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Inter-simple sequence repeat analysis of somaclonal variation in *Sechium edule* var. *virens levis* resistant to *Phytophthora capsici*

Análisis mediante repeticiones de secuencias intersimples de la variación somaclonal en *Sechium edule* var. *virens levis* resistentes a *Phytophthora capsici*

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ABSTRACT

Veracruz is the main producer of *Sechium edule* in Mexico, which is severely affected by *Phytophthora capsici*, a phytopathogen that affects roots, stems, leaves, and fruits, causing plant death. The molecular variation of *S. edule* vitroplants resistant to *P. capsici* was evaluated using inter-simple sequence repeat (ISSR) markers. Genomic DNA was extracted from *S. edule* mother plants and ten vitroplants showing resistance to *P. capsici.* Twenty primers were used to select those showing clarity, reproducibility and high number of amplified bands. Three primers were selected to analyze genetic variability. The profiles obtained from polymerase chain reactions (PCR) were recorded in a binary matrix, with 1 representing the presence of a band and 0 absence. The total number of amplified bands, the percentage of polymorphic bands and the size of each band were recorded. The analysis showed variation in *S. edule* plants resistant to *P. capsici* compared to the mother plants. Primers ISSRCR-8, UBC 808, and UBC-834 produced 13, 21, and 18 polymorphic bands with sizes of 250–2,500 bp, and 81.25, 100, and 100 % polymorphism, respectively, confirming the

molecular variation in the somaclonal variants.

Keywords: Indirect organogenesis*, in vitro* culture, ISSR, somaclonal variation

RESUMEN

Veracruz es el principal productor de *Sechium edule* en México, el cual es severamente afectado por *Phytophthora capsici*, un fitopatógeno que afecta raíces, tallos, hojas y frutos, causando la muerte de las plantas. Se evaluó la variación molecular en vitroplantas de *S. edule* resistentes a *P. capsici* utilizando marcadores de repetición de secuencias intersimples (ISSR). El ADN genómico se extrajo de plantas madre de *S. edule* y diez vitroplantas que mostraban resistencia a *P. capsici*. Se utilizaron 20 cebadores para seleccionar aquellos que mostraran claridad, reproducibilidad y un alto número de bandas amplificadas. Tres cebadores se seleccionaron para analizar la variabilidad genética. Los perfiles obtenidos de las reacciones en cadena de la polimerasa (PCR) se registraron en una matriz binaria, con 1 representando la presencia de una banda y 0 ausencia. Se registró el número total de bandas amplificadas, el porcentaje de bandas polimórficas y el tamaño de cada banda. El análisis mostró variación en las plantas de *S. edule* resistentes a *P. capsici*, comparadas con las plantas madre. Los cebadores ISSRCR-8, UBC 808 y UBC-834 produjeron 13, 21 y 18 bandas polimórficas con tamaños de 250-2,500 pb y 81.25, 100 y 100 % de polimorfismo, respectivamente, lo que confirma la variación molecular en las variantes somaclonales.

Palabras clave: Organogénesis indirecta, cultivo *in vitro*, ISSR, variación somaclonal.

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1. INTRODUCTION

Mexico is the main producer and exporter of the smooth green variety of chayote. The states that stand out in its production are Veracruz, Michoacan, Jalisco and San Luis Potosi, with Veracruz being the main producer of this vegetable (SIAP, 2024). Due to the commercial success of *S. edule* var. *virens levis*, production as a monoculture has increased significantly in recent years (Cadena-Íñiguez, 2005; SIAP, 2024). This has led to the appearance of phytosanitary problems such as that caused by *P. capsici*, one of the main phytopathogens that affect the crop. This oomycete is cosmopolitan, highly dynamic, and destructive, causing serious damage, like crown rot in plants, yellowing and wilting of foliage, and infected fruits are characterized by presenting watery lesions, and the appearance of white mycelium, eventually leading to plant death (Lamour *et al*., 2012; Olguín-Hernández *et al*., 2013). Soto-Contreras *et al*. (2024) reported a protocol to obtain *in vitro* plants of *S. edule* regenerated by indirect organogenesis, in which vitroplants grown in media supplemented with culture filtrates of *P. capsici* were selected. These vitroplants showed increased resistance with a decrease in yellowing and necrosis at the base of the stem. It has been reported that plants regenerated via indirect organogenesis exhibit somaclonal variation with respect to the parent plants (Ramírez-Mosqueda & Iglesias-Andreu, 2015). This genetic variation originated in plants is a biotechnological tool used to search for disease resistance (Torres, 2010). Somaclonal variation has also been an effective tool for obtaining promising genotypes from an agronomic point of view, tolerant to different biotic factors (pathogenic cells, insects, and herbivores) and abiotic factors (heavy metals, salts, herbicides, phytotoxins, fungal filtrates) and of high quality (Lestari, 2006; Lebeda & Svábová, 2010).

