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VIRUS IDENTIFICATION, ELIMINATION AND MICROPROPAGATION IN
Tropaeolum tuberosum* and *Arracacia xanthorrhiza

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EXECUTIVE SUMMARY

Mashua, *Tropaeolum tuberosum*, and white carrot, *Arracacia xanthorrhiza*, are tuber and root crops indigenous to the Andes and are both considered to be "lost crops" of the Incas The tubers of *T tuberosum* are a highly prized staple food of Ecuadorian and other Andean indigenous peoples and can be grown on the poorest soils of the region and have the potential to produce large yields with little input The large storage root of *A xanthorrhiza* is an important source of carbohydrate for the indigenous peoples at lower Andean elevations and both crops have great potential for commercialization At the beginning of this grant, mashua productivity had fallen so low that it was no longer a cash crop but only a "food" crop for self-consumption by the poorest indigenous people and was rarely available in the marketplace Both crops had at least one virus known to infect them and observations of plants in the field and preliminary tests indicated that at least one virus was present in mashua Neither of these plant species had received much research attention and very little if any expertise in the area of plant virology and virus elimination was available in Ecuador prior to the beginning of this grant Mashua plants in the field often exhibited symptoms typical of virus infection, including chlorosis, leaf cupping, distortion, and stunting Using mechanical transmission tests, a virus was determined to be present in a high percentage of the Ecuadorian germplasm collection at the National Institute for Agricultural Research (INIAP) at Santa Catalina with 34 of 46 lines testing positive for the virus All eight production fields tested were determined to be infected by this virus Isolates of this virus were characterized and determined to be a new and unique member of the potyvirus group and tentatively named Tropaeolum mosaic potyvirus (TropMV) Polyclonal antiserum was developed to highly purified preparations of TropMV and used to screen all accessions and production fields via enzyme-linked immunosorbent assays (ELISA) This polyclonal antiserum and monoclonal antisera to potyvirus epitopes were used to determine the relationship of TropMV to other potyviruses Tropaeolum mosaic virus was determined to be unique but related to maize dwarf mosaic potyvirus, potato Y potyvirus, tobacco etch potyvirus, potato A potyvirus, and watermelon mosaic 2 potyvirus No virus was detected in white carrot Methods for the tissue culture regeneration of both mashua and white carrot were developed and shared with INIAP and the Center for International Potato Research (CIP) at Santa Catalina Virus-elimination methods using apical domes and meristems were developed and employed to generate a total of twelve virus-free lines of mashua and ten tissue cultured lines of white carrot This germplasm was turned over to INIAP Over 50 individuals from government, university and private sector laboratories were trained in basic methods in virus detection and elimination using tissue culture through three national plant virology workshops One Ecuadorian student, training at the University of Delaware, received her M S degree in Plant Science in the areas of plant virology and plant tissue culture Extension programs and demonstration plots were used to educate indigenous peoples as to the value of using virus-free tubers to establish mashua plantings Approximately, 100 3-pound bags of virus-free mashua tubers of mixed lines, to simulate landraces of the plant, were provided to indigenous peoples of the provinces of historical mashua production, Chimborazo, Bolivar, Tungurahua, and Cotopaxi This

was the reintroduction of mashua to Bolivar and Tungurhua Provinces and was facilitated by CARE, Catholic Relief Services and the extension service of The Polytechnic University of Chimborazo

RESEARCH OBJECTIVES

The research objectives of this project were 1) to train one Ecuadorian graduate student in plant virus detection and elimination methodolgies and determine the presence or absence of viruses in Ecuadorian germplasm of mashua and white carrot 2) develop techniques for virus elimination from mashua and white carrot 3) develop methods for the micropropagation of virus-free mashua and white carrot using tissue culture and 4) initiate an educational-extension program to insure that Ecuadorian farmers will utilize virus-free germplasm

METHODS AND RESULTS

Introduction Isañu, añu, cubio or mashua *(T tuberosum)* is probably the Andean region's fourth most important root crop after potato (*Solanum tuberosum*), oca (*Oxalis tuberosa*), and ulluco (*Ullucus tuberosus*) (National Research Council, 1989) Mashua (pronounced mah-shoo-ah) is closely related to the garden nasturtium, and has been cultivated by people of the Andean highlands since Incan time Among Andean tubers, mashua is one of the highest yielding, easiest to grow, and most resistant to cold It also has been demonstrated to have repellent properties against insects, nematodes and other pathogens, making it a valuable plant to inter crop with other plant species (National Research Council, 1989, FAO, 1990)

Tubers of mashua are the size of small potatoes with shapes ranging from curved to conical and colors from yellow to purple to red Mashua is a very nutritious root crop Solids comprise about 20% and protein as much as 16% of the dry matter, with a good balance between carbohydrates and proteins and a high content of ascorbic acid, ß-carotene and minerals (King and Gershoff, 1987, Tapia, 1990) Mashua tubers should have wide applicability in the food industry as dried slices, for flour and starch processing or for baby food mixtures (Tapia, 1990)

Despite its potential productivity and pest resistance, mashua is not widely commercialized internationally Mashua tubers in Ecuador are found in the highland markets during the harvest season from the end of May until September, whereas in the highlands of Colombia, mashua is well commercialized, especially in the cities of Tunja, Bogota, and Pasto (Villamizar, 1985)

Mashua can be viewed as an endangered crop in Ecuador because few farmers cultivate the tubers due to the low yields, long growing season, and low market price Over the past 10 years, the number of hectares of mashua planted and yield per hectare

have both drastically decreased due to cultural and environmental changes Lack of productivity also has been cited as a reason for reducing interest in growing the crop (National Research Council, 1989, FAO, 1990, Tapia, 1990)

Virus infection of tuber crops is known to have an effect on productivity (Van der Zaag, 1987) and at least one mechanically transmitted virus has been reported to infect mashua (Delhey and Monasterios, 1977) and the occurrence of viruses in mashua is believed to effect its productivity (National Research Council, 1989) Over the past 10 years, the number of hectares of mashua planted and yield per hectare have both decreased drastically, and this reduced productivity has been cited as a reason for reduced interest in growing mashua (National Research Council, 1989) Field plants exhibit virus-like symptoms which include smaller leaves with cupping and distortion and interveinal chlorosis Plants exhibiting any of these symptoms generally were thought to be infected with at least one mechanically transmissible virus (personal observation) Worldwide, a number of viruses have been reported to infect mashua's close relative, garden nasturtium Nasturtium ringspot virus appears to be closely related to broad bean wilt virus (Horvath and Szirmai, 1975) and has been detected in California (Jensen, 1959) and Brazil (Silberschmidt, 1953) Other viruses detected in *T majus* include cabbage black ringspot tymovirus (Smith, 1950), beet curly top geminivirus (Severin and Freitag, 1933) cucumber mosaic cucumovirus (Schmelzer, 1960), pea early browning tobravirus (Beemster, 1960) and tobacco rattle tobravirus (Uschdraweit and Valentin, 1956) Only a single virus disease, mosaic, has been reported in *T tuberosum* in Bolivia (Delhey and Monasterios, 1977) Plants typically exhibited symptoms which included a mosaic and malformation of leaves The causal virus was reported to be a flexuous rod with a mean length of 800 nm from leaf dip preparations but no serological work was reported

METHODS

Virus Identification and Characterization

Virus host range Initially, ten mashua accession (ECU 1145, ECU 1141, ECU 1084, ECU 1093, ECU 1115, ECU 1101, ECU 1102, ECU 1096, ECU 1147, and ECU 1098) were introduced as tubers to the quarantine greenhouse at USDA-ARS Ft Detrick, Frederick, MD from the Ecuadorian highlands under APHIS-PPQ permit Ten virus isolates, one from each accession (Tt 1, Tt 2, Tt 3, Tt 4, Tt 5, Tt 6, Tt 7, Tt 8, Tt 9 and Tt 10) respectively, were established by mechanical inoculation onto selected herbaceous indicator plants (Table 1) Symptomatic leaf tissue was triturated in 0 1 M sodium citrate buffer, pH 7 4, and rub-inoculated onto leaves of indicator plants that had been first dusted with 600 mesh carborundum Plants were observed over a 6-week-period for symptom development Inoculated leaves of symptomless indicator plants were back-inoculated to *Nicotiana benthamiana* L, a systemic host that produces distinct vein clearing, downward curling of leaves and stem, and necrosis of leaves and stem tissue over a 2-week period, and *Gomphrena globosa* L, a bioassay host that produces red-rimmed local lesions with necrotic centers in 10 days, to test for latent infection Virus isolates Tt 4 and/or Tt 9 were selected for further testing

Physical properties Longevity *in-vitro* (LIV), thermal inactivation point (TIP), and dilution end-point (DEP) were determined for the Tt 4 and Tt 9 isolates using standard protocols (Walkey, 1991) For TIP determination, systemically infected leaves of *N benthamiana* in 0 1 M sodium citrate buffer, pH 7 4, were heated for 10 minutes at temperatures from 50° to 100° C at 10 degree intervals using a heat-block For LIV, crude sap prepared from *N benthamianum* was held at 20° C in the dark for 0, 1, 5, 7, 14 and 30 days For DEP, crude sap was serially diluted with 0 1 M sodium citrate buffer, pH 7 4 All samples were tested for infectivity using *N benthamianum* plants

Virus purification The Tt 4 isolate was purified from systemically infected leaf tissue of *N benthamiana* using a modification of the method developed by Hammond and Lawson (1988) for potyviruses Systemically infected leaf tissue (187 g) was triturated in liquid nitrogen then homogenized in 1000 ml of 0 5 M K_2H/KH_2PO_4 buffer, pH 8 4, with 0 5 % (w/v) Na_2SO_3, and filtered through cheesecloth The filtrate was centrifuged at 8,000g for 10 min at 4°C in a GSA rotor and the supernatant collected Triton X-100 was added to 2% and the solution stirred for 15 min at 4°C Polyethylene glycol (PEG) 8000 was added to 6% and NaCl to 2% and the solution stirred for 1 hr at 4°C and centrifuged at 10,000 g for 10 min at 4°C in a GSA rotor Pellets were resuspended in 50 ml of 100 mM borate/KCl buffer, pH 8 0, and homogenized, transferred to a beaker and stirred at least 1 hr at 4°C A 25 ml aliquot of the suspension was measured into R30 tubes, underlayed with 5 ml/tube 30% (w/v) sucrose in 0 1 m borate/KCl buffer, pH 8 0, and centrifuged at 70,000 rpm for 2 5 hr in an Ti50 rotor Pellets were resuspended in either 100 mM borate/KCl buffer, pH 8 0, or 10 mM sodium phosphate buffer, pH 7 6 After centrifuging at 10,000 g for 10 min at 4°C for clarification, the suspensions were layered onto 10-40% sucrose gradients and centrifuged at 70,000 rpm for 2 hrs in a SW-41 rotor and fractionated using a ISCO (ISCO Inc, Lincoln, NB) UA-5 ultraviolet fractionator at 258 nm Ultraviolet absorbing fractions were collected, pooled and diluted 10-fold with borate/KCl buffer, pH 8 0, or 10 mM sodium phosphate buffer, pH 7 6, respectively, centrifuged at 70,000 g for 2 5 h in a Ti75 rotor, supernatant discarded and pellets resuspended in 250 µl of the same buffer

Electron microscopy The morphology of virions was determined by placing partially purified preparations of isolate Tt 4, from the above procedure, onto Formvar-backed, carbon-coated grids, staining them with 2% uranyl acetate or 2% phosphotungstic acid, and examining them with a Phillips 201 electron microscope at 80 kV Two hundred particles were measured and particle size determined by compression with photographs taken at the same magnification of germanium-shadowed carbon replicas made from diffraction gratings with 54,864 lines per inch and/or grids containing purified preparations of tobacco mosaic tobamovirus as standard size markers

Polyclonal antibodies and indirect enzyme-linked immunosorbent assay Polyclonal antibodies were produced to highly purified preparations of the Tt 4 isolate Two rabbits were given intramuscular injections of 100 µg of virus in Hunter's TiterMax (TiterMax, Inc , Altanta, GA) adjuvant every 10 days Test bleeds began 30 days after initial injection Indirect enzyme-linked immunosorbent assay (ELISA) tests were carried

out using purified γ globulin to the Tt 4 isolate and goat anti-rabbit γ-globulin conjugated with alkaline phosphatase (Sigma Co St Louis, MO) Virus isolates Tt 4 and Tt 9 were tested in indirect ELISA against antiserum (Agdia Inc , Elkhart, IN) to the following potyviruses watermelon mosaic potyvirus 1 (WMV-1), watermelon mosaic potyvirus 2 (WMV-2), potato potyvirus Y- common strain (PVY-C), potato potyvirus A (PVA), pepper mild mottle potyvirus (PMMV), turnip mosaic potyvirus (TuMV), lettuce mosaic potyvirus (LMV), dasheen mosaic potyvirus (DaMV), soybean mosaic potyvirus (SMV), tobacco etch potyvirus (TEV), pea seedborne mosaic (PSMV), maize dwarf mosaic potyvirus A (MDMV-A), and maize dwarf mosaic potyvirus B (MDMV-B) A positive threshold absorbance reading of four times the mean of the healthy control was used

