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CENTRO DE INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA DE ARAGÓN
INIA
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
Ana Palacio-Bielsa
Isabel Mª Berruete
CITA, Zaragoza (Spain)
Jaime Cubero
INIA, Madrid (Spain)
Miguel A. Cambra
Raquel Collados
CPV, Zaragoza (Spain)
María M. López
IVIA, Valencia (Spain)
GOBIERNO DE ARAGON
Departamento de Agricultura y Alimentación
IVIA
INSTITUTO VALENCIANO DE INVESTIGACIONES AGRARIAS

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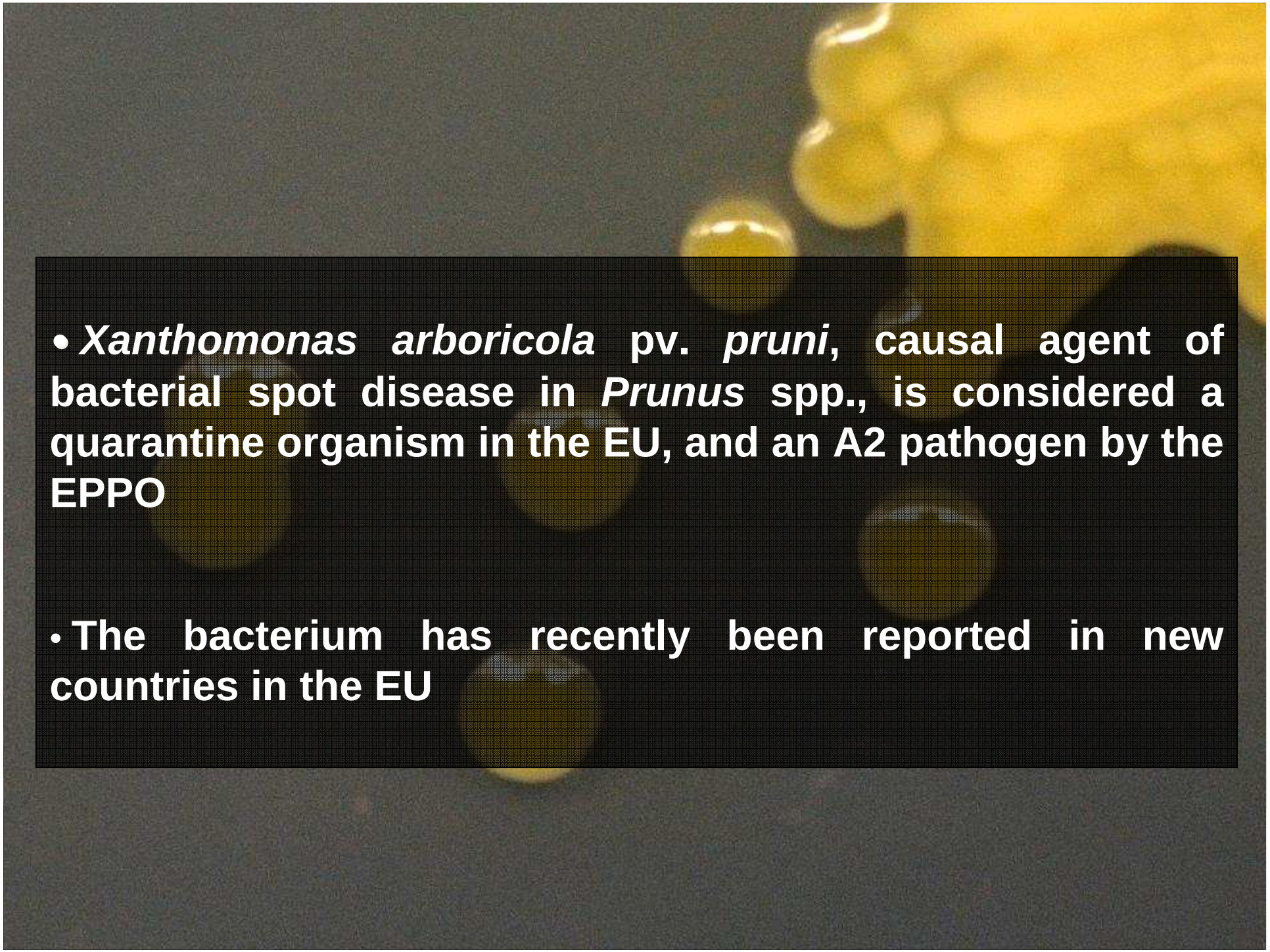
Development of an efficient real-time quantitative PCR protocol for detection of *Xanthomonas arboricola* pv. pruni in *Prunus* species