Larkin & Scowcroft (1981) were the first to define somaclonal variation, which refers to the genetic variability generated during plant *in vitro* culture. Although the causes of this phenomenon have not been fully elucidated, it is known to be caused by genetic and epigenetic changes present in the explant (genotype), as well as by the stress that *in vitro* conditions impose on explants (Kaeppler *et al*., 2000; Azizi *et al*., 2020), factors influencing this phenomenon are: explant source, culture medium, type and concentration of plant growth regulators (PGRs), and number and duration of subcultures (Krishna *et al*., 2016). The PGR, 2,4-dichlorophenoxyacetic acid (2,4-D) is a mutagenic agent that is directly related to the induction of somaclonal variation (Ramírez-Mosqueda & Iglesias-Andreu, 2015; Baklouti *et al*., 2022).

The analysis of somaclonal variation can be conducted by different methods: physiological, morphological, biochemical, and molecular (Bairu *et al*., 2011). Within these, molecular markers have been widely used for the analysis of somaclonal variation in different crops; like pea (*Pisum sativum* L.) (Kuznetsova *et al*., 2005), rice (*Oryza sativa* L.) (Ngezahayo *et al*., 2007), orchid hybrids (*Aerides vandarum* Reichb. f x *Vanda stangeana* Reichb. f) (Kishor & Devi, 2009), rye (*Secale cereale* L.) (Linacero *et al*., 2011), hydrangea (*Hydrangea macrophylla* (Thunb.) Ser.) (Liu *et al*., 2011), date palm (*Phoenix dactylifera* L.) (Ahmed *et al*., 2012), lily (*Lilium orientalis* L.) (Liu & Yang, 2012), vanilla (*Vanilla planifolia* Jacks.) (Ramírez-Mosqueda & Iglesias-Andreu, 2015), maral root (*Raphonticum carthamoides* (Willd.) Iljin) (Skała *et al*., 2015) and tuberose (*Polianthes tuberosa* L.) (Nalousi *et al*., 2019).

The most widely used molecular markers are ISSR (Núñez-Pastrana *et al*., 2023). ISSRs have the advantage of providing reproducible results at a low cost, and no prior knowledge of the sequences is required for detection (Ng & Tan, 2015). ISSRs are a tool based on the Polymerase Chain Reaction (PCR), described by Zietkiewicz *et al*. (1994), these markers were developed based on the use of microsatellite repeats with di, tri, tetra or penta nucleotide repeats, through the fixation (5' or 3') of arbitrary primers (Pradeep Reddy *et al*., 2002; Ranghoo-Sanmukhiya, 2020). ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta *et al*., 1994; Tsumura *et al*., 1996; Ratnaparkhe *et al*., 1998; Wang *et al*., 1998). However, they also segregate as co-dominant markers in some cases, thus enabling distinction between homozygotes and heterozygotes (Wu *et al*., 1994; Akagi *et al*., 1996; Wang *et al*., 1998; Sankar & Moore, 2001), and are highly polymorphic (Pradeep Reddy *et al*., 2002; Ranghoo-Sanmukhiya, 2020). These properties make ISSRs suitable markers for analyzing somaclonal variation that originates during *in vitro* culture (Peng *et al*., 2015; Pastelín-Solano *et al*., 2019). Genotypes with desirable agronomic characteristics (resistant to biotic and/or abiotic factors) can be obtained by inducing somaclonal variation as a biotechnological tool for plant breeding and analyzing it using molecular markers. The objective of this study was to evaluate the somaclonal variation of *S. edule* var. *virens levis* obtained by indirect organogenesis with resistance to *P. capsici* using ISSR markers. The results obtained will contribute to *S. edule* genetic improvement purposes.