Monoclonal antibody panel Twenty-six monoclonal antibodies (McAbs) from a previous study (Jordan, 1992) designated PTY McAbs, were used separately to compare the Tt 4 and Tt 9 isolates of the unknown potyvirus from mashua and other characterized potyviruses (courtesy R Jordan and J Hammond) Members of this panel of McAbs recognize epitopes that are (a) specific to bean yellow mosaic potyvirus (BYMV) strains, (b) common to members of the BYMV subgroup, (c) distinctive for unique potyviruses, or (d) common to many distinct potyviruses (Jordan and Hammond, 1991) The format is an indirect ELISA with the viral antigen being coated directly onto the microtiter plate These broad spectrum McAbs react with antigenic determinants on viral subunit regions not exposed on the virion surface Virions were disrupted during the coating process to expose cryptotopes

Antigen-coated plate enzyme-linked immunosorbent assays Indexing of mashua tissue samples for the Tt 4 and Tt 9 isolates was carried out using the PTY McAbs in indirect antigen-coated plates (ACP) ELISA (Jordan and Hammond, 1991) One gram composite samples of symptomatic leaves were triturated in 10 ml of 0 05 M sodium carbonate-bicarbonate buffer (CB) plus polyvinylpyrrolidone (PVP), pH 9 6, containing 0 2% sodium diethyldithiocarbamate (DIECA) The samples were incubated at room temperature for 2 hr to disrupt virions and 100 µl of each sample dispensed to duplicate wells of Nunc MaxiSorp polystyrene ELISA plates Plates were incubated for one hour at room temperature, washed three times with phosphate buffered saline plus Tween 20 (PBS-Tween) before 100 µl of an admixture of McAbs to potyvirus epitopes 1+2+3+4+8+10+21 (10,11) was added The plates were incubated for 2 hr at room temperature or overnight at 4° C and washed 4 times with PBS-Tween Later, 100 µl of goat antirabbit γ-globulin conjugate was added and incubated for 1 hr at room temperature Finally, 100 µl of p-nitrophenol phosphatase (Sigma Chemical Co , St Louis, MO) diluted in diethanolamine buffer was added to each well Reactions were stopped with 50 µl of 3 M sodium hydroxide and optical density at 405 nm determined after 1 hr The positive threshold used was four times the mean of the healthy control value

Survey of mashua in Ecuador for the occurrence of potyviruses A survey of 46 accessions of mashua, maintained at the National Institute for Agricultural Research's (INIAP) Santa Catalina Research Station (SCRS), Quito, Ecuador and plants from 8 fields representing the major production areas in the Ecuadorian highlands, for the presence of Tt

4 and Tt 9-like isolates was carried out during April of 1993 Virus testing included both indirect ACP-ELISA, with an admixture of PTY McAbs described above, and bioassay to *N benthamianum* and *G globosa* For each accession and production field, a composite sample of symptomatic young leaves from a minimum of five plants was collected Leaf samples were triturated in either CB/PVP containing 0 2% DIECA for ACP-ELISA or in 0 01 M sodium phosphate buffer, pH 7 0, for bioassay on indicator plants Sap extracts were diluted 10-fold and tested using indicator plants as before Plants were maintained in a greenhouse and observed over a four-week period for symptom development

Aphid transmission Green peach aphids (*Myzus persicae* [Sulzer]) were reared on curly dock (*Rumex crispus* L) in a quarantine greenhouse Several hundred adults and nymphs were shaken onto moist filter paper and starved for 4 hrs Approximately 100 adults and nymphs (3rd and 4th instar) were transferred to detached leaves of *T tuberosum* (ECU 1093) and given an acquisition access period (AAP) of 30 seconds to 5 minutes As soon as aphids probed, they were moved in sets of 25 or more to 3-week-old *N benthamiana* seedlings for a 24-hr inoculation access period (IAP) An identical group of green peach aphids were allowed the same period of time to probe on virus-free curly dock and transferred onto *N benthamiana* for a 24 hr IAP as a control Plants were sprayed with a dilute solution of malathion (O,O-dimethylphosphorodithioate of diethylmercaptosuccinate) (0 2 g a i /L) and placed on a greenhouse bench for a 30-day observation period To confirm virus transmission, leaf tissue was bioassayed using healthy *N benthamiana* as previously described

Tissue Culture Methods

Plant material Four potyvirus-infected lines (1093, 1115, 1141, and 1147) from the mashua collection at the Ecuadorian Institute for Agricultural Investigation (INIAP), Santa Catalina Research Station, Quito, Ecuador were selected based on geographic distribution within the country, and morphological characteristics such as tuber form and color Shoot apices (SA), ca 0 2mm in size (meristem plus 1 or 2 leaf primordia), were isolated using a razor-blade sliver from virus-infected, *in vitro* maintained, microcuttings Lines initiated from SA were designated as potentially-virus-free lines (PVFL) PVFL and virus-infected stock lines (VIL) were maintained in vitro by subculturing monthly to proliferation medium

Culture media The basal medium contained the inorganic salts and vitamins of Murashige and Skoog (1962), supplemented with 3% sucrose and 0 64 % Phytagar For proliferation, basal medium was supplemented with 5 mg l^{-1} BA and 1 mg l^{-1} kinetin according to Fadiño *et al* (1987) For in vitro rooting, 5 mg l^{-1} (or 2 mg/l in field Experiment I) of NAA was added to basal medium Media pH were adjusted to 5 7-5 8 prior to autoclaving

Proliferation versus virus infection The effect of virus infection on in vitro proliferation was tested using lines 1141 and 1093 The PVFL-derived clones for each line were selected based on negative results obtained from a bioassay using in vitro

derived microcuttings Each jar contained two explants, an apical shoot tip cultured vertically, and a lateral shoot tip cultured horizontally Cultures were placed under a 24-hour photoperiod (50 μmol m^{-2} s^{-1}) and evaluated after one month in culture

Rooting and reestablishment. The effect of virus infection on rooting ability in vitro and survival during acclimation was compared for PVFL and VIL Five microcuttings were cultured per jar Cultures were placed under a 16-hour photoperiod (50 μmol m^{-2} s^{-1}) for one month before potting and placement in the greenhouse Rooted mashua plants were transplanted into 3 5 cm cell packs having 72 cells using a pasteurized soil mixture that contained 32% Pro-mix BX, 16% field soil (clay loam), 16% peat moss, 16% vermiculite, 12% perlite and 8% sand plus fertilizer Plants were placed in a mist tent for one week after transplanting, after which they were moved to a greenhouse bench and watered as needed Artificial lights (32-95 μmol m^{-2} s^{-1}) were used to provide a 12-hour day length, at a temperature range of 18-25°C Survival of PVFL and VIL were recorded after 2 weeks when plants were transplanted to 10 cm pots using the same soil mixture

Indexing of in vitro material PVFL-derived clones were bioassayed for the presence of virus using *Nicotiana benthamiana* as an indicator plant Microcuttings generated after 2 or 3 culture cycles were triturated in 1 ml of a buffer (0 01 M sodium phosphate, pH 7 0), using a mortar and pestle and rub inoculated onto young leaves of *N benthamiana* that had been dusted with 600 mesh carborundum As a positive control, microcuttings of VIL were assayed at the same time

Indexing of greenhouse established material A greenhouse bioassay was performed using tissue samples from 2-month-old potted plants from each PVFL Composite samples of 4 to 5 mashua leaves were collected from available plants and triturated in 10 ml of 0 01 M sodium phosphate buffer pH 7 4, and rub inoculated onto *C quinoa* as a host species

Field Experiments in Ecuador The Effect of Virus Infection

Plant material Microcuttings of PVFL and VIL were rooted in vitro (12-hour photoperiod, 50 μmol m^{-2} s^{-1}) and were transplanted into pumice in plastic boxes, covered with a humidity dome and placed in a greenhouse for 21-25 days PVFL were kept separate from VIL to avoid cross contamination After keeping covered for a week to avoid dehydration, the humidity dome was removed progressively until plants were acclimated Plants were then potted into a sterile soil mixture that contained 30% of sand, 30% of composted humus and 40% field soil (clay-loam) until final transplanting to the field After one month in pots, plants were bioassayed for the presence of virus Two or three leaves per plant were macerated in a chilled mortar with one ml of 0 5 M sodium citrate solution Leaves of *N benthamiana* were evenly covered with carborundum powder (500-600 mesh) followed by the crude leaf extract and gently swabbed to avoid mechanical damage

Detection of potyvirus in the field For virus reinfestation, composite samples of symptomatic or asymptomatic tissue were bioassayed using *N benthamiana* and also tested using DAS (Double-Antibody-Sandwich)-ELISA for Potyvirus Group after 7 months in the field A composite sample of symptomatic, young leaves was collected and triturated with coating buffer plus polyvinylpyrrolidone (CB/PVP) Microtiter plates were coated with an admixture of 16 potyvirus antibodies The positive/negative threshold was 4 times the mean of the background mean value The background mean was determined by the average of a negative control of a healthy indicator plant

Effect of virus infection in mashua on yield Field experiment I was set up in La Merced S W of Tunurahua Province at an altitude of 3,000 m, and for 1993-1994, an annual average rainfall of 509 to 690 mm and temperature of 10 5C in February 1994 using PVFL and VIL of 1147

There were 8 plants per row, and 6 plants per evaluation The distance between rows was 1m and 0 5m between plants The experimental design was a randomized complete block with 4 treatments and 3 replications Plots were furrow irrigated during January to March after which natural rainfall was sufficient Plants were harvested 1) at seed set and 2) two weeks later at the end of the vegetative cycle The tubers were classified by the size and weight

Survival in the field was determined 30 days after transplanting by counting the number of cuttings that remained vigorous and initiated new shoots Time-to-flowering was determined when 50% of the plants of a specific line were flowering Plant height in cm (from the soil surface to the most apical leaf) from two randomly selected plants was measured twice at 30 and 60 days after transplanting The number of new shoots/plant after covering with soil was recorded at harvest

Field experiment 2 using both PVFL and VIL of 1093, 1141, and 1147 was established at the Querochaca farm of the Agricultural Engineering Faculty of the Technical University of Ambato, Ecuador at an altitude of 2868 meters above sea level in October 1994 A completely randomized design using a split plot, where the main plot was mashua line and the split plot was virus-infection status, was applied as the experimental design, with a randomized arrangement within main plots and split plots The experiment had 3 treatments per block with 3 replications Mashua plants were spaced at 0 75 m between plants and 1 00 m between rows Tubers of purple mashua were planted around the edge of each replicate to minimize border effects Each plot was 50 m^2 (10 m long x 5 m wide) and blocks had 322 m^2 (23 m long x 14 m wide)

RESULTS

Virus Identification and Characterization

Host range The host range of all ten isolates of the unknown potyvirus were identical, with most species being only locally infected Good diagnostic hosts were identified as *Chenopodium quinoa* L , *C amaranticolor* L , and *G globosa* L serving as bioassay or local lesion hosts and *N benthamiana* as both a diagnostic and propagative host Isolates Tt 4 and Tt 9 were selected for further study

Physical properties In crude extracts, isolates Tt 4 and Tt 9 were infective after treatment at 50° C for 10 minutes but not after treatment at 60° C for 10 min The DEP for both isolates was between 10^5 to 10^6 and the LIV for both isolates was determined to be 5 days at 20°C

Purification and electron microscopy Purification of the Tt 4 isolate from infected *N benthamiana* yielded approximately 10 mg of virus per kilogram of fresh weight of leaf tissue, an absorbance maximum of 260 nm and an $A_{260/280}$ of 1 08 In negatively stained preparations, Tt 4 appeared as flexuous particles 600 to 800 nm, with a modal length of 750 nm and approximate diameter of 10 nm

Aphid transmission The green peach aphid (*Myzus persicae*) transmitted virus to *N benthamiana* from *T tuberosum* accession ECU 1093 infected with isolate Tt 4 Controls were negative Symptoms on *T tuberosum* began as vein-clearing by 10 days and progressed to chlorotic mosaic, followed by leaf deformation, petiole curvature and necrosis of leaf and stem tissues within 2 weeks

Polyclonal antibody indirect ELISA The Tt 4 and Tt 9 isolates reacted positively to PTY-1 McAb and polyclonal antiserum to BCMV and WMV-2 but not to the healthy control or WMV-1, PVY-C, PVA, PMMV, TuMV, LMV, DaMV, TEV, PSMV, MDMV-A and B, and SMV In reciprocal tests, polyclonal antiserum to the Tt 4 isolate reacted positively in indirect ELISA against isolates of Tt 4, Tt 9, PVY-C and less strongly to TEV and PVA, but not to MDMV-A, MDMV-B, BCMV, DaMV, WMV-1, WMV-2, PMMV, TuMV, LMV, SMV, and PSMV The Tt 4 and Tt 9 isolates were determined to be closely related strains of the same aphid-transmitted potyvirus The virus has been tentatively named Tropaeolum mosaic potyvirus (TropMV)

Detection of unique and potyvirus group-common epitopes The reactivity patterns of a panel of twenty-six virus-specific and group-cross reactive monoclonal antibodies (McAbs) with selected virus isolates from *T tuberosum* and other potyviruses were compared in ACP-ELISA This panel of McAbs has been useful in examining the intra-virus, inter-virus, and intra-group serological relationships among and between diverse potyviruses (Jordan, 1992 and Jordan and Hammond, 1991)

