko iz pridobljenih rezultatov izluščimo zgolj splošne trende, ki lahko služijo kot izhodišče za nadaljnje študije. Rezultati nakazujejo, da simptomatske rastline obeh sort akumulirajo

primerljive količine virusa ZYMV. V asimptomatskih rastlinah obeh sort je bilo detektiranega manj virusa ZYMV kot v simptomatskih rastlinah. Preliminarna analiza izražanja gena CAT1 je pokazala, da se tolerantna in občutljiva sorta na okužbo z virusom ZYMV odzivata različno v primeru asimptomatskih rastlin, kjer smo pri tolerantni sorti 'GL Opal' zaznali znižanje ravni izražanja CAT1, medtem ko je bilo v občutljivi sorti izražanje višje kot v kontroli. V primeru simptomatskih rastlin je bilo v obeh sortah opazno znižanje ravni ekspresije gena CAT1. Na osnovi naših preliminarnih rezultatov lahko povzamemo, da kvantifikacija virusa ZYMV verjetno lahko zgolj pripomore k standardizaciji opisa odpornosti, saj nismo zaznali očitnih razlik med sortama. Za bolj zanesljive zaključke bi potrebovali tako analizo večjega števila bioloških ponovitev kot tudi več vključenih akcesij.

4.2 SUMMARY

Despite growing interest for the production of styrian oil pumpkin, the development of biotechnological breeding approaches lags behind many other representatives of *C. pepo* and other species of the Cucurbitaceae family. Recently released hybrids are displacing open-pollinated cultivars due to their superior performance such as higher yield and better resistance to major diseases. Our aim was the development of hull-less genotype-specific biotechnological methods which could be applied to accelerate styrian oil pumpkin breeding.

A suitable alternative to the lengthy process of pure line development for hybrid breeding via backcrossing is haploid induction followed by genome doubling and final regeneration of DH. Given the number of successful reports in the Cucurbitaceae family, gynogenesis via pseudofertilization with irradiated pollen seems to be the method of choice for haploid induction. Most published protocols are based on gamma-irradiation, whose use is increasingly restricted due to rigid security and safety regulations. Therefore, we adapted the protocol for X-ray irradiation, which has been successfully applied and resulted in haploids in most of the tested hull-less pumpkin accessions. The highest haploid frequency was observed in 'Turkey #2' (10.0 %), followed by 'Gleisdorfer Ölkürbis' (4.4 %), and 'Naked Seed' (3.9 %) used as female donors. Of all tested accessions used as female donors, we have not been able to induce haploids only from the open-pollinated cultivar 'Slovenska golica'. The efficiency of haploid induction protocols also depends on the accessions used as the pollen donor. In our study, haploids were obtained when female flowers were pollinated with pollen of 'GL Opal' or 'White Acorn', whereas pollination with other donors was not successful or did not result in the regeneration of haploids. The optimum X-ray dose has been determined to be 200 and 300 Gy. In total, we determined the ploidy of 3830 putatively parthenogenic regenerants of which the majority was diploid. However, haploids, triploids and, interestingly, also tetraploids were detected. Tetraploid regeneration was clearly unexpected but was associated with pollination with pollen irradiated at higher doses. Using polymorphic microsatellite markers, the heterozygosity of 99.6 % analyzed diploids was confirmed, which suggests that there is no further need of testing diploid regenerants. These results clearly show that genome doubling of haploids is required for DH development. In this study, a method for haploid induction in hull-less pumpkin accessions was established for the first time.

The obtained haploid frequencies were insufficient for viable commercial application, therefore we aimed for further optimization of the haploid induction protocol. As known from literature, more haploids can be produced if higher doses are applied for pollen irradiation. Even under natural conditions, *C. pepo* pollen is prone to viability loss due to drying, therefore the maintenance of humidity during irradiation is crucial to ensure germinability after the prolonged irradiation needed to reach sufficiently high doses. For accurate germinability testing, we optimized the *in vitro* germination medium and found that liquid Brewbaker and Kwack (1963) medium with 12.5 % sucrose and pH 9 is optimal for styrian oil pumpkin pollen. Media with other sucrose concentrations, pH values and components (mannitol and polyethylene glycol) were not as efficient. After irradiation at HH conditions, the observed pollen germinability was higher than germinability of pollen irradiated at RH conditions. However, a significant drop in germinability was observed at 100 Gy, regardless of the humidity conditions during irradiation. Significant differences between the two irradiation conditions were observed at doses higher than 350 Gy (prolonged irradiation times). Based on the results of the haploid induction trial, in which irradiation at RH was used and a comparison of germination rates after irradiation under both conditions, embryo formation is expected for doses as high as 600 Gy using HH, whereas using RH only doses up to 500 Gy seem feasible. The pollen diameter measurements revealed two size subgroups in the total pollen population. In liquid medium, pollen near the median of the population was more likely to germinate. Generally, irradiated germinating pollen was larger than non-irradiated germinating pollen. A slight shift towards larger pollen was observed in germinating pollen irradiated under HH conditions. All these findings suggest that pollen of a certain diameter range is favoured in stress conditions and that variability in size might serve as a survival strategy in adverse environments.

