UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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School of Agricultural Engineering and Environment

Evaluation of resistance to the Moroccan Watermelon Mosaic Virus (MWMV) in a collection of accessions of the genus Cucurbita

End of Degree Project

Bachelor's Degree in Biotechnology

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BIOTECHNOLOGY DEGREE FINAL THESIS

EVALUATION OF RESISTANCE TO THE *MOROCCAN WATERMELON MOSAIC VIRUS* (MWMV) IN A COLLECTION OF ACCESSIONS OF THE GENUS *CUCURBITA*

ACADEMIC COURSE 2021/2022

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Title	Evaluation of resistance to the *Moroccan Watermelon Mosaic Virus* (MWMV) in a collection of accessions of the genus *Cucurbita*.
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Summary

Cucurbit species are considered economically important crops worldwide and breeding for disease resistance is a major objective to increase the annual yield of these crops. *Potyviruses*, such as *Zucchini Yellow Mosaic Virus* (ZYMV) or the *Watermelon Mosaic Virus* (WMV) can cause significant damage to cucurbits. Additionally, four decades ago, a new aphid-transmitted species within the genus was reported, the *Moroccan Watermelon Mosaic Virus* (MWMV), which is considered an emergent threat to cucurbit production in Mediterranean countries. As an insect transmitted disease, the most efficient method to avoid it is through genetic resistance. Thus, screening for sources of resistance to this emerging virus would help avoiding significant economic losses. To do so, natural resources of resistance should be searched in both cultivated and related wild species, which is the main objective of this study, and has been focused on the genus *Cucurbita* (pumpkins, squashes, and gourds). Thirty-five different cucurbit accessions, selected among a germplasm collection maintained by the Cucurbits breeding group and the germplasm bank within COMAV, were mechanically inoculated with a MWMV isolate provided by Geves Group. Source of viral inoculum was obtained by infecting plants of *Cucurbita pepo*, MU-CU-16 accession, known to be susceptible to the disease. Plants were grown in a climatic chamber with controlled temperature and symptoms were assessed for four weeks. Tissue samples were collected for further viral RNA analyses by tissue printing/dot-Blot hybridization or qPCR. Results indicate that tissue printing is not suitable for viral detection, but symptomatology, dot-Blot and qPCR are efficient methods to identify MWMV infection. Most of the *Cucurbit*a spp. accessions studied were susceptible to MWMV. However, two accessions of *C. maxima* were found as candidates to be tolerant, while one accession from a wild species, *C. pedatifolia*, was classified as resistant. Future prospects include the evaluation of tolerance in these accessions and the genetic study of MWMV resistance inheritance from *C. pedatifolia*.

Key words: Squash and gourds, screening, diversity, mechanical inoculation, tissue printing, dot-Blot, qPCR.

Resumen

Las cucurbitáceas se consideran una familia de gran importancia económica a nivel global, y uno de los objetivos principales para incrementar el rendimiento anual de estos cultivos se basa en la mejora de resistencia a enfermedades. Los *Potyvirus*, como el *Zucchini Yellow Mosaic Virus* (ZYMV) o el *Watermelon Mosaic Virus* (WMV) pueden provocar daños significativos a las cucurbitáceas. Adicionalmente, hace cuatro décadas, se reportó una nueva especie de virus transmitida por pulgones dentro del género, denominada *Moroccan Watermelon Mosaic Virus* (MWMV), considerada una amenaza emergente para la producción de cucurbitáceas en los países mediterráneos. Como se trata de una enfermedad transmitida por insectos, el método más eficiente para evitarla es mediante resistencia genética. Así pues, la búsqueda de fuentes de resistencia frente a este virus ayudaría a evitar pérdidas económicas considerables. Para ello, se deben buscar fuentes naturales de resistencia tanto en especies cultivadas como silvestres relacionadas, siendo este el objetivo de este trabajo, que se ha enfocado en el género *Cucurbita* (calabazas y calabacines). Treinta y cinco entradas diferentes del género *Cucurbita*, seleccionadas de la colección de germoplasma mantenida por el grupo de mejora de cucurbitáceas y el

banco de germoplasma del COMAV, fueron inoculadas de forma mecánica con un extracto de un aislado de MWMV proporcionado por el grupo Geves. La fuente de inóculo viral se obtuvo mediante infección de plantas de la especie *Cucurbita pepo*, entrada MU-CU-16, que es susceptible a la enfermedad. Las plantas se cultivaron en una cámara climatizada, a una temperatura controlada y los síntomas se monitorizaron durante cuatro semanas. Se recogieron muestras de tejido para posteriores análisis del RNA viral mediante hibridación basada en tissue printing/dot-Blot o qPCR. Los resultados indican que la técnica de tissue printing no es adecuada para la detección del virus, pero la visualización de síntomas, dot-Blot y qPCR son métodos eficaces para identificar infección por MWMV. La mayoría de las especies de *Cucurbita* estudiadas fueron susceptibles al MWMV. Sin embargo, dos entradas de *C. maxima* se encontraron como posibles candidatas a ser tolerantes, mientras que una entrada de una especie silvestre, *C. pedatifolia*, se clasificó como resistente. Los prospectos de futuro incluyen la evaluación de tolerancia en las dos primeras entradas y estudios genéticos de la heredabilidad de la resistencia a MWMV de *C. pedatifolia*.

Palabras clave: Calabazas, cribado, diversidad, inoculación mecánica, tissue printing, dot-Blot, qPCR.

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After four years of laughs, tears and stress, this bachelor thesis means that I have finally finished this academic formation period of my life, in which I have put all my energy and efforts.

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INTRODUCTION

1. INTRODUCTION

1.1 Cucurbitaceae family

The Cucurbitaceae family comprises ca. 1000 species within 96 different genera, many of which are commercially relevant (Grumet et al., 2017). Globally, 10 are considered major crops, cultivated worldwide and thus regarded as economically important, while another 23 species fall into being minor crops, often cultivated locally (Wehner et al., 2020). The major domesticated crops include *Citrullus lanatus* (Thunb.) Matsum & Nakai (watermelon), *Cucumis melo* L. (melon), *Cucumis sativus* L. (cucumber), *Cucurbita argyrosperma* Huber (cushaw), *Cucurbita maxima* Duchesne (winter squash), *Cucurbita moschata* (Duchesne ex Lam.) Duchesne ex Poiret (pumpkin, butternut squash), *Cucurbita pepo* L. (zucchini), *Benincasa hispida* (Thunb.) Cogn. (wax gourd), *Lagenaria siceraria* (Molina) Standl. (bottle gourd) and *Momordica charantia* L. (bitter gourd) (Chomicki et al., 2020). Additionally, many wild taxa are potentially valuable, which means that this family is extremely important for human exploitation (Wehner et al., 2020).

According to The United Nation's Food and Agriculture Organization (FAO) statistics, cucumbers and melons, pumpkins, and squash (*Cucurbita* spp.), and watermelons are located among the leading crops in quantity of production, area planted and monetary value. The worldwide production of these cucurbits was estimated in 249,309,354 tonnes from a harvested area of 2,019,564 hectares in 2020 and a total yield of 1,141,341 hg/ha (FAOSTAT, 2022). Within cucurbits, surface of cultivation and production is led by watermelons, while cucumber yield is the most significant worldwide (**Figure 1**).

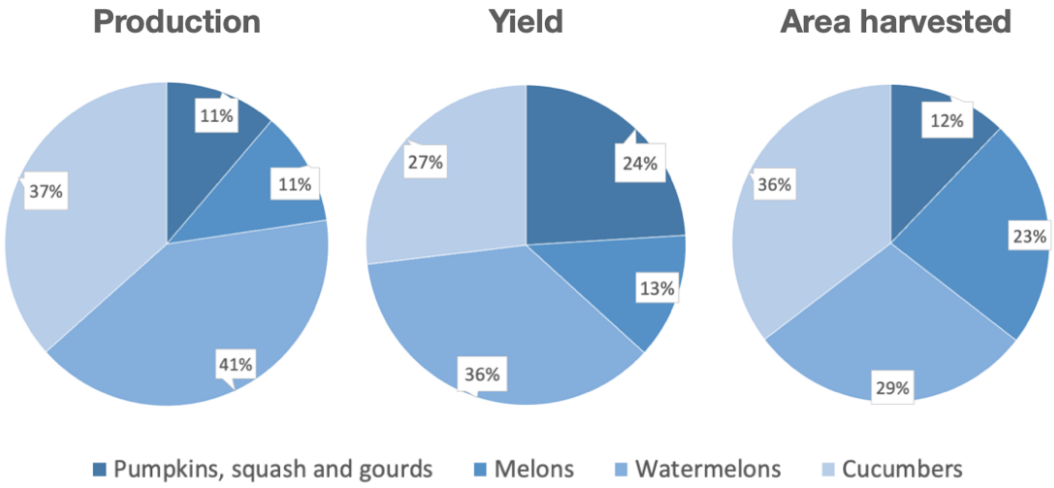


Figure 1. World production (tonnes), yield (hg/ha) and area harvested (hectares), respectively, of main cucurbits; pumpkins, squash, and gourds (*Cucurbita* spp.), melons and cucumbers (*Cucumis* spp.), and watermelons (*Citrullus lanatus*). Average percentage of each cultivar indicated to the total is depicted on all graphs (FAOSTAT, 2022).

Species within Cucurbitaceae are usually subtropical or tropical and are distributed globally. Many of them are endemic to the Americas (40%), while others occur in Africa (28%) and Asia (26%) (Art et al., 2021). Individuals in this family are frost-sensitive and have tendril-bearing vines, being all of them dichotomous.

1.2 *Cucurbita* spp.

The *Cucurbita* genus comprises 14 species (**Figure 2**), being all native to Americas (Grumet et al., 2017; Nesom, 2011). They are usually divided into mesophytic annuals, including cultivated and wild species with fibrous roots and xerophytic annuals, representative of the ancestral *Cucurbita*, composed by wild species with fleshy storage roots (Art et al., 2021).

Figure 2. Phylogeny and domestication of *Cucurbita*, based on the maximum likelihood (ML) inferred from 44 nuclear loci (Kates et al., 2017). Varieties within same species are equally colored. ML bootstrap support values are shown for the backbone and the main clades (Chomicki et al., 2020).

Only five *Cucurbita* species have been domesticated (Grumet et al., 2017); *Cucurbita argyrosperma, C. ficifolia* Bouché, *C. maxima*, *C. pepo* and *C. moschata* being the last three species extensively cultivated globally, which indicates that most of the species within this genus are wild resources (Zhu et al., 2021). Pumpkins, squashes, and gourds are edible ubiquitous fruits from *Cucurbita* species, easily grown in different climates and with a considerable worldwide annual production (Grumet et al., 2017) specially in China and India, which are the main producers of these cucurbits (**Figure 3**).

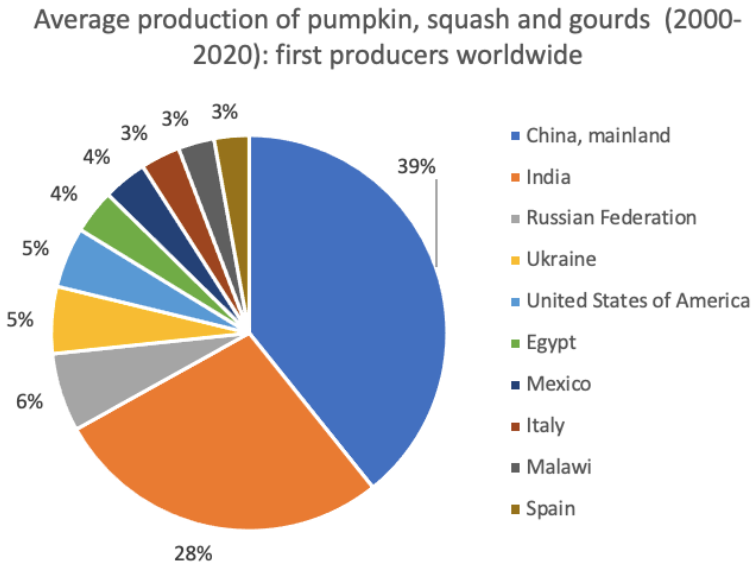


Figure 3. Main producer countries of pumpkin, squash, and gourds, according to an average production between years 2000 and 2020 in tonnes (t). Production values are the following: China, mainland, 6,364,218.81 t; India, 4,486,913.48 t; Russian Federation, 1,040,289.19 t; Ukraine, 886,511.43 t; United States of America, 803,173.33 t; Egypt, 585,838.19 t; Mexico, 584,479.9 t; Italy, 523,807.43 t; Malawi, 479,240.6 t and Spain, 462,018 t (FAOSTAT, 2022).

1.2.1 Origin and domestication

Many important crop species within the Cucurbitaceae family are the world's oldest domesticates (Art et al., 2021). The earliest pumpkin domestication, initiated in northern South America and Central America, occurred ca. 10,000 years ago (Chomicki et al., 2020). Movement of cucurbits from their respective centers of origin is the main outcome of human interaction (Grumet et al., 2017), and followed by diversification, the species currently known arised (Art et al., 2021). *Cucurbita* species were early employed by humans (Kates et al., 2017) and through cultivation and domestication, nowadays they exhibit traits distinguishable from other cucurbits, such as a great variety of fruit shapes, sizes and colours. Furthermore, adaptation to different abiotic and biotic stresses characterizes these species, especially to cold, salinity and some viruses (Zhu et al., 2021).

Domestication involves a change in crop's abiotic and biotic environments, resulting in new modified traits that differentiate the currently cultivated species from their wild ancestors. The main interest in this human-driven gradual process is to provide crops with enhanced characteristics, that suit the needs of both growers and consumers. However, not only the interest in enhanced traits by human gatherers, but certain characteristics commonly associated with colonizing species or weeds, enabled cucurbit adaptation to human environments (Wehner et al., 2020). People probably started by consuming only the gourd seeds, which were nutritious and rich in oils, while fruits started to be used after an initial domestication, due to their bitterness (Chomicki et al., 2020). Selection of plants lacking bitter fruits was facilitated with the occasional encounter by native people, which allowed the fruit flesh to become a food source, however, these were not only useful as food, but as ornaments or utensils. It is thought that six or more independent domestication events occurred within the genus and that the same species could have been domesticated more than once, as occurs with C. *pepo* (Grumet et al., 2017).

Comparisons between wild and domesticated cucurbit resources indicate that domestication initiated with the loss of bitterness and selection affected the sugar and carotenoid content, as well as the seed coat (Chomicki et al., 2020).

Nowadays, crop improvement is based on finding new genes and alleles that confer valuable traits, which makes germplasm resources essential to ensure high quality, yield, shelf life and sustainable production of cucurbits (Grumet et al., 2017). After many years of independent domestication events within the genus, especially considering the five domesticated species, a great pool of genetic diversity has arisen. These were distributed differently through allopatric cultivation, having their own native ranges, and being adapted to distinct climate (Grumet et al., 2017).

1.2.1.1 Bitterness

Even though some wild cucurbit species have sweet pulps, in most of them the fruits are bitter. This bitterness depends on the presence of cucurbitacins, which are terpenoid compounds present in roots, leaves, and fruits that confer defense mechanisms against herbivores (Zhou et al., 2016). In most *Cucurbita* species, bitter fruits are produced because of genetics and environment, and these bitter compounds can occur both in foliage and fruits. Several genes control the bitterness caused by cucurbitacins and for fruits of C. *pepo,* the *Bi* gene has been found to be the cause of exhibition of this trait, which encodes an enzyme responsible for the initiation of cucurbitacin C biosynthesis (Chomicki et al., 2020). However, fruit bitterness is usually conferred by both *Bi* and *Bt* genes, the later one being selected during domestication (Shang et al., 2014). Nevertheless, the environment is also related with the fruits being bitter, especially in dry periods, but can be managed with adequate irrigation (Wehner et al., 2020).

This trait was essential in the initial domestication of Cucurbitaceae fruit crops, and the bitterness was lost by a mutation in the promoter that regulates the *Bt* gene, causing a significant reduction in the production of cucurbitacins (Art et al., 2021).

1.2.1.2 Sweetness and carotenoid content

This feature, independently from the loss of bitter fruits, was also a key within cucurbits domestication, especially in melon and watermelon. Sweetness is a trait that depends on a combination of genes involved in sugar metabolism and transport, as well as depends on environmental conditions (Chomicki et al., 2020). In *Cucurbita maxima*, sucrose content is the principal determinant of sweetness, but for the heritability of the trait, the environment in which the plants grow, rather than the genotype itself, has a major impact on it (Hurst et al., 2006). Carotenoids are antioxidants usually in the form of ß-carotene or lycopene, which confer the characteristic orange or red fruit pulp (Art et al., 2021) and provide nutritional benefits, thus determining the fruit quality (Hurst et al., 2006). In pumpkins, the composition of carotenoids is affected by maturation stage, the environment and the edaphoclimatic conditions (Abbas et al., 2020). Its accumulation is due to the regulation of many metabolic genes, 12 recognized for *C. moschata* (Abbas et al., 2020) and as occurs with fruit size, selection for this trait is complicated (Art et al., 2021). However, it is important to note that since some fruits are consumed when mature, while others before maturity, these traits are not as relevant as others within pumpkins, squashes, and gourds.