The reactivity patterns of Tt 1, Tt 2, Tt 4, Tt 9 and pepper mottle potyvirus (PepMoV-NC), potato potyvirus Y (PVY-3), tobacco etch potyvirus (TEV-PV69), and bean yellow mosaic potyvirus (BYMV-GDD) with ten of the McAbs (Table 2) The 10 selected McAbs define 10 different potyviral epitopes (Jordan, 1992 and Jordan and Hammond, 1991) McAbs PTY 1 through PTY 12 each define unique potyvirus group-common epitopes present on 33 distinct potyviruses McAbs PTY 21 defines a unique epitope shared between specific isolates of BYMV and PepMoV NC McAbs PTY 35 defines an epitope shared between 6 of 9 BYMV isolates The reaction profiles of Tt 1, Tt 2 and Tt 9 are indistinguishable from each other but differ from the profile of Tt 4 Both reactivity profiles are distinct from any of the other potyviruses tested The reaction profile of Tt 1, Tt 2, and Tt 9 against the panel of 26 McAbs also are distinct from any of the other potyviruses tested with this panel of McAbs (Jordan, 1992 and Jordan and Hammond, 1991) The reaction profile of Tt 4 is similar to that of maize dwarf mosaic potyvirus A (MDMV-A) reported in the literature (Joidan, 1992) indicating that the epitopes recognized by McAbs PTY 1,3 and 4 are apparently shared between MDMV-A and isolate Tt 4

Survey for potyvirus in mashua in Ecuador Forty-three of 46 accessions of *T tuberosum* at the INIAP's-SCRS and plants from all eight production fields surveyed exhibited interveinal chlorosis, leaf cupping, distortion and stunting symptoms typical for virus infection Thirty-four of 46 accessions tested positive for TropMV using ACP-ELISA, and 39 accessions tested positive by bioassay to either *N benthamianum* or *G globosa* (Table 3) Four accessions tested negative in both ACP-ELISA and bioassay tests Composite samples from all eight production fields tested positive for TropMV in ACP-ELISA and bioassay

Plant Tissue Culture

Virus elimination The culture of shoot apices (SA) ca 0 2 mm in size was effective for the elimination of potyvirus TropMV from mashua lines as determined through the screening of in vitro generated microcuttings and 2-month-old generated plant material on *N benthamiana* in the greenhouse and confirmed subsequently by DAS-ELISA when plants were in the field Of 58 SA excised, 30 survived and grew After two months of growth, 24 of the SA-derived clones (PVFL) were bioassayed for the presence of virus by mechanically inoculating *N benthamiana* with sap preparations from in vitro maintained microshoots Twenty-two of these 24 clones tested virus-free

Bioassays using 2-month-old greenhouse generated plant tissue of the PVFL and VIL confirmed the results obtained from the bioassays that used in vitro generated microcuttings Based on observations 19 days after inoculation of *C quinoa*, VIL of 1093 and 1147 tested positive while lines 1115 and 1141 were not tested due to poor plant growth and mortality VIL plants of 1147 were asymptomatic, however, inoculation onto *N benthamiana* resulted in the development of symptoms 8 days later

Proliferation versus virus infection Line 1093 proliferated significantly better than 1141 Although there was no clear pattern of significance among the PVFL and VIL, virus infection appeared to have a detrimental effect on general performance The total number of shoots was not significantly different among the PVFL and the VIL within a line, even though the PVFL were consistently more proliferative The majority of the difference between the VIL and the PVFL was evident in the 1-2 cm shoot counts There were no differences in the number of shoots > 2 cm which were not present as often as the other shoot lengths Shoots < 1 cm were the greatest contributors to the proliferation difference between the two lines The FW, DW, FW-DW trends for the relationship among PVFL and VIL were found to be similar to the trends associated with the proliferation rate Tissue of 1093 had more biomass than that of 1141 for both PVFL and VIL Explant orientation was found to be correlated with shoot fresh weight, lateral buds cultured horizontally had significantly more biomass than apical buds cultured vertically

Rooting in vitro and reestablishment There were no differences in vitro rooting ability among VIL and PVFL Rooted microcuttings of VIL were significantly longer than rooted microcuttings of PVFL Percentages of mortality were lower for lines 1093 (1 %), 1147 (1 %) and 1141 (7 %) compared to 1115 (7%) Both VIL and PVFL of 1093 and 1147 and PVFL of 1141 and 1115 survived and grew over a 2-month period after transplanting At the same time, VIL of lines 1141 and 1115 did not develop well and finally deteriorated drastically, having 33% and 50% of the plants dead, respectively, 2 months after transplanting

Field Experiments in Ecuador The Effect of Virus Infection

Plant mortality In experiment 1 there was no difference between virus-free and -infected plants for survival after transplanting to the field, for plant height at 30 or 60 days, for number of new sprouts, or for number of tubers harvested per plant Virus-free plants had significantly greater tuber weight (928 gm) compared to virus-infected plants (235 gm) In experiment #2 the mortality of all plants was high due to environmental conditions after transplanting to the field Lines 1093 and 1141 had survived significantly better than did line 1147 There were no differences in survival rate between the virus-infected and virus-free plants

Plant vigor Measurements of leaf length, width and area of the 3rd and 10th fully-developed leaves were not significantly different for the lines tested However, length, width and area of the 10th fully-developed leaves from virus-free plants were significantly reduced Mashua plant vigor evaluation in the field demonstrated that there were no differences between the tested lines whether virus-infected or not

Flowering Virus-free plants flowered earlier (100 days) and were harvested earlier (220 days) than virus-infected plants (155 days and 254 days, respectively) Differences in flowering time were due mainly to differences in lines Line 1147 (virus-infected and -free) flowered first at 145 days after field transplanting, followed by line

1093 at 154 days and finally line 1141 at 168 days Flowering increased over a period of 45 days, at which time the first flowers had already set fruit

Yield evaluation There were no statistical differences in field performance for the three lines examined Although there were no differences in overall yield between the virus-infected and -free lines, virus-infected lines produced a significantly greater number of large tubers (3 4 vs 1 4)

Indexing of field plants Thirty-three percent of the virus-free plants became reinfected by the end of Field Experiment 2 based on DAS-ELISA tests Virus transmission through aphids may have been the cause, as there was a large population of aphids present in the plots from January to the end of March Surprisingly, 24% of the virus-infected plants tested negative using DAS-ELISA

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

During the time span of the project several scientific activities were held at AMDE and/or sponsored by the project

- The Second National Tissue Culture Congress for 125 participants and international scientist
- The Second and Third National Plant Virology Workshops for approximately 70 participants and international scientists
- 5 Ecuadorian laboratories, University of Cuenca, INEXA, INIAP, Laboplanto and the Technical University of Ambato (UTA) received free consulting services and chemicals for use in their research activities
- 3 students from UTA conducted their senior thesis research required for their graduation as agricultural engineers
- AMDE Corp scientists have presented talks at many events in Ecuador, as well as Internationally
- One student received training in plant virology and plant tissue culture at the University of Delaware and received her M S degree during the life of the project and returned to Ecuador to work at UTA in the area of plant pathology and horticulture
- 12 virus-free mashua lines were returned to INIAP for maintainence, propagation and dispersal to the agricultural industry and indigenous peoples
- 10 lines of white carrot were provided in tissue culture to INIAP which was having a difficult time placing the crop *in vitro* This will allow them to maintain the germplasm longterm
-2 demonstration plots of virus-free mashua were established in Bolivar and Tunguruhua Provinces and presentations made to indigenous peoples as to the value of starting with virus-free material
- 100 3-pound bags of a mixture of virus-free lines of mashua, simulating landraces of the crop, were provided to indigenous peoples of Bolivar, Tunguruhua, Cotopaxi and Chimorazo Provinces through CARE, Catholic Relief Services and the extension service of the Polytechnic University of Chimborazo

- AMDE has expanded its reputation for being a well qualified laboratory specializing in the tissue culture of native Ecuadorian/Andean plants to a laboratory able to develop and certify virus-free plant materials AMDE's role in research, education and extension has been enhanced through the training and research carried out during this project
- At the end of the project, AMDE had two operating tissue culture laboratories and two greenhouse facilities, one in Ambato and one in Samanga Each facility having a computer center, and fax services available to Ecuadorian and international scientists The laboratory facilities in Ambato also maintained the ability to perform ELISA tests using an automated mini-plate reader
- Research personnel at AMDE, including Ing Jaime Rojas, continue to work on indigenous tuber crops The focus of their work has shifted to methods of production of other Andean tubers such as oca and ullucus and is being carried out in conjunction with UTA and CIP at Quito
- The trained personnel have and will continue to provide training services for in country needs Laboratory facilities will continue to be used by the focus of the services has shifted to meet the needs of the region At the present time, the ELISA facility is being used to screen both boiler and egg laying chickens for a number of important virus diseases They have given at least one seminar since the end of the project to poultry industry personnel in how to carry out ELISA tests for detection of avian viruses We believe that this facility and infrastructure will continue to be important to the agricultural sector of Ecuador in a variety of different ways

PROJECT ACTIVITIES/OUTPUTS

Workshops and Congresses

Organized, developed and hosted

- Second National Plant Virology Workshop, April 12-15, 1993 Ambato, Ecuador - with the participation of Dr V D Damsteegt and Dr T A Evans and more than 25 participants from University, government and private laboratories

- Second National Congress on Plant Tissue Culture, June 16-17, 1994 Machala del Oro, Ecuador with the participation of Dr T A Evans, Dr S L Kitto and Dr V D Damsteegt and over 125 students, faculty and scientists primarily from the lowlands of Ecuador

- Third National Plant Virology Workshop, June 9-11, 1995, Catholic University, Quito, Ecuador with the participation of Dr Evans, Dr Kitto and Dr V D Damsteegt and 45 students, faculty and scientists from University, government and private laboratories

Workshops and Trainings Attended

- Victor Cortez participated in a 15 day workshop on Biotechnology Applications of Integrated Pest Control for Horticultural Crops in Cuernavava, Mexico beginning on Sept 25, 1994 sponsored by ONUDI-ONU-UNAM, with full scholarship

- Carmita Taboada was sponsored by AMDE to participated in a 7 day training on Integrated Pest Management in Riobamba organized by the Agricultural Ministry beginning on Oct 7, 1994

THESES

Masters

Soria, Sandra 1996 Identification and elimination of viruses from mashua (*Tropaeolum tuberosum*, Ruiz and Pavon) and methods for its micropropagation, University of Delaware, Thesis, Newark, DE

Undergraduate

Taboada, Carmita Micropropagation and effect of virus infection on productivity of mashua (*Tropaeolum tuberosum*) University of Cuenca, 1994

Montero, Lorena Micropropagation of *Arracacia xanthorrhiza*, Technical University of Ambato 1993

Bermudez, Elsa Efficiency of production of cuttings from mashua tubers Technical University of Ambato 1992

FACULTY INVOLVED IN PROJECT

- Dr Thomas A Evans, Associate Professor of Plant Virology, University of Delaware
- Dr Sherry L Kitto, Professor of Horticulture, University of Delaware
- Dr Vernon D Damsteegt, Research Plant Virologist, USDA-ARS, Ft Detrick, MD
- Dr Ramiro Velastegui Professor of Plant Pathology, Technical University of Ambato
- Ing Rebeca Vega de Rojas, Director and Plant Tissue Culture Specialist, AMDE Corp
- Ing Jaime Rojas, Professor of Horticulture, Technical University of Ambato
- Ing Edwardo Fiallos, Professor of Horticulture, Technical University of Ambato
- Biol Ligia Ayala, Research Plant Virology, INIAP, Quito

GERMPLASM COLLECTION

- Provided twelve virus-free lines of mashua to the germplasm collection AMDE, the Technical University of Ambato and INIAP including some new accessions from Bolivar and Tunguruhua Provinces Additionally, ten lines of white carrot were provided to the germplasm collection at INIAP's Santa Catalina Research Laboratory in tissue culture for long term maintenance

CONSULTANT BENEFITS

- Dr Sherry Kitto, International consultant, advised in tissue culture to representatives of the University of Cuenca, University of Machala, INEXA, FUNDAGRO, CIP and INIAP
- Dr Evans, International consultant, advised on plant virology to representatives of the Technical University of Ambato, Catholic University (PUCE), University of Cuenca , INEXA, CIP, INIAP, FUNDAGRO and PROEXANT
- Dr Vernon Damsteegt, International consultant, advised on plant virology to representatives of PUCE, INIAP and CIP

PUBLICATIONS

Refereed

Soria, S L , R Vega, V D Damsteegt, L L McDaniel, S L Kitto and T A Evans 1998 Occurrence and Partial Characterization of a New Mechanically Transmissible Virus in Mashua from the Ecuadorian Highlands Plant Disease 82 69-73

Soria, S L , C Taboada, R Vega, T Evans, V D Damsteegt and S Kitto 1999 In Vitro and Field Performances of Virus-freed and Virus-infected Plants of *Tropaeolum tuberosum* Ruiz & Pavon (in review)

Abstracts

Evans, T A , Soria, S L , Kitto, S L, Damsteegt, V D , McDaniels, L , and Rojas, R 1996 Occurrence and partial characterization of a mechanically transmissible virus in mashua (*Tropaeolum tuberosum*) from Ecuador Second International Congress on Underutilized Crops, Southampton, England