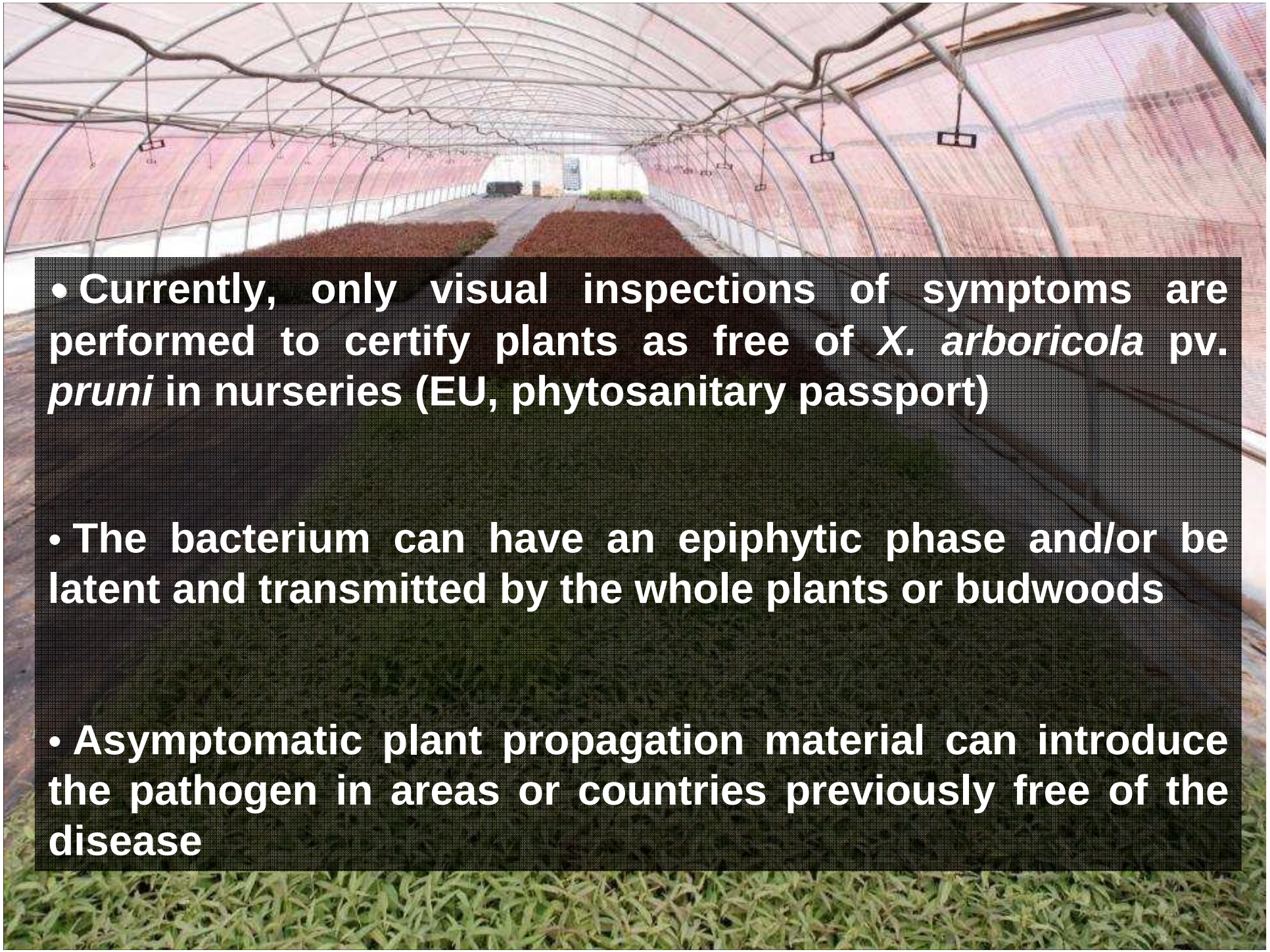
Ana Palacio-Bielsa*, Jaime Cubero, Miguel A. Cambra, Raquel Collados, Isabel M. Berruete, and María M. López
Centro de Investigación y Tecnología Agroalimentaria de Aragón, Av. Montañana, 930, 50059 Zaragoza, Spain; Departamento de Protección Vegetal, INIA, Carretera de La Coruña km 7, 28040 Madrid, Spain; Centro de Protección Vegetal, Av. Montañana, 930, 50059 Zaragoza, Spain; and Instituto Valenciano de Investigaciones Agrarias, Carretera Moncada a Náquera km 4.5, 46113, Moncada, Valencia, Spain
*To whom correspondence should be addressed. Email: **apalaciob@aragon.es**