2. MATERIALS AND METHODS

2.1. Plant material

The analysis of somaclonal variation involved three mother plants of *S. edule*, germinated *in vitro* from zygotic embryos. The plants were established following the protocol of Cruz-Martínez *et al*. (2017). Also, 10 *S. edule* plants obtained by Soto-Contreras *et al*. (2024), that were regenerated by indirect organogenesis from callus obtained from the mother plants, and that were resistant to *P. capsici* were included in the study.

2.2. Genomic DNA extraction from *S. edule* plants

The cetyltrimethylammonium bromide (CTAB) method (Stewart & Via, 1993) was employed for genomic DNA extraction. Duplicate extractions were performed using 0.25 g of leaf tissue from both, *S. edule* mother plants and *S. edule* plants from indirect organogenesis resistant to *P. capsici*. DNA extractions were carried out individually for each plant. DNA concentration was calculated using a spectrophotometer (Genesys 10S UVVIS, Thermo Scientific, IL, USA) at an absorbance (A) of 260 nm. The absorbance ratios A260/280 and A260/230 were utilized to assess the purity of the samples. The DNA concentration was 650 to 1,600 ng μL^{-1}, with $A_{260}/_{230}$ and $A_{260}/_{280}$ ratios indicating good purity. Furthermore, DNA integrity was confirmed through 1% (w/v) agarose gel electrophoresis, stained with 4.3 µg μL^{-1} ethidium bromide, and visualized on an ultraviolet (UV) light transilluminator coupled to a GelDoc-It photodocumentation system (UVP, Upland, Canada). The extracted samples were stored at -20 °C to prevent DNA degradation.

2.3. ISSR analysis

ISSR analysis was performed on *S. edule* mother plants and plants derived from indirect organogenesis and resistant to *P. capsici.* The analysis was carried out individually for each plant. First, 20 ISSR primers (Integrated DNA Technologies, IDT®) were tested to screen DNA polymorphism using DNA from mother plants (Table 1). Based on the results, three ISSR primers were used to assess genetic variability in *S. edule* plants obtained from indirect organogenesis and resistant to *P. capsici*.

Table 1. ISSR primers used to select reproducible primers to detect somaclonal variation in *S. edule.*

ISSR Primers	Nucleotide Sequences (5´ - 3´)	Alignment temperature (°C)
UBC 825	ACACACACACACACACT	46.4
UBC 864	ATGATGATGATGATGATG	38.6
UBC 816	CACACACACACAGG	41.2
UBC 846	CACACACACACACACART	46.8
UBC 865	CCGCCGCCGCCGCCGCCG	71.8
UBC 866	CTCCTCCTCCTCCTCCTC	50.7
ISSRCR-8	GTGGTGGTGGTGGTGGC	54.7
ISSRCR-2	CACACACACACACACACAAG	46.6
UBC 808	AGAGAGAGAGAGAGAGC	42.8
UBC 811	GAGAGAGAGAGAGAGAC	41.8
UBC 812	GAGAGAGAGAGAGAGAA	40.7
UBC 813	CTCTCTCTCTCTCTCTT	40.7
UBC 815	CTCTCTCTCTCTCTCTG	41.8
UBC 834	AGAGAGAGAGAGAGAGYT	44.2
UBC 840	GAGAGAGAGAGAGAGAYT	42.4
UBC 842	GAGAGAGAGAGAGAGAYG	43.8
UBC 857	ACACACACACACACACYT	48.1
UBC 860	TGTGTGTGTGTGTGTGRA	48.1
UBC 879	CTTCACTTCACTTCA	36.3
UBC 880	GGAGAGGAGGAGAGGAGA	49.3

Y = C or T.

PCRs were carried out in a final volume of 25 µL. The reaction mixture included 25 ng of DNA, 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primer, and 1U of Taq DNA polymerase (Sigma-Aldrich Chemical Company, MO, USA). The PCR products were amplified using a MaxyGeneTM thermal cycler (Axygen® Inc., Union City, CA), with the following program: 95°C for 2 min, followed by 30 cycles at 95°C for 30 sec, different alignment temperatures for 30 sec, 72°C for 1.5 min, and a final extension at 72°C for 10 min. Alignment temperatures were 5°C below the melting temperature of each primer. The amplification process was repeated three times for each primer.