Kitto, S L , Soria, S L Evans, T A , Damsteegt, V D , and Rojas, R L 1996 Micropropagation of mashua (*Tropaeolum tuberosum*) and the elimination of Tropaeolum mosaic virus via shoot tip culture Second International Congress on Underutilized Crops, Southampton, England

Evans, T A , Soria, S L , Kitto, S L, Damsteegt, V D , McDaniels, L , and Rojas, R 1996 Effect of potyvirus infection on productivity of mashua (*Tropaeolum tuberosum*) in Ecuador Phytopathology 86 0000 Presented at National APS Annual Meeting, Indianapolis, IN

Evans, T A , Damsteegt, V D , Soria, S , Vega de Rojas, R , Stone, A S , Jordan, R L and Kitto, S L 1993 Occurrence of a mechanically transmissible virus in *Tropaeolum tuberosum* from Ecuador Phytopathology 83 0000 Presented at National APS Annual Meeting, Nashville, TN

Soria, S and Rojas, R 1993 Rooting and acclimatization procedures for mashua Quito, Ecuador

Non-refereed

Evans, T A Impact of plant viruses on germplasm collection, Second National Congress on Plant Genetic Resources, Quito, Ecuador, April 12 1991

Vega, R Identification and virus elimination in mashua First National Biotechnology Workshop, Quito, Ecuador, March 30, 1994

Taboada, C Bioassays for the certification of virus-free micropropagated mashua Ecuadorian Biology Journey 18, November, 1994

Soria, S L The classification and histology of mashua Ecuadorian Biology Journey 18, November, 1994

PROJECT PRODUCTIVITY

The project has accomplished all of the goals for the project Approximately 200 individuals were trained in basic methods in virus detection and elimination using tissue culture through three national workshops held in Machala, Quito and Ambato The individuals trained were from the private sector, university and government laboratories One Ecuadorian student, Ing Sandra Soria, training at the University of Delaware, received her M S degree in Plant Science in the areas of plant virology and plant tissue culture and returned to Ecuador to work for the Technical University of Ambato Methods for the regeneration of mashua and white carrot via tissue culture were developed Twelve virus-free mashua cultivars and ten virus-free white carrot cultivars in tissue culture and as tubers or stem cuttings were turned back to the Ecuadorian National Germplasm program at INIAP, Santa Catalina Extension programs and demonstration plots were used to educate indigenous peoples as to the value of using virus-free tubers to establish mashua plantings Approximately, 100 3-pound bags of virus-free mashua tubers were provided to indigenous peoples of the provinces of historical mashua production, Chimborazo, Bolivar, Tungurhua, and Cotopaxi This was the reintroduction of mashua to Bolivar and Tungurhua Provinces and was facilitated by CARE, Catholic Relief Services and the extension service of The Polytechnic University of Chimborazo

FUTURE WORK

At the present, no future work is planned in our laboratories on these two crops AMDE continues to work on Andean tubers other than mashua and white carrot with CIP A project funded by the McKnight Foundation to Pennsylvania State University and the International Center for Potato Research (CIP) in Lima, Peru is targeted at the re-development of the lost crops of the Incas including mashua and white carrot and other important Andean tubers We have provided both antiserum and isolates of TropMV to the Pennsylvania State University component of their project We believe that the value and cultural importance of these two crops has now been firmly established as indicated by the funding of the Andean tuber project which has substantial economic support from the McKnight Foundation

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APPENDICES

Table 1 Host range of the potyvirus isolated from mashua (*Tropaeolum tuberosum*) in the highlands of Ecuador

TEST SPECIES	REACTION[A]
Chenopodium quinoa	Red rimmed local lesions with necrotic centers
C amaranticolor	Red rimmed local lesions with necrotic centers
Gomphrena globosa	Red rimmed local lesions with necrotic centers
Nicotiana debneyi	Chlorotic local lesions
N rustica	Chlorotic local lesions
N occidentalis	Dark rimmed local lesions with necrotic centers
N benthamiana	Systemic vein clearing, downward curling of leaves, stem distortion and necrosis of leaf and stem tissue
Nicotiana tabacum	
cv Samsun	No reaction[B]
cv B-21	No reaction
cv Havana	No reaction
cv Xanthi	No reaction
Cucumis sativum	
cv National Pickling	No reaction
Phaseolus vulgaris	
cv Top Crop	No reaction
cv Bountiful	No reaction
cv Pinto	No reaction
cv Blackturtle Soup	No reaction
cv Monroe	No reaction
Capsicum annum	No reaction
Brassica rapa	No reaction
Vigna unguiculata	
cv California Blackeye	No reaction
Amaranthus hybridus	No reaction
Abelmoschus esculentus	
cv Clemson spineless	No reaction
Lycopersicum esculentum	No reaction

[A] Isolates Tt 1, Tt 2, Tt 3, Tt 4, Tt 5, Tt 6, Tt 7, Tt 8, Tt 9, Tt 10 of Tropaeolum mosaic potyvirus (TropMV) gave identical reactions when mechanically inoculated to herbaceous hosts

[B] Inoculated leaves of asymptomatic indicator plants were triturated and inoculated onto leaves of *N benthamianum* to test for latent infection

TABLE 2 Differential reactivity of PTY monoclonal antibodies with selected virus isolates from *Tropaeolum tuberosum* (Tt) and other potyviruses in antigen coated plate (ACP) enzyme-linked immunosorbent assay (ELISA)[A]

	PTY Monoclonal Antibody									
Potyvirus[B]	1	2	3	4	5	8	10	12	21	35
Tt 1	+++	-	+	+	+	+	-	-	-	-
Tt 2	+++	-	+	+	+	+	-	-	-	-
Tt 9	+++	-	+	+	+	+	-	-	-	-
Tt 4	+++	-	+	+	-	-	-	-	-	-
BYMV-GDD	+++	+++	+++	+++	-	++	(+)	+	+++	+++
PepMoV-NC	+++	-	+++	+	-	-	+++	++	+++	-
PVY-3	+++	+++	-	+++	-	++	+++	-	-	-
TEV-PV69	+++	+++	-	-	-	++	++	+	-	-
MDMV-A	+++	+++	-	+++	-	++	-	-	-	-

[A]Indirect ELISA using potyvirus-infected plant extracts (final dilution 1 30, w/v) diluted in carbonate buffer and direct coating of Nunc ELISA plates
Relative ELISA A405 values after 1 hr substrate incubation were (+++) = > 1 2, (++) = 0 6 to 1 2, (+) = 0 6 to 0 2, (-)=<0 1
[B]Acronyms Tt 1, Tt 2, Tt 9 and Tt 4 represent Tropaeolum mosaic potyvirus isolates from mashua (*Tropaeolum tuberosum*) accessions ECU 1145, ECU 1141, ECU 1147 and ECU 1093, respectively and BYMV GDD = bean yellow mosaic potyvirus strain GDD, PepMoV = pepper mottle virus isolate NC, PVY strain 3 = potato virus Y 3, and TEV PV69 = tobacco etch virus isolate PV69, MDMV-A = maize dwarf mosaic potyvirus strain A

TABLE 3 Incidence of mechanically transmitted potyvirus in Ecuadorean accessions of mashua (*Tropaeolum tuberosum)* at INIAP's Santa Catalina Research Station, Quito, Ecuador as determined by bioassay and ACP-ELISA

Sample[A]	Bioassay[B]		ACP-ELISA[C]
	N b	G g	
14 accessions	+	+	+
7 accessions	-	+	+
10 accessions	+	-	+
2 accessions	+	+	-
4 accessions	-	+	-
2 accessions	+	-	-
3 accessions	-	-	+
4 accessions	-	-	-

[A]Six to 13 symptomatic plants were tested of each accession and with few exceptions 100% of the source plants tested positive

[B]Indicator species include N b = *Nicotiana benthamiana*, G g = *Gomphrena globosa* Symptoms were as described in Table 1

[C]Indirect ELISA using admixture of McAbs to reactive to TropMV Tt 4 and Tt 9 isolates with virus-infected plant extracts (final dilution 1 30, w/v) diluted in carbonate buffer and direct coating of Nunc ELISA plates A positive/negative threshold A405 value of 4 times the healthy control was used

Occurrence and Partial Characterization of a New Mechanically Transmissible Virus in Mashua from the Ecuadorian Highlands

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ABSTRACT

Soria S L Vega R Damsteegt V D McDaniel L L Kitto S L and Evans T A 1998 Occurrence and partial characterization of a new mechanically transmissible virus in mashua from the Ecuadorian Highlands Plant Dis 82 69 73

Mashua *(Tropaeolum tuberosum)* is a tuber crop indigenous to the Andean highlands and of economic value to its native people as a food and medicinal crop Field plants often exhibit symptoms typical of virus infection including interveinal chlorosis leaf cupping distortion and stunting Using an herbaceous host range at least 1 mechanically transmissible virus was detected in each of 10 accessions All virus isolates tested reacted to potyvirus group antiserum in indirect enzyme linked immunosorbent assays (ELISA) Results of monoclonal antibody testing using antigen coated plate (ACP) ELISA where virions are disrupted to expose cryptotopes indicated that isolate Tt 4 has epitopes in common with maize dwarf mosaic potyvirus A (MDMV A) Polyclonal antiserum produced to purified preparations of the Tt 4 isolate reacted in indirect ELISA to Tt 4 the common strain of potato potyvirus Y (PVY C) less strongly to tobacco etch potyvirus (TEV) and potato potyvirus A (PVA) but not to 11 other potyviruses including MDMV A Conversely the Tt 4 isolate reacted strongly in indirect ELISA to antisera to bean common mosaic potyvirus (BCMV) and watermelon mosaic potyvirus 2 (WMV 2) but not antisera to 12 other potyviruses Our data suggest that isolate Tt 4 is a distinct potyvirus closely related to and sharing external epitopes with PVY C TEV PVA WMV 2 and BCMV and buried epitopes with MDMV A The virus has been tentatively named Tropaeolum mosaic potyvirus (TropMV) A survey of *T tuberosum* in Ecuador revealed that TropMV was widespread being detected by ACP ELISA in 34 of 46 accessions at the National Institute for Agricultural Research s Santa Catalina Research Station Quito and all eight production fields surveyed

Mashua also known as isanu cubio or anu (*Tropaeolum tuberosum* Ruiz & Pavon) is one of the lost crops of the Inca and currently ranks fourth in importance among root crops of the Andean region after potato oca and ulluco (10) Mashua is often grown in a multicrop system along with other crops and is a hardy plant that is often the prevalent root crop in the poorest regions where pesticides and fertilizers are too costly to use (Fig 1) In the past mashua tubers could be found in almost any rural Andean market Mashua (pronounced mah shoo ah) is closely related to the garden nasturtium (*Tropaeolum majus* L) and has been cultivated for its tubers by the people of the Andean highlands since Incan time Among Andean tubers mashua is one of the highest yielding easiest to grow and most tolerant of cold temperatures It also repels many insects nematodes and other pathogens making it a valuable plant for intercropping Despite its potential productivity and pest resistance mashua is not widely commercialized

Tubers of mashua are the size of small potatoes with shapes ranging from conical to carrot-like and colors from yellow to purple to red (Fig 2) Eaten raw some have a peppery taste reminiscent of hot radishes When boiled they become mild and in some instances sweet Tubers are usually boiled with meat to form a stew or eaten as baked or fried vegetables In New Zealand, where the plant has been newly introduced mashua sets tubers well in open fields during a normal spring to-autumn growing season (10) Mashua is a nutritious root crop with 20% solids and protein as much as 16% of the dry matter (10)

The occurrence of viruses in mashua affects productivity (10) Over the past 10 years the number of hectares of mashua planted and yield per hectare have both decreased drastically and this reduced productivity has been cited as a reason for reduced interest in growing mashua (10) Field plants exhibit virus-like symptoms which include smaller leaves with cupping distortion and interveinal chlorosis Plants exhibiting any of these symptoms generally were thought to be infected with at least one mechanically transmissible virus (*personal observation*) Worldwide, a number of viruses have been reported to infect mashua s close relative garden nasturtium (2 3) Nasturtium ringspot virus appears to be closely related to broad bean wilt virus (6) and has been detected in California (7) and Brazil (13) Other viruses detected in *T majus* include cabbage black ringspot tymovirus (14) beet curly top geminivirus (12) cucumber mosaic cucumovirus (11) pea early browning tobravirus (1) and tobacco rattle tobravirus (16) Only a single virus disease mosaic has been reported in *T tuberosum* in Bolivia (4) Plants typically exhibited symptoms which included a mosaic and mal-

Fig 1 Mashua (*Tropaeolum tuberosum* foreground) is often grown in a multicrop system along with other crops such as corn (*Zea mays* background)

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formation of leaves Based on leaf dip preparations the causal virus was reported to be a flexuous rod with a mean length of 800 nm but no serological work was reported

The objective of the present research was to isolate and characterize the causal virus of this mosaic disease of mashua and to determine its incidence within mashua plantings in the highlands of Ecuador