Based on our observations obtained by ploidy determination of putatively parthenogenic regenerants, we decided to analyze the endopolyploidy of different organs of styrian oil pumpkin plants. Flow cytometric analysis revealed leaf tissue as the least endoreduplicated, with a majority of cells with 2C and 4C nuclei, whereas the hypocotyl and epicotyl contained nuclei with DNA content of up to 64C. Similarly, the cotyledon was determined as one of the most endoreduplicated organs. It has previously been shown that the basal part of the cotyledon is the most responsive for adventitious regeneration in *Cucurbita* spp. Our analysis of cotyledonary tissue revealed that the basal part is the least endoreduplicated among the 3 sections analyzed (basal, central, and distal) in 7 day old seedlings. Basal cotyledonary explants were therefore subjected to regeneration on Murashige and Skoog (1962) media with different supplements. The highest regeneration was observed on media supplemented with BA, PABA, and FA. FA was added to media for in *in vitro* selection pressure and should strongly limit regeneration and allow survival of regenerants which developed as a result of induced variation. However, our results show that the addition of a low concentration of FA to regeneration media even increased the regeneration rate. We therefore propose the use of higher FA concentrations or 2iP-supplemented media, since PABA might alter the normal tissue response to the toxin, as it was shown to be involved in systemic aquired resistance. Interestingly, FA added to the media in intermediate concentrations (10 and 20 mg/l) resulted in the regeneration of

tetraploid regenerants. Since no tetraploids were detected on media without FA, we conclude that FA induces genome doubling.

For virus quantification, qPCR is commonly applied to support classical phenotyping and classification of disease resistance. We aimed to develop a qPCR based quantification method for ZYMV, which is one of the major pathogens in styrian oil pumpkin production. For valid qPCR analysis, an accurate normalization of data is required. In this study, the expression profiles of 9 reference genes (RPL36aA, ACT, UFP, PP2A, EF-1A, CAC, HELI, CUC18S, COX) were determined and those with most stable expression levels were selected for ZYMV quantification and expression analysis of the CAT1 gene in virus-infected leaf tissue. The expression stability of the reference genes was analyzed by RefFinder software, which includes analysis by 4 algorithms: geNorm, NormFinder, BestKeeper, and Delta Ct method. According to the comprehensive ranking of all 4 algorithms, the most stably expressed reference genes were UFP, PP2A, CAC, EF-1A, and RPL36aA. ACT and COX were consistently ranked as the least reliable reference genes. For normalization of ZYMV quantification and CAT1 expression, we used the 2 most stably expressed reference genes as determined by geNorm – UFP and EF-1A. Since the number of biological replicates in our study was insufficient to make reliable conclusions, we can only derive some general trends from our data. Our preliminary results show that the tolerant and susceptible cultivar accumulated comparable quantities of ZYMV in systemically infected leaves of symptomatic plants. However, less virus was detected in asymptomatic plants of both cultivars. The preliminary analysis of CAT1 gene expression revealed a differential response to ZYMV infection in asymptomatic plants of the two cultivars analyzed; the tolerant cultivar responded to infection with ZYMV with a downregulation of the CAT1 gene, whereas the susceptible one responded with an upregulation. In symptomatic plants, both cultivars responded with a downregulation of the CAT1 gene. Although our results are only of a preliminary nature we can conclude that based on the fact that no major differences in the accumulation of ZYMV were found, qPCR analysis can serve only as a supporting technique of classical phenotyping, owing to the fact that the observed ZYMV quantities did not correspond to resistance levels of analyzed cultivars. A detailed analysis using a higher number of biological replicates and accessions would allow for the obtention of more accurate conclusions.

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