2.4. Agarose gel electrophoresis

The amplification products were separated by electrophoresis on 2.0% (w/v) agarose gels using 1X TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA, and pH 8.4) at 90 V for 1.4 h. Gels were stained with 4.3 µg μL^{-1} of ethidium bromide for 30 min. A DNA ladder (250–10,000 bp, Promega®) was used as a molecular weight marker. Finally, the bands were visualized on a UV light transilluminator, coupled to the photo-documentation system described above.

2.5. Molecular data analysis

The three ISSR primers that produced clear and reproducible bands in the mother plant and in the plants obtained from indirect organogenesis were recorded in a binary data matrix, with value 1 for band presence and 0 for absence. GenAlEx 6.5 software (Genetic Analysis in Excel, Peakall and Smouse, 2012) was used to calculate the total number of amplified bands, the percentage of polymorphic bands, and the size of each band.

3. RESULTS

3.1. ISSR analysis on *S. edule* mother plants

From the 20 ISSR primers used to standardize in mother plants, eight primers generated a total of 78 bands; the number of bands detected per primer varied from 4 to 14, with an average of 9.75 bands per primer. The molecular weights of the bands ranged from 300 to 2,000 bp (Table 2). The remaining 12 primers did not produce clear and reproducible bands, and some showed few bands (2 or 3).

Table 2. Number and size of ISSR bands amplified in the *S. edule* mother plants.

ISSR Primers	Total number of amplified bands	Band size **(base pairs)**
UBC-825	6	600-1,500
UBC-865	7	400-1,300
UBC-866	4	900-1,500
ISSRCR-8	14	500-2,000
ISSRCR-2	13	500-1,900
UBC-808	14	500-1,400
UBC-834	14	300-1,500
UBC-880	6	300-900
Total	78	300-2,000
Number of bands/ primer	4-14	-
Number of bands/primer (mean)	9.75	-

Figures 1 and 2 show the ISSR band profiles of *S. edule* mother plants obtained using the eight selected primers. Primers ISSRCR-8, UBC-808, and UBC-834 produced the highest number of bands (14) with sizes ranging from 500-2,000, 500-1400, and 300-1,500 bp, respectively. Conversely, primer UBC-866 generated the fewest bands (4) with sizes of 900-1,500 bp (Fig. 2). Primers, ISSRCR-8, UBC-808 and UBC-834 were selected to analyze somaclonal variation

in *P. capsici*-resistant *S. edule* plants since they were the ones that produced the highest number of bands.

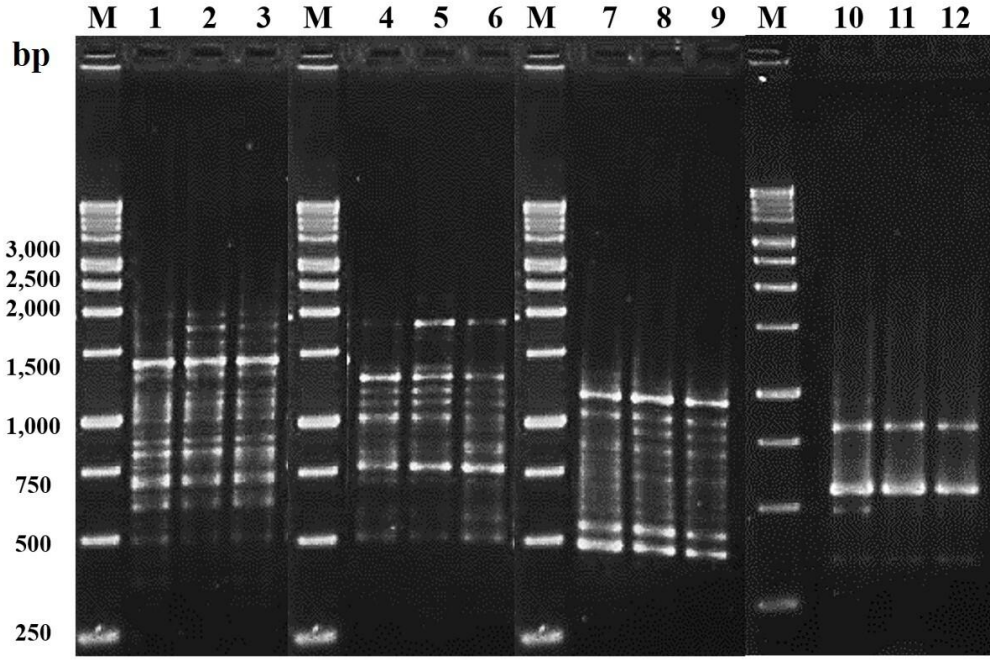

Fig. 1. ISSR banding pattern in 2% (w/v) agarose gel of *S. edule* mother plants amplified with primers ISSRCR-8 (1-3), ISSRCR-2 (4-6), UBC-808 (7-9) and UBC-880 (10-12). M = molecular weight marker, bp = base pairs.