1.2.1.3 Fruit size and seed traits

Cell differentiation, division and expansion are the processes that control fruit size. This indicates that to acquire larger fruits, shifts within cell division and expansion are needed and there is still uncertainty in how transcriptional regulators act on this matter due to its complexity (Art et al., 2021). Several studies, such as (Montero-Pau et al., 2017) in *Cucurbita pepo*, have been focused on the identification of genes and QTLs (Quantitative Trait Locus) involved in fruit shape and size. However, it is known that phytohormones, microtubules and cyclins play a key role in fruit size. Not many cucurbit crops are

grown for seed usage, but the most economically important ones come from *C. pepo* and in some cultivars, a single recessive locus has been identified to control the seed traits (Chomicki et al., 2020).

1.2.2 Diversity and morphology

Plants within Cucurbitaceae are mainly found in subtropical and tropical regions, rather than in temperate climate, being sensitive to freezing temperatures (Wehner et al., 2020). *Cucurbita* spp. are monoecious, having mostly large, orange, and showy unisexual flowers, (**Figure 4**), which anthers produce considerable amounts of pollen. The fruit is referred as pepo and the variety of shapes, sizes and colors is diverse across cultivars. Bitter fruits are usually only found in wild species and seeds from domesticated species tend to be large (Wehner et al., 2020). In general, there is a great variety within cucurbits and species can be easily distinguished by foliage, growth habit, flowering, peduncle, fruit, and seed characteristics (ECPGR, 2008).

Figure 4. Flowers from different *Cucurbita* species. (a,b) *C. maxima* male flower. (c) *C. argyrosperma* male flower. (d) *C. maxima* female flower. (e,f,g) *C. pepo* female flower. (h) *C. moschata* female flower.

1.2.2.1 Cultivated species

1.2.2.1.1 *Cucurbita pepo*

Cucurbita pepo includes summer and winter squashes (consumed unripe and ripe, respectively), pumpkins, ornamental gourds, and even unique cultivars, indicating that this species is highly polymorphic. In fact, it includes 3 subspecies, being ssp. *pepo* and ssp. *ovifera*, both containing cultivated types, and ssp. *fraterna*, which only includes wild populations (Paris et al., 2015). Peduncles are generally angular and hard, but sometimes these are slightly flared next to the fruit, depending on their domestication centre, either the North American (**Figure 5d.1**) or the Mexican (**Figure 5d.2**). From the first domestication, the modern Pumpkins and Zucchini squash (ssp. *pepo*) arise, while from a second time arise the Scallop, Crookneck, Acorn and Straightneck squash (spp. *ovifera*) (Paris, 2016). The stem is usually hard, angular, prickly, and grooved, and leaves are palmately lobed, often deeply

cut (**Figure 5c**). Seeds are characterized by a dull white to tan coloration, with a smooth surface and prominent margins (**Figure 5e**). Within Cucurbitaceae, it appears to have the larger fruit genetic diversity, regarding color, texture, size, and shape. Fruits can be white, yellow, orange, or green, even striped, or variegated. Their surface can be smooth, furrowed, warty or ribbed and their shape varies from being flat to round, going through oval, elongated or necked forms (**Figure 5a, 5b**). Diameter of the fruits is also diverse being the smaller of 5 cm in some gourds and going up to 50 cm in pumpkins (Wehner et al., 2020).

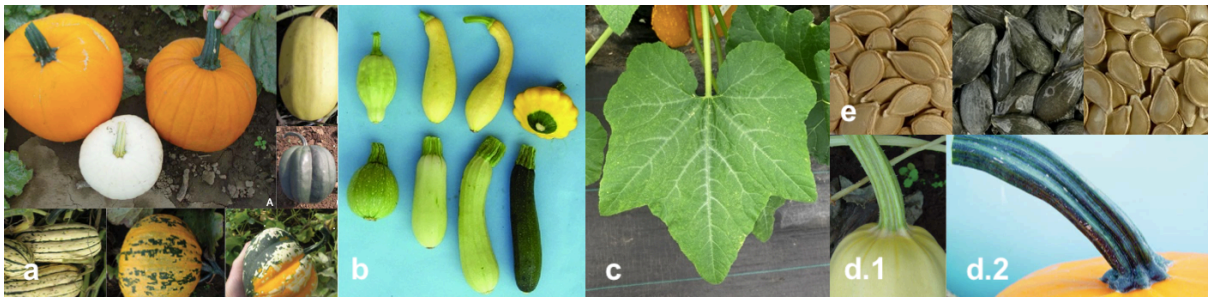


Figure 5. (a) *Cucurbita pepo* Winter squashes: Jack-o'lantern ornamental and pie Pumpkins, Spaghetti, Acorn, Delicata, Styrian oilseed pumpkin and Delicata-acorn type (Wehner et al., 2020). (b) Summer squashes; Zucchini, bicolor Straightneck, Crookneck, Ball and Patty pan, from left to right (Lust & Paris, 2016). (c) *C. pepo* leaf. (d.1, d.2) *C. pepo* from Mississippi river valley (left) and Mexican centre of domestication (right) peduncles (Wehner et al., 2020). (e) *C. pepo* seeds.

1.2.2.1.2 *Cucurbita moschata*

Cucurbita moschata, commonly referred as butternut squash, is considerably adapted to warm, humid tropics, even though it is known that many cultivars can thrive in temperate and arid climates (Paris, 2016). Three horticultural types are classified as commercial cultivars, being Butternut-Bell, Cheese, and Crooknecks (**Figure 6a.1, a.2, a.3**), but the global diversity within *C. moschata* is much more extense (Paris, 2016). Fruits within this species are highly diverse and represent an important source of nutrients for the diet (Paris, 2016). They are usually large, often being green or with a buff-colouration and even yellow-skinned. Fruit surface is smooth or ribbed, with wrinkled, warted or smooth rinds. Peduncles of this species are hard, smoothly angled and broadly flared (**Figure 6b**), while the stem is hard and smoothly grooved. Leaves are nearly round to moderately lobed and soft (**Figure 6c**). Seed coloration goes from dull white to brown, with a rough surface and a prominent margin (Wehner et al., 2020).

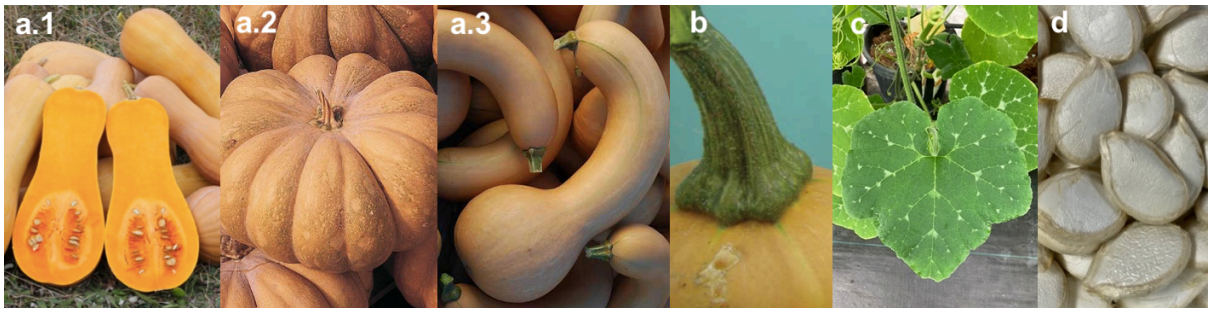


Figure 6. (a.1, a.2, a.3) *Cucurbita moschata* fruits; Butternut-Bell, Cheese and Crooknecks. (Chernilevsky, 2022; Wooldridge, 2015) (b) Peduncle morphology (Wehner et al., 2020). (c) *C. moschata* leaf. (d) *C. moschata* seeds.

1.2.2.1.3 *Cucurbita maxima*

Some *Cucurbita maxima* are known to produce the largest squashes within the genus. They can have green, orange, or grey coloration, a round or oval shape, being the last one smooth or ribbed (**Figure**

7a.1, a.2). Even though most cultivars within *C. maxima* are used as winter squash, a few are consumed as immature vegetable, together with seeds. The peduncle is generally soft, round and often corky (**Figure 7b**), while the stem is soft and round. Leaves tend to be unlobed, nearly round and soft (**Figure 7c**) and seed coloration goes from white to brown, being often plump and split or wrinkled in their surface (**Figure 7d**). Squashes within this species are the largest among all *Cucurbita* spp., being typically oval or round and sometimes including a protuberance when blossom ends. Fruits tend to be green, orange or even grey, with plump and large seeds (Wehner et al., 2020). Whereas ssp. *maxima* includes the cultivated types, ssp. *andreana* is considered the wild ancestor of the cultivated forms (López-Anido, 2021).

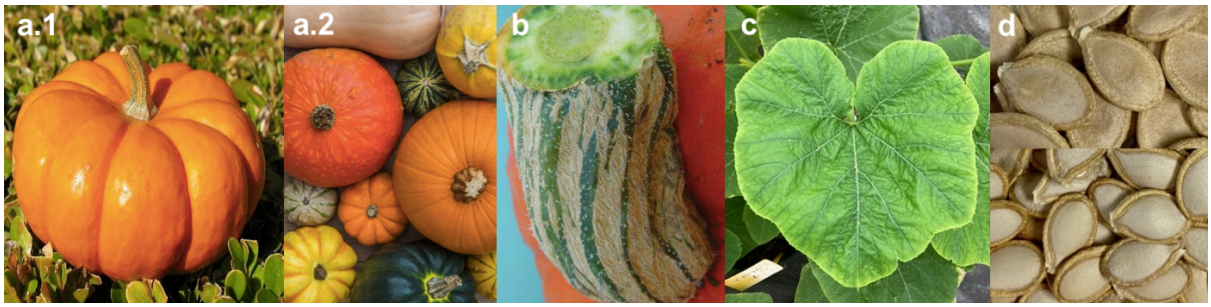


Figure 7. (a.1, a.2) *Cucurbita maxima* fruit diversity (Maroon, 2020). (b) Peduncle morphology (Wehner et al., 2020). (c) *C. maxima* leaf. (d) Seeds.

1.2.2.1.4 *Cucurbita argyrosperma*

Commonly referred as cushaw or silver-seeded gourd, this species is usually grown for the seeds or as forage, mainly because the quality of the fruit flesh is low. Additionally, the phenotypic diversity appears to be the lowest one among *Cucurbita* spp. (Art et al., 2021) (**Figure 8a**). Cultivated forms belong to ssp. *argyrosperma*, supposed to derive from the wild ssp. *sororia* (Sanjur et al., 2001). The peduncle is hard, angular but becoming round at maturity, corky in the valleys, only slightly flared at fruit attachment (**Figure 8b**). Stems are hard, angular, and grooved, while leaves are moderately lobed and softly pubescent (**Figure 8c**). Seeds are usually white, possibly very large and with a smooth or split surface (**Figure 8d**). The margin is prominent, smooth to ragged and sometimes dark (Wehner et al., 2020).

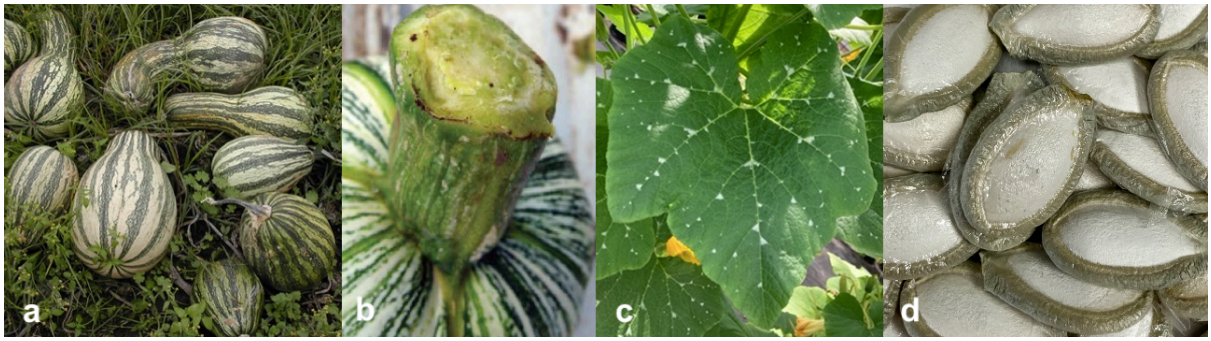


Figure 8. *(a) Cucurbita argyrosperma* fruits (Lopez, 2006). (b) Peduncle morphology (Wehner et al., 2020). (c) *C. argyrosperma* leaf. (d) Seeds.

1.2.2.1.5 *Cucurbita ficifolia*

Fig-leaf gourd is a monoecious climbing vine, cold-tolerant and adapted to cultivation at high altitudes species. However, its distribution is much more narrowed if compared with the other cultivated species. Since it is poorly diffused outside the tropics due to its ecological requirements, this crop presents little diversity. Fruits are uniform, usually mottled green and white, with a round to oblong shape (Art et al.,

2021) (**Figure 9a**). The peduncle is hard, smoothly angled with slight flaring (**Figure 9b**), while the stem is hard and smoothly grooved. Leaves are lobed, nearly round, and slightly prickly (**Figure 9c**). Seeds are usually black and sometimes tan, with a minutely pitted surface, having a narrow and smooth margin (Wehner et al., 2020) (**Figure 9d**). Even though not many cultivars are commercialized, they serve as rootstocks for grafting, and it is tolerant to abiotic stresses as cold and salinity, so it represents a natural resource for resistance breeding (Art et al., 2021).

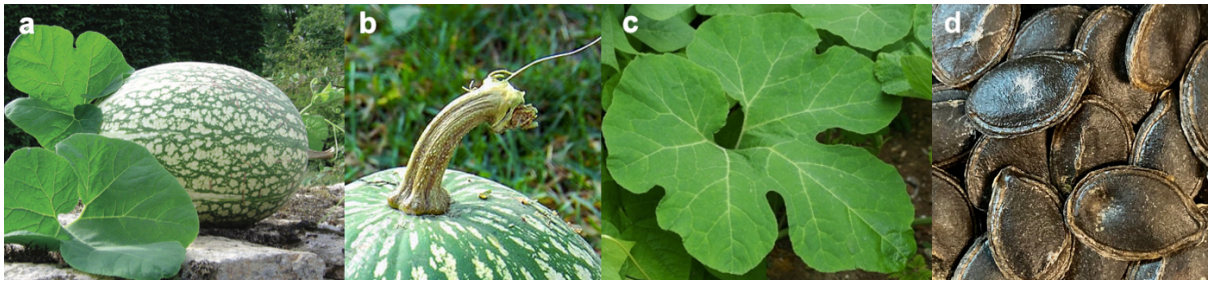


Figure 9. (a) *Cucurbita ficifolia* fruit (Salliet, 2006). (b) Peduncle morphology (Weha, 2020) . (c) *C. ficifolia* leaf (Spedona, 2008). (d) Seeds.

1.2.2.2 Wild species

In comparison with the cultivated *Cucurbita*, wild species are relatively uniform in surface, coloration, shape, and size, being usually small (**Table 1**). Distribution of many of these species is restricted to small regions of America, except for a minority that is less geographically limited. Taxa found in this group include *Cucurbita ecuadorensis* Cutler & Whitaker, *C. okeechobeensis* (Small) L.H. Bailey, *C. pedatifolia* L.H. Bailey, *C. foetidissima* Kunth, *C. lundelliana* L.H. Bailey, *C. palmata* S. Watson, *C. cordata* S. Watson, *C. digitata* A. Gray, *C. radicans* Naudin and *C. x scabridifolia* L.H. Bailey (Saade, 1995). However, *C. x scabridifolia* is probably a natural hybrid between *C. foetidissima* and *C. pedatifolia,* so it is not usually included within the 14 species in the genus (Kates et al., 2017).

Table 1. Morphology of some *Cucurbita* wild species, according to type of plant, stems, leaves, peduncle, and fruits (Bemis et al., 1978; Cutler & Whitaker, 1968; Saade, 1995).