MATERIALS AND METHODS

Host range Initially 10 mashua accessions (ECU 1145 ECU 1141 ECU 1084 ECU 1093 ECU 1115 ECU 1101 ECU 1102 ECU 1096 ECU 1147 and ECU 1098) were introduced as tubers to the quarantine greenhouse at United States Department of Agriculture Agricultural Research Station (USDA-ARS) Fort Detrick Frederick Maryland from the Ecuadorian highlands under Animal and Plant Health Inspection Service (APHIS-PPQ) permit Ten virus isolates 1 from each accession (Tt 1 Tt 2 Tt 3 Tt 4 Tt 5 Tt 6 Tt 7 Tt 8 Tt 9 and Tt 10) respectively were established by mechanical inoculation onto selected herbaceous indicator plants (Table 1) Symptomatic leaf tissue was triturated in 0 1 M sodium citrate buffer pH 7 4 and rub inoculated onto leaves of indicator plants that first had been dusted with 600 mesh Carborundum Plants were observed over a 6 week period for symptom development Inoculated leaves of symptomless indicator plants were back inoculated to *Nicotiana benthamiana* L a systemic host that produces distinct vein clearing downward curling of leaves and stem and necrosis of leaves and stem tissue over a 2 week period and *Gomphrena globosa* L a bioassay host that produces red-rimmed local lesions with necrotic centers in 10 days to test for latent infection Isolates Tt 4 and/or Tt 9 were selected for further testing

Fig 2 The tubers of mashua (*Tropaeolum tuberosum*) are the size of small potatoes and often conically shaped

Physical properties Longevity in vitro (LIV) thermal inactivation point (TIP) and dilution end point (DEP) were determined for the Tt 4 and Tt 9 isolates using standard protocols (17) For TIP determination systemically infected leaves of *N benthamiana* in 0 1 M sodium citrate buffer pH 7 4 were heated for 10 min at temperatures from 50 to 100°C at 10 degree intervals using a heat block For LIV crude sap prepared from *N benthamiana* was held at 20°C in the dark for 0, 1 5 7 14 and 30 days For DEP crude sap was serially diluted with 0 1 M sodium citrate buffer pH 7 4 All samples were tested for infectivity using *N benthamiana* plants

Virus purification The Tt 4 isolate was purified from systemically infected leaf tissue of *N benthamiana* using a modification of the method developed by Hammond and Lawson (5) for potyviruses Systemically infected leaf tissue (187 g) was triturated in liquid nitrogen then homogenized in 1 000 ml of 0 5 M K_2H/KH_2PO_4 buffer pH 8 4 with 0 5% (wt/vol) Na_2SO_3 and filtered through cheesecloth The filtrate was centrifuged at 8 000 × *g* for 10 min at 4°C in a GSA rotor and the supernatant collected Triton X-100 was added to 2% and the solution stirred for 15 min at 4°C Polyethylene glycol (PEG) 8000 was added to 6% and NaCl to 2% and the solution stirred for 1 h at 4°C and centrifuged at 10 000 × *g* for 10 min at 4°C in a GSA rotor Pellets were resuspended in 50 ml of 100 mM borate/KCl buffer pH 8 0 and homogenized transferred to a beaker and stirred at least 1 h at 4°C A 25 ml aliquot of the suspension was measured into R30 tubes underlayed with 5 ml/tube 30% (wt/vol) sucrose in 0 1 m borate/KCl buffer pH 8 0 and centrifuged at 70 000 × *g* for 2 5 h in an Ti50 rotor Pellets were resuspended in either 100 mM borate/KCl buffer pH 8 0 or 10 mM sodium phosphate buffer pH 7 6 After centrifuging at 10 000 × *g* for 10 min at 4°C for clarification the suspensions were layered onto 10 to 40% sucrose gradients and centrifuged at 70,000 × *g* for 2 h in a SW-41 rotor and fractionated using an ISCO (ISCO Inc Lincoln NB) UA-5 ultraviolet fractionator at 258 nm Ultraviolet absorbing fractions were collected pooled and diluted 10-fold with borate/KCl buffer pH 8 0 or 10 mM sodium phosphate buffer pH 7 6 respectively centrifuged at 70 000 × *g* for 2 5 h in a Ti75 rotor supernatant discarded and pellets resuspended in 250 µl of the same buffer

Electron microscopy The morphology of virions was determined by placing partially purified preparations of isolate Tt 4 from the above procedure onto Formvar-backed carbon coated grids, staining them

Table 1 Host range of the potyvirus isolated from mashua (*Tropaeolum tuberosum*) in the highlands of Ecuador

Test species	Reaction[a]
Chenopodium quinoa	Red rimmed local lesions with necrotic centers
C amaranticolor	Red rimmed local lesions with necrotic centers
Gomphrena globosa	Red rimmed local lesions with necrotic centers
Nicotiana debneyi	Chlorotic local lesions
N rustica	Chlorotic local lesions
N occidentalis	Dark rimmed local lesions with necrotic centers
N benthamiana	Systemic vein clearing downward curling of leaves stem distortion and necrosis of leaf and stem tissue
Nicotiana tabacum	
cv Samsun	No reaction[b]
cv B 21	No reaction
cv Havana	No reaction
cv Xanthi	No reaction
Cucumis sativum	
cv National Pickling	No reaction
Phaseolus vulgaris	
cv Top Crop	No reaction
cv Bountiful	No reaction
cv Pinto	No reaction
cv Blackturtle Soup	No reaction
cv Monroe	No reaction
Capsicum annum	No reaction
Brassica rapa	No reaction
Vigna unguiculata	
cv California Blackeye	No reaction
Amaranthus hybridus	No reaction
Abelmoschus esculentus	
cv Clemson spineless	No reaction
Lycopersicon esculentum	No reaction

[a] Isolates Tt 1 Tt 2 Tt 3 Tt 4 Tt 5 Tt 6 Tt 7 Tt 8 Tt 9 Tt 10 of Tropaeolum mosaic potyvirus (TropMV) gave identical reactions when mechanically inoculated to herbaceous hosts

[b] Inoculated leaves of asymptomatic indicator plants were triturated and inoculated onto leaves of *N benthamiana* to test for latent infection

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with 2% uranyl acetate or 2% phosphotungstic acid and examining them with a Philips 201 electron microscope at 80 kV Two hundred particles were measured and particle size determined by comparison with photographs taken at the same magnification of germanium shadowed carbon replicas made from diffraction gratings with 54 864 lines per inch and/or grids containing purified preparations of tobacco mosaic tobamovirus as standard size markers

Polyclonal antibodies and indirect enzyme linked immunosorbent assay Polyclonal antibodies were produced to highly purified preparations of the Tt 4 isolate Two rabbits were given intramuscular injections of 100 µg of virus in Hunter s TiterMax (TiterMax Inc Atlanta GA) adjuvant every 10 days Test bleeds began 30 days after initial injection Indirect enzyme linked immunosorbent assay (ELISA) tests were carried out using purified γ globulin to the Tt 4 isolate and goat anti rabbit γ globulin conjugated with alkaline phosphatase (Sigma Chemical Co St Louis) Virus isolates Tt 4 and Tt 9 were tested in indirect ELISA against antiserum (Agdia Inc Elkhart IN) to the following potyviruses watermelon mosaic potyvirus 1 (WMV 1) watermelon mosaic potyvirus 2 (WMV-2) potato potyvirus Y-common strain (PVY C) potato potyvirus A (PVA) pepper mild mottle potyvirus (PMMV) turnip mosaic potyvirus (TuMV) lettuce mosaic potyvirus (LMV) dasheen mosaic potyvirus (DaMV) soybean mosaic potyvirus (SMV) tobacco etch potyvirus (TEV) pea seedborne mosaic (PSMV) maize dwarf mosaic potyvirus A (MDMV-A) and maize dwarf mosaic potyvirus B (MDMV-B) A positive threshold absorbance reading of four times the mean of the healthy control was used

Monoclonal antibody panel Twenty six monoclonal antibodies (MAbs) from a previous study (4) designated PTY MAbs were used separately to compare the Tt 4 and Tt 9 isolates of the unknown potyvirus from mashua and other characterized potyviruses (courtesy R Jordan and J Hammond) Members of this panel of MAbs recognize epitopes that are (i) specific to bean yellow mosaic potyvirus (BYMV) strains (ii) common to members of the BYMV subgroup (iii) distinctive for unique potyviruses or (iv) common to many distinct potyviruses (9 10) The format is an indirect ELISA with the viral antigen being coated directly onto the microtiter plate These broad spectrum MAbs react with antigenic determinants on viral subunit regions not exposed on the virion surface Virions were disrupted during the coating process to expose cryptotopes

Antigen coated plate enzyme linked immunosorbent assays Indexing of mashua tissue samples for the Tt 4 and Tt 9 isolates was carried out using the PTY MAbs in indirect antigen coated plates (ACP) ELISA (10) One gram composite samples of symptomatic leaves were triturated in 10 ml of 0 05 M sodium carbonate bicarbonate buffer (CB) plus polyvinylpyrrolidone (PVP) pH 9 6 containing 0 2% sodium diethyldithiocarbamate (DIECA) The samples were incubated at room temperature for 2 h to disrupt virions and 100 µl of each sample dispensed to duplicate wells of Nunc MaxiSorp polystyrene ELISA plates Plates were incubated for 1 h at room temperature and washed three times with phosphate buffered saline plus Tween 20 (PBS Tween) before 100 µl of an admixture of MAbs to potyvirus epitopes 1+2+3+4+8+10+21 (10 11) was added The plates were incubated for 2 h at room temperature or overnight at 4°C and washed four times with PBS-Tween Later 100 µl of goat anti rabbit γ-globulin conjugate was added and incubated for 1 h at room temperature Finally 100 µl of p nitrophenol phosphatase (Sigma Chemical Co) diluted in diethanolamine buffer was added to each well Reactions were stopped with 50 µl of 3 M sodium hydroxide and optical density at 405 nm determined after 1 h The positive threshold used was four times the mean of the healthy control value

Survey of mashua in Ecuador for the occurrence of potyviruses A survey of 46 accessions of mashua maintained at the National Institute for Agricultural Research s (INIAP) Santa Catalina Research Station (SCRS) Quito Ecuador and plants from 8 fields representing the major production areas in the Ecuadorian highlands for the presence of Tt 4 and Tt 9 like isolates was carried out during April 1993 Virus testing included both indirect ACP-ELISA with an admixture of PTY MAbs described above and bioassay to *N benthamiana* and *G globosa* For each accession and production field a composite sample of symptomatic young leaves from a minimum of 5 plants was collected Leaf samples were triturated in either CB/PVP containing 0 2% DIECA for ACP ELISA or in 0 01 M sodium phosphate buffer pH 7 0 for bioassay on indicator plants Sap extracts were diluted 10 fold and tested using indicator plants as before Plants were maintained in a greenhouse and observed over a 4-week period for symptom development

Aphid transmission Green peach aphids (*Myzus persicae* (Sulzer)) were reared on curly dock (*Rumex crispus* L) in a quarantine greenhouse Several hundred adults and nymphs were shaken onto moist filter paper and starved for 4 h Approximately 100 adults and nymphs (3rd and 4th instar) were transferred to detached leaves of *T tuberosum* (ECU 1093) and given an acquisition access period (AAP) of 30 s to 5 min As soon as aphids probed they were moved in sets of 25 or more to 3 week old *N benthamiana* seedlings for a 24 h inoculation access period (IAP) An identical group of green peach aphids was allowed the same period of time to probe on virus free curly dock then transferred onto *N benthamiana* for a 24 h IAP as a control Plants were sprayed with a dilute solution of malathion (*O O*-dimethylphosphorodithioate of diethylmercaptosuccinate 0 2 g a i /liter) and placed on a greenhouse bench for a 30 day observation period To confirm virus transmission leaf tissue was bioassayed using healthy *N benthamiana* as previously described

RESULTS

Host range The host range of all 10 isolates of the unknown potyvirus were identical with most species being only locally infected (Table 1) Good diagnostic hosts were identified as *Chenopodium quinoa* L *C amaranticolor* L and *G globosa* L serving as bioassay or local lesion hosts, and *N benthamiana* as both a diagnostic and propagative host Isolates Tt 4 and Tt 9 were selected for further study

Physical properties In crude extracts isolates Tt 4 and Tt 9 were infective after treatment at 50°C for 10 min but not after treatment at 60°C for 10 min The DEP for both isolates was between 10^5 to 10^6 and the LIV for both isolates was determined to be 5 days at 20°C

Purification and electron microscopy Purification of the Tt 4 isolate from infected *N benthamiana* yielded approximately 10 mg of virus per kilogram of fresh weight of leaf tissue an absorbance maximum of 260 nm and an $A_{260/280}$ of 1 08 In negatively stained preparations Tt 4 appeared as flexuous particles 600 to 800 nm with a modal length of 750 nm and approximate diameter of 10 nm

Aphid transmission The green peach aphid (*Myzus persicae*) transmitted virus to *N benthamiana* from *T tuberosum* accession ECU 1093 infected with isolate Tt 4 Controls were negative Symptoms on *T tuberosum* began as vein clearing by 10 days and progressed to chlorotic mosaic followed by leaf deformation petiole curvature and necrosis of leaf and stem tissues within 2 weeks