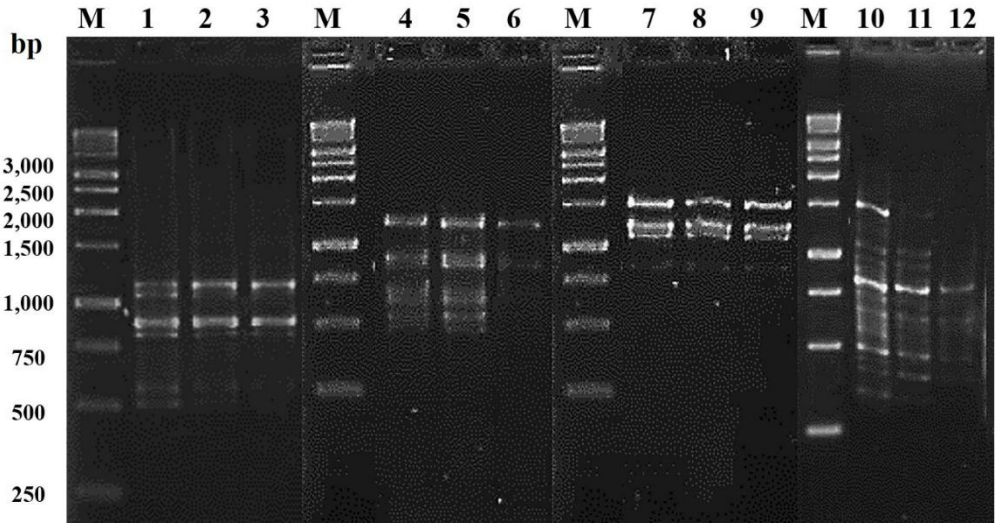

Fig. 2. ISSR banding pattern in 2% (w/v) agarose gel of *S. edule* mother plants amplified with primers UBC-825 (1-3), UBC-865 (4-6), UBC-866 (7-9) and UBC-834 (10-12). M = molecular weight marker, bp = base pairs.

3.2. ISSR analysis on *S. edule* plants from indirect organogenesis and resistant to *P. capsici*

A total of 52 different bands were found, with each primer producing between 13 and 21 bands. On average, each primer produced 17.33 different bands (Table 3). Primers UBC-808 and UBC-834 produced the highest numbers of bands, with 21 and 18, respectively. Both primers also had the highest percentage of different bands, with 100% polymorphism for each (Figs. 3 and 4). On the other hand, the primer ISSRCR-8 had the lowest number and percentage of polymorphic bands, with 13 bands and a polymorphism rate of 81.25% (Table 3) (Fig. 5).
The 11 *in vitro* plants (a mother plant and 10 plants from indirect organogenesis) showed differences between them in the number of amplified bands and the size of each band (Table 3).

Table 3. Analysis of ISSRs in *S. edule* plants derived from indirect organogenesis resistant to *P. capsici*.

ISSR primers	Total number of amplified bands	Percentage of polymorphic bands (%)	Band size (base pairs)
ISSRCR-8	16	81.25	450-2,500
UBC-808	21	100	250-2,100
UBC-834	18	100	300-2,100
Total	55	94.54	250-2,500
Number of bands/Primer	16-21	-	-
Number of bands/Primer (mean)	18.33	-	-