Trait	*C. ecuadorensis*	*C. okeechobeensis*	*C. pedatifolia*	*C. foetidissima*
Plant	Herbaceous, vigorous annual climbers.	Climbers, herbaceous, annuals.	Herbaceous, climbing perennial, not extremely vigorous.	Essentially creeping plants, usually with a strong smell, with radial colony growth.
Stems	Striate with scattered spicules and hairs.	Slightly angled and striate, glabrescent.	Usually geniculate, with long or short internodes, being puberulent to glaberescent.	Teretes to slightly angular-sulcate, densely scabrous-pubescent-aculeolate.
Leaves	Broadly ovate to reniform, with often shallower lateral lobes, being the central one larger. Lobes are mucronate with irregular margin.	Cordate-orbicular to reniform, with triangular lobes, from obtuse to sharp and irregular margins.	Ovate-cordate, usually wider than long, lobed to sectate, with primary or superior lobes narrowly elliptical, wavy-lobate or auriculate. Obtuse to sharp apex, mucronate, with denticulate to dentate margins.	Usually triangular, entirely, or slightly lobed close to the base (heart shaped), rarely 3-5-angulose-lobed, with a well-defined central lobe. The apex is obtuse to sharp, mucronate.

Peduncle	Slender, long, angled, spiculate and slightly enlarged at attachment.	Rigid, slightly angular-sulcate, widened at the union to the fruit.	Slim, almost as long as fruits, slightly angular, widened at the union with the fruit.	Angular and sulcate, widening on the attachment to the fruit. Scabrous-pubescent-aculeolate, glaberescent with age.
Fruits	Globose, with smooth and hard shell, being green with creamy irregular longitudinal lines, turning yellow at maturity. Pulp is white and usually bitter.	Globose, smooth, and rigid shell, green with white-greenish longitudinal and abundant blemishes, turning light brown at maturity. The pulp is white, fibrous, and bitter.	Subglobose to commonly oblate, usually small. Hard and smooth shell, being shiny dark green, with longitudinal lines. The pulp is white greenish, rarely pale orange, fibrous and bitter.	Globose to oblate, rarely slightly ovoid, with smooth, hard, and light green shell, with longitudinal lines and small blemishes being of a lighter green to pale cream or white greenish. The pulp is fibrous, white, and bitter.
Seeds	Ovate, white to tan at the centre, with conspicuous margin that is darker than the body.	Ovate, blue-greenish-greyish, with slightly widened margins and obtuse to truncate-oblique apex.	Ovate-elliptic, smooth, white, or creamy, with poorly differentiated margins and obtuse apex.	White to pale creamy, ovate-elliptic, slightly compressed, without highlighted margins or conspicuously differentiated and with an obtuse apex.

1.2.3 Cultivation and uses

Even though there is a huge diversity in cucurbits usage, they are extensively used for food and drink purposes, being seeds, flowers, leaves, and shoot tips also used as part of the human diet in some regions. While many cucurbit species are employed as storage containers or as ornaments (Art et al., 2021), others are devoted to the production of oils, candles, soaps, shampoos, and other industrial products from seeds. In some cultures, they even function to obtain gunpowder, fuses, and tinder. Additionally, cucurbit fruits and their extracts are known for their therapeutic applications, functioning as emetics, purgatives, and anti-parasites. Presence of cucurbitacins, which trigger drastic effects in the digestive system, is what makes these crops important within medicine, but also the existence of free amino acids, saponins and alkaloids (Wehner et al., 2020). It is also important to mention that some *Cucurbita* spp., mainly *C. moschata* and *C. maxima* hybrids, are used as rootstocks to cope with several stresses in other cucurbits, such as watermelon or melon. Rootstocks of different cucurbit accessions have been previously tested for *Fusarium* spp. resistance, providing significant results not only in watermelon, but also in melon and cucumber (Lee et al., 2010; Miguel et al., 2004).

1.2.4 Threats to cucurbit production

Cucurbita crops are affected by both biotic, including several diseases caused by viruses, bacteria, fungi or oomycetes, nematodes and phytoplasmas, and abiotic stresses, as adaptations to local environments (Grumet et al., 2021). Every stage of cucurbit development is prone to be attacked by pathogens, from germination to even postharvest of the fruits and thus control measures are essential to maintain yield and quality (Wehner et al., 2020).

Numerous abiotic stresses can impact the crops, and these are specially related with the soil type, wind, solar radiation, irrigation systems, elevation, fertility, and the genotype of the plants. The first problem

that cucurbits face is the cold since temperatures below 10 ºC produce chilling injuries. However, the existence of tolerant accessions and the presence of tolerance genes both in the nucleus and cytoplasm, allows to create hybrids that can survive these harsh conditions. Another important issue is a water deficit caused by drought, which can reduce fruit yield and quality due to shorter vines, delayed flowering, and shift to maleness. As happens for low temperatures, there also exist tolerant accessions to drought that can serve to develop new adapted cultivars. Not only drought is a concerning problem, but flooding, which leads to hypoxia in soils and can reduce the nutrient content in the soil, and thus plant growth is reduced. The ideal solution for an excessive presence of water is the development of crops with adventitious roots, especially in the hypocotyl region to enhance flooding tolerance. Sustainable vegetable production is also dependent on heat tolerance and many tropic production countries suffer from high temperatures. Even though the origin of cucurbits is in tropical regions, not all of them can thrive in those environments. In some species heat induces flower abortion and sterility, reduced fruit size and even sunburn and desiccation of leaves. Nutrient availability also represents a major issue in cucurbit production, both deficiencies and excesses. Calcium, manganese, nitrogen, phosphorous and potassium are the most important ones, and their accessibility is essential to ensure adequate crop productivity. The last abiotic factor that influences cucurbit growth is salinity, which is a heritable trait that has been characterized in cucumber (Wehner et al., 2020).

Within biotic factors, soil-borne pathogenic fungi and plant-parasitic nematodes are the primary pathogens causing disease in the root system in cucurbitaceous crops. Both coexist in the rhizosphere and cause disruption of the vascular system in the host plant and interfere in the water and nutrient uptake. In the first group, *Phytophthora* spp., *Pythium* spp. and *Fusarium* spp. are remarkably important, but also others such as *Rhizoctonia solani*, *Acremonium cucurbitacearum*, *Phomopsis* spp., *Monosporascus* spp., *Olpidium bornovanus* or *Verticillium dahlia*. All these soil-borne fungi are distributed worldwide, leading in many cases to plant death and thus to a significant decline in yield. Nematodes associated with cucurbits include many genera, being *Meloidogyne* the most important due to its damage, wide distribution, and economic importance. Even though cucurbit crops are all susceptible to species within this genus, the suitability differs, which indicates that the interaction between the host plant and nematode species is extremely specific (Ayala-Doñas et al., 2020). These root knot nematodes act by invading the root tissue, which causes galling of roots, stunting and wilt in plants, but they can also transmit viruses, such as the *Tobacco ringspot virus* (TRSV) (Wehner et al., 2020).

Bacterial wilt is one of the main threats to cucurbit crop production in Asia and America, caused by *Erwinia tracheiphila* or bacterial fruit blotch caused by *Acidovorax citrulli* (Art et al., 2021). These are transmitted by striped and spotted beetles, and cause severe losses, mainly in *Cucurbita* and *Cucumis*. Management against it relies on the use of insecticides, but the approach also involves humans, pollinators, birds, and other ecosystem providers endangerment, apart from being costly (Liu et al., 2018).

Pests, as occurs with insects as whiteflies, thrips, and aphids, apart from carrying potential pathogens, can themselves represent an obstacle to cucurbit production yield. Cucurbits are affected by insect pests through their different growth stages, suffering from defoliation, damage in roots or flowers and being more susceptible to receive viral, bacterial, or fungal diseases (Sharma et al., 2016). For that reason, integrated pest management (IPM) is essential, including the use of control methods as crop rotation, ploughing or trap crops. Even though, cultural, and physical methods are in many cases complemented with chemicals, there is an increased interest in the use of beneficial insects for biological management, such as the use of the parasitic wasp *Encarsia formosa* to control the whitefly population. Additionally, resistance breeding has also been suggested and tested, but progress is scarce and slow, especially because conferring resistance against a specific insect can involve susceptibility to others (Wehner et al., 2020).

Viruses are considered the most representative threat to cucurbit production, and more than 60 have been identified to infect cucurbits in natural conditions, being at least 10 of them identified in Spain (Pérez-De-Castro et al., 2020). Many of these remain agronomically important and introduction of new

strains in several countries is leading to an increased economic impact on cucurbit yield. Even though the prevalence and stability of many recurrent viruses is apparently adequate, these newly transmitted strains are rapidly replacing the pre-existing virus populations. This emergence appears to be related with changes in climate and cultural practices, which in fact favor long-distance spread by natural vectors, but also due to commercial exchanges of plant material (Desbiez, 2020).

Viruses from the genera *Potyvirus, Cucumovirus, Poleovirus, Begomovirus, Ipomovirus, Crinivirus* and *Tobamovirus* are known to infect cucurbits, being transmitted either through insect vectors, direct contact, seeds, or soil (Desbiez et al. 2020). Among the most important ones, due to their distribution and damage, *Cucumber mosaic virus* (CMV), *Cucurbit aphid-borne yellows virus* (CABYV), *Beet pseudo-yellows* (BPYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Cucurbit chlorotic yellows virus* (CCYV), *Cucumber vein yellowing virus* (CVYV) and *Tomato Leaf Curl New Delhi Virus* (ToLCNDV) can be found (Martín-Hernández & Picó, 2021).

Potyviruses are particularly important worldwide, and at least 20 have been described to infect cucurbits, causing severe damage to cucurbits, and involving significant economic losses, such as *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV) and *Papaya ringspot virus* (PRSV) (Kabelka & Grumet, 1997). They are transmitted in a non-persistent manner by several aphid species, but also through pruning tools or plant contact. However, this type of transmission is not very specific, the virus remains in the stylet from minutes to hours and many species can transmit a given virus. These insects also constitute agronomic issues, since they produce direct damage through sap-sucking feeding, which consequently induces weakening, photosynthesis reduction and leaf crispation (Desbiez, 2020).

1.3 Moroccan Watermelon Mosaic Virus

Four decades ago in Morocco, a new aphid-transmitted species within the genus *Potyviruses* was reported, the *Moroccan Watermelon Mosaic Virus* (MWMV), considered an emergent threat to cucurbit production in Mediterranean countries (Chatzivassiliou et al., 2016). Even though it was firstly described as a strain from WMV, it was recently identified as a distinct virus within the genus, part of the PRSV cluster (Ibaba et al., 2016). Since its discovery, it has been detected in many countries within Africa, as Niger, South Africa, Sudan, Zimbabwe, Cameroon, Congo, Tunisia, Tanzania, Nigeria and Kenya, but also in countries as Italy, Portugal, France, Greece, Iraq and Spain (Miras et al., 2019; Mumo et al., 2022).

The viral genome is a single-stranded positive sense RNA, 9.7kb in size, with a single Open Reading Frame (ORF), that gives rise to a large polyprotein which is cleaved by the virus-encoded proteases into individual functioning proteins (Wylie et al., 2017). Studies indicate that the MWMV core protein shares 73% of similarity with PRSV, while it shares 61% of sequence identity with MWV and ZYMV (Kabelka & Grumet, 1997). The host range of this virus is narrow and mainly affects cucurbits (Chatzivassiliou et al., 2016) and, interestingly, possibly due to it and its absence in alternative weeds, its prevalence is short, and it no longer appears to be present in Spain since it was first reported in 2018 (de Moya-Ruiz et al., 2021).

1.3.1 Transmission

Since MWMV was identified, it has rapidly spread to many Mediterranean countries and Africa (Chatzivassiliou et al., 2016). Its transmission is through a few aphid species in a non-persistent manner, such as *Myzus persicae, Aphis spiraecola, A. fabae* and *A. nerii* (Mumo et al., 2022). Additionally, no seed transmission has been reported so far (Chatzivassiliou et al., 2016). Since wounds are required to propagate the virus, for research purposes, mechanical inoculation of the viral isolate is an effective and simple method to transmit the virus to the desired host and monitor how the infection proceeds (Mumo et al., 2022). In Spain, the occurrence of aphid-borne viruses is expanding, possibly because there is a lack of enough measures against viruses or their vectors. Not only because of that, but an increase in organic cultivation, making these cultivars more vulnerable to the attack by aphids. Thus, the current

status of viruses, together with their genetic structure and epidemiology should be continuously assessed (de Moya-Ruiz et al., 2021).

1.3.2 Symptoms

Identification of viral symptoms is an essential issue to avoid misinterpretation with nutritional deficiencies. These may depend on the species, the plant cultivar, the growth conditions or, in some cases, coexistence of viruses in the same individual (de Moya-Ruiz et al., 2021). Cucurbits infected by MWMV show mosaic patterns, interveinal chlorosis, with dark green blisters on the leaves. Filimorphism is also observed in leaves (Mumo et al., 2022), as well as leaf and fruit malformation (Ibaba et al., 2016). If the infection appears early, severe stunting may occur, leading to reduced fruit yield or even complete crop failure.

1.3.3 Detection methods

Since treating the plants after infection is not feasible, accurate diagnosis is the basis of disease management to eliminate sources of transmission in fields (Rubio et al., 2020). Several methods have been developed to allow plant viral detection, including those based on biological properties as evaluation of symptomatology, microscopy, serological and molecular techniques, as well as high-throughput sequencing (Jeong et al., 2014).

1.3.3.1 Symptomatology

Many symptoms are characteristic of specific diseases, but many factors can have influence on the exhibition of these symptoms, such as virus strain, host, or environmental conditions. Additionally, there could be no apparent symptoms or that the plant is symptomless, but still infected. This indicates that in many cases, evaluation of symptoms by itself is not completely reliable to indicate if a plant is infected or not by a certain virus, and other complementary analyses should be employed (Naidu & Hughes, 2003).

1.3.3.2 Microscopy

Morphology of the viral particles is commonly useful to identify many plant viruses, especially if they induce characteristic inclusions or crystalline aggregates. In the case of *Potyviruses*, with techniques as Orange-Green or negative staining, characteristic fibrous inclusions can be distinguished (Christie & Edwardson, 1986). Electron microscopy (EM) is a powerful tool, but tedious and expensive, which means that it is not affordable by some research institutions. If available, it can provide a simple and rapid method to confirm viral infection. Nevertheless, it is not as specific as serological procedures, so it is sometimes coupled with these techniques to increase the efficiency of virus visualization (Naidu & Hughes, 2003).

1.3.3.3 Serological techniques

All techniques within this category are based on an antigen-antibody reaction, being the antigen the viral protein of interest. Many methods have been developed in this case, including immunosorbent electron microscopy (ISEM), enzyme-linked immunosorbent assays (ELISA) and dot or tissue immunoblotting (DIBA or TIBA) (Jeong et al., 2014; Naidu & Hughes, 2003). ISEM combines the sensitivity provided by serological assays with the visualization capabilities of EM, which is suitable for confirmatory tests when analysing a small number of samples (Naidu & Hughes, 2003). ELISAs are based on the visualization of an enzyme-substrate reaction given by the binding of a specific antibody to the corresponding viral particle, and these have been used to detect plant viruses providing high sensitivity and allowing multiple samples at once (Jeong et al., 2014). DIBA can be used to detect viruses in plants, but also vectors carrying them. Extracts from the plant are spotted on a membrane and

the detection is based on a precipitating or chemiluminescent substrate. This technique is as sensitive as ELISA, but also simple and inexpensive (Naidu & Hughes, 2003). TIBA is performed on nylon and nitrocellulose membranes, and in terms of cost, time, convenience, and sensitivity has considerable benefits over ELISA (Jeong et al., 2014).

1.3.3.4 Techniques based on nucleic acids detection

These molecular methods are used due to their accuracy and sensitivity, as an alternative diagnosis technique to the serological ones. Methods based on nucleic acid amplification can be the polymerase chain reaction (PCR) and its variants, as multiplex, nested, real-time PCR, or isothermal amplification (Jeong et al., 2014). Since the MWMV is an RNA virus, these techniques are based on the reverse transcription of the nucleic acid and amplification of the cDNA generated in vitro. RT-PCR is commonly used for *Potyviruses* such as ZYMV or WMV and combined with other methods as sequencing, allows to perform diversity studies or genomic characterization of new isolates or species (Verma et al., 2020).

An alternative option is the nucleic acid hybridization assays, based on complementarity between the viral nucleic acid and sequence-specific labelled DNA or RNA probes. Binding is consequently identified through fluorescence, colorimetry, chemiluminescence, radioactivity and through serological techniques based on antibodies (Rubio et al., 2020). This allows the analysis of many samples and relative quantification of the virus but has not been extensively used with *Potyviruses*. Tissue printing is based on the hybridization of a crude extract of plant material, which is pressed onto a positively charged nylon membrane, with specific probes usually designed from the capsid protein sequence. This technique is sensitive and rapid, but even though it has been extensively used with *Geminiviridae*, it is not commonly used for *Potivyridae* (Alfaro-Fernández et al., 2016; Rubio et al., 2003). To obtain more sensitive results, the RNA can be extracted and put directly onto the membrane, in a technique commonly called dot-Blot. As occurred with the previous method, this is also sensitive and rapid, but a nucleic acid extraction is required. However, this involves an advantage, since the accessibility of the probe to RNA is higher and the resulting signal is clearer and more specific (Saeed et al., 2007).