Polyclonal antibody indirect ELISA The Tt 4 and Tt 9 isolates reacted positively to PTY 1 MAb and polyclonal antiserum to BCMV and WMV-2 but not to the healthy control or WMV-1 PVY-C PVA PMMV TuMV LMV DaMV TEV PSMV MDMV-A and B and SMV In reciprocal tests polyclonal antiserum to the Tt 4 isolate reacted positively in indirect ELISA against isolates of Tt 4 Tt 9 PVY C less strongly to TEV and PVA but not to MDMV A MDMV B BCMV DaMV WMV 1 WMV-2 PMMV TuMV LMV SMV and PSMV The Tt 4 and Tt 9 isolates were determined to be closely related strains of the same aphid-transmitted potyvirus The virus has been tentatively named Tropaeolum mosaic potyvirus (TropMV)

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Detection of unique and potyvirus group common epitopes The reactivity patterns of a panel of 26 virus-specific and group cross reactive MAbs with selected virus isolates from *T tuberosum* and other potyviruses were compared in ACP ELISA This panel of MAbs has been use ful in examining the intra virus inter virus and intra group serological relationships among and between diverse potyviruses (8 9)

The reactivity patterns of Tt 1 Tt 2 Tt 4 Tt 9 pepper mottle potyvirus (PepMoV NC) potato potyvirus Y (PVY-3) tobacco etch potyvirus (TEV-PV69) and bean yel low mosaic potyvirus (BYMV GDD) with 10 of the MAbs are shown in Table 2 The 10 selected MAbs define 10 different poty viral epitopes (8 9) MAbs PTY 1 through PTY 12 each define unique potyvirus group common epitopes present on 33 distinct potyviruses MAbs PTY 21 defines a unique epitope shared between specific isolates of BYMV and PepMoV NC MAbs PTY 35 defines an epitope shared between 6 of 9 BYMV isolates The reac tion profiles of Tt 1 Tt 2 and Tt 9 are indistinguishable from each other but differ from the profile of Tt 4 Both reactivity profiles are distinct from any of the other potyviruses tested (Table 2) The reaction profile of Tt 1 Tt 2 and Tt 9 against the panel of 26 MAbs (*data not shown*) also are distinct from any of the other potyvi ruses tested with this panel of MAbs (8 9) The reaction profile of Tt 4 is similar to that of MDMV A reported in the literature (8) indicating that the epitopes recognized by MAbs PTY 1 3 and 4 are apparently shared between MDMV A and isolate Tt 4

Survey for potyvirus in mashua in Ecuador Forty three of 46 accessions of *T tuberosum* at the INIAP s SCRS and plants from all eight production fields sur veyed exhibited interveinal chlorosis leaf cupping distortion and stunting symptoms typical for virus infection (*data not shown*) Of the 46 accessions 34 tested positive for TropMV using ACP ELISA and 39 accessions tested positive by bioassay to either *N benthamiana* or *G globosa* (Table 3) Four accessions tested negative in both ACP ELISA and bioassay tests Composite samples from all eight production fields tested positive for TropMV in ACP ELISA and bioassay (*data not shown*)

Table 2 Differential reactivity of potato virus Y (PTY) monoclonal antibodies with selected virus isolates from *Tropaeolum tuberosum* (Tt) and other potyviruses in antigen coated plate enzyme linked immunosorbent assay (ELISA)[a]

	PTY monoclonal antibody									
Potyvirus[b]	1	2	3	4	5	8	10	12	21	35
Tt 1	+++	–	+	+	+	+	–	–	–	–
Tt 2	+++	–	+	+	+	+	–	–	–	–
Tt 9	+++	–	+	+	+	+	–	–	–	–
Tt 4	+++	–	+	+	–	–	–	–	–	–
BYMV GDD	+++	+++	+++	+++	–	++	(+)	+	+++	+++
PepMoV NC	+++	–	+++	+	–	–	+++	++	+++	–
PVY 3	+++	+++	–	+++	–	++	+++	–	–	–
TEV PV69	+++	+++	–	–	–	++	++	+	–	–
MDMV A	+++	+++	–	+++	–	++	–	–	–	–

[a] Indirect ELISA using potyvirus infected plant extracts (final dilution 1 30 wt/vol) diluted in car bonate buffer and direct coating of Nunc ELISA plates Relative ELISA A405 values after 1 h sub strate incubation were (+++) = > 1 2 (++) = 0 6 to 1 2 (+) = 0 6 to 0 2 and (–) = < 0 1

[b] Acronyms Tt 1 Tt 2 Tt 9 and Tt 4 represent Tropaeolum mosaic potyvirus isolates from mashua (*Tropaeolum tuberosum*) accessions ECU 1145 ECU 1141 ECU 1147 and ECU 1093 respec tively BYMV GDD = bean yellow mosaic potyvirus strain GDD PepMoV = pepper mottle virus isolate NC PVY 3 = potato virus Y strain 3 TEV PV69 = tobacco etch virus isolate PV69 and MDMV A = maize dwarf mosaic potyvirus strain A

Table 3 Incidence of mechanically transmitted potyvirus in Ecuadorian accessions of mashua (*Tropaeolum tuberosum*) at the National Institute for Agricultural Research (INIAP) Santa Catalina Research Station Quito Ecuador as determined by bioassay and antigen coated plate enzyme linked immunosorbent assay (ACP ELISA)

	Bioassay[b]		
Sample[a]	*Nicotiana benthamiana*	*Gomphrena globosa*	ACP ELISA[c]
14 accessions	+	+	+
7 accessions	–	+	+
10 accessions	+	–	+
2 accessions	+	+	–
4 accessions	–	+	–
2 accessions	+	–	–
3 accessions	–	–	+
4 accessions	–	–	–

[a] Six to 13 symptomatic plants of each accession were tested with few exceptions 100% of the source plants tested positive

[b] Indicator species Symptoms were as described in Table 1

[c] Indirect ELISA using admixture of MAbs reactive to Tropaeolum mosaic potyvirus Tt 4 and Tt 9 isolates with virus infected plant extracts (final dilution 1 30 wt/vol) diluted in carbonate buffer and direct coating of Nunc ELISA plates A positive/negative threshold A_{405} value of 4 times the healthy control was used

DISCUSSION

This work shows that TropMV is wide spread among accessions of *T tuberosum* at INIAP s SCRS and in all production fields surveyed in the highlands of Ecua dor Because several symptomatic acces sions tested negatively in ACP ELISA and bioassay for TropMV other viruses are probably present Results of physical prop erty tests and the determination of particle shape (flexuous rod) and modal length (750 nm) are consistent with the potyvirus group TropMV appears to be a unique member of the potyvirus group that shares epitopes with PVY C TEV PVA WMV-2 and BCMV and in the case of Tt 9 cryp totopes with MDMV A

Tropaeolum mosaic potyvirus is readily transmitted mechanically and because mashua is vegetatively propagated via tubers TropMV may be spread during propagation of the crop It is reasonable to assume that TropMV has been spread among locations in the Andean highlands by infected tubers As no attempt has been made to eliminate viruses from this crop it is possible that TropMV has been accumulating in mashua germplasm for centuries Our data indicates that the majority (more than 50%) of mashua germplasm accessions are contaminated by TropMV and that all production fields surveyed where tubers are often saved from year to year contained infected plants While aphids are generally not present at the higher elevation range of mashua production aphids were noted on weeds and on mashua plants at the lower elevation range of the crop (*personal observation*) As *C quinoa* is a common weed in the Andean highlands and is known to be a host for TropMV (Table 1) it may act as a reser voir for the virus Control of aphids and weed species that may act as virus reservoirs could be an important means to man age the disease for growers if mashua germplasm is to be maintained virus free for prolonged periods of time

We do not known if TropMV is the same as the virus described by Delhey and Monasterios (4) from mashua in Bolivia because their study did not include the physical properties host range or serological data Further they made no comment as to the incidence of infection within mashua Our results are the first report of the incidence and partial characterization of a new potyvirus tentatively named Tropaeolum mosaic potyvirus infecting mashua in the Andean highlands Because of the lack of

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definitively virus-free mashua germplasm TropMV was not returned to mashua to determine if it in fact causes the symptoms described in this paper

To date no attention has been given to the development of virus-free mashua and the effect of TropMV on mashua growth and productivity Subsequent work in our laboratories has demonstrated that virus free germplasm can be generated via the culture of apical domes and that the growth and productivity of virus infected mashua may be adversely affected under certain conditions (15) Four virus free accessions have been developed and are available to Ecuadorian farmers Mashua is being grown in the United States Pacific Northwest and in New Zealand (10) and has potential for commercialization The widespread occurrence of TropMV in Ecuador and the possibility of additional viruses occurring within germplasm collections must be addressed if plant material is to move freely between countries and mashua is to be successfully commercialized

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In Vitro and Field Performance of Virus-freed and Virus-infected Plants of Tropaeolum tuberosum, Ruiz & Pavon

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Plant Pathology/Pest Management

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ABSTRACT

Mashua, (Tropaeolum tuberosum), has been a high yielding, low maintenance tuber crop, however, the number of hectares planted and yield per hectare have drastically decreased over the past 10 years Ecuadorean germplasm has been found recently to be widely infected with the potyvirus TropMV The main objectives of this research were to produce virus-freed mashua plants in order to compare growth in vitro (proliferation, rooting, reestablishment) and in the field (vigor, yield) for virus-freed (VF) versus virus-infected (VI) germplasm Twenty-three apical dome-derived clones tested VF based on bioassays using Nicotiana benthamiana and Chenopodium quinoa Plant tissue from in vitro-generated microcuttings was just as effective for determining VF status as was greenhouse-generated plant tissue Mashua line rather than virus-infection status appeared to have greater effect on in vitro proliferation There were no differences in in vitro rooting ability amongst the mashua lines or between the VF clones compared to the VI clones, with at least 90% of the microcuttings rooting

However, rooted microcuttings of VI clones were significantly longer than rooted microcuttings of VF clones Plants, 99% of VF and 79% of VI, readily reestablished in the Ft Detrick greenhouse In field experiment 1, ca 75% of the plants survived transplanting to the field and VF plants of line 1147 had greater tuber weight (928 gm) than VI plants (235 gm) In field experiment 2, plant mortality was high one month after transplanting to the field Lines 1093 (59%) and 1141 (54%) had higher survival than line 1147 (44%), however, there were no survival rate differences between the VF (46%) and VI (59%) plants of all these lines There were no differences in field performance for the three lines examined after ten months of field growth Length, width and area of the 10th fully developed leaf of VF plants were smaller than those of VI plants Although there were no differences in overall tuber yield among the VI, VF, and reinfected-VF plants, VI plants produced heavier large tubers compared to VF plants Surprisingly, 33% of the VF plants became reinfected and 42% of the VI plants tested negative after ten months in the field based on DAS-ELISA tests Regardless of the effect of TropMV on mashua yield, the development of relatively simple protocols for producing TropMV-free mashua will be beneficial for the international movement of germplasm

INTRODUCTION

Isañu, añu, cubio or mashua (T tuberosum), probably the Andean region's fourth most important root crop after potato (Solanum tuberosum), oca (Oxalis tuberosa), and

ulluco (Ullucus tuberosus) (National Research Council, 1989), is related closely to the garden nasturtium (Tropaeolum majus) and is believed to have insect repellent properties Traditionally, mashua has been a high yielding, low maintenance crop commonly grown in small plots for consumption by women and children Tubers of mashua are the size of small potatoes with shapes ranging from curved to conical to long-conical and colors from yellow to purple to red Mashua tubers are a very nutritious root crop containing about 20% solids and up to 16% protein (based on dry matter), a good balance between carbohydrates and proteins and a high content of ascorbic acid, ß-carotene and minerals (King and Gershoff, 1987, Tapia, 1990) Therefore, mashua tubers should have wide application in the food industry as dried slices, for flour and starch processing or for baby food mixtures (Tapia, 1990) Due to its strong flavor, mashua is not eaten raw, but is used in stews with other Andean tubers or faba beans, or is used in desserts with milk and sugar (National Research Council, 1989)

In the highlands of Colombia, mashua is well commercialized, especially in the cities of Tunja, Bogota, and Pasto (Villamizar, 1985) where large tubers, washed and packed to offer better quality, are requested weekly in farmer's markets and supermarkets Although mashua tubers used to be commonly found in markets during the harvest season, typically from the end of May until September (National Research Council, 1989), they presently are a rare commodity in Ecuadorean markets (personal observation) Few farmers presently cultivate mashua owing to reduced tuber productivity (National Research Council, 1989, FAO, 1990, Tapia, 1990), long

growing season, and low market price Thus, mashua may be viewed as an endangered crop in Ecuador

Over the past 10 years, the hectares of mashua planted and yield per hectare have both drastically decreased Decreasing yields in mashua is a complex issue (National Research Council, 1989) Mashua is cultivated at altitudes above 3500 m where environmental conditions are not optimal for crop development Above 3,200 m, mashua fields may be exposed to drought during the developmental cycle, extreme temperature variations in the same day, frost, and poor soil fertility as fertilizer use is infrequent Mashua market prices are not a good incentive for growers because they are the lowest compared with other Andean tubers (Tapia, 1990) For all of these reasons, mashua is exposed to constant germplasm erosion

Virus infection of tuber crops is known to have an effect on productivity (Walkey et al , 1982, Wright, 1970, 1977, Van Der Zaag, 1987) An uncharacterized, mechanically transmitted virus (Delhey and Monasterios, 1977) and potato leaf roll virus (National Research Council, 1989) have been reported to infect mashua We recently have isolated and characterized a potyvirus, TropMV, that appears to be widespread in the mashua germplasm of Ecuador (Soria et al , 1996) The widespread distribution of TropMV throughout Ecuadorean germplasm is not surprising as T tuberosum has been vegetatively propagated via tubers by people of the Andean highlands since Incan time (National Research Council, 1989, FAO, 1990)

