

Extraction methodologies of seed borne viruses in beans (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* (L.) Walp.).

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Abstract

In Venezuela, from 1980, the presence of pathogenic microorganisms like bacteria, fungi and viruses has been indicated in most of the seeds of studied fabaceas, such as, commercial, registered, foundation and experimental; and the used methods to guarantee the safety of the seed do not include tests to accomplish the extraction of the viruses; at this moment, in our country do not exist registries nor knowledge in general about how viral diseases are introduced through seeds, in spite of the relevance of this way of transmission. More than 50% of the important diseases in bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* (L.) Walp.) with more than 15 viruses, are transmitted by seeds. For this reason, it was taken a sample of 53 seeds of bean and cowpea experimental, certified and common type from different states of Venezuela: *Aragua*, *Falcón*, *Guárico*, *Lara*, *Portuguesa*, and *Sucre*; also from different countries: Argentina, Chile, Colombia, Canada and the USA; to be processed for the extraction of viruses. Two procedures were used: The first one is an observation of viral symptoms in the first fully expanded trifoliate leaf; the second one is a mechanical inoculation of indicator plants using seed flour. Both methodologies confirmed the presence of viruses. For bean seeds, 25% of the total experimental type showed viral infection, certified bean seeds 28.6%, common bean seeds 23.1% and 16.6% of the total imported seeds. In cowpea seeds, viral infection was detected in the common ones (55.5%). The methodologies verify the percentage of viral infection of the sowing material of different areas from the country and its application will lead to establish controls that prevent the dissemination and use of material with a high viral degree of contamination.

Key words: virus, transmission seed borne, extraction, fabaceas.

Introduction

Edible fabaceas that are traditionally produced in Venezuela, bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* (L.) Walp.), represent an important and economic source of proteins among the popular social groups, who consume them in a massive way and due to their adaptability to agroclimatic variable conditions, they may have a noticeable future in the national market. Maybe, the dramatic decrease in the production of fabaceas (83% in bean and 43 % cowpea) is the biggest prove for agricultural failure in Venezuela, since this species are very important protein sources for the population in general; and for popular social groups, in particular. As a consequence of stagnant benefits and decreasing harvested areas, the production of fabaceas was diminished between the years 1988 and 2001, going from 31.376 t to 9.356 t (70%) for bean and from 11.986 t to 7.867 t (34.4%) for cowpea (12).

In spite of their adaptability to a great variety in agroclimatic conditions, in the country, it is been pointed out that production has gone down gradually in the last years (15). The reason is that most producers use as sowing material seeds from previous harvests or interchange with their neighbors, sometimes, they even use grains that are ready to consume as seeds (17); also, the imported grains that are destined for human consumption are also used as seeds; this is in favor, among other things, of the proliferation of sicknesses, most

of all, those transmitted through seeds. This circumstance leads to a perpetuation of sicknesses from one generation to another, which plays a very important role when towards the epidemiology of sicknesses (13).

In Venezuela, from 1980, the presence of pathogenic microorganisms like bacteria, fungi and viruses, has been indicated in most of seeds of studied fabaceas, such as, commercial, registered, foundation and experimental (20). The used methods to guarantee the safety of the seeds do not include tests to accomplish the extraction of the viruses. Those viruses transmitted through seeds, can be detected making germination tests, which consist in sowing representative samples of seeds big enough (100 seeds) in trays, to visually evaluate viral symptoms manifestation at least in one seedling around 15 or 30 days after been sowed (16). In Venezuela, it is been determined the percentage of transmissions through seeds from three types of cowpea cultivars (7). In another research, it is recommended to sow seeds and evaluate viral symptoms so then, unsafe samples are separate; this test method requires time, at least two weeks to obtain results and infrastructure, such as shelter against insects and controlled temperatures; nevertheless, this seems to be the simplest and most profitable method to determine the presence or not of viruses in seeds (20).

Another method that has been applied to determine the presence of viruses, is the use of flour of infected

seeds, as an inoculum was used this methodology using fabaceas (10, 21) obtaining a noticeable infection even in plants where used fresh flour as inoculum and flour that was storage during 31 months at -2°C ; with the flour the inoculum is formed as a dough, mixing one part of flour and two of distilled water (5).