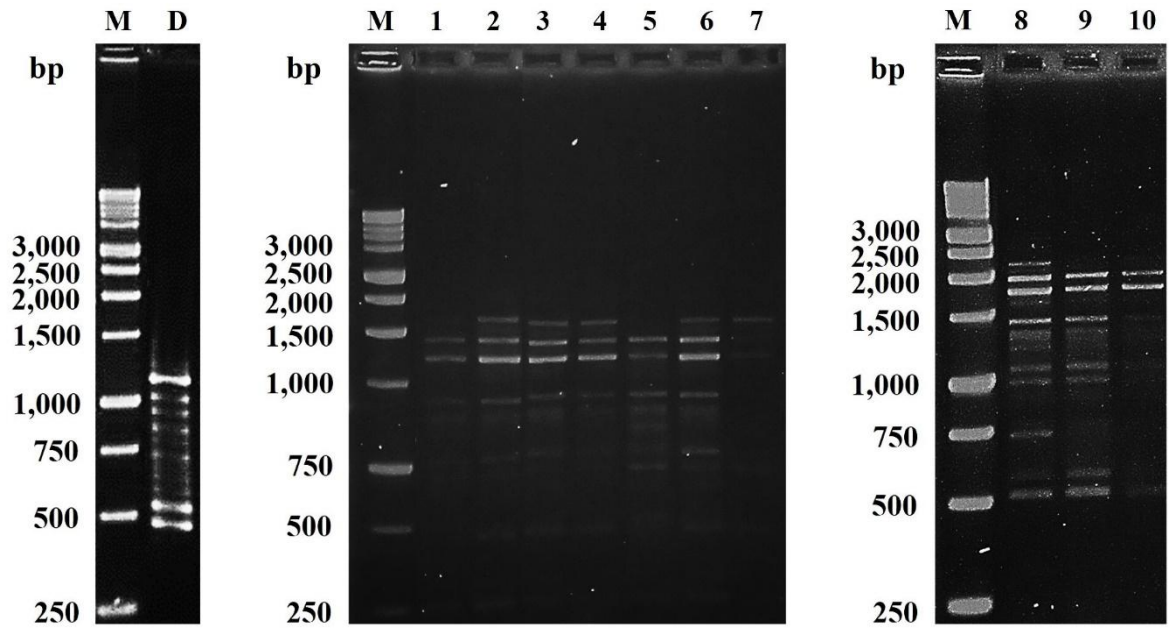

Fig. 3. ISSR banding pattern in 2% (w/v) agarose gel of *S. edule* plants resistant to *P. capsici*, amplified with primer UBC-808. M: molecular weight marker (bp = base pairs), D: mother plant, 1-10: *S. edule* plants resistant to *P. capsici*.

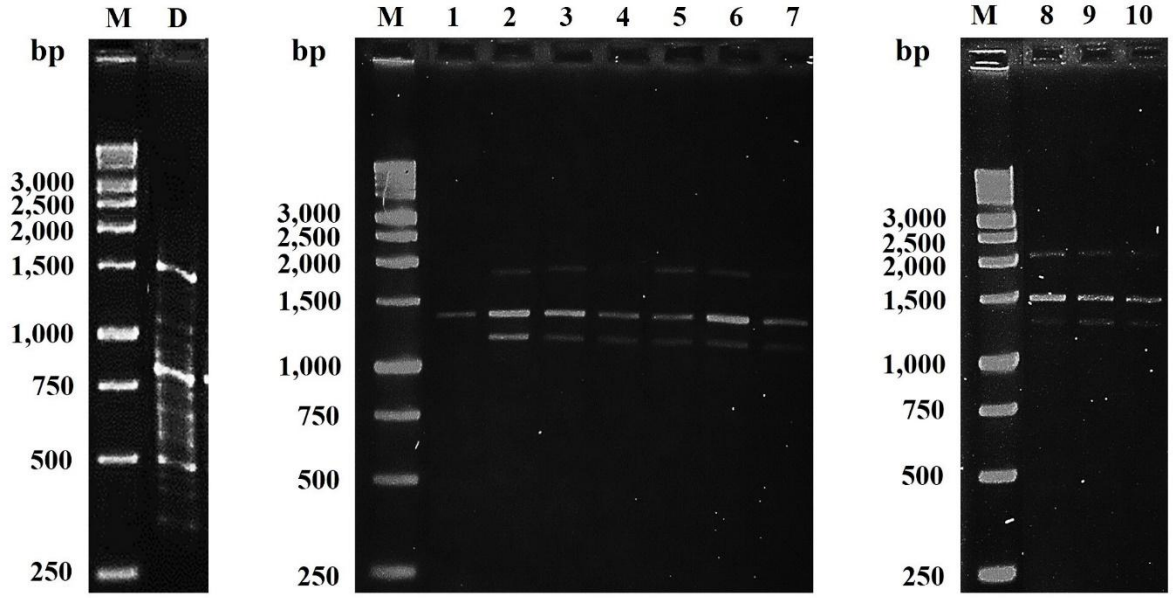

Fig. 4. ISSR banding pattern in 2% agarose gel of *S. edule* plants resistant to *P. capsici,* amplified with primer UBC-834. M: molecular weight marker (bp = base pairs), D: mother plant, 1-10: *S. edule* plants resistant to *P. capsici*.