Apical dome culture is one technique that has been used to produce virus-free germplasm in a number of crops (Zapata et al ,1995, De Vries-Paterson et al ,1992)

Protocols for initiating and maintaining mashua in vitro have been developed (Estrada et al , 1986, Perea-Dallos et al , 1986) The main objectives of this research were to produce virus-freed mashua plants in order to compare growth in vitro (proliferation, rooting, reestablishment) and in the field (vigor, yield) for virus-freed (VF) versus virus-infected (VI) germplasm

MATERIALS AND METHODS

Plant material

Four TropMV-infected lines (1093, 1115, 1141, 1147) from the mashua collection at the Ecuadorean Institute for Agricultural Investigation (INIAP), Santa Catalina Research Station, Quito, Ecuador were selected based on geographic distribution within the country, and morphological characteristics such as tuber form and color (Soria, 1996) Tubers from the lines were collected, sent to the Fort Detrick, Frederick, MD, quarantine facility under permit, and maintained there for testing

Apical domes (meristem plus 1 to 2 leaf primordia), ca 0 2 mm in size, were isolated using a razor blade sliver from virus-infected, in vitro maintained, microcuttings of the four mashua lines Apical domes were cultured in petri plates (60 x 15 mm) containing 10 ml of culture medium Clones initiated from apical domes were designated as virus-freed (VF) clones VF and virus-infected (VI) clones were maintained in vitro by subculturing monthly to proliferation medium

Culture media

The basal medium contained the inorganic salts and vitamins of Murashige and Skoog (1962), supplemented with 3% sucrose and 0 64% Phytagar (Life Technologies, Inc , New York) For proliferation, basal medium contained 5 mg l^{1} BA (6-benzyl adenine) and 1 mg l^{1} kinetin (6-furfurylaminopurine) according to Fadiño et al (1987) For in vitro rooting, the basal medium contained 5 mg l^{1} of NAA (naphthalene-6-acetic acid) Media pH were adjusted between 5 7-5 8 prior to autoclaving

Proliferation versus virus infection

The effect of virus infection on in vitro proliferation was tested using two lines, 1141 and 1093 VF clones for each line were selected based on negative results obtained from bioassays (see virus indexing section below) Jars (25 x 50 mm) containing 25 ml of medium were used for the proliferation stage Each jar contained two explants, an apical shoot tip, cultured vertically, and a lateral shoot tip, cultured horizontally Cultures were placed under a 24-hour photoperiod (50 μmol m^{2} s^{1}) and evaluated after one month

Rooting and reestablishment

The effect of virus infection on rooting ability in vitro and survival during acclimation was compared for VF and VI clones Five microcuttings were cultured in jars (25 x 50 mm) containing 25 ml of rooting medium Cultures were placed under a 16-hour photoperiod (50 μmol m^{2} s^{1}) for one month before potting and placement in the

Ft Detrick greenhouse Rooted mashua plants were transplanted into 3 5 cm cell packs having 72 cells using a pasteurized soil mixture that contained 32% Pro-mix BX, 16% field soil (clay loam), 16% peat moss, 16% vermiculite, 12% perlite and 8% sand plus fertilizer Plants were placed in a mist tent for one week after transplanting, after which they were moved to a greenhouse bench and watered as needed Artificial lights (32-95 $\mu mol\ m^{2}\ s^{1}$) were used to provide a 12-hour day length, at a temperature range of 18-25 °C Survival of VF and VI clones were recorded after 2 weeks when plants were transplanted to 10 cm pots using the same soil mixture

Field experiments in Ecuador

VF and VI clones were rooted in vitro in Magenta GA-7 boxes (Magenta Corp , Chicago, Ill) containing 30 ml of medium (9 microcuttings/box, 12-hr photoperiod, 50 $\mu mol\ m^{2}\ s^{1}$), transplanted into plastic boxes containing pumice, covered with a humidity dome and placed in a greenhouse for 21-25 days VF clones were kept separate from VI clones to avoid cross contamination After being covered for a week to avoid dehydration, the humidity dome was removed progressively until plants were acclimated Plants were then potted into a sterile soil mixture that contained 30% sand, 30% composted humus and 40% field soil (clay-loam) until final transplanting to the field Field plots were furrow irrigated when rainfall was insufficient

Experiment 1 Field experiment I was set up in La Merced S W of Tungurahua Province at an altitude of 3,000 m in February 1994 (for 1993-1994 an annual rainfall

of 509 to 690 mm, 10 5 °C) using VF and VI clones of 1147 One-month-old plants were bioassayed for the presence of virus prior to planting in the field Plants were harvested after 7 5 to 8 5 months

Experiment 2 Field experiment 2 was established at the Querochaca farm of the Agricultural Engineering Faculty of the Technical University of Ambato, Ecuador at an altitude of 2,868 m in October 1994 [from December 1994 to July 1995 avg temperature 13 °C ± 0 7 (S D), avg high temperature 23 °C ± 1 6 (S D), avg rainfall 46 ± 31 (S D) mm] Both VF and VI plants of 1093, 1141, and 1147 were planted, harvested and serologically assayed for the presence of TropMV after 10 months (Vf_r = virus-free plants that became reinfected in the field)

Virus indexing

Biological assays Apical dome-derived clones from four mashua lines were bioassayed for the presence of virus using Nicotiana benthamiana as an indicator plant Microcuttings generated after 2 or 3 subcultures from an individual apical dome-derived clone were pooled, macerated in 1 ml of buffer (0 01M sodium phosphate, pH 7 0) using a mortar and pestle and rub-inoculated onto young leaves of N benthamiana (systemic-infection indicator) that had been dusted with 600 mesh silicon carbide As a positive control, microcuttings of virus-infected parent lines (VI clones) were assayed at the same time

A bioassay of greenhouse-generated tissue used samples from 2-month-old plants from each apical dome-derived VF clone (There were 3 to 8 individual apical dome-derived VF clones per line, e g , line 1093 had 8 VF clones each derived from an individual apical dome) Composite samples were collected from individual plants (4 leaves for VF and 2 to 3 leaves for VI clones), macerated in buffer (10ml of 0 01 M sodium phosphate buffer pH 7 4), rub-inoculated onto Chenopodium quinoa (local-lesion indicator) and evaluated 19 days later

In field experiment 1, two or three leaves per field-grown plant were macerated in a chilled mortar with one ml of 0 5 M sodium citrate solution and rub-inoculated onto N benthamiana as described above

Serological assays In experiment 2, leaf tissue from individual, 10-month-old plants was tested using DAS (Double-Antibody-Sandwich)-ELISA for potyvirus A composite sample of young leaves was collected and macerated with coating buffer plus polyvinylpyrrolidone (CB/PVP) Microtiter plates were coated with an admixture of 16 potyvirus antibodies (Soria, 1996) Sap extracts were diluted approximately 10-fold, incubated for 2 hours at room temperature, and 100 µl were added to duplicate wells of the polystyrene microtiter plates and incubated for 1 hour at room temperature Plates were washed 3 times with phosphate buffered saline plus Tween 20 (PBS-Tween) before 100 µl of gamma globolin (I_sG) were added to each well The plates were incubated 2 hours at room temperature or overnight at 4°C and washed 4 times with PBS-Tween Later 100 µl per well of the I_sG enzyme conjugate (alkaline phosphatase)

was added, and incubated 1 hour at room temperature Finally, 100 µl of p-nitrophenol (Sigma Chemical Co , St Louis, MO) diluted in diethanolamine buffer was added to each well The reaction was stopped after one hour with 50 µl of 3M sodium hydroxide and OD_{405} readings were taken using a Bio-Tech (Winoosk, VT) microplate reader model EL307C The positive/negative threshold was 4 times the mean of the background mean value

Field experiments

Field experiment 1 Survival in the field was determined 30 days after transplanting by counting the number of cuttings that remained vigorous and initiated new shoots Time-to-flowering was determined when 50% of the plants of a line were flowering Plant height in cm (from the soil surface to the most apical leaf) from two randomly selected plants was measured at 30 and 60 days after transplanting The number and weight of tubers and the number of new shoots generated per plant were recorded at harvest

There were 8 plants per row, and 6 plants per evaluation The distance between rows was 1 m and 0 5 m between plants The experimental design was a randomized complete block with 3 replications

Field experiment 2 Microcuttings were rooted in Magenta GA-7 boxes (four boxes per VF clone, 16 boxes for the VI clones), and the experiment was repeated Due to lack of balance in the experimental design, the Satterthwaite (1946) procedure was

applied to adjust error terms and obtain approximate distribution of estimates of variance components

A completely randomized design using a split plot, where the main plot was mashua line and the split plot was virus-infection status, was applied as the experimental design, with a randomized arrangement within main plots and split plots The experiment had 3 treatments per block with 3 replications (plots) Mashua plants were spaced at 1 0 m between rows and 0 75 m between plants Tubers of purple mashua were planted around the edge of each replicate to minimize border effects Each plot was 50 m^2 (10 m long x 5 m wide) and blocks were 322 m^2 (23 m long x 14 m wide) The total area of the field experiment was 1058 m^2 (46 m long x 23 m wide) A total of 60 plants were used for each plot, 30 VF and 30 VI (180 total plants) Ten plants were chosen per split plot for data collection

Data collection and statistical analysis

Tissue culture Proliferation, in vitro rooting, and acclimation experiments were set up in completely randomized designs, with a nested arrangement for the VF and VI clones per mashua line The proliferation study was repeated, and each treatment had 10 experimental units (jars), and 20 observations (2 explants/jar) The following in vitro shoot growth variables were collected proliferation, vigor (size), and fresh and dry weights Proliferation of the explant was divided into the following categories 1) the total number of shoots (TOTSHT), 2) shoots that measured less than 1 cm (SHT<1), 3)

shoots between 1-2 cm (SHT1-2) and 4) shoots greater than 2 cm (SHT>2) Fresh (FW) and dry weights (DW) and ratios between water content and dry weight (FW-DW/FW) were determined

Field experiments Data collected were plant survival one month after transplanting, number of days from transplanting to flowering, plant vigor, yield and reinfection Plant vigor was evaluated by measuring the length and width in centimeters, of the 3rd fully developed leaf and the 10th leaf from the apex of the main stem that was at least 25 cm long Number of days from transplanting to flowering and fruit set was recorded Yield was determined by measuring total yield of the mashua plants including fresh weight of foliage and tuber size [large (more than 8 cm in length), medium (4-8 cm in length) and small (less than 4 cm in length) and microtubers] and weight

RESULTS

Virus elimination

Of 58 apical domes excised, 30 survived and grew (Table 1) After two months of growth, 25 of the apical dome-derived clones (VF) were bioassayed for the presence of virus by mechanically inoculating N benthamiana with sap preparations from in vitro-maintained microshoots Twenty-three of the clones were apparently virus- free

The 23 VF clones were then established for two months in a Ft Detrick greenhouse and assayed using C quinoa, all 23 VF clones again tested negative for virus

Proliferation versus virus infection

Although there was no clear pattern of significance among the VF and VI clones, virus infection within a line appeared to have a detrimental effect on general performance in vitro (Table 2) The total number of shoots was not significantly different among the VF and VI clones within a line, even though the VF clones were consistently more proliferative The majority of the difference between the VF and VI clones was evident in the number of microshoots >1 cm, however, most microshoots were <1 cm in length The FW and DW trends for the relationship among VF and VI clones were found to be similar to the trends associated with the proliferation rate

Line 1093 proliferated better than 1141 (data not shown) Shoots less than 1 cm were the greatest contributors to the proliferation difference between the two lines, 1141 and 1093 Line 1093 had more biomass than line 1141 for both VF and VI clones (Table 2) Explant source orientation was found to be correlated with biomass, lateral buds cultured horizontally had more biomass than apical buds cultured vertically (data not shown)

Rooting and reestablishment

There were no differences in in vitro rooting ability among lines 1093, 1141, 1115, and 1147, or between the VF and VI clones with at least 90% of the microcuttings rooting, however, rooted microcuttings of VI clones were longer that rooted microcuttings of VF clones (data not shown) Plants, 99% of VF and 79% of VI, readily became reestablished in the Ft Detrick greenhouse (Table 3)

Field experiments in Ecuador

Once it was determined that all apical dome-derived clones were apparently virus free within a mashua line, VF clones within a line were pooled for the field plantings

Experiment 1 Field survival after transplanting (85% VF vs 65% VI), plant height at 30 (ca 5 cm) or 60 (ca 7-8 cm) days, number of new sprouts (6 VF vs 3 VI), and number of tubers harvested per plant (65 VF vs 55 VI) were not different between VF and VI plants of line 1147 VF plants had significantly greater tuber weight (928 gm) compared to VI plants (235 gm) VF plants flowered earlier (100 days) and were harvested earlier (220 days) than VI plants (155 days and 254 days, respectively)

Plants were not reindexed for virus status at the end of the study

Experiment 2

Survival after transplanting Plant mortality was high one month after transplanting to the field (Table 4) Lines 1093 (59%) and 1141 (54%) survived better than did line 1147 (44%), however, there were no survival rate differences between the VF (46%) and VI (59%) plants (Table 3)