Abstract
Xanthomonas arboricola pv. pruni, causal agent of bacterial spot disease of stone fruit, is a quarantine organism for the European Union (EU) and the European and Mediterranean Plant Protection Organization (EPPO). The bacterium can undergo an epiphytic phase and/or be latent and can be transmitted by plant material but currently, only visual inspections are used to certify plants as *X. arboricola* pv. pruni-free. A novel and highly sensitive real-time TaqMan PCR detection protocol was designed based on a sequence of a gene for a putative protein related to an ABC transporter ATP-binding system in *X. arboricola* pv. pruni. Pathogen detection can be completed within a few hours with a sensitivity of 10^2 CFU ml^{-1} thus surpassing the sensitivity of the existing conventional PCR. Specificity was assessed for *X. arboricola* pv. pruni strains from different origins, as well as for closely related *Xanthomonas* species, non-*Xanthomonas*, saprophytic bacteria and healthy *Prunus* samples. The efficiency of the developed protocol was evaluated in field samples of 14 *Prunus* species and rootstocks. In symptomatic leaf samples, the protocol was very efficient even when washates of the leaves were directly amplified, without any previous DNA extraction. In samples of 117 asymptomatic leaves and 285 buds, the protocol was more efficient after a simple DNA extraction and *X. arboricola* pv. pruni was detected, respectively in 9.4 and 9.1% of the 402 samples analyzed, demonstrating its frequent epiphytic or endophytic phase. This newly developed real-time PCR protocol can be used as quantitative and offers a reliable and sensitive test for *X. arboricola* pv. pruni, and is suitable as a screening test in symptomatic as well as asymptomatic plant material.



- ***Xanthomonas arboricola*** **pv. *pruni*, causal agent of bacterial spot disease in *Prunus* spp., is considered a quarantine organism in the EU, and an A2 pathogen by the EPPO**

- **The bacterium has recently been reported in new countries in the EU**



- **Currently, only visual inspections of symptoms are performed to certify plants as free of *X. arboricola* pv. *pruni* in nurseries (EU, phytosanitary passport)**

- **The bacterium can have an epiphytic phase and/or be latent and transmitted by the whole plants or budwoods**

- **Asymptomatic plant propagation material can introduce the pathogen in areas or countries previously free of the disease**


Inspection in nurseries
(Asymptomatic, May- July)

Inspection in nurseries

(Symptoms, September)