These methodologies have some advantages, mainly in a practical way over traditional mechanical methods. In most of the cases, symptoms are evident in sick plants; except for

viruses that do not present symptoms in some genotypes or in certain environmental conditions, making necessary the indexation of fabaceas plants through indicator plants that are sensitive to viruses (8, 18, 19).

Taking into consideration what has been exposed before, the objective of this research is to evaluate methodologies to extract viruses from fabaceas seeds, specifically bean and cowpea, which may be implemented easy and safely.

Material and methods

Location. The research was developed in a shelter protected from insects and under controlled environmental conditions by the vegetal virology lab from the agronomy faculty, Universidad Central de Venezuela.

Material. From the main fabaceas producers of the country, it was collected 53 materials used as seeds (*Aragua*, *Falcón Guárico*, *Lara*, *Portuguesa* and *Sucre*), to determine through extraction, viruses incidence that are transmitted through seeds. Material was classified, according to its origin, the following way:

Experimental bean seed. 8 materials from experimental production were evaluated, from advanced line evaluations and others, from the improvement program of the INIA (Instituto Nacional de Investigaciones Agrícolas / National Institute of Agricultural Researches).

Certified seed of bean and cowpea. A set of 9 materials from the private industry were evaluated,

represented by basic or foundation type, and registered seeds, subjected to the process of certification and comply the necessary requirements of these categories for the National Service of Seeds (Servicio Nacional de Semillas) (14).

Common seed of bean and cowpea. 22 materials were evaluated as seeds from previous harvests, represented by seeds with no identification, normally used by producers in different zones of the country.

Imported seed of bean and cowpea (grains). In this study, the term imported is defined as those materials that are currently used in some productive areas to sow; most of these materials are grains that are in the market for human consumption and used by producers as seeds; 14 materials were evaluated (Argentina, Chile, Colombia, Canada and the USA) and they were summit by private establishments.

Plants maintenance. 2 daily irrigation processes and every eight days sprinklings with liquid foliar fertilizer with pesticides.

Method I: Observation of symptoms in the first trifoliolate leaf in bean and cowpea plants

400 seeds were evaluated for each sample of chosen types; using two examining trays per sample. The trays were previously disinfected with sodium hypochlorite in a 3%; seeds were covered with a thin sheet of substrate (coconut sawdust and black sand).

Symptoms evaluation. When the first trifoliolate leaf was big enough, it was determined the percentage of plants infected with the virus, evaluating the presence of viral symptoms according to a scale (Boswell and Gibbs (2)) to identify viruses in fabaceas, then, the symptoms were reproduced by mechanical inoculation over safe plants of the same variety.

Method II: Inoculation with flour of bean and cowpea

For this test, the flour was taken from the following types of cowpea and bean: certified, experimental, common and imported. With each sample of processed flour, eight plants were inoculated for every used indicator, for a total of 40 plants per sample.

Obtaining the flour. Cafati methodology was used (5) with certain modifications, 72 g of seed were pulverized (average weight of 400 seeds) in a food processor for 10 min., then it was filtrated through organza

fabric and a very fine dust was obtained, that was applied for the inoculation of bean and cowpea plants and for virus indicators; in dissolution 1:5 (w/v) of flour and distilled sterile water, it was shaken and was spun to 3000 g during 10 min., then the overflowing was used.

Inoculation and maintenance of plants. Young plants were inoculated with 7 days emerged, rubbing the main simple leaves with the overflowing, previously dusted with mesh carborundum 600; the excess was cleaned with distilled water.

Detection and quantification of viral symptomatology. The presence of viral symptoms was evaluated and the percentage of plants with viruses between the 5th and the 14th day, after the inoculation.

Reproduction of viral symptoms by mechanical inoculation. For the multiplication of the viral symptomatology, was applied the French and Herbert methodology (9), it was inoculated with raw juice from the sick sample a set of 48 safe plants; between 7 and 10 days were emerged. To obtain the raw juice of sick tissue, leaves were weighted and cut thinly, and then they were marinated in a cold sterilized container, in the presence of buffer phosphate of potassium, pH 7.5 and 0.1 M, in proportion 1:4 (w/v). With only the first leaves, plants were dusted with the corrosive mesh carborundum 600 and with the tip of the pointing finger soaked in flour, the leaves were touched; then, with distilled water the inoculated leaves

were cleaned to eliminate the rest of the flour. As a negative control, there were inoculated only 5 plants with distilled water, after the inoculation, three evaluations were carried out at the 5th, 10th and 15th day.