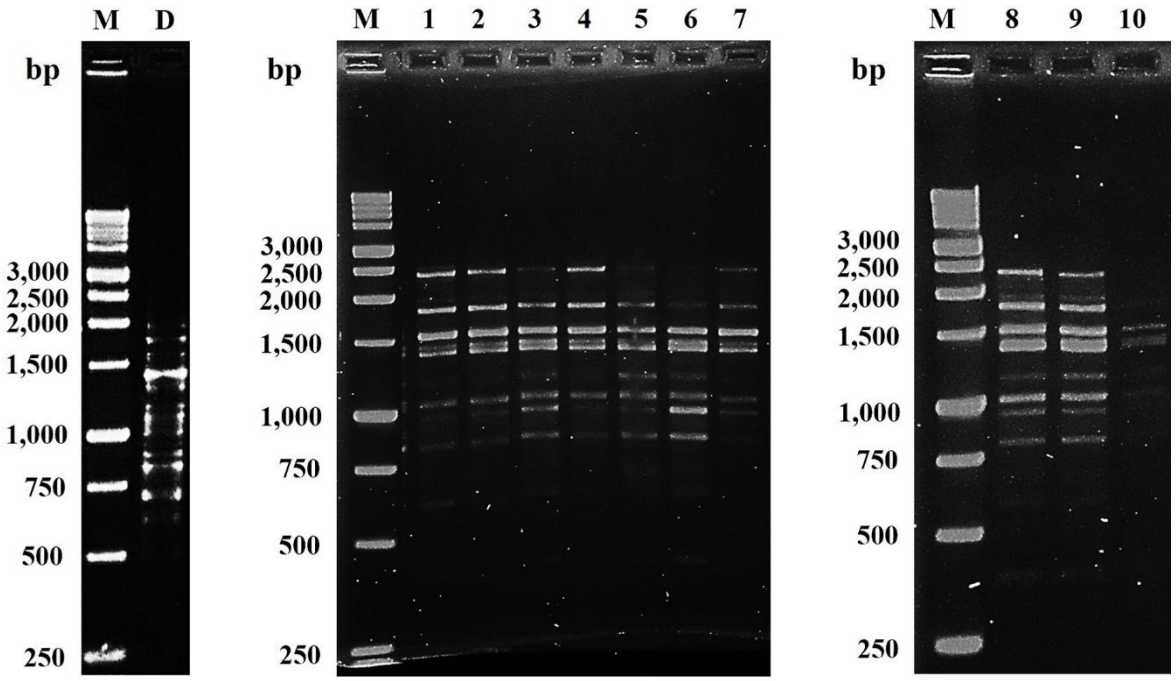

Fig. 5. ISSR banding pattern in 2% agarose gel (w/v) of *S. edule* plants resistant to *P. capsici*, amplified with the ISSRCR-8 primer. M: molecular weight marker (bp = base pairs), D: mother plant, 1-10: *S. edule* plants resistant to *P. capsici*.

