Transformação Genética em Fungos

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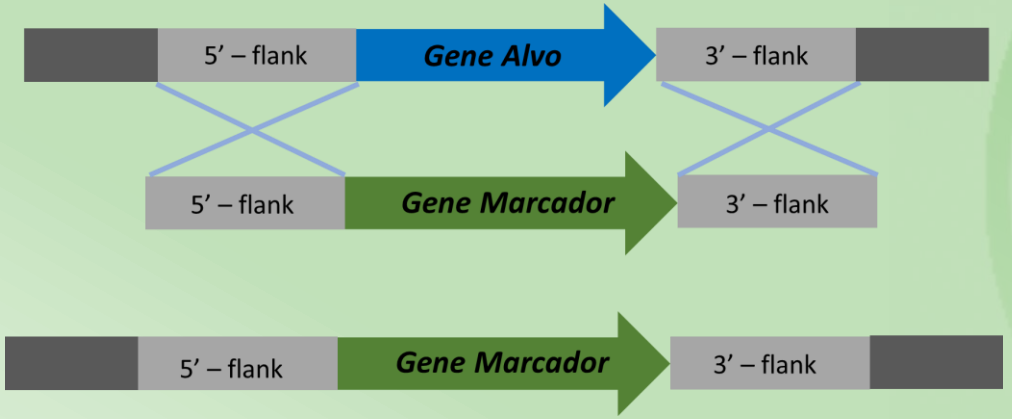
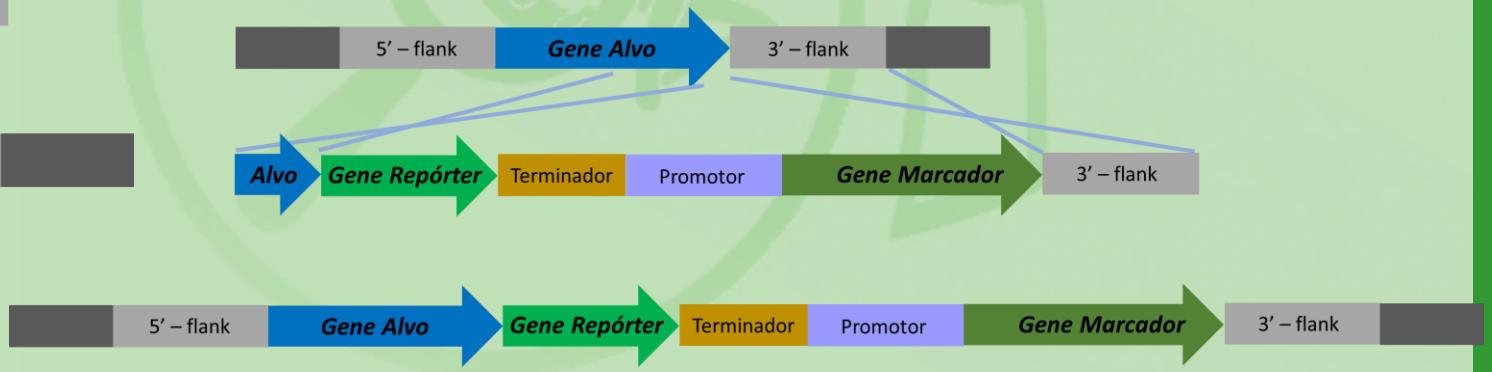
Aulas 14 e 15

Tipos mais comuns de transformação

- Deleção
  - Eliminar gene alvo, substituindo por gene marcador seletivo
  - O fenótipo mutante pode indicar a função do gene

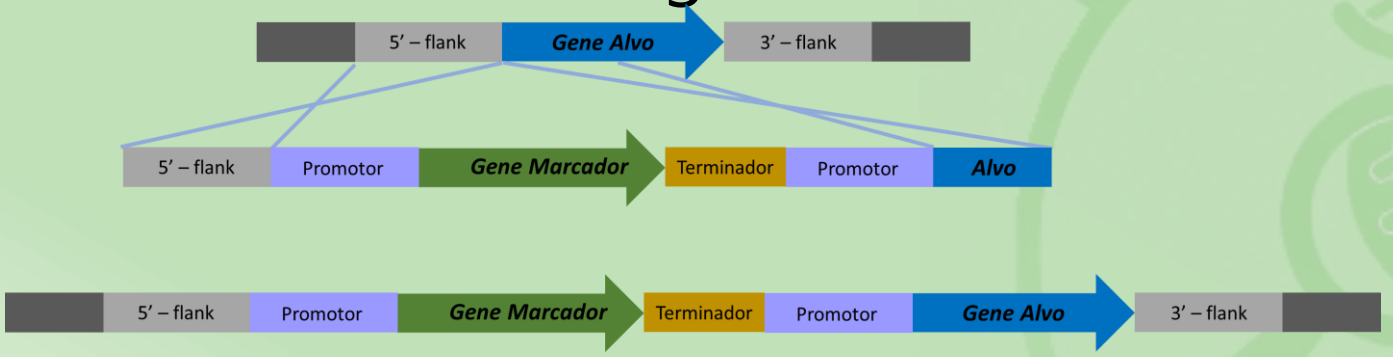


- Fusão
  - Incluir uma proteína repórter ou epítopo de anticorpo à sequência codificante de um gene
  - Observar o momento da expressão, co-localização subcelular, identificar interações via anticorpo.

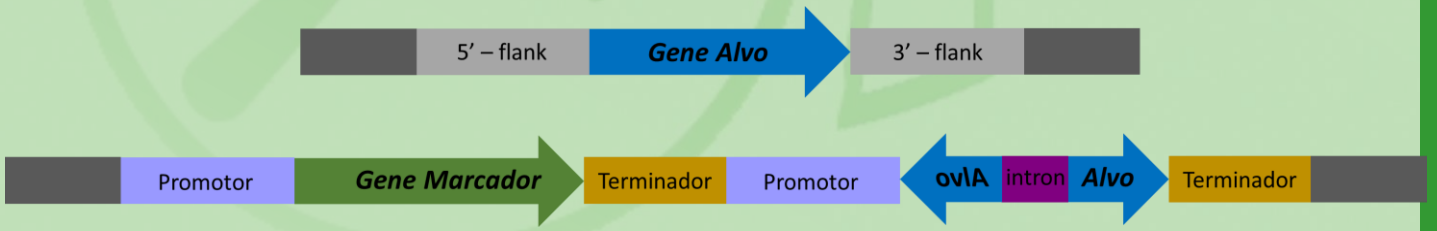


Tipos mais comuns de transformação

- Substituição de Promotor
  - Trocar o promotor original do gene por um constitutivo e/ou de expressão forte
  - Observar o efeito da expressão constitutiva do gene



- RNAi
  - Utilizar 400-500 pb senso e antisenso do CDS de um gene para produção de RNAs de interferência
  - Observar o efeito da redução na expressão do gene