1.3.3.5 High-throughput sequencing

High-throughput sequencing (HTS) methods are divided into second generation, as Illumina and third generation, as Nanopore sequencing. These methods do not require knowledge of the viral sequence, which means that they are useful to identify unknown viruses and allow sequencing of up to billions of nucleic acid molecules in parallel (Rubio et al., 2020).

1.3.4 Resistance sources within *Cucurbita*

Resistance sources to *Potyviruses* such as ZYMV, WMV, PRSV and MWMV have been found in cucurbit species. MWMV resistance was identified in *Citrullus ecirrhosus* (Cogn), *Cucumis metuliferus* E. mey ex Naudin and *Cucumis sativus* L. (Kabelka & Grumet, 1997). However, within *Cucurbita* spp., genetic studies of resistance are scarce and focused mainly in *Potyviruses*, *Cucumoviruses* and *Geminiviruses* (Martín-Hernández & Picó, 2021). In *C. sativus*, resistance is known to be conferred by a single recessive gene, but this species is not crossable with *Cucurbita* spp. (Martín-Hernández & Picó, 2021; Miras et al., 2019). Thus, resistance to MWMV needs to be identified within this genus, especially considering that gene editing is not allowed in the European Union according to the present legislation. Also, some *Cucurbita* accessions have already been found to be tolerant or resistant, belonging to *C. moschata* (PI653064 and PI199014 African cultivars), *C. foetidissima*, and *C. ecuadorensis*, respectively (Miras et al., 2019). Wild *Cucurbita* are especially promising when it comes to resistance screening, but are genetically distant from the domesticated species, which can negatively influence the breeding procedure. In addition, not all the crosses are possible within the genus (**Figure 10**). Resistance from crosses between C. *ecuadorensis* and *C. maxima* can be transferred to other cucurbits, but there

are still some challenges to face with *C. pepo*, due to genetic background effects and segregation (Martín-Hernández & Picó, 2021).

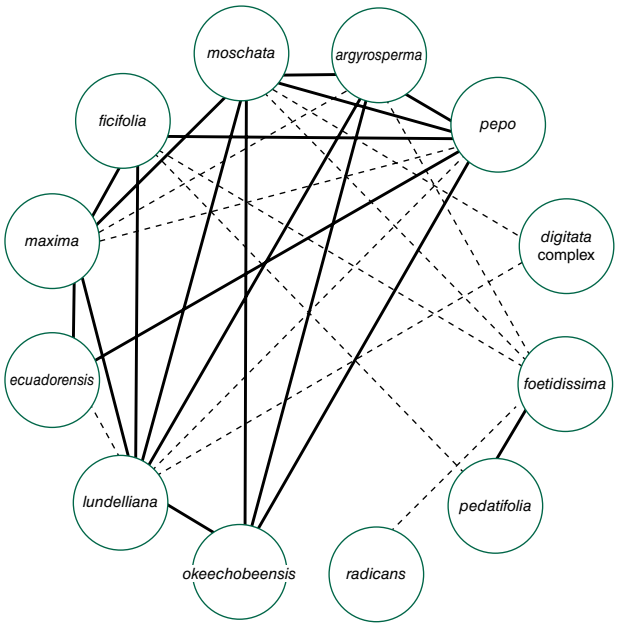


Figure 10. Crossability polygon of *Cucurbita* species. 'Digitata Complex' includes *C. digitata, C. palmata, C. cylindrata* and *C. cordata.* Solid lines indicate an F_1 hybrid that is at least partially fertile; dashed lines indicate a viable but sterile F_1 plant (Wehner et al., 2020).

Resistance genes against *Potyviridae* are usually recessively inherited, and many of them have been reported to encode translation initiation factors equivalent to eukaryotic ones, such as eIF4E (Miras et al., 2019). These are essential for the viral cycle, which involves that resistance is commonly associated with the absence or mutations in eIFs. Based on this information, it is believed that knocking-out these factors from the plant will provide resistance to several viruses (Miras et al., 2019). Additionally, since viruses are commonly transmitted through polyphagous sucking aphids, resistance to infestation by these vectors would help avoiding certain viral diseases. In melon, a dominant locus, referred as *Vat* has been identified to confer a high level of resistance to the aphid *Aphis gossypii*, which causes damage to several crops, specially within cucurbits. In *Cucurbita*, no resistance gene against these vectors has been discovered, but transfer of the *Vat* gene to other species could be a potential source of aphid resistance for the genera (Dogimont et al., 2014). Through investigation of paleohistory of the *Vat* gene cluster, it is known that a *Vat*-related gene was present in the ancestral cucurbit *Momordica charantia*, but with time it was lost in *Luffa* and *Cucurbita*, even it is still conserved in the Benincasae linage (Chovelon et al., 2021).

Even though cucurbits are a common crop worldwide, there is still a lack on *Cucurbita* research to allow improvement in cultivation and breeding of important species within the genus (Zhu et al., 2021). However, there is a great variety of *Cucurbita* collections stored at germplasm banks, such as the National Plant Germplasm System (NPGS)-USDA or the Institute for the Preservation and Improvement of Valencian Agrodiversity (COMAV), which are essential to preserve the diversity and serve as a variation source for breeding programs. For that reason, the characterization of the cucurbit accessions, searching for resistance against viruses or pathogenic fungi, among others, is continuously being done in several research institutions, such as in the Cucurbits genetic breeding group within COMAV. This group, in the framework of an Emerging project, funded by 'Conselleria de Innovación, Universidades, Ciencia y Sociedad Digital', has carried out several screenings for diverse pathogens and the characterization of a *Cucurbita* nuclear collection previously analyzed by RNA-seq (Leiva-Brondo et al., 2021) (**Figure 11**). This germplasm collection includes accessions from diverse origins, especially from American countries and Spanish ones, and belonging to 12 *Cucurbita* species. Evaluation for the response to MWMV in this collection has not been done to date.

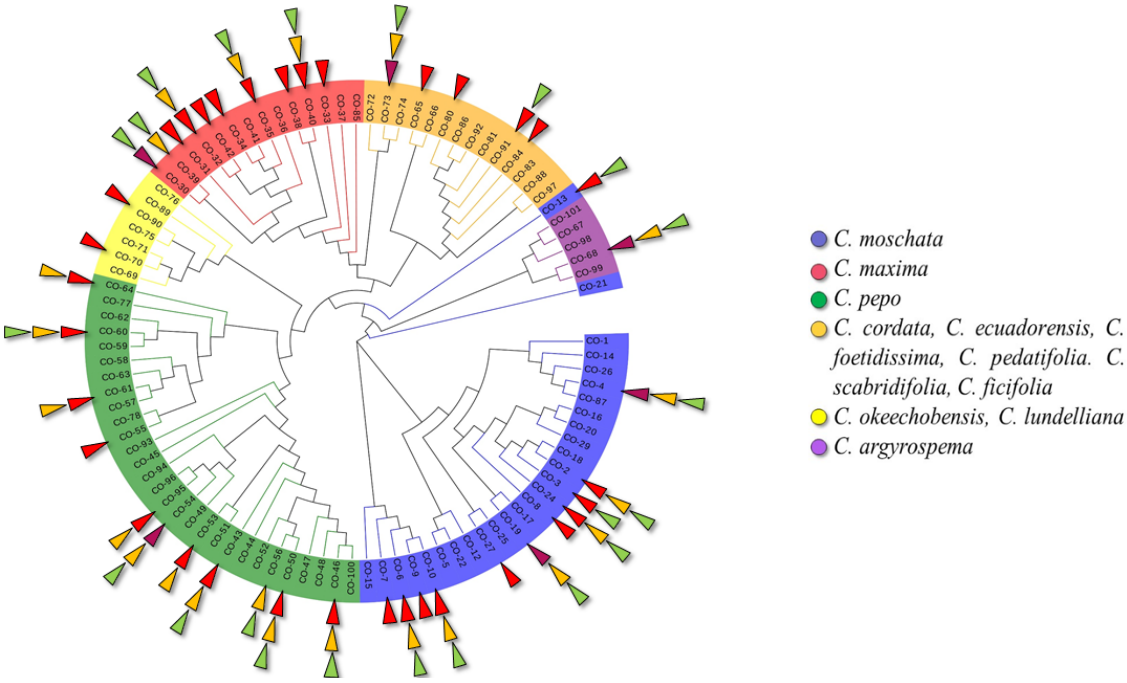


Figure 11. Neighbour Joining, tree with the 96-*Cucurbita* accessions collection sequenced by RNAseq. Red (33) and maroon (6) arrows indicate the entries screened for ZYMV resistance, the orange ones (22) for Fusarium solani species complex (FSSC) and the green ones (22) for Powdery mildew (Leiva-Brondo et al., 2021).

OBJECTIVES

2. OBJECTIVES

In this context, highlighting that species within the genus *Cucurbita* (pumpkins, squash, and gourds) are of remarkable agronomic interest due to their considerable annual production, and thus, the importance of breeding programs trying to improve crop's yield, limiting the effect of pathogens and pests through genetic resistance, we have focused on the control of *Moroccan Watermelon Mosaic Virus* (MWMV), an emerging virus in the Mediterranean countries.

Therefore, the aim of this work is to screen for MWMV resistance in a subset of 35 accessions from a *Cucurbita* nuclear collection previously molecularly characterized, including both cultivated and wild species, 12 belonging to *C. pepo*, 11 to *C. maxima*, 7 to *C. moschata*, and one accession of *C. argyrosperma, C. okeechobensis, C. ecuadorensis, C. foetidissima* and *C. pedatifolia* species. As a second objective, several detection methods such as tissue printing, dot-Blot and quantitative PCR will be tested to assess tolerance/resistance and identify the presence of the virus in the collected samples.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Plant material

For the evaluation of resistance to MWMV, 35 *Cucurbita* spp. accessions maintained by the Cucurbits breeding group of COMAV were selected based on seed availability and genetic relationships previously detected (**Figure 11**) (**Table 2**). Ten seeds per accession were used, except from the accession CO-102, in which 20 seeds were collected.

Table 2. *Cucurbita* spp. accessions. Bank code, origin, and corresponding species of the selected seeds for the study are also depicted. Accessions from COMAV's genebank or kindly provided by (NPGS)-USDA germplasm bank in USA ([1]), N.I. Vavilov All Russian Institute of Plant Genetic Resources (VIR) in Russia ([2]), Tropical Agricultural Research and Higher Education Center (CATIE) in Costa Rica ([3]), International Plant Genetic Resources Institute (IPGRI) in Bulgaria ([4]) and subsequently reproduced by COMAV's Cucurbits breeding group.

ACCESSION	BANK CODE	ORIGIN	SPECIE
CO-002	AFR-CU-1	Morocco	*C. moschata*
CO-003	AN-CU-45	Spain (Andalucía)	*C. moschata*
CO-006	KUROKAWA	Japan	*C. moschata*
CO-010	PI-419083[1]	China	*C. moschata*
CO-013	PI-482527[1]	Zimbawe	*C. moschata*
CO-024	CA-CU-30	Spain (Canary Islands)	*C. moschata*
CO-030	SUD-CU-6	Argentina	*C. maxima*
CO-031	VAV-1860[2]	Australia	*C. maxima*
CO-032	VAV-2422[2]	Central African Republic	*C. maxima*
CO-033	VAV-3202[2]	Chile	*C. maxima*
CO-035	VAV-4381[2]	Peru	*C. maxima*
CO-037	AN-CU-59	Spain (Andalucía)	*C. maxima*
CO-038	CATIE 9824[3]	Colombia	*C. maxima*
CO-039	MAX 306/98	Argentina	*C. maxima*
CO-040	PI-543227[1]	Bolivia	*C. maxima*
CO-042	ANG1-3	Angola	*C. maxima*
CO-043	Styriam pumpkin	Austria	*C. pepo ssp. pepo (Pumpkin)*
CO-046	CATIE 11368[3]	Guatemala	*C. pepo ssp. pepo (Pumpkin)*
CO-050	AS-CU-3	Spain (Asturias)	*C. pepo ssp. pepo (Pumpkin)*
CO-053	AFR-CU-12	Morocco	*C. pepo ssp. pepo (Marrow)*
CO-054	MU-CU-16	Spain (Murcia)	*C. pepo ssp. pepo (Zucchini)*
CO-055	V-CU-196	Spain (Valencia)	*C. pepo ssp. ovifera (Scallop)*
CO-056	V-CU-142	Spain (Valencia)	*C. pepo ssp. ovifera (Acorn)*
CO-060	PI-615111[1]	USA	*C. pepo ssp. ovifera (Acorn)*
CO-061	NSL-5206	USA	*C. pepo ssp. ovifera (Croockneck)*
CO-064	V-CU-202	USA	*C. pepo ssp. ovifera (Straightneck)*
CO-068	PI-512115[1]	Guatemala	*C. argyrospema ssp. argyrosperma*
CO-069	PI-532363[1]	Mexico	*C. okeechobensis ssp. martinezzi*
CO-073	PI-432443[1]	Ecuador	*C. ecuadorensis*
CO-078	PI-614701[1]	Mexico	*C. pepo ssp. fraterna*
CO-080	PI-442197[1]	Mexico	*C. foetidissima*
CO-085	PI-458653[1]	Argentina	*C. maxima ssp. andreana*
CO-092	PI-540737[1]	Mexico	*C. pedatifolia*
CO-095	ISI-3	Italia	*C. pepo ssp. pepo*
CO-102	B4-E0-087[4]	Bulgaria	*C. moschata*

3.2 Seed disinfection, germination, and culture

Seeds from all accessions, except CO-102, were treated according to the short disinfection procedure for melon and pumpkin. Seeds from CO-102 came from a considerably infected greenhouse, which indicated that a large disinfection procedure was required to prevent the presence of virus on the coat.

In the first procedure, seeds were soaked in commercial bleach (40g active chloride/L) diluted to 30% for 1 hour, upon continuous agitation. Once finished, seeds were gently washed with distilled water 9 times, with a 5-minute gap between each wash. Nevertheless, the procedure for CO-102 was more extensive, starting with heat treatment at 78-80 ºC for 24 hours. After that, seeds were soaked in a 10% Na_3PO_4 solution (trisodium phosphate) for 3 hours, upon continuous agitation, then washed with distilled water 5 times. The last two steps corresponded to the ones employed for the first disinfection procedure.

Seeds from all the accessions were placed in moisturized plates to enhance germination. Petri dishes included humid cotton and filter paper, previously autoclaved. These were introduced in a heater at 37 ºC for 48 hours, moved to an illuminated place and consequently monitored, to be transplanted to 7x7x9 cm substrate-containing pots, once the radicle was visible. Pots were marked with marked tags, corresponding to *Cucurbita* accession and plant number, and located in a climactic chamber at 25 ºC during the day, and 18 ºC at night, for 16 and 8 hours, respectively.

3.3 Source of inoculum and virus inoculation

A MWMV isolate provided by Geves Group was biologically cloned in 15 *C. pepo* plants, corresponding to accession CO-054, known to be susceptible to the disease. For the viral multiplication, 0.02 g of active carbon, 0.4 g of frozen infected tissue and 2 mL of inoculum buffer (containing 1% (w/v) PVP-10, 1% (w/v) PEG-6000, 10% (v/v) KH_2PO_4 0.5 M pH 8) were employed. All these components were grinded in a mortar to obtain a homogeneous final solution. The material required for the whole procedure was previously autoclaved. The inoculation was performed mechanically by rubbing with ear cotton swabs one cotyledon and the first true leaf, previously sprinkled with carborundum, with the extract from MWMV-infected plants and the plants were reinoculated 7 days later. Fifteen days after inoculation, tissue was collected to inoculate the 35 *Cucurbita* spp. accessions, using the same protocol as indicated for CO-054 but with fresh infected tissue.

3.4 Evaluation of symptoms

Symptoms were evaluated at 15, 21 and 28 (week 2, 3 and 4), days post-inoculation (dpi) based on a scale 0-4 (Miras et al., 2019), in which 0 means that the plant does not show any disease symptoms and 4 that the plant is highly affected by MWMV (**Figure 12**).

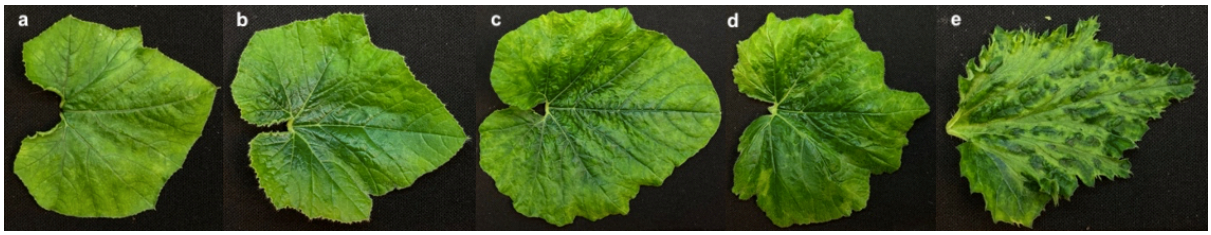


Figure 12. Symptoms upon MWMV infection on scale 0 to 4. (a) No symptoms (0). (b) Mild symptoms (1). (c) Moderate symptoms (2). (d) Severe symptoms (3). (e) Very severe symptoms or plant death (4).