4. DISCUSSION

The analysis of somaclonal variation using ISSR markers in *S. edule* genotypes resistant to *P. capsici* is an essential tool for designing breeding programs in this species, for which, diverse sources of genetic resistance and agronomic elite characteristics must be incorporated, as this is a prerequisite for the production of superior varieties with increased productivity by resisting diseases or other biotic and abiotic stresses (Rajan & Singh, 2021). The methodology used to obtain *S. edule* vitroplants resistant to *P. capsici* has been successfully reported in other plant species to induce genetic variability, through the application of 2,4-D, followed by the indirect organogenesis route (Ferreira *et al*., 2023). Furthermore, using a selective agent during *in vitro* culture allows to obtain plants with the desired characteristics. Selection agents include pathogen elicitors, pathogen culture filtrates, and purified pathotoxins (Anil *et al*., 2018).
Pre-selected somaclonal variants that show a significant resistance response to the pathogen or its inducer are subsequently analyzed using molecular marker methods to determine that the observed resistance adaptation is indeed due to genetic changes (Anil *et al*., 2018). Currently, ISSR markers have been widely used to assess somaclonal variation because they are simple, fast, inexpensive and detect high levels of polymorphism (Ng & Tan, 2015). ISSR molecular markers were used because of their advantages. Fourteen different-sized bands (500-2,000, 500-1,400, and 300-1,500 bp) were identified using ISSRCR-8, UBC-808, and UBC-834 primers in the mother plants. This allowed for an accurate analysis of somaclonal variation in *S. edule* plants that were obtained through indirect organogenesis and were resistant to *P. capsici* compared to the mother plants. These three primers exhibited high levels of polymorphism (81.25%, 100%, and 100%, respectively). ISSRCR-8 primer has been used to evaluate the

genetic diversity of other cucurbits, yielding 13 bands ranging in size from 818 to 1,873 bp (Sikdar *et al*., 2010), while in this study the largest band was approximately 2,500 bp. This demonstrates that this primer can be widely used to analyze genetic variability in this plant family. UBC-808 and UBC-834 primers were successfully used in different cucumber genotypes to determine genetic diversity (Punetha *et al*., 2017; Sahoo *et al*., 2020) and UBC-808 primer was used to assess diversity in five melon cultivars (Daryono *et al*., 2020).

The observed somaclonal variation in the *S. edule* plants resistant to *P. capsici* may be attributed to the method of plant acquisition and the *in vitro* selection pressure used to induce resistance to the pathogen. The differences between the 11 *in vitro* plants analyzed were due to the somaclonal variation that each one possesses and may also be due to the variations in the resistance to *P. capsici* that each plant acquired.

The results obtained in this study agree with studies reported on *S. cereal* (Linacero *et al*., 2011), *H. macrophylla* (Liu *et al*., 2011), *L. orientalis* (Liu & Yang, 2012), *Capsicum chinense* Jacq. (Bello-Bello *et al*., 2014), *V. planifolia* (Ramírez-Mosqueda & Iglesias-Andreu, 2015), *R. carthamoides* (Skała *et al*., 2015) and *P. tuberosa* (Nalousi *et al*., 2019), in terms of the pathway used to obtain somaclonal variants and the analysis of somaclonal variation using ISSR markers. In cucumber, 20 ISSR primers were used to evaluate plant resistance to downy mildew disease, a total of 17 primers (85%) amplified in 40 cucumber plants, with a percentage of polymorphic bands per primer ranging from 55.56% to 100% (Innark *et al*., 2014). The number of primers used (20) and the high percentage (100%) reported in cucumber coincides with that reported in this study with the chayote crop. Hamza *et al*. (2024) used ISSR markers to evaluate the resistance of cucumber plants to *Fusarium oxysporum*, they selected five primers for analysis, one showed a polymorphism of 60%, while three primers showed 75% and the fifth no polymorphism. Based on the results obtained in this study, it is suggested to use at least 20 ISSR primers for a correct analysis of somaclonal variation in disease resistant plants.

5. CONCLUSION

The eight primers (UBC-825, UBC-865, UBC-866, ISSRCR-8, ISSRCR-2, UBC-808, UBC-834, and UBC-880) that were assessed in *S. edule* mother plants exhibited an adequate number and size of amplified bands, showing ISSR are suitable for assessing somaclonal variation. Primers ISSRCR-8, UBC-808, and UBC-834 allowed to compare polymorphism between *S. edule* plants derived from indirect organogenesis and resistant to *P. capsici*, and mother plants. This polymorphism indicates the presence of somaclonal variation in *S. edule* plants resistant to *P. capsici*. Nonetheless, these plants should be analyzed in greenhouse and field conditions to corroborate their resistance to the oomycete and investigate whether this resistance is preserved in subsequent generations.

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AUTHORS CONTRIBUTION

Anell Soto-Contreras: Methodology, software, research, writing of original draft, visualization, writing, review and editing. Lourdes G. Iglesias-Andreu: Writing, review and editing, supervision

and project management. Rosalía Núñez-Pastrana: Conceptualization, validation, formal analysis, resources, writing of original draft, writing, review and editing, visualization, supervision and project management.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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