SÍNTOMAS EN HOJAS



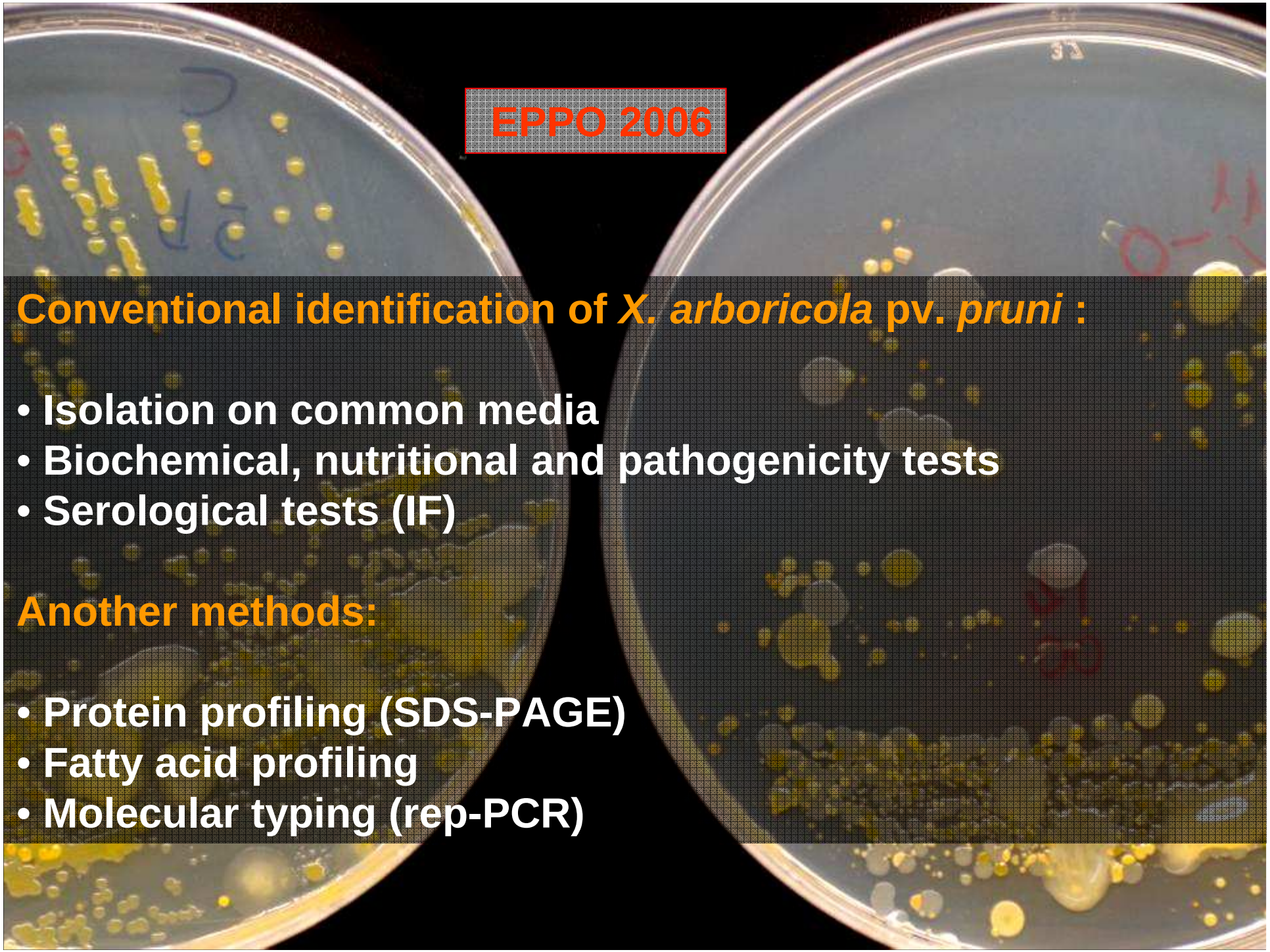
SÍNTOMAS EN FRUTOS



Diagnostic and detection of *Xanthomonas arboricola* pv. *pruni*

European and Mediterranean Plant Protection Organization PM 7/64 (1)

2006 *Bulletin OEPP* 36, 129-133



Conventional identification of *X. arboricola* pv. *pruni* :

- Isolation on common media
- Biochemical, nutritional and pathogenicity tests
- Serological tests (IF)

Another methods:

- Protein profiling (SDS-PAGE)
- Fatty acid profiling
- Molecular typing (rep-PCR)

RAPID IDENTIFICATION AND DETECTION BY PCR

• Improvement of a conventional PCR for specific detection of a 943-bp DNA fragment of a gene sequence for a putative protein related to an ABC transporter ATP-binding system in *X. arboricola* pv. *pruni* (Pagani, PhD thesis, 2004)

Laboratories involved (Peñalver *et al.*, 2009. COST 873)

IVIA, Valencia, Spain (Peñalver J., Llop P., López M.M.)

Laboratorio de Diagnóstico, Valencia, Spain (Roselló M.)

C.R.A., Roma, Italy (Ferrante P., Scortichini M.)

Detection limit with different *Prunus* spp. material: 10^3-10^6 CFU/ml

• A more sensitive TaqMan real-time PCR for detection in both symptomatic and asymptomatic plant material has been developed and used for diagnosis as well as for *in planta* screening

TaqMan REAL-TIME PCR PROTOCOL DESIGN

- **Primers and TaqMan probe**

  Designed from the sequence of a putative protein related to an ABC transporter ATP-binding system in *X. arboricola* pv. *pruni* used for conventional PCR (Pagani, PhD thesis, 2004)

- **Reaction optimization**

  Primers and probe concentrations
  Denaturation and annealing steps (temperature and time)
  SmartCycler: 45 two-step cycles of 95ºC and 59ºC

TaqMan REAL-TIME SPECIFICITY EVALUATION (Bacterial pure cultures)

- **50 strains of other 20 plant pathogenic bacterial species:** ***Xanthomonas arboricola*** **pv.** ***corylina, X. arboricola*** **pv.** ***fragariae, X. arboricola*** **pv.** ***juglandis*****,** ***X. arboricola*** **pv.** ***populi*****,** ***X. axonopodis*** **pv.** ***vesicatoria*****,** ***X. citri*** **subsp.** ***citri*****,** ***X. campestris*** **pv.** ***campestris*****,** ***Pseudomonas syringae*** **pv.** ***syringae*****,** ***P. syringae*** **pv.** ***tomato*****,** ***P. syringae*** **pv.** ***mori*****,** ***P. savastanoi*** **pv.** ***savastanoi*****,** ***P. corrugata*****,** ***Agrobacterium tumefaciens*****,** ***A. vitis*****,** ***Brenneria quercina*****,** ***Erwinia amylovora*****,** ***E. billingiae, E. pyrifoliae, E. tasmaniensis, Clavibacter michiganensis*** **subsp.** ***michiganensis*****,** ***C. michiganensis*** **subsp.** ***sepedonicus***

- **2 strains of saprophytic bacteria:** ***Pseudomonas fluorescens, Pantoea agglomerans***

- **159** ***X. arboricola*** **pv.** ***pruni*** **strains:** **different hosts and geographicall origins (Italy, Spain, Argentina, Brazil, Canada, USA, South Africa and New Zealand)**

TaqMan REAL-TIME PCR SPECIFICITY EVALUATION (Plant material)

- ✓ Healthy *Prunus* samples (peach, almond, Japanese plum and GF-677 rootstock)
- ✓ *Prunus* samples spiked with *X. arboricola* pv. *pruni*

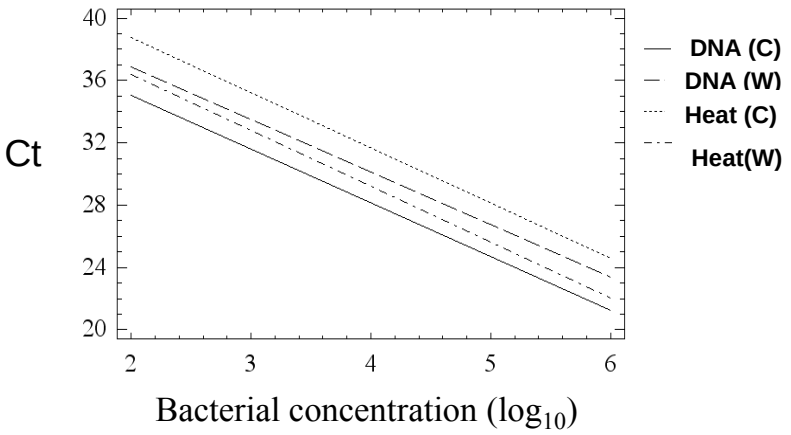
TaqMan REAL-TIME PCR SENSITIVITY EVALUATION

			Heat treated	DNA extraction (Llop *et al.*, 1999)
X. arboricola pv. *pruni* (ISPaVe-B4)*			10^3 CFU/ml	10^2 CFU/ml
Prunus spp. leaves spiked with Xap (ISPaVe-B4)	Peach (cv. Catherine)	Comminuted	10^4 CFU/ml	10^2 CFU/ml
		Washed	10^2 CFU/ml	10^2 CFU/ml
	Almond (cv. Guara)	Comminuted	10^3 CFU/ml	10^2 CFU/ml
		Washed	10^2 CFU/ml	10^2 CFU/ml
	Japanese plum (cv. Golden Japan)	Comminuted	-	10^2 CFU/ml
		Washed	10^5 CFU/ml	10^2 CFU/ml
	GF-677 rootstock	Comminuted	10^3 CFU/ml	10^2 CFU/ml
		Washed	10^2 CFU/ml	10^2 CFU/ml

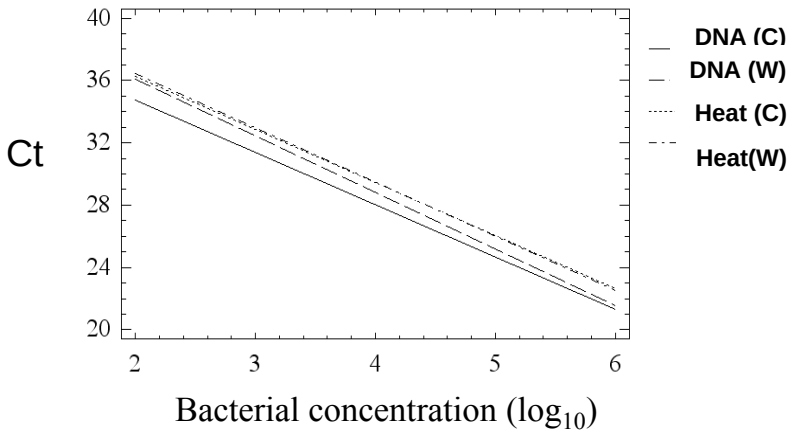
*Istituto Sperimentale per la Patologia Vegetale, Roma, Italy

Calibration curves of different samples treatments

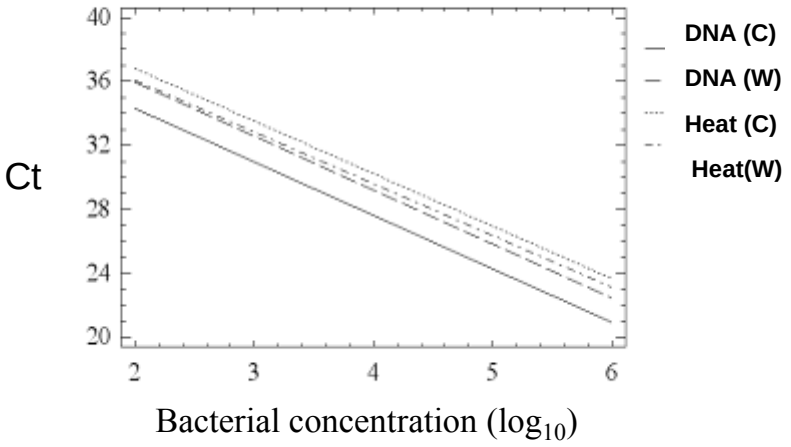
Peach



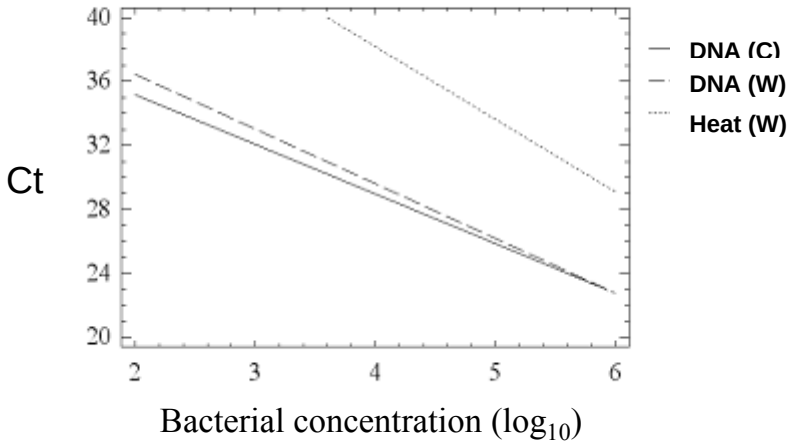
Almond



GF-677



Japanese plum



TaqMan real-time and conventional PCR sample processing methods assayed on plants with symptoms

FIVE SYMPTOMATIC PEACH LEAVES (M1 to M5)

- Sterile distilled water tissue washes
- Comminuted tissues
- Five serial dilutions of comminuted tissues
- Comminuted tissues with DNA extraction

TaqMan real-time PCR

	W	C (ADN)	C	Comminuted serial dilutions				
				- 1	- 2	- 3	- 4	- 5
M1	20	16	-	21	25	28	31	33
M2	21	17	18	22	26	29	31	36
M3	19	16	17	21	24	27	30	34
M4	21	18	19	22	27	30	32	36
M5	22	19	20	23	27	30	32	45

Convencional PCR

	W	C (ADN)	C	Comminuted serial dilutions				
				- 1	- 2	- 3	- 4	- 5
M1	+	+	-	+	-	-	-	-
M2	+	+	+	+	-	-	-	-
M3	+	+	-	+	-	-	-	-
M4	+	+	+	+	-	-	-	-
M5	+	+	+	+	-	-	-	-

TaqMan real-time PCR higher sensitivity level

Asymptomatic samples (orchards and nurseries)

Washed tissues (leaves)

Nº positive TaqMan PCR / Total
33 / 280

Comminuted with DNA extraction (dormant buds)

Nº positive TaqMan PCR / Total
26 / 285

Good correlation between positive TaqMan rt PCR analysis and isolation

Asymptomatic samples (orchards and nurseries)

Washed / Comminuted with DNA extraction

Washed Nº positive / Total	Cominuted with DNA extraction Nº positive / Total
0 /117	11 / 117

9.4% positive samples “recovered” after DNA extraction

WASHED TISSUES:

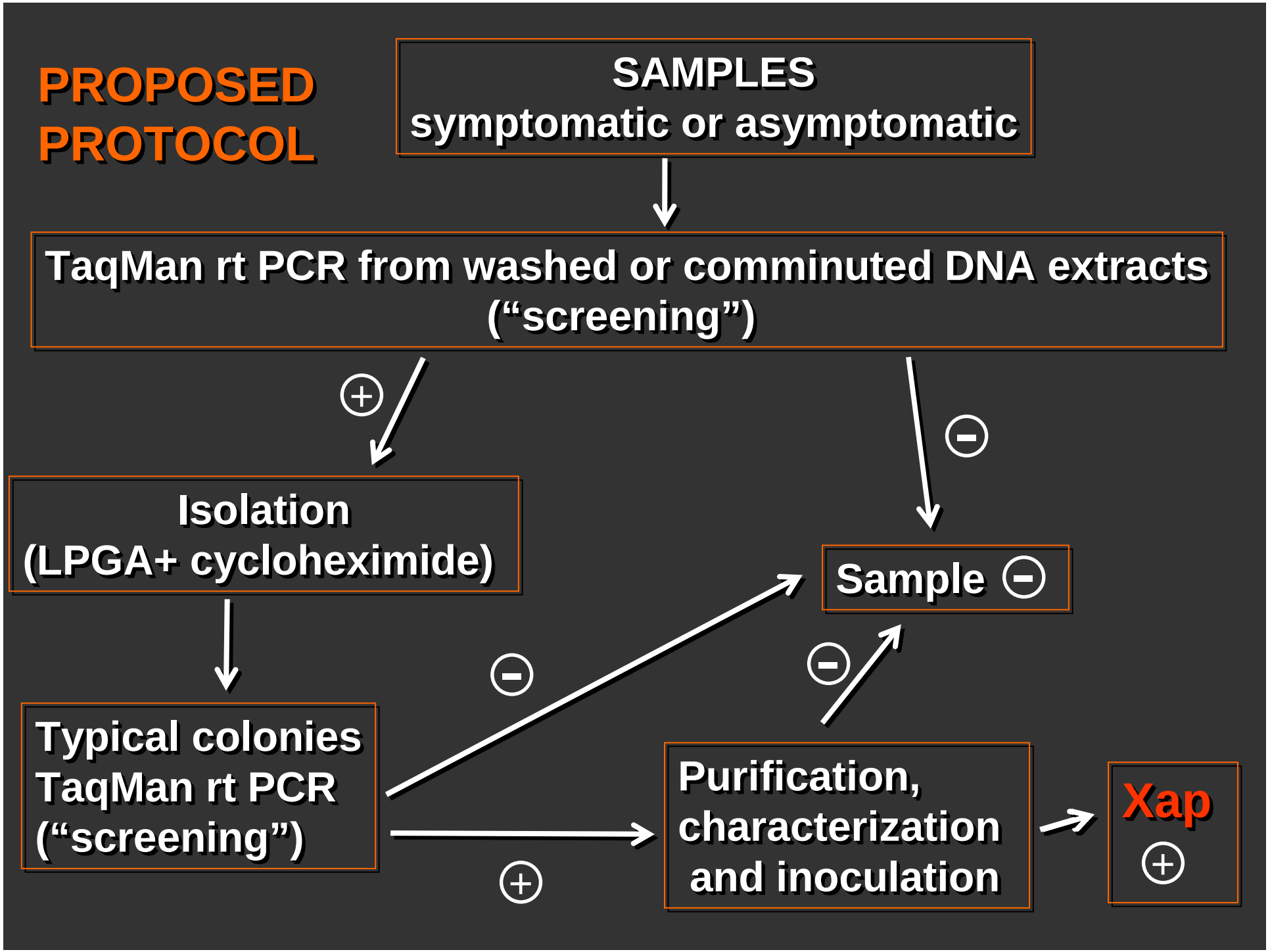
- Lower sensibility (high inhibitors concentrations)
- Offers high-throughput potential

Suitable for large-scale screening (nurseries)

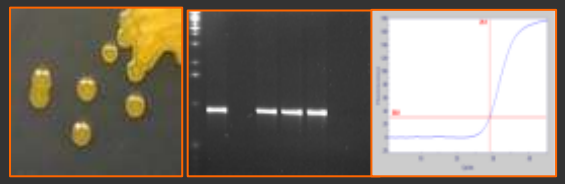
COMMINUTED TISSUES AND DNA EXTRACTION:

- Reducing the likelihood of false-negative
- Limits large-scale applications

Suitable for Certification Programs (plant propagation material)


PROPOSED PROTOCOL
SAMPLES
symptomatic or asymptomatic
TaqMan rt PCR from washed or comminuted DNA extracts
("screening")
⊕
⊖
Isolation
(LPGA+ cycloheximide)
Sample ⊖
⊖
⊖
Typical colonies
TaqMan rt PCR
("screening")
⊕
Purification,
characterization
and inoculation
Xap
⊕

CONCLUSIONS



✓ The TaqMan real-time PCR protocol developed is specific for *X. arboricola* pv. *pruni*. A sensitivity of 10^2 CFU/ml is achieved with plant material.

✓ Amplification is even achieved from washed tissues without DNA extraction.

✓ A good correlation between results of TaqMan real-time PCR analysis and isolation of the bacterium is obtained.

✓ The method has been tested for diagnosis and detection of the pathogen in symptomatic and asymptomatic naturally infected plant samples in orchards and nurseries. It is proposed for the accurate screening of contaminated nursery propagation material.

THANKS FOR YOUR ATTENTION

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