Plant vigor Measurements of length, width, and area (length x width ratio) of the 3rd and 10th fully developed leaves were not significantly different among the mashua lines (Table 5), however, length, width and area of the 10th fully developed leaves from VF plants were reduced compared to VI plants (Table 5)

Yield evaluation After ten months of field growth, line 1147 yielded more small and micro tubers (Table 6) Although there were no differences in total yield among the VI, VF_r and VF plants, VI plants produced heavier large tubers compared to VF plants (253 vs 70 gm/plant), and VF_r plants produced heavier microtubers compared to VF plants

Indexing of field plants Thirty-three percent of the VF plants became reinfected and 42% of the VI plants tested negative after ten months in the field based on DAS-ELISA tests A large population of aphids was present in the plots from January to the end of March

Flowering Differences in flowering time was due mainly to lines and not to virus-infection status Line 1147 flowered first at 145 days after field transplanting, followed by line 1093 at 154 days and finally by line 1141 at 168 days Flowering increased for all lines over a period of 45 days, at which time, the first flowers had already set fruit

DISCUSSION

Tissue culture

The culture of apical domes ca 0 2 mm in size was effective for the elimination of TropMV from mashua lines 1141, 1093, 1147 and 1115 as determined through bioassays using N benthamiana plants rub inoculated with sap from in vitro-maintained microcuttings To be assured there were no false negative readings, a subsequent screening, using C quinoa as the indicator plant, of these same apical dome-derived clones using tissue from established 2-month-old greenhouse-grown plants confirmed the virus-free nature of the apical dome-derived plants There was 100% corroboration between the two bioassays Brown et al (1988) also have shown that in vitro-generated tissue is efficacious for the determination of virus-free status

Nearly 50% of the apical domes cultured did not survive (Table 1) which could have been due to apical dome size, some apical domes may have been too small (< 0 2

mm), or could have been due to the use of a medium not developed for apical dome outgrowth

Apparently TropMV is an easy virus to eliminate from mashua compared to other plant-virus combinations (Zapata et al , 1995, De Vries-Paterson et al , 1992) as 83% of the apical dome-derived plants were virus free This may have been due to the ease with which mashua apical domes could be cultured, characteristics of TropMV, and/or to the size of apical dome we were able to isolate Although apical dome excision is very labor intensive, and may result in delayed outgrowth and low survival (Matthews, 1992), the use of apical domes alone for virus elimination from mashua is beneficial as it avoids the use of antiviral/chemotherapy chemicals that can be phytotoxic and eliminates the time and the space required for thermotherapy (Zapata et al , 1995) The ease with which VF plants of mashua were obtained may in part be due to using tissue culture-derived shoots as the apical dome source In some plants, it is possible to isolate smaller apical domes from tissue culture-derived shoots compared to greenhouse-derived shoots (Brown et al , 1988)

Tissue culture is a good system for the study of some plant-virus interactions (Greño, et al 1988, Duran-Vila, et al 1989, 1991, and De Vries-Paterson et al 1992) and, in the case of mashua, the influence of TropMV on the ability of explants to proliferate, root in vitro and acclimate ex vitro was tested Mashua line had a greater effect on proliferation in vitro than did virus-infection status, with line 1093 out

performing line 1141 in shoot number and shoot weight (Table 2) Although there was no clear pattern of significance among the VF and VI clones, virus infection appeared to have a detrimental effect on the general performance of the microshoots

Microcuttings of mashua readily rooted in vitro and survived potting and greenhouse acclimation conditions, however, field site had an impact on subsequent survival In field experiment 1, greenhouse-acclimated plants survived transplanting in the field quite well (75% overall), however, in field experiment 2, survival after transplanting in the field was much lower (ca 52% overall)

Field experiments

In field experiment 1, VF plants out yielded VI plants of line 1147 in tuber weight Although not significant, the larger number of lateral shoots generated from below the soil surface (tuber production requires the production of lateral shoots below the soil surface) and the larger number of tubers produced by VF plants compared to VI plants surely may have contributed to greater tuber weight as found by others (National Research Council, 1988)

In field experiment 2, 33% of the VF mashua plants became reinfected with TropMV after 10 months in the field Virus transmission via aphids may have been the cause as there was a large population of aphids present in the plots from January to the

end of March TropMV has been shown to be readily transmitted by the green peach aphid (Myzus persicae, Sulzer) in a non-persistent manner when given an acquisition access period of 30 sec to 5 min (Soria et al , 1996)

It was surprising that such a large percentage (42%) of the VI plants tested negative during the final screening using DAS-ELISA, however, virus titer has been shown to vary based on season and/or explant evaluated within plants of apple (Malus pumila, tomato ringspot virus) (Bitterlin et al , 1984), rhubarb (Rheum rhaponticum cv Timperley Early, aphid-borne turnip mosaic virus) (Walkey et al , 1982), and tobacco (Nicotiana tabacum cv Samsun, potato-A-potyvirus) (Bartels, 1954)

In field experiment 2, although not significant, mashua line rather than virus-infection status appeared to have a greater effect on field performance (Tables 5 & 6) VF and VI plants performed similarly in the field except VI plants produced heavier large tubers and had larger 10th fully developed leaves compared to VF plants Although there was no difference in FW of foliage among VF, VF_r and VI plants in this study, plant biomass has been correlated with tuber yield in mashua (National Research Council, 1989)

It is difficult to draw broad conclusions about the effect of TropMV on yield based on these two field experiments especially if one examines the variance in yield between the VF and VI plants of line 1147 While the National Research Council

(1989) reports increased yields when virus-freed mashua plants are grown, field experiments with virus-freed vs virus-infected potatoes (Solanum tuberosum), a much more widely grown and studied tuber crop, have not produced consistent correlative results at all field locations with regard to increased yields and virus-freed plants (Wright 1970, 1977) In rhubarb, yield was indirectly correlated with the rate of reinfection of virus-free field plants (Walkey et al , 1982) After three years, virus-free plots having 36% reinfection had significantly lower yields compared to virus-infected stock plants Walkey et al postulate that reduction in yield associated with reinfection may be related to long term selection of germplasm that is tolerant to the virus Mashua, like rhubarb, is vegetatively propagated, and high yielding, virus-tolerant clones may have been selected over the years In the present study, both field sites were chosen in the best interest of mashua Field experiment 1 was set up in a location that, for 1993-1994, was more environmentally similar to that recommended for growing mashua (National Research Council, 1989) The conditions at the second site (field expt 2) were not optimal in 1994-1995 for growing mashua Mashua prefers a misty, moist environment for optimal growth (National Research Council, 1988) and although the plots were furrow irrigated to compensate for the droughty soil conditions, the relative humidity was surely lower than optimal The unexpected drought directly after planting no doubt affected survival after transplanting and may have had long term effects on general growth and vigor and may account in part for the crop maturing late Normally, mashua tubers are harvested after 6 to 8 months (National Research Council,

1988) and the fact that in field experiment 2 plants were not ready to be harvested until after 10 months suggests that this was a less than perfect field site

Regardless of the effect of TropMV on mashua yield, the development of relatively simple protocols for producing TropMV-free mashua will be beneficial for the international movement of germplasm

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Table 1 Virus elimination via culture of the shoot apex of four accessions of Tropaeolum tuberosum

	No. of shoot apices				
				Virus free[z]	
Line	Cultured	Survived	Indexed	In Vitro	Greenhouse
1193	21	11	8	8	8
1141	12	5	5	5	5
1147	15	11	9	7	7
1115	10	3	3	3	3

[z]In vitro = negative bioassay results using in vitro-derived microcuttings, greenhouse = negative bioassay results using 2-month-old greenhouse-established plants

Table 2 Proliferation, and fresh and dry weights of microcuttings obtained from apical dome-derived and virus-infected clones of *Tropaeolum tuberosum* lines 1141 and 1093

LINE	ID[z]	N[y]	TSHT[x]	SHT<1[w]	SHT1-2[v]	SHT>2[u]	FW[t]	DW[s]	FW-DW/FW
1093	VF2	40	6 9a	3 6abc	2 0a	1 4a	0 30a	0 023ab	0 89ab
	VF3	40	6 2ab	3 4abcd	1 6ab	1 2a	0 29a	0 025a	0 91ab
	VF4	40	6 3ab	4 4ab	1 6ab	0 4a	0 30a	0 026a	0 91ab
	VF8	39	6 5ab	4 7a	1 3abcd	0 5a	0 32a	0 24ab	0 92a
	VI	32	5 0abc	3 7abc	0 7cd	0 6a	0 22ab	0 20abc	0 89ab
1141	VF1	40	4 8bc	1 5e	1 5ab	1 8a	0 13bc	0 019abc	0 85b
	VF2	40	4 7bc	2 5cde	1 0bcd	1 2a	0 13bc	0 015bcd	0 88ab
	VF5	40	4 7bc	2 2cde	1 4abc	1 0a	0 16bc	0 013dc	0 90ab
	VF6	37	5 3ab	3 0bcd	1 2abcd	1 0a	0 18bc	0 017abcd	0 90ab
	VI	38	3 1c	1 9de	0 6d	0 6a	0 10c	0 008d	0 91ab

[z] ID = apical dome-derived (VF) clones vs virus-infected (VI) stock

[y] Number of jars, 2 microcuttings/jar, one horizontal and one vertical, experiment repeated once

[x] TSHT Avg total number of shoots proliferated from each microcutting

[w] SHT<1 Avg no of axillary shoots smaller than 1 cm

[v] SHT 1-2 Avg no of axillary shoots between 1 and 2 cm

[u] SHT>2 Avg no of axillary shoots greater than 2 cm

[t] FW Avg fresh weight of microcuttings

Table 3 Survival of four mashua lines that were rooted in vitro and then potted and placed in a greenhouse for two weeks

Line	Status[z]	N[y]	Survival (%)
1093	VF	93	98 7
	VI	8	100
1115	VF	34	100
	VI	6	50
1114	VF	55	100
	VI	6	67
1147	VF	73	98 7
	VI	6	100

[z]VF = virus free, VI = virus infected

[y]N = number of plants potted 1093 VF = 8 apical dome-derived cones tested, each clone had 10-13 plants bioassayed, 1115 VF = 3 apical dome-derived clones tested, each clone had 9-13 plants bioassayed, 1141 VF = 5 apical dome-derived clones tested, each clone had 10-12 plants bioassayed, 1147 VF = 7 apical dome-derived clones tested, each clone had 8-12 plants bioassayed

Table 4 Survival of three lines of mashua plants (*Tropaeolum tuberosum*) 1 month after transplanting in the field

Trt[z]	N[y]	Survival[x]
1093	60	17 8a
1141	60	16 3a
1147	60	13 2b
VF	90	13 8a
VI	90	17 8a

[z]VF = virus free, VI = virus infected

[y]N=number of plants per plot

[x]Mean number of plants to survive out of three replications

Table 5 Mashua (*Tropaeolum tuberosum*) vigor by leaf measurement

		Length[y]		Width[x]		Area[w]	
Trt	N[z]	Lf3	Lf10	Lf3	Lf10	Lf3	Lf10
1093	6	2 88a	5 08a	2 16a	3 89a	6 33a	20 78a
1141	6	2 87a	4 67a	2 25a	3 53a	6 33a	16 95a
1147	6	3 02a	5 24a	2 38a	4 11a	7 44a	23 35a
VF[v]	9	2 89a	3 53b	2 26a	4 56b	6 72a	17 17b
VI	9	2 95a	4 16a	2 26a	5 36a	6 88a	23 55a

[z] N = number of split plots, 1-10 plants/split plot

[y] Length in cm of the 3rd and 10th fully-developed leaves

[x] Width in cm of the 3rd and 10th fully-developed leaves

[w] Leaf area (length x width) expressed in cm^2

[v] VF=virus free at the beginning and end of the field study, VI=virus infected at the beginning and end of the field study

Table 6 Mashua (Tropaeolum tuberosum) yields 10 months after field planting in Ecuador

			Tubers[x]								
Trt	N[z]	Foliage[y]	No big	Wt big	No med	Wt med	No small	Wt small	No micro	Wt micro	Total yield
1093	6	1292a[w]	3 7a	265a	17a	678a	22b	400a	31b	194a	1537a
1141	6	870a	1 6a	82a	12a	313a	28b	300a	43b	126a	822a
1147	6	1334a	2 2a	134a	23a	593a	54a	568a	62a	190a	1484a
VF[v]	9	1150a	1 4a	70b	14a	376a	34a	374a	37a	124b	944a
VFr	9	1302a	2 5a	145ab	20a	544a	45a	499a	60a	232a	1420a
VI	9	1045a	3 5a	253a	18a	649a	28a	405a	42a	158ab	1466a

[z]N = number of split plots, 1-10 plants/split plot

[y]Foliage = weight of foliage in grams

[x]Big tubers = > 8cm in length, medium tubers = 4-8 cm, small tubers = 2-4 cm, micro tubers < 2cm, weight in grams Total yield = tubers in grams

[w]Mean separation in columns by Duncan's multiple range test, 5% level

[v]VF = virus free at the beginning and end of the field study, VFr = virus free at the beginning but virus infected at the end of the field study, VI = virus infected at the beginning and end of the field study

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