Also, the viral symptomatology was evaluated in the different indicator plants. Four plants for each indicator selected were mechanically inoculated, based on that most viruses infect one or more of these species (2, 11). The indicator plants were:

Chenopodium amaranticolor Coste & Reyn., *Ch. quinoa* Willd. *Nicotiana tabacum* L., *N. glutinosa*, *Cucumis sativus* L., *Vigna unguiculata* L. Walp, *Phaseolus vulgaris* L. and *Gomphrena globosa* L. (2, 9).

In the case of fabaceas, inoculation was made when they were 7 days old and when indicator plants of other families were 20 days old. 5, 10 and 15 days after inoculation, three evaluations for symptoms were performed.

Results and discussion

Percentage of sick plants by seed type. Out of the evaluated sowing material, the 22.5% of samples of bean and 38.5% of cowpea, presented viral symptoms. When bean behavior was studied according to the type and with method I, a high percentage of viral infection was found in certified seeds (28.6%); if we compare with the other obtained results: 25% for experimental seeds, 23.1% for common seeds and 16.6% for imported seeds. For cowpea, it was obtained a high percentage of infection for the common seeds (55.5%); experimental seeds were not included, for certified and imported seeds, viral infection was not found, even though, the number of samples was little to arrive to a definitive conclusion. Nevertheless, when calculating the total of infected plants for bean and cowpea samples, it was observed that the obtained results for certified seeds of bean are under the 2%, this means that it complies with the necessary requirements for the

Bean Common Mosaic Virus demanded by the National Service of Seeds' norms for certification, specifically (14); high levels of infection were obtained for common seeds (6.06 until 20.2%), experimental seeds (5.53 until 12.64%) and for imported seeds (2.19 until 11.47%).

Effectiveness of the proposed methods. The application of the two methods to detect viruses that are transmitted through seeds, allow determining the percentage of viral infection in the evaluated sowing material. This application has been effective to reduce, substantially, the infection in sets of commercial seeds (1). Even though, limitations in the methods have been shown, such as, the interaction between strains of viruses, another one is that some crops show no symptoms, it is necessary to have from 15 to 20 days to observe the symptoms and enough physical space, the procedure is slow (13-19 days) and it does not detect varieties that are tolerant to viruses

(16). It is true that, in the present, these two methods are rarely used by producers since there are modern ones that are faster and more sensitive toward viruses, for example: The ELISA (Enzyme Linked Immunosorbent Assay). In spite of limitations, these two methodologies may help detect material with a high viral infection and might be applied by organisms in charge of the safety of seeds, when there is lack of resources to acquire new equipments.

Method I. Pros and cons of the application. The percentage of plants with viruses, with the use of this methodology, turn out to be important, since it allow knowing how transmissible they are (9). With this method, only evident viruses symptoms were detectable, becoming a disadvantage, because it is impossible to detect latent and disguised viruses; even though, it is affirmed that it is not frequent the disguising of viruses in this kind of proves or growing-on tests (6). Literature makes reference to emphasize method I (1, 16), this lead to infer that this is the most used.

Method II. Pro and cons of the application. When comparing both methodologies, in spite of being different, it is unquestionable the fact that both allow to know how transmissible are viruses carried in the evaluated seed. With method II, percentage of infection is evaluated through sample and not through plants, like method I. Besides, characterized viruses are detected, because most of their activity is retained in dry tissue, since they

survive the desiccation for long periods; being in evidence when using the seed flour and detecting the viruses. This was proved by McKinney, who indicated that the mosaic virus of oats was transmissible with difficulty when using extracts of fresh tissue, and present a little activity after desiccation; while flour made of infected seeds from recent harvests, provides a great source of viruses, demonstrating the high efficiency transmitting through seeds, when detecting the biggest part of infected samples using this flour (21). The application of this method have some advantages, specially in the practice over the traditional inoculation methods, since the inoculum can be preserved for a long time when adding preservatives, it can be applied in any moment, it is profitable, simple and easy; it assures a high percentage of transmission. There are some limitations, such as, interaction among strains of viruses, which requires space because of the number of plants to evaluate, to have 15 or 20 days to observe the symptoms, since procedure requires it, approx.; and method I takes 16 days.