3.5 Tissue samples collection

The evaluated leaf was also used to collect tissue sample for future RNA isolation and tissue printing. Two tissue discs per plant assayed were sampled into 2 mL-tubes, introduced into liquid nitrogen, and stored in a freezer at -80 ºC until use.

3.6 RNA viral detection

3.6.1 RNA extraction

The RNA extraction was done with EXTRAzol (Blirt). The first step included the homogenization of the tissue samples by the addition of 700 µL of EXTRAzol and vigorous agitation, followed by incubation at room temperature for 10 minutes. Then, 140 µL of chloroform were added, the tubes were shaken and incubated 3 minutes at room temperature. Samples were then centrifuged for 15 minutes at 12000xg at 4 ºC. The sample separates into a colored organic phase, an interphase, and a colorless upper aqueous phase in which the RNA is located. The next step was to recover the aqueous phase and introduce it into new 1.5 mL Eppendorf tubes (ca. 300 µL). To precipitate the RNA, 300 µL of isopropyl alcohol were added to the tubes, which were incubated 10 minutes at room temperature after inversion to mix both solutions. After incubation, the tubes were centrifuged 10 minutes at 12000xg at 4 ºC. Once done, the supernatant was discarded and 1 µL of ethanol was added per 1 µL of EXTRAzol used, to wash the pellet, followed by vortex. The tubes were centrifuged 5 minutes at 7500xg and 4 ºC. The ethanol was discarded, and all the remains of the alcohol were removed using a pipette and leaving the tubes open a couple of minutes. Once the ethanol was removed, 40 µL of miliQ autoclaved water were added and the samples could be analyzed or stored at -80 ºC.

The integrity of the extracted RNA was tested though 1.2% agarose gel electrophoresis in TAE 1X (40 mM Tris pH 7,5, 20 mM sodium acetate, 1 mM EDTA). Five µL of each sample with 1 µL loading buffer 6X (50% glycerol, 10 mM de EDTA pH 8.0, 0,0025% bromophenol blue and 0,0025% de xylencianol) were loaded on the gel, which was run at 95V for 45 minutes. To determine the length of each fragment, the molecular marker DNA GeneRuler™ 100 pb Ladder Plus (Fermentas) was used. To visualize the RNA, the gel was incubated in an ethidium bromide solution (10mg/mL) for 15 minutes and illuminated with UV light in a transilluminator (BIORAD). Then, the RNA was quantified, and its quality was measured (ratio A260/A280 ≥ 1,7) with a ThermoFischer Scientific NanoDrop™ 1000 spectrophotometer (Waltham, Massachusetts, United States).

3.6.2 Tissue printing

There exist several methods to study the presence or absence of viral RNA in a sample. Tissue printing is based on the transfection of the cell contents to an adhesive or absorptive surface by contact through a freshly cut tissue section. In this case, leaf peduncles were pressed against each cell of a positively charged nylon membrane (Hoffman-La Roche, Basel, Switzerland) until it became moist. Two controls were used, which were RNA from a non-inoculated plant (p318) as a negative and the inoculated susceptible (MU-CU-16) as a positive control and a total of four membranes, two per week 2 and week 4 post-inoculation were done (**Annex I; Figure I & II**). Membranes at week 3 (21 dpi) were not done to avoid compromising the plant's survival by removal of photosynthetic organs, such as the leaves. Then, the membrane was placed inside a UV cross-linker device on each side with UV light at 120 mJ/cm^2, so RNA molecules were fixed to the surface and stored for further analysis.

Nylon membranes previously fixed with UV light were treated to obtain an image according to a protocol divided into four phases: prehybridization, hybridization, washing steps and development, which are detailed below.

3.6.2.1 Prehybridization and hybridization

To reveal the results, a first prehybridization must be performed at 68 ºC. Membranes were put inside the rotating oven tubes with tweezers, avoiding contact between them and formation of bubbles. The membrane's side into which the plant material was pressed should face the interior of the tube and they should remain wet through the process. 5-10 mL of prehybridization solution was added, which is formed by 50% formamide, 5x SSC, 0.1% SLS, 002% SDS, 2% blocking and miliQ water. Prehybridization proceeded 2 hours at 65-68 ºC. Composition of buffers is detailed in **Annex I** (**Table I**).

Once completed, the solution was removed and a new one was added, with the same composition as the previous one but with the denatured RNA probe, corresponding to the CP gene (capsid) in the virus,

which is labeled with digoxigenin and stored at -20 ºC. Hybridization proceeded overnight at 65-68 ºC since it is an RNA virus (RNA-RNA hybridization).

3.6.2.2 Washing and incubation

After this, the membranes were washed adequately with several buffers (**Annex I; Table I**), starting with 5 minutes with 10 mL of 2x SSC/0.1% SDS buffer at room temperature, which was done twice. Then, 15 minutes in 0.5x SSC/0.1% SDS at 68 ºC, also twice, followed by 5 minutes with TL buffer and 30 minutes with T2 blocking buffer. After that, T2 was used together with 1 µL of antidigoxigenin antibody (dilution 1:10000), previously centrifuged 5 minutes at 13.000 rpm. The last steps include washing with 10 mL of TL buffer twice for 15 minutes, 10 mL of T3 buffer for 5 minutes and T3 buffer with CSPD (chemiluminiscence substrate) (dilution 1:100, 1 mL of buffer and 10 µL of substrate) for 5 minutes. This later step was performed in a cassette in which the membranes were located upon an acetate. When the T3-CSPD solution was added, the cassette was closed and exposed to darkness for 5 minutes. Once completed, the membranes were put inside a plastic bag, a developing film was placed on them, and the cassette was closed again. This was performed in a dark chamber, where membranes stayed during two hours before they could be developed.

3.6.2.3 Membrane development

The film was introduced into a revealing liquid in agitation, followed with a washing step with distilled water. Finally, the film was exposed to fixation liquid and let to dry inside the dark chamber. All these steps were done upon red light to avoid damage through the development process. Viral load ranged from null (0) to very intense (4) according to spot intensity visualized on the membrane.

3.6.3 Dot-Blot

A dot-Blot membrane was done pipetting 1 µL of RNA extracted from each *Cucurbita* accession onto the nylon membrane and visualized, as was performed with the tissue printing membranes.

3.6.4 Relative quantification through RT-qPCR

3.6.4.1 RNA dilution, DNAse treatment and reverse transcription

Once the RNA was extracted and its concentration was known, it could be reverse transcribed into cDNA. The samples were diluted to a concentration of 120 ng/µL, according to their initial concentration measured with Nanodrop. Before starting the reaction, the samples were treated with DNAse I. For that reason, 16 µL of RNA, 2 µL of buffer 10X, 1.92 µL of DNAseI and 0.06 µL of miliQ water were mixed and incubated at 37 ºC for 30 minutes. Then, 2 µL of EDTA were added to each tube, which were incubated at 65 ºC for 5 minutes. After that, samples were ready to be reverse transcribed.

For the reverse transcription reaction, the RevertAid RT kit of ThermoFischer Scientific (Waltham, Massachusetts, United States) was employed. Firstly 11 µL of RNA were mixed with 1 µL of a primer random hexamer and incubated at 65ºC for 5 minutes. Then 8 µL of the reaction mix (4 µL of buffer 5X, 1 µL of Ribolock, 2 µL of dNTPs 10 mM and 1 µL RevertaidRT) were added to each sample and these were incubated 5 minutes at 25 ºC, 1 hour at 42 ºC and 70 ºC for 5 minutes to inactivate the enzyme. Then, these could be stored at -20 ºC until qPCR was performed.

3.6.4.2 MWMV primer design for qPCR

3.6.4.2.1 CP gene amplification

For the qPCR reaction, specific primers to the MWMV isolate employed in the study were required. Previously described primers (de Moya-Ruiz et al., 2021), could hybridize with diverse species or involved problems with the hybridization to some MWMV isolates. For this reason, through amplification of a region of the viral isolate with PCR and its sequencing with Sanger, new primers for qPCR were designed.

For the PCR reaction, cDNA from a MWMV isolate was used, to amplify a genomic region of 639 bp (capsid gene). Twenty-three µL of PCR mix (2.5 µL of buffer 10x (+20 mM MpCl2), 0.75 µL of forward and reverse primers (MWMV-CPF, MWMV-CPR), 1 µL of dNTPs 10 mM, 0.16 µL of dream Taq. 5 U/µL and 17.84 µL of H_2O) were used to perform the PCR reactions, each one containing 2 µL of target cDNA, from three different samples, one being from *C. pepo*, all infected with the same MWMV isolate. The primers used in the amplification were the following:

MWMV-CPF: GATCTTGCCTAGAGTCAGAG (T_M 58ºC)

MWMV-CPR: CACTTACGCATGCCCAGGAG (T_M 63ºC)

The PCR thermal profile was 5 minutes at 94 ºC, 30 cycles that include 20 seconds at 94 ºC, 20 seconds at 54 ºC and 40 seconds at 72 ºC, and at the end samples were exposed for 5 minutes at 72 ºC. Gel electrophoresis was performed with the amplified samples at 90V for 40 minutes, using a 1% agarose gel and a molecular marker up to 1000 bp. Once done, the gel was introduced for 15 minutes in ethidium bromide (10 mg/mL) and visualized under UV light in a transilluminator (BIORAD).

3.6.4.2.2 PCR product purification, sequencing, and primer design for qPCR

When the amplification of the cDNA was verified, it was isolated from the PCR samples with the Extractme DNA-clean-up kit (Blirt). The first step was to add 2 volumes of the CB buffer to 1 volume of the cDNA sample and vortex for 3 seconds. Then, the cDNA was transferred to a column placed into a collection tube, centrifuged for 30 seconds at 11000xg, and the filtrate was discarded. The column was placed into a new collection tube, and 700 µL of CW buffer were added to the tube, which was centrifuged 30 seconds at 11000xg. The filtrate was discarded, and the step was repeated. After that, the tube was centrifuged to remove any remains of the CW buffer 1 minute at 11000xg. This ensured that the alcohol present in the buffer was eliminated from the microcolumn before elution. The last step included the placement of the column into an Eppendorf tube of 1.5 mL and the addition of 30 µL of elution buffer (previously heated up to 70 ºC). The tube with the microcolumn was incubated 4 minutes at room temperature and centrifuged 1 minute at 11000xg. Finally, the microcolumn was removed and the cDNA was ready for further analyses.

The sequencing of the purified cDNA, using the same primers corresponding to the capsid protein, was carried out in the 'Sequencing Core Service' at the Institute for Plant Molecular and Cellular Biology (IBMCP). Forward and reverse sequences were visualized using Chromas 2.6.6 software, aligned with BLAST tool (NCBI) to eliminate less accurate regions, and Primer3 software (v. 0.4.0) (https://bioinfo.ut.ee/primer3-0.4.0/primer3/) was used to design new primers for an amplicon about 100 bp.

3.6.4.3 qPCR

Relative quantification compares the levels of two different target sequences in a single sample to finally express the result as a ratio or relation of these targets, for example, the target CP gene for MWMV and

an endogenous gene which displays a constant expression to function as a reference. For the relative quantification through qPCR reaction, the FastStart Essential DNA Green Master of Roche kit was used, introducing 7.5 µL of Green master 2x, 1.5 µL of each primer forward and reverse (10 uM), 3 µL of H_2O and 1.5 µL of each cDNA per well. Each qPCR plate contains 96 wells, which were filled with the cDNA samples, using two different reaction mixes, one containing MWMV specific primers and the other primers for the endogenous ubiquitin fusion protein (UFP) gene, used as an endogenous calibrator (UFP-F:CGGACCAGCAGAGGCTTATC, UFP-R:GAGAGTTCGCCCATCCTCAA), previously tested in *Cucurbita* spp. (Obrero et al., 2011a). cDNA samples were analyzed in two technical replicates per reaction mix used. Additionally, different controls were tested, including the susceptible accession CO-054 (from the experiment itself) and MU-CU-16 cDNA as positive, while cDNA from non-inoculated plants (p318, PI38, MU-CU-16) as negative controls. A negative control not including cDNA but water was also added.

The instrument used to run the qPCR was *LightCycler*® 480 System (Roche), using the program WMV standards, which includes 45 amplification cycles, with the following thermal profile: 92 ºC for 10 seconds, 59 ºC for 15 seconds and 72 ºC for 20 seconds.

Ct is the intersection between an amplification curve and a threshold line for the fluorescence, being set in the linear phase of the amplification plot (**Figure 13**), before starting the exponential phase. This cycle is a relative measure of the concentration of target sequence in the PCR reaction and the Ct gradient (ΔCt), normalized with Ct from the reference gene gives information about the viral accumulation in the analyzed tissue.

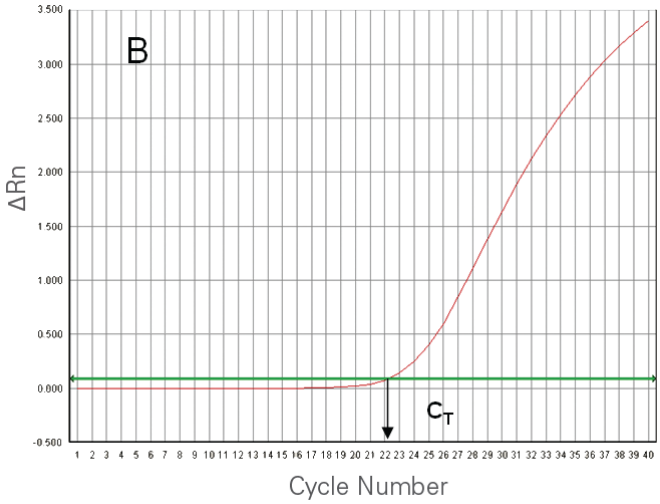


Figure 13. Graphical representation of qPCR data. Rn is the fluorescence of the indicator dye divided by the fluorescence of a passive reference dye, which means that it is the reporter signal normalized to the fluorescence signal of the Applied Biosystems™ ROX™ dye. ΔRn is Rn minus baseline and is plotted against the PCR cycle number (ThermoFischer Scientific, 2022).

ΔCt was calculated as $\Delta Ct = Ct_{MWMV} - Ct_{UFP}$

The average and standard deviation were calculated for each pair of samples. Low and negative values indicate susceptibility, and consequently high viral load, while high values indicate lower viral load and thus tolerance or resistance.

3.6.4.4 Statistical analysis

Means of ΔCt were analyzed by ANOVA and least significance difference (LSD) multiple range test with STATGRAPHICS Centurion XVIII (Statpoint technologies, Inc.) with a level of confidence of 95%, to evaluate statistically significant differences between them. Linear regression analysis was also performed with the same software, to evaluate correlation between the different detection methods.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Seed germination

From the 35 *Cucurbita* accessions, only viable seeds from 28 accessions were obtained (**Figure 14**), mainly from the cultivated species. Germination of the wild species was considerably lower or absent in comparison with *C. pepo*, *C. moschata*, *C. maxima* and *C. argyrosperma*. Accessions corresponding to CO-024, CO-032, CO-035, CO-069, CO-073, CO-085 and CO-102, were not able to be added into the study, due to lack of germination. These include *C. okeechobensis* and *C. ecuadorensis*, species in which tolerance and resistance to MWMV have been reported, such as the resistant *C. ecuadorensis* PI432441 (Miras et al., 2019). This indicates that a similar an accession of the same species used in this study, PI432443 or referred as CO-073, could be a potential source of resistance as well, but unfortunately this accession did not germinate. Both accessions were collected in 1979 in Ecuador (USDA, 1979), meaning that these are probably related, which involves that future resistance screening assays against MWMV with CO-073 would serve to possibly identify resistance in this *Cucurbita* accession.

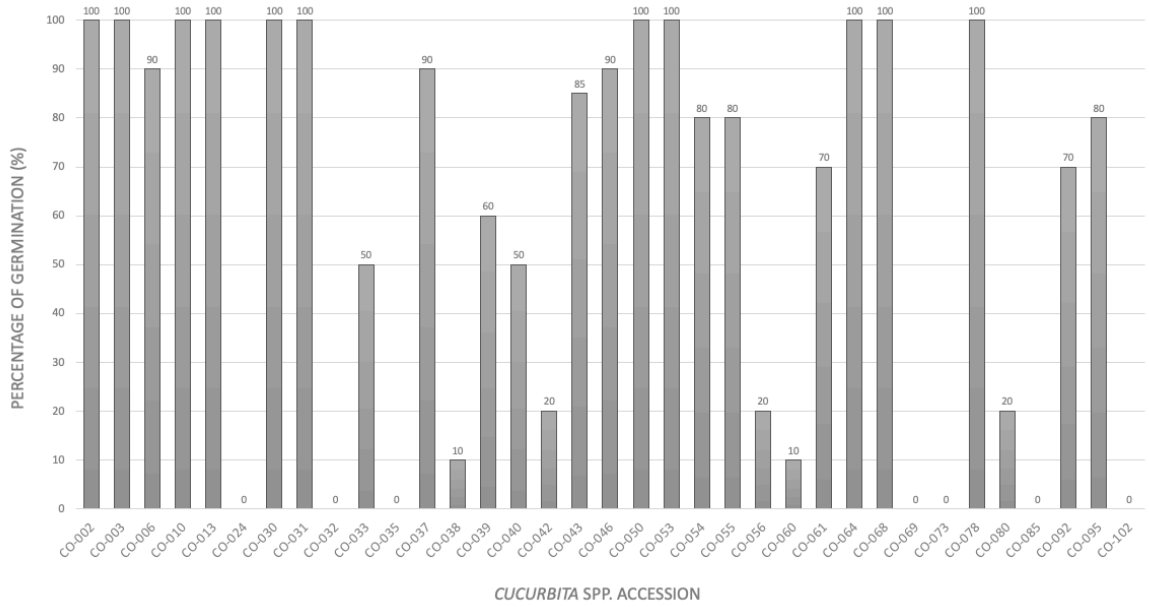


Figure 14. Percentage (%) of seed germination for the 35 *Cucurbita* accessions studied.

In previous assays, one accession of *C. moschata* (CO-102) seemed tolerant to other *Potyviruses*, such as WMV. However, according to the disinfection treatment, seeds from CO-102 were initially expected to have low percentage of germination, which consequently happened when incubated, so no seeds were available for the study. Since growth for the entries CO-038 and CO-080 was considerably slower than for the other entries, those were excluded from the study, as two batches of inoculation had already been performed. At the end, only 26 of the 35 *Cucurbita* accessions were employed for the assay and due to non-uniform germination, two different inoculation batches were needed. Initially, if available, 5 plants were inoculated for each given accession (**Table 3**).

Table 3. *Cucurbita* accessions inoculated with MWMV, both first and second batch respectively. Plants for entry CO-006 and CO-053 appear in both batches to evaluate a maximum total of 5 plants if available.

1st Batch	Plants	2nd Batch	Plants
CO-002, CO-003, CO-010, CO-013 CO-030, CO-031, CO-033, CO-037	5	CO-039, CO-043, CO-046, CO-055 CO-092	5
CO-050, CO-054, CO-061, CO-064		CO-040	3
CO-068, CO-078, CO-095, CO-006, CO-053	4	CO-042	2
CO-060	1	CO-006, CO-053, CO-056	1

4.2 Evaluation of symptoms

Monitored symptoms of infection at 15, 21 and 28 dpi (**Table 4**) are expressed as an average of the symptoms showed by the plants infected from each accession. At week 2, corresponding to 15 days after the inoculation with the viral isolate, some accessions showed considerable damage caused by the infection, showing severe mosaic, vein banding, bubbling, malformation of leaves and filimorphism (**Figure 16**), ranging from level 2-3 to 4. The others showed milder or moderate symptoms, ranging from level 0 to 2, as occurs for 5 of the 26 accessions studied. It is important to note that some plants did not show symptoms of disease due to slow growth (**Annex II; Table I, II, III & IV**) or others, which influences symptom average, as can be seen with the standard deviation (**Table 4**). All these results indicate that none of the accessions tested is totally resistant to MWMV infection, but there could still exist tolerance or partial resistance to the virus. Two accessions of *Cucurbita moschata*, PI653064 and PI199014 (African cultivars), have been discovered as tolerant in previous studies (Miras et al., 2019), but its origin is different from PI419083 (CO-010, from China) and PI482527 (CO-013, from Zimbabwe) (USDA, 1977), which are the ones included in this study, so it is unlikely that these are found to be tolerant or resistant. Additionally, symptom average of the studied plants indicate that these two accessions are susceptible to the disease, reaching level 4 of infection at week 4 post-inoculation.

Table 4. Symptom average and corresponding standard deviation for weeks 2, 3 and 4 after inoculation with MWMV for both accession batches, on a scale 0 to 4, from symptomless to completely infected, respectively. Grey shading indicates symptom average < 2.

Accession code	Symptoms average ± standard deviation		
	15 dpi (week 2)	21 dpi (week 3)	28 dpi (week 4)
CO-002	2.50 ± 1.00	2.90 ± 1.14	3.20 ± 1.04
CO-003	1.20 ± 1.20	4.00 ± 0.00	3.80 ± 0.45
CO-006	2.30 ± 0.45	3.30 ± 0.84	4.00 ± 0.00
CO-010	3.50 ± 0.50	3.50 ± 0.71	4.00 ± 0.00
CO-013	2.60 ± 0.42	3.90 ± 0.22	4.00 ± 0.00
CO-030	0.80 ± 0.57	0.60 ± 0.42	0.60 ± 0.42
CO-031	2.10 ± 0.82	1.70 ± 0.45	1.40 ± 0.65
CO-033	3.10 ± 1.24	3.80 ± 0.45	4.00 ± 0.00
CO-037	2.70 ± 0.84	4.00 ± 0.00	4.00 ± 0.00
CO-039	2.00 ± 0.61	2.80 ± 0.57	2.10 ± 1.08
CO-040	2.00 ± 0.50	2.67 ± 0.29	3.33 ± 0.58
CO-042	1.75 ± 0.35	3.50 ± 0.00	4.00 ± 0.00
CO-043	1.40 ± 1.47	0.40 ± 0.89	1.60 ± 2.19
CO-046	3.10 ± 1.52	4.00 ± 0.00	4.00 ± 0.00
CO-050	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
CO-053	3.40 ± 0.89	3.10 ± 1.47	3.60 ± 0.65
CO-054	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
CO-055	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
CO-056	3.50 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
CO-060	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
CO-061	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
CO-064	3.60 ± 0.89	2.40 ± 2.19	4.00 ± 0.00
CO-068	3.25 ± 0.50	3.30 ± 0.97	4.00 ± 0.00
CO-078	3.20 ± 1.79	3.50 ± 1.12	4.00 ± 0.00
CO-092	0.00 ± 0.00	0.40 ± 0.22	0.50 ± 0.61
CO-095	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00

Symptom severity increased with time, reaching at week 4 remarkably elevated levels of infection in most of the accessions (**Figure 15**), except for CO-030, CO-031, CO-043 and CO-092, reaching a symptom average of 0.6, 1.4, 1.6 and 0.5, respectively. However, it is important to note that some of

the plants belonging to the first three entries, CO-030, CO-031 and CO-043 had physiological problems, such as chlorosis or lack of leaves (**Figure 16**), which indicates that symptom evaluation was not completely reliable in these ones.

Figure 15. Leaves of *Cucurbita* accessions with a level 4 of infection at 28 dpi.

Figure 16. Plant appearance at week 4 (28 dpi). (a) Accession CO-030. (b) Accession CO-031. (c) Accession CO-043. (d) Accession CO-092.

Both CO-030 and CO-031 are *Cucurbita maxima* accessions (SUD-CU-6 from Argentina and VAV-1860 from Australia). Average of symptoms in the first accession remained lower than 1 at 15, 21 and 28 dpi (**Table 4**), showing only slight bubbling on some leaves (**Figure 17a**), but no mosaic or filiphormism was observed through time. At 28 dpi, the plants showed dryness due to a drastic humidity reduction within the climatic chamber, which consequently caused yellowing of the leaf's margins (**Figure 17a**), and this was also visible for CO-031 and CO-043 (**Figure 17b, 17c**). For CO-031, at 28 dpi moderate bubbling was seen in one of the plants (**Figure 17b**), but no other severe disease symptoms were observed. Whole plant disease symptoms for CO-031, not only the selected leaf (**Figure 17a, 17b**), were more distinguishable than for CO-030, which is the reason why its symptom average is higher. Additionally, symptoms diminished through time, which could indicate that the accession is able to recover from disease. For CO-043, a *C. pepo* accession from Austria (Styriam pumpkin), symptoms were clearly moderate or severe for two of the five plants employed (**Figure 17c**), showing bubbling, mosaic and filiphormism, but for the others these symptoms of disease were possibly masked by physiological problems as described previously, so it is unlikely that this accession is tolerant or resistant to MWMV.

Figure 17. Leaves of different *Cucurbita* accessions at week 4 (28 dpi). (a) CO-030. (b) CO-031. (c) CO-043.

For that reason, the following diagnosis methods used in the study would serve to evaluate if CO-030 and CO-031 accessions are candidates to be tolerant or partially resistant to MWMV. Since both accessions are *C. maxima*, existence of tolerance or partial resistance would benefit breeding programs due to crossability with other *Cucurbita* spp. (**Figure 10**), especially if compared with the wild species.

For accession CO-092 which corresponds to *Cucurbita pedatifolia* coming from Mexico (PI-540737), no severe symptoms of disease were seen, as most accessions developed with time, and no significant physiological problems were observed, as occurred with CO-030, CO-031 and CO-043. Other *Cucurbita* spp. have been identified as tolerant if they accumulate virus to a level comparable to susceptible controls but did not show symptoms or as total or partially resistant if no or low virus titer is present, compared to the susceptible controls and symptoms are not visible (Miras et al., 2019). In this case, symptoms appeared mostly at 28 dpi, which were mild and included vein banding (**Figure 18d**) and slight bubbling in some leaves but was variable between the inoculated plants (**Figure 18**). Additionally, it is important to note that some plants showed yellowing that is not related with MWMV infection, but to abiotic factors (temperature, humidity, irrigation) within the climatic chamber (**Figure 18e**).

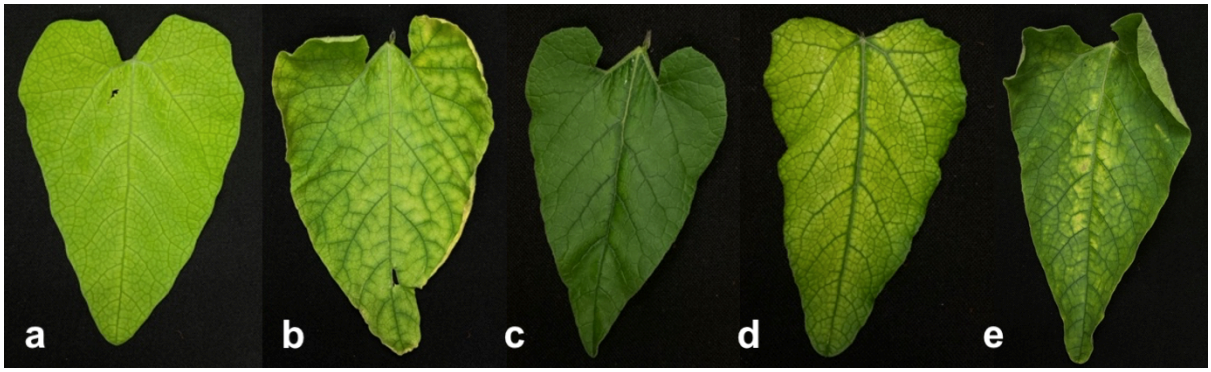


Figure 18. Leaves of *C. pedatifolia* (CO-092) at week 4 after MWMV inoculation. (a) CO-092:1. (b) CO-092:3. (c) CO-092: 4. (d) CO-092:5. (e) CO-092:6.

All these results indicate that these accessions, especially CO-092, could show tolerance or partial resistance to the disease, but the level of infection will be further evaluated with more sensitive diagnostic techniques, such as hybridization assays or qPCR.

4.3 Diagnostic techniques

4.3.1 RNA extraction and evaluation

After the extraction, quality, and quantity of the RNA for samples taken at 15, 21 and 28 was verified through gel electrophoresis, considering of enough quantity those samples showing the bands corresponding to the ribosomal RNA (**Figure 19**). For accessions showing the maximum average level of infection (4), only 3 samples were used, while for the remaining samples in which symptoms were variable between plants, the extraction was carried for all of them. For RNA samples which failed or seemed degrades such as for example in lane 1 (**Figure 19**), a new isolation was carried out if possible. All samples were employed in the dot-Blot detection method, even with a slight degradation. However, only samples of high quality, clearly visualized on the gels and with a minimum concentration of 120 ng/µL and good ratios 260/280 and 260/230, evaluated using Nanodrop (**Annex III; Table I, II & III**), were selected for qPCR.

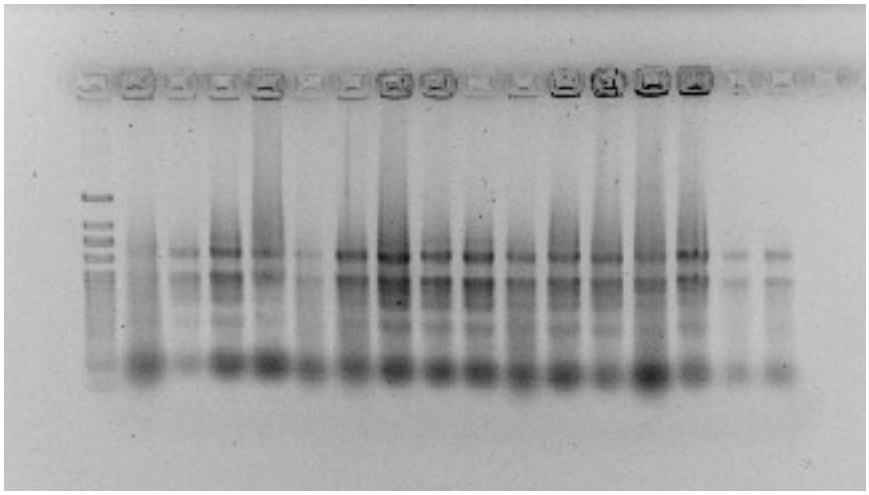
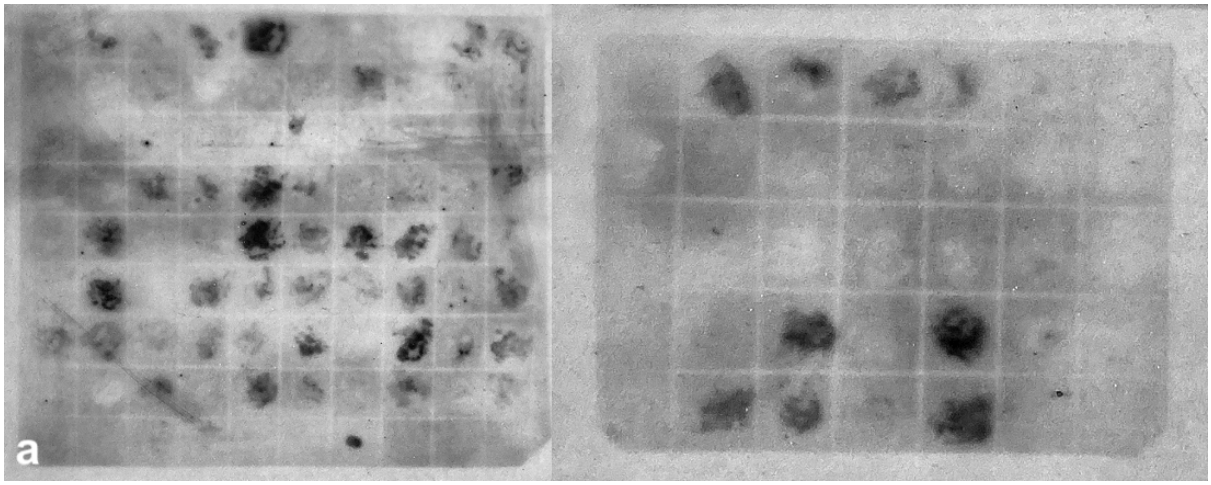


Figure 19. Gel electrophoresis of the samples after RNA extraction using EXTRAzol.

4.3.2 Tissue printing

Results of membrane hybridization with the MWMV probe and subsequent film development for peduncle-phloem prints of assayed plants at 15 and 28 dpi (**Figure 20**), show black dark spots when viral RNA was present and thus, hybridization with MWMV probe had occurred. Many plants were infected by the virus (**Table 5**), but in some of them, the hybridization procedure did not work properly (**Figure 20**), probably due to distortions in the printed regions and undesired noise background, which difficult the visualization of results (Álvarez et al., 2011). It is known that the technique has not been widely used to detect *Potyviruses* and that in previous assays, the method was not successful, possibly due to inhibitors present in the tissue or secondary structures of RNA if not denatured (Alfaro-Fernández et al., 2016; Rubio et al., 2003). Theoretically, the intensity of the spots is proportional to the virus titer present in the phloem of each plant peduncle at the time when the membranes were done and for that reason, they can be evaluated on a scale 0-4 according to spot darkness on the membrane (**Figure 20**).

Both membranes were hybridized with the MWMV probe and developed at the same time and the positive control present in the first membrane (**Figure 20**), indicates that the probe and reagents employed during the process worked accurately.



b

CO-002:3	CO-002:4	CO-002:7	CO-002:6	CO-002:9	CO-003:3	CO-003:8	CO-003:1	CO-003:7	CO-003:6
CO-006:2	CO-006:6	CO-006:5	CO-006:4	CO-010:2	CO-010:6	CO-010:5	CO-010:4	CO-010:7	CO-013:1
CO-013:5	CO-013:4	CO-013:3	CO-013:2	CO-030:2	CO-030:7	CO-030:5	CO-030:9	CO-030:8	CO-031:2
CO-031:3	CO-031:10	CO-031:8	CO-031:9	CO-033:4	CO-033:3	CO-037:3	CO-033:2	CO-033:5	CO-033:1
CO-037:6	CO-037:4	CO-037:1	CO-037:2	CO-050:2	CO-050:6	CO-050:5	CO-050:1	CO-050:3	CO-053:2
CO-053:1	CO-053:3	CO-053:4	CO-054:3	CO-054:1	CO-054:4	CO-054:5	CO-054:2	CO-060:1	CO-061:5
CO-061:3	CO-061:2	CO-061:1	CO-061:4	CO-064:2	CO-064:1	CO-064:4	CO-064:06	CO-064:03	CO-068:4
CO-068:2	CO-068:3	CO-068:1	CO-068:8	CO-078:2	CO-078:8	CO-078:10	CO-078:1	CO-078:6	CO-095:2
CO-095:3	CO-095:4	CO-095:5	CO-095:1		p318	MU-CU-16			

c

CO-053:7	CO-046:1	CO-046:2	CO-046:4	CO-046:7	CO-046:3	CO-042:1
CO-042:2	CO-092:1	CO-092:3	CO-092:4	CO-092:5	CO-092:6	CO-040:4
CO-040:2	CO-040:3	CO-039:1	CO-039:2	CO-039:3	CO-039:4	CO-039:5
CO-043:1	CO-043:6	CO-043:5	CO-043:8	CO-043:9	CO-006:1	CO-056:1
CO-055:1	CO-055:2	CO-055:3	CO-055:4	CO-055:5		

Figure 20. (a) Tissue printing membranes corresponding to week 4 after MWMV inoculation, with the corresponding negative and positive controls, p318 and MU-CU-16, respectively. (b,c) Intensity scale (0-4) of the tissue printing membrane results for the tissue samples corresponding to week 3 and week 4 after MWMV inoculation, with the corresponding negative and positive controls, p318 and MU-CU-16, respectively. Intensity is shown as (0) white, (1) light yellow, (2) light orange, (3) orange and (4) dark orange.

Accessions that have at least one positive result include CO-002, CO-003, CO-010, CO-030, CO-031, CO-033, CO-037, CO-043, CO-046, CO-050, CO-053, CO-054, CO-055, CO-061, CO-064, CO-068, CO-078 and CO-095. However, accessions CO-006, CO-013, CO-039, CO-040, CO-042, CO-056, CO-060 and CO-092 were not positive for the hybridization assay. In the case of CO-092, it could be possible that due to low degree of infection, as previously revealed by scarce disease symptoms, the viral load present on the phloem is not enough to be detected with this method. Additionally, for the remaining accessions, it could be possible that the disease is so advanced that detection of MWMV is not possible anymore, or that due to irregular distribution of the virus within the host, the pressed

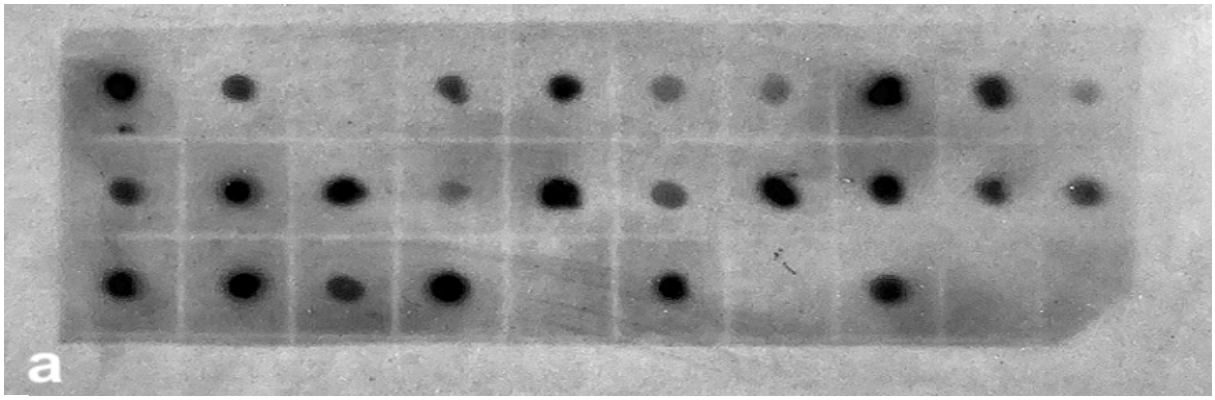
peduncle lacked viral particles to be detected (Matic et al., 2008). Another hypothesis for the negative results obtained could be the existence of interference with other phloem components, such as inhibitors, as previously mentioned.

Apart from the fact that many samples have been negative for the assay, the intensity of some spots does not exactly coincide with the symptoms observed in the plants 4 weeks after the inoculation, which also indicates that the detection method is not accurate for relative quantification of infection. Since results were not clear, membranes corresponding to week 2 (15 dpi) were not developed. Additionally, statistical analysis through linear regression models indicates that the correlation between symptom and tissue printing scores is weak, with a correlation value of 0.27 (**Annex IV; Figure I**), which corroborates that the technique is not completely adequate for MWMV detection.

Taking everything into account, even though the method is simple and rapid, the direct printing of the plant tissue material reaches a considerably low or inexistent level of detection in many cases. For that exact reason and especially considering the high levels of infection, based on symptoms present on most of the *Cucurbita* accessions studied, this technique is not sensible enough to be used as a reliable and accurate relative quantitative detection method for MWMV diagnosis. Therefore, it could serve as a first screening method to evaluate the status of the plants, with the advantages of minimum manipulation of samples, no special conservation requirements before analysis and that it allows large surveys to be performed (de Fátima Rosas-Cárdenas et al., 2015; Matic et al., 2008), but since the hybridization, washing and development of the membranes is extensive and time-consuming, other methods would be more appropriate for the identification of MWMV.

4.3.3 Dot-Blot

Firstly, since the positive RNA control used for the tissue printing membranes was perfectly visualized, an initial dot-Blot test was consequently performed by developing a membrane containing one representative RNA sample of each of the *Cucurbita* accessions studied, to evaluate if this technique is more sensible and accurate than tissue printing. Results (**Figure 21**) clearly indicate that this procedure gives more visual and interpretable results if compared with the previous method.



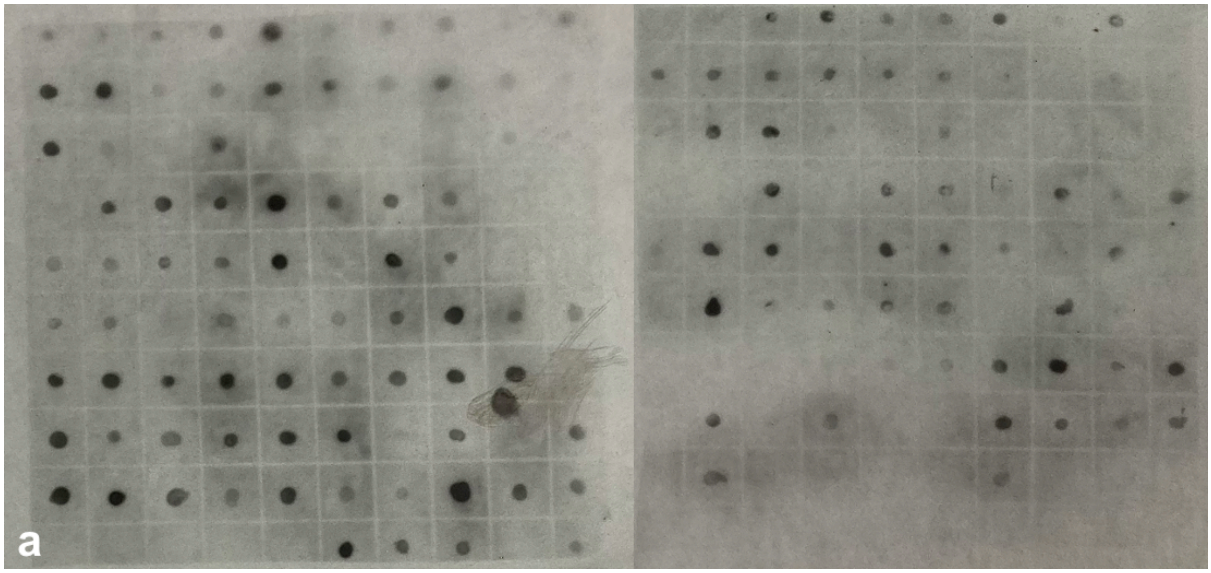
b

CO-002:7	CO-003:3	CO-006:6	CO-010:4	CO-013:2	CO-030:5	CO-031:2	CO-033:4	CO-037:1	CO-039:4
CO-040:3	CO-042:2	CO-043:5	CO-046:1	CO-050:3	CO-053:7	CO-054:2	CO-055:2	CO-056:1	CO-060:1
CO-061:2	CO-064:3	CO-068:2	CO-078:8	CO-092:1	CO-095:2	p318	MU-CU-16		

Figure 21. (a) Dot-Blot membrane for the extracted RNA samples corresponding to week 2, week 3 and week 4 after MWMV inoculation, with the corresponding negative and positive controls, p318 and MU-CU-16, respectively. (b) Intensity scale (0-4) of the dot-blot membrane results for the extracted RNA samples corresponding to week 2, week 3 and week 4 after MWMV inoculation, with the corresponding negative and positive controls, p318 and MU-CU-16, respectively. Intensity is shown as (0) white, (1) light yellow, (2) light orange, (3) orange and (4) dark orange.

Only two samples were negative for the test, CO-006 and CO-092, being the second one hypothesized as tolerant or partially resistant against MWMV. However, CO-006 had severe symptoms of disease, but no hybridization occurred, which could be due to failure in RNA extraction or a problem when performing the hybridization assay. As occurred for tissue printing, the intensity of the dots was different for each sample, and it is theoretically supposed to be proportional to the amount of virus present in each plant. For that reason, these were evaluated on a scale 0-4 according to darkness of the spot (**Figure 21**).

After analyzing the results from that trial, the technique was found successful for the detection of MWMV, so two bigger membranes equivalent to corresponding to week 3 and week 4 post-inoculation with all the available RNA samples were developed. If the plants from the same accession showed different disease symptoms, all tissue samples belonging to it were extracted, but if symptoms were all equal and showing maximum levels of infection (4), only three plant tissue samples were used for the RNA extraction. Together, all RNA samples, concretely 98 and 94, for week 3 for week 4 respectively, were put on the dot-Blot membranes (**Figure 22**).



b

CO-002:3	CO-002:4	CO-002:6	CO-002:7	CO-002:9	CO-003:1	CO-003:3	CO-003:6	CO-006:1	CO-006:2
CO-006:4	CO-006:5	CO-006:6	CO-010:2	CO-010:4	CO-010:5	CO-010:6	CO-010:7	CO-013:2	CO-013:3
CO-013:5	CO-030:2	CO-030:5	CO-030:7	CO-030:8	CO-030:9	CO-031:2	CO-031:3	CO-031:8	CO-031:9
CO-031:10	CO-033:3	CO-033:4	CO-033:5	CO-037:1	CO-037:2	CO-037:6	CO-039:1	CO-039:2	CO-039:3
CO-039:4	CO-039:5	CO-040:2	CO-040:3	CO-040:4	CO-042:1	CO-042:2	CO-043:1	CO-043:5	CO-043:6
CO-043:8	CO-043:9	CO-046:1	CO-046:2	CO-046:3	CO-046:4	CO-046:7	CO-050:1	CO-050:2	CO-050:3
CO-050:6	CO-053:1	CO-053:2	CO-053:3	CO-053:4	CO-053:7	CO-054:1	CO-054:2	CO-054:5	CO-055:1
CO-055:2	CO-055:3	CO-055:4	CO-055:5	CO-056:1	CO-060:1	CO-061:1	CO-061:2	CO-061:3	CO-064:2
CO-064:3	CO-064:4	CO-068:1	CO-068:2	CO-068:3	CO-068:4	CO-068:8	CO-078:6	CO-078:8	CO-078:10
CO-092:1	CO-092:3	CO-092:4	CO-092:5	CO-092:6	CO-095:1	CO-095:2	CO-095:5	p318	MU-CU-16

c

CO-002:3	CO-002:4	CO-002:6	CO-002:7	CO-002:9	CO-003:1	CO-003:3	CO-003:6	CO-003:7	CO-006:2
CO-006:4	CO-006:5	CO-006:6	CO-010:2	CO-010:4	CO-010:5	CO-010:6	CO-010:7	CO-013:1	CO-013:2
CO-013:3	CO-013:4	CO-013:5	CO-030:2	CO-030:5	CO-030:7	CO-030:8	CO-030:9	CO-031:2	CO-031:3
CO-031:9	CO-031:10	CO-033:1	CO-033:2	CO-033:3	CO-033:4	CO-037:1	CO-037:2	CO-037:3	CO-039:3
CO-039:4	CO-039:5	CO-040:2	CO-040:3	CO-040:4	CO-042:1	CO-042:2	CO-043:1	CO-043:5	CO-043:6
CO-043:8	CO-043:9	CO-046:1	CO-046:2	CO-046:4	CO-050:1	CO-050:2	CO-050:3	CO-050:5	CO-050:6
CO-053:1	CO-053:2	CO-053:3	CO-053:4	CO-054:2	CO-054:3	CO-054:5	CO-055:2	CO-055:3	CO-055:4
CO-055:5	CO-056:1	CO-060:1	CO-061:3	CO-061:4	CO-061:5	CO-064:1	CO-064:2	CO-064:3	CO-064:4
CO-068:1	CO-068:3	CO-068:4	CO-078:2	CO-078:6	CO-078:8	CO-095:3	CO-095:4	CO-095:5	CO-092:1
CO-092:3	CO-092:4	CO-092:5	CO-092:6	p318	MU-CU-16				

Figure 22. (a) Dot-blot membrane for the extracted RNA samples corresponding to week 3 (left) and week 4 (right) after MWMV inoculation, with the corresponding negative and positive controls, p318 and MU-CU-16, respectively. (b,c) Intensity scale (0-4) of the dot-blot membrane results for the extracted RNA samples corresponding to week 3 and week 4 after MWMV inoculation, with the corresponding negative and positive controls, p318 and MU-CU-16, respectively. Intensity is shown as (0) white, (1) light yellow, (2) light orange, (3) orange and (4) dark orange.

Membrane for week 4 appeared to have problems with its development, probably at the time of contacting the developing film with the membrane, since some square lines are not clearly visible, including the positive control MU-CU-16. All entries resulted positive for MWMV infection at week 3, except for CO-092. However, it is important to note that results are also related with the state of the plants at the time of getting samples. Additionally, the sample from CO-006 that was used for the initial dot-Blot membrane (**Figure 21**), which was negative, gave positive results in the new membrane (**Figure 22**), which indicates that there was a problem with the RNA loading in the first one, and thus that it should have been positive. Comparisons between week 3 and week 4 results indicate that at 28 dpi the plant tissue condition is worse and RNA extraction was not as precise as occurred at 21 dpi, which explains why the membrane yield is lower for these samples.

Statistical significance and correlation between symptom and dot-Blot scores was also performed (**Annex IV; Figure II & III**). In this case, the correlation is better than for tissue printing, especially for week 3 than for week 4. In both cases, there is a statistically significant relationship between the variables, according to a P-value (<0.05), but for 21 dpi, the correlation coefficient is higher, being 0.48, while for 28 dpi it is 0.34.

Even though total extraction of nucleic acids is required, this technique has been proved as efficient for the detection of *Potyviruses*, even reaching higher detection levels than with ELISAs (Álvarez et al., 2011). So, it could serve as a detection method with good sensitiveness and specificity and from this study, the yield of this method has been proved and thus expanded to the emerging MWMV. In addition, dot-Blot results obtained from week 3 (21 dpi) are more reliable than for week 4 (28 dpi), and this could be considered for further screenings.

4.3.4 Relative quantification through RT-qPCR

4.3.4.1 Primer design for qPCR

Results from the sequencing of part of the MWMV CP gene, including forward and reverse strands, were the following:

>cpf

AAAAATTTGGGACATCTGTTGCAGTACAATCCTAATCAAATTGACCTGTCCAACACCAGGGCAACTCAGAATCAG
TTTGATAGGTGGCACGATGGAGTCAAGAATGACTATGGTCTTGATGATGAAGAAATGGCTATAGTACTCAATGGT
TTCATGGTATGGTGCATTGAAAATGGCACATCTCCGAATGTTAATGGAGTTTGGACCATGATGGACAATGGGGAG
CAGGTGGAGTACTTACTGAAGCCAATGATAGAACATGCATCTCCGACTCTGCGACAGATTATGGCTCATTATAGC
AATGCAGCAGAGGCGTACATTGCTAAGAGAAATGCAACGGAGCGTTACATGCCTCGATATGGACAAAAACGAAAC
CTCAGGGACATCAGTTTGGCCAGATATGCTTTCGATTTCTATGAGATGACTTCCAAGACTCCTGAGAGAGCGCGA
GAAGCACACATGCAGATGAAGGCAGCAGCAATTAGAGGTGCGAACACTCGATTGTTTGGTATTGATGGAAATGTT
GGTGGGGGAGAAGAGAACACGGAGAGACACACTGTTGATGATGTTGAGCGCGATATGCATAGCCTCCTGGGTG

>cpr

GCGCCTACAATCATCAACAGTGTGTCTCTCCGTGTTCTCTTCTCCCCCACCAACATTTCCATCAATACCAAACAA
TCGAGTGTTCGCACCTCTAATTGCTGCTGCCTTCATCTGCATGTGTGCTTCTCGCGCTCTCTCAGGAGTCTTGGA
AGTCATCTCATAGAAATCGAAAGCATATCTGGCCAAACTGATGTCCCTGAGGTTTCGTTTTTGTCCATATCGAGG
CATGTAACGCTCCGTTGCATTTCTCTTAGCAATGTACGCCTCTGCTGCATTGCTATAATGAGCCATAATCTGTCG
CAGAGTCGGAGATGCATGTTCTATCATTGGCTTCAGTAAGTACTCCACCTGCTCCCCATTGTCCATCATGGTCCA
AACTCCATTAACATTCGGAGATGTGCCATTTTCAATGCACCATACCATGAAACCATTGAGTACTATAGCCATTTC
TTCATCATCAAGACCATAGTCATTCTTGACTCCATCGTGCCACCTATCAAACTGATTCTGAGTTGCCCTGGTGTT
GGACAGGTCAATTTGATTAGGATTGTACTGCAACAGATGTTCAAGATTTAATGCAATTTTACCTCTGACTCAGGG
GGAAAGAGATCAC

For the primer design, less accurate regions in the sequences provided, based on the electropherogram and alignment of both forward and reverse sequences (**Annex V; Figure I, II & III**) were eliminated and two sets of primers were developed by using Primer3 Output tool:

qPCR_MWMV_F: CATGATGGACAATGGGGAGC
qPCR_MWMV_R: TGAGCCATAATCTGTCGCAG

qPCR_MWMV_F2: CATCTCCGACTCTGCGACAG
qPCR_MWMV_R2: ATCGAGGCATGTAACGCTCC

4.3.5.2 Evaluation of MWMV primer specificity

Through BLAST alignments, hits with other species were obtained when employing the primers described by (de Moya-Ruiz et al., 2021), so two new sets of primers were designed to avoid non-specific hybridization. These were tested to deliberate which pair functions best for MWMV amplification and results indicated that both sets of primers were able to hybridize with the MWMV. However, the second pair of primers (qPCR_MWMV_F2, qPCR_MWMV_R2) gave higher fluorescence signal and better melting curve profiles than the first set (**Annex V; Figure IV & V**). Additionally, with the first pair (qPCR_MWMV_F, qPCR_MWMV_R) there was amplification of the negative RNA control (p318), which should not occur. For these reasons, the second pair of primers was selected for further qPCR reactions.

4.3.5.3 qPCR

Quantitative real-time PCR (qPCR) is rapid, sensitive, specific, and reproducible, which can be used for detection and quantification of plant viruses (Rubio et al., 2022) if compared with the previous methods. Analysis of the melting curve (**Annex VI; Figure I**) to ensure specificity in the amplification was performed previous to the analysis using the absorbance quantification/second derivative max method to obtain the value for Ct (threshold cycle), for each sample.

In this case, relative quantification of viral load was done by evaluating the Ct gradient between the virus and the endogenous gene UFP. In previous studies, the Ct for UFP in *Cucurbita* spp. was found to be around 18-19 cycles (Obrero et al., 2011b), however, in this study, Ct_{UFP} varies over and under a value of 25 cycles (**Annex VI; Table I, II, III, IV, V & VI**), which could be related with wide range of UFP gene expression levels in the *Cucurbita* species and varieties employed. The designed primers worked properly at 21 dpi, but another endogenous gene could be employed to obtain lower threshold cycles. Technical replicate threshold cycles indicate that the assay was consistent and valid (**Annex VI; Figure II**). Results indicate that in most accessions the virus was able to successfully reproduce, and thus these accumulate high virus titer (**Figure 23**). Mean differences for most accessions are not statistically significant, except for CO-002, CO-030, CO-031 and CO-092 accessions (**Annex VI; Table VI**). As done for the previous detection methods, qPCR results can be divided into four categories, according to ΔCt values: low (< -3.35), medium (3.35 to 6.71), high (6.71 to 10.06) and very high (10.06 to 13.41) virus titer. According to these values, accession CO-092 would show low virus titer and accessions CO-030 and CO-031 would show medium virus titer, while the remaining accessions fall either into high or very high virus titer and thus are considered fully susceptible in terms of virus replication. Interestingly, there is also variation of virus titer within each accession (**Annex VII; Table VII**), but in those cases the virus titer was considerably high, so all those accessions were described as susceptible.

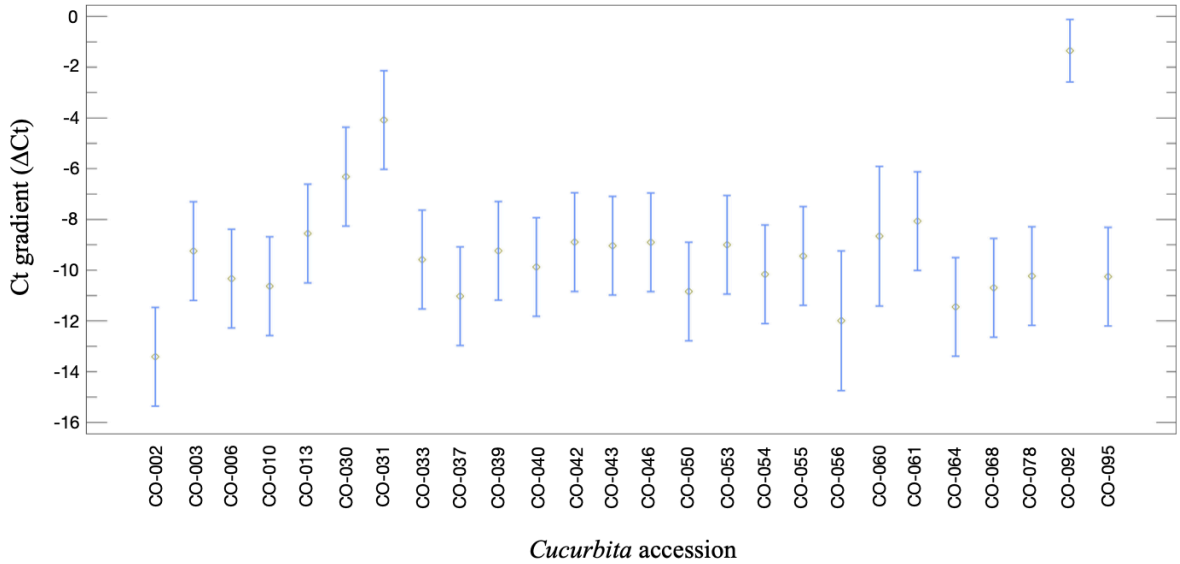


Figure 23. ΔCt means and standard deviation with a level of confidence of 95% from the 26 *Cucurbita* accessions evaluated through qPCR. Statistical analysis was performed with STATGRAPHICS Centurion XVIII.

Even though accession CO-092 has the lowest virus titer, its ΔCt mean is not statistically distinct from CO-030 and CO-031, as seen from an LSD multiple rage test (**Annex VI; Table VI**). However, from what has been seen in previous detection methods, CO-092 would be resistant, while CO-030 and CO-031 could be considered as tolerant to MWMV. Regarding the detection method, there is a significant (P-value < 0.05) and moderately strong relationship between symptoms and ΔCt means, according to a correlation coefficient of -0.66 (**Annex VI; Figure III**).

4.4 Final assessment of susceptibility, tolerance, or resistance to MWMV

As a whole, qPCR was the most sensitive and reliable method to detect MWMV presence on the inoculated plants, followed by dot-Blot hybridization at 21 dpi.

To assess resistance/tolerance to MWMV, all four detection methods were evaluated (**Table 5**). As said, 22 accessions showed severe disease symptoms (2-4), and even if they were negative in tissue printing, high or very high viral load was identified through dot-Blot and qPCR, so these were classified as

susceptible to MWMV infection. Accession CO-043 showed no symptoms in 3/5 plants evaluated, but 2/5 were highly infected, reaching level 4 of infection at 28 dpi. Furthermore, the virus presence was identified through tissue printing ($\bar{x}$ = 2.00 ± 2.83), dot-Blot ($\bar{x}$ = 1.50 ± 2.12) and qPCR ($\bar{x}\Delta$Ct = -9.04 ± 2.26), which confirms that plants lacked symptoms if they were physiologically affected by external abiotic factors and that thus, the accession is susceptible to the disease. Accessions CO-030 and CO-031 showed mild or moderate symptoms of disease ($\bar{x}$ = 0.50 ± 0.71, $\bar{x}$ = 1.75 ± 0.35), no virus presence was identified through tissue printing but through dot-Blot ($\bar{x}$ = 1.50 ± 0.71, $\bar{x}$ = 1.00 ± 0.00) and qPCR ($\bar{x}\Delta$Ct = -6.32 ± 2.91, $\bar{x}\Delta$Ct = -4.08 ± 3.10). However, the viral load registered by these methods is lower than the average of almost the whole collection and especially than the susceptible entry CO-054. This means that virus replication and disease symptom appearance is considerably lower than for susceptible accessions, so these could be regarded as tolerant to MWMV. However, it is important to note that only two samples of these accessions were analyzed through qPCR, so it would be convenient to perform a larger study to totally confirm that these two accessions show tolerance to the virus, especially considering its crossability with cultivated *Cucurbita* spp., such as *C. moschata* (**Figure 10**). In the case of CO-092, symptoms at 21 dpi were mild or absent in the 5 plants studied ($\bar{x}$ = 0.40 ± 0.22) and no virus presence was identified either through tissue printing or dot-Blot. Additionally, results from the qPCR indicate that the virus was able to replicate within the plants, but that virus titer was very low ($\bar{x}\Delta$Ct = -1.35).

Table 5. Assessment of susceptibility, tolerance or resistance to MWMV in the *Cucurbita* accessions, according to symptomatology, tissue printing, dot-Blot and qPCR results. [a] S = susceptible accession (high disease symptoms at 21 dpi >2, dot-Blot readings at 21 dpi >1.5, qPCR readings at 21 dpi >6.71). T = tolerant accession (mild symptoms at 21 dpi <1.8, dot-Blot readings at 21 dpi <1.5, qPCR readings at 21 dpi <6.71). R = resistant accession (mild or no symptoms at 21 dpi <0.5, dot-Blot at 21 dpi = 0, qPCR readings at 21 dpi <-3.35).

Accession	Symptoms 21 dpi		Viral load						Class[a]
			Tisue printing		dot-Blot		qPCR		
	Average ± Stdev		Average ± Stdev		Average ± Stdev		ΔCt ± Stdev		
CO-002	4.00	0.00	2.00	2.83	3.00	1.41	-13.41	0.54	S
CO-003	4.00	0.00	1.00	1.41	2.00	0.00	-9.25	3.26	S
CO-006	2.63	0.88	0.00	0.00	4.00	0.00	-10.33	0.13	S
CO-010	3.50	0.71	0.00	0.00	3.00	1.41	-10.63	0.59	S
CO-013	4.00	0.00	0.00	0.00	3.00	1.41	-8.56	1.05	S
CO-030	0.50	0.71	0.00	0.00	1.50	0.71	-6.32	2.91	T
CO-031	1.75	0.35	0.00	0.00	1.00	0.00	-4.08	3.10	T
CO-033	4.00	0.00	3.00	1.41	4.00	0.00	-9.58	1.33	S
CO-037	4.00	0.00	0.00	0.00	3.50	0.71	-11.03	1.07	S
CO-039	3.25	0.35	0.00	0.00	2.50	0.71	-9.24	0.36	S
CO-040	2.50	0.00	0.00	0.00	3.00	0.00	-9.88	0.70	S
CO-042	3.50	0.00	0.00	0.00	2.50	2.12	-8.90	3.49	S
CO-043	0.50	0.71	2.00	2.83	1.50	2.12	-9.04	2.26	S
CO-046	4.00	0.00	1.00	1.41	2.00	1.41	-8.90	0.08	S
CO-050	4.00	0.00	2.50	0.71	3.50	0.71	-10.84	1.02	S
CO-053	3.75	0.35	0.00	0.00	3.50	0.71	-9.00	1.71	S
CO-054	4.00	0.00	2.00	0.00	3.50	0.71	-10.16	0.52	S
CO-055	4.00	0.00	1.00	1.41	3.50	0.71	-9.44	0.59	S
CO-056	4.00	0.00	0.00	0.00	4.00	0.00	-11.99	0.00	S
CO-060	4.00	0.00	0.00	0.00	4.00	0.00	-8.66	0.00	S
CO-061	4.00	0.00	2.00	0.00	1.50	2.12	-8.07	2.16	S
CO-064	4.00	0.00	1.50	0.71	3.50	0.71	-11.45	0.40	S
CO-068	3.13	1.24	0.00	0.00	3.00	1.41	-10.70	0.69	S
CO-078	4.00	0.00	1.00	1.41	3.00	0.00	-10.23	1.85	S
CO-092	0.40	0.22	0.00	0.00	0.00	0.00	-1.35	2.80	R
CO-095	4.00	0.00	0.50	0.71	3.50	0.71	-10.26	1.04	S

Together, these results indicate that accession CO-092 is partially resistant to MWMV since even though very mild symptoms were observed, viral replication is remarkably low.

The future prospects that follow this study would include the evaluation of tolerance in accessions CO-030 and CO-031 with a larger population size and genetic studies of the MWMV resistance inheritance for accession CO-092. In the second case, since CO-092 belongs to *Cucurbita pedatifolia*, a wild *Cucurbita* species which has outside species crossability with the rest of the genus, except for *C. foetidissima*, it is unlikely to achieve an F_1 population from directed crosses between any cultivated species of interest (*C. pepo*, *C. maxima* and *C. moschata*) and *C. pedatifolia* (PI540737). This involves that conventional breeding programs would not serve to naturally introduce resistance to MWMV, but there is a possibility to use embryo rescue through in vitro culture techniques. If successful, pyramiding of resistance genes would be a suitable approach to combine multiple resistance genes within the same cultivar. However, if crosses are not possible, transgenesis would be the ideal solution to introduce resistance to MWMV in the cultivated species, but the current legislation does not allow the use of CRISPR/Cas, which considerably difficult the resistance breeding program.

CONCLUSIONS

5. CONCLUSIONS

1. Within the *Cucurbita* collection, 23 accessions showed high susceptibility to MWMV.

2. In two accessions of cultivated species belonging to *Cucurbita maxima*, certain levels of tolerance were observed. These accessions should be inoculated again with a higher sample size to confirm the response against MWMV infection.

3. One accession, corresponding to *C. pedatifolia*, showed resistance to MWMV. In this case, studies of crossability between the wild and cultivated species should be addressed to determine the possible use of this accession in breeding programs.

4. For MWMV diagnosis, visual symptom evaluation is the most rapid method for a first disease screening, while tissue printing was not adequate for viral load detection, as usually occurs for *Potyviruses*.

5. Despite processing of the tissue samples is required, dot-Blot hybridization was fast, simple, sensitive, and provided better results than tissue printing, so it serves for screening of MWMV, especially when there is a need to analyze a significant number of samples.

6. Quantitative real-time PCR (qPCR) was the most efficient method to detect the virus presence in plants that showed low or no symptoms of disease and gave negative results with both tissue printing and dot-Blot techniques.

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