Behavior of the guests (viruses). Applying a series of differential guests in method II, allow identifying viruses that stay hidden and to define variants (18). Even though, environmental conditions and the different combinations of guests/viruses, influence the expression of symptoms, it is necessary to maintain the pavilions where the plants are, free from plagues to guarantee the results (9). Species of fabaceas and

quenopodiaceas showed viral symptoms, it is important to highlight that, *P. vulgaris* y *V. unguiculata* constitute the main indicators, due to their susceptibility to the known viruses that affect bean and cowpea (2). Fabaceas used for this study were *P. vulgaris* var. "*Tacarigua*" y *V. unguiculata* var. "*Apure*", since they can be sowed very easily, reducing test time; among the employed quenopodiaceas, *Ch. Quinoa* showed a higher number of local wounds in a brief time. It is significant to emphasize that, the quenopodiaceas and *D. stramonium* are complementary guests that isolate viruses and help in the identification; this is why they have been applied in several researches (2, 3, 4, 9, 11).

Epidemiologic consequences. These results reflect quality when referring to fitosanitary safety, that owns the sowing material that is being used. The common seed evidences high percentages of infection, when comparing with national certified seed, and these differences are logical, since for their production, certified seed do not receive any kind of control like inspections or elimination of infected plants; this seed, generally, comes from previous harvests. Taking into account the experimental seed, the obtain results indicate that for its production, inspections, elimination of infected plants, vectors control, etc; have not been successful as fitosanitary measures; while, for imported seed the percentages of viral

infection were very high, so, it can be said that when sowing grains that come from abroad for human consume, viral sicknesses are promoted and its incidence increase in the productive zones of fabaceas, besides, the apparition of other viral infections non reported in the country. High percentages of viral infections may affect crops in a drastic way with economical losts, causing breaks, formed by an ecological complex where viruses, vectors, plants and environment play important roles. Applying these detection methods, obtained percentages for viral infection through observation of symptomatology, do not stop to be relevant, since this is about seeds and material used with this objective, which are distributed in the main productive areas and indicate the potential threaten of viruses and the extension to new areas. Besnier (1) points out that seeds play a big role in the introduction of new sicknesses in the national territory, nevertheless, it is not possible in practice to forbid the coming in, because radical measures may trigger great perturbations for the international commerce; it would be inefficient in most of the cases, since they can be introduced by other means less controlled; It is imperative to say that grains, such as, cereals, fabaceas and oil products can be utilized as seeds, although they are not meant for sowing, in some occasions, this is the last performance.

Conclusions and recommendations

The methods that were carried out for this study, allow knowing the presence of viruses in lots of seeds, resulting very easy and low cost procedures, in minimal conditions.

The national certified seed presented viral infections, although its percentages were the lowest, so, it can be said that required controls are fulfilled, giving guarantees to producers.

In relation to experimental seed, the high percentage of viral infection, indicates that practices to control on the field have been unsuccessful; for

common seed, the presence of virus is expected, since, generally, it comes from previous harvests with no selection; about imported grain, it can be said that percentages of viral infection are alarming, therefore, this grains should not be used as seeds.

Implementation methods applied in this study, organisms in charge of guaranteeing the safety of the seed, will allow detecting and taking the necessary actions to certificate the fitosanitary quality of the sowing material before taken to the field.

Literature cited

1. Besnier, R. F. 1989. Ensayos fitosanitarios. Cap. 20: Semillas, biología y tecnología. Ediciones Mundiprensa, Madrid, España, 540 p.
2. Boswell K. F. y A. J. Gibbs. 1983. Viruses of Legumes 1983. Descriptions and keys from vide. Research School of Biological Sciences. Australia. 117-123 p.
3. Bruening, G. 1989. En: Brunt, A. A., K. Crabtree, M. J. Dallwitz, A. J. Gibbs, L. Watson, y E. J. Zurcher. 1997. Plant viruses on line: Descriptions and lists from VIDE database. Version 16 th. January (eds.) (1996 Onwards) URL. [http://biology.anu.edu.au/Groups/MES/ vide](http://biology.anu.edu.au/Groups/MES/vide)
4. Büchen-Osmond, C. 1998. Plant viruses on line: Descriptions and lists from DELTA-Format. Abril, 1998 .<http://ife.anu.edu.au/viruses/ICTVdB/1801000.htm>.
5. CAFATI, K. 1968. Inoculación de frijoles con *Phaseolus Virus 1* a partir de harina de semillas de plantas enfermas. Rev. Agric. Téc. (Santiago) 28:130-131.
6. De Tempe, J. y J. Binnerts. 1979. Introduction to methods of seed health testing. Seed Sci. & Technol. (7): 601-636.
7. Debrot, E., M. Alfaro, E. Brown, y F. Centeno. 1993. Detección de un virus transmitido a través de la semilla de frijol (*Vigna unguiculata*). XIII Congreso Venezolano de Fitopatología. Fitopatol. Venez. 6 (2): 66.
8. Fonturbel, F. 2001. Los vitropatógenos: consideraciones generales, detección y eliminación. Rev. Biología. org. N° 6, octubre.
9. French, E. y T. Hebert. 1982. Métodos de investigación fitopatológica. Instituto Interamericano de Cooperación para la Agricultura. San José, Costa Rica. 135 p.
10. George, J. A. 1962. A technique for detecting virus-infected montmorency cherry seeds. En: WILSON, V. E. and L.L. DEAN, 1964. Flour of infected bean seed as source of virus. Phytopathology 54: 489.

11. Hampton, R., L. Beczner, D. Hagedorn, L. Bos, T. Inouye, O. Barnett, M. Musil y J. Meiners. 1978. Host reactions of mechanically transmissible legume viruses of the northern temperate zone. *Phytopathology* 68: 989-997.
12. Marin, D. 2002. Rendimiento y producción agrícola vegetal: un análisis del entorno mundial (1997 - 1999) y de Venezuela (1988 - 2001). *Rev. Agroalim.* V. 15, Mérida.
13. Maury, Y., C. Duby y R. K. Khetarpal. 1998. Seed certification for viruses. Cap 18. p. 237. En: HADIDI, A., R. K. KHETARPAL and H. KOGANEZAWA. Plant virus diseases control. American Phytopathological Society. Minnesota, USA. p. 1034.
14. MINISTERIO DE AGRICULTURA Y CRÍA. 1986. Servicio a la producción. Normas generales sobre semillas. Cap. III, p. 3.: De la certificación de semillas. Artículo 13. Gaceta Oficial de la República de Venezuela. Núm. 33.456. Caracas, Venezuela.
15. MINISTERIO DE AGRICULTURA Y CRÍA. 1997. El reto de la agricultura Venezolana: Dificultades y opciones. Dirección General Sectorial de Planificación y Políticas. Caracas, Venezuela. 123 p.
16. Morales, J. F. 1983. El mosaico común del frijol: Metodología de investigación y técnicas de control. Edición revisada. Centro Internacional de Agricultura Tropical (CIAT). Cali, Colombia, 26 p.
17. Morros, M.E. 2001. Cultivo de la caraota con énfasis en el estado Lara. Maracay, Ven., Instituto Nacional de Investigaciones Agrícolas. Centro de Investigaciones Agropecuarias del Estado Lara. 74 p.
18. Sarasola, A. y M. S. Rocca. 1975. Fitopatología. Primera Edición, Editorial Hemisferio sur. Argentina, 192 p.
19. Schwartz H. y F. Morales. 1994. Patología de la Semilla. Cap. 19. p. 473-494. En: Corrales, M. y F. Howard. Problemas de la producción del frijol en los trópicos. Centro Internacional de la Agricultura Tropical. Segunda Edición Cali, Colombia.
20. Trujillo G. 1989. La problemática de las semillas de leguminosas comestibles en relación con los patógenos de planta en Venezuela. Facultad de Agronomía, Universidad Central de Venezuela. *Rev. Agronomía al día*, 3: 30.
21. Wilson, V. E. y L.L. Dean. 1964. Flour of infected bean seed as source of virus. *Phytopathology* 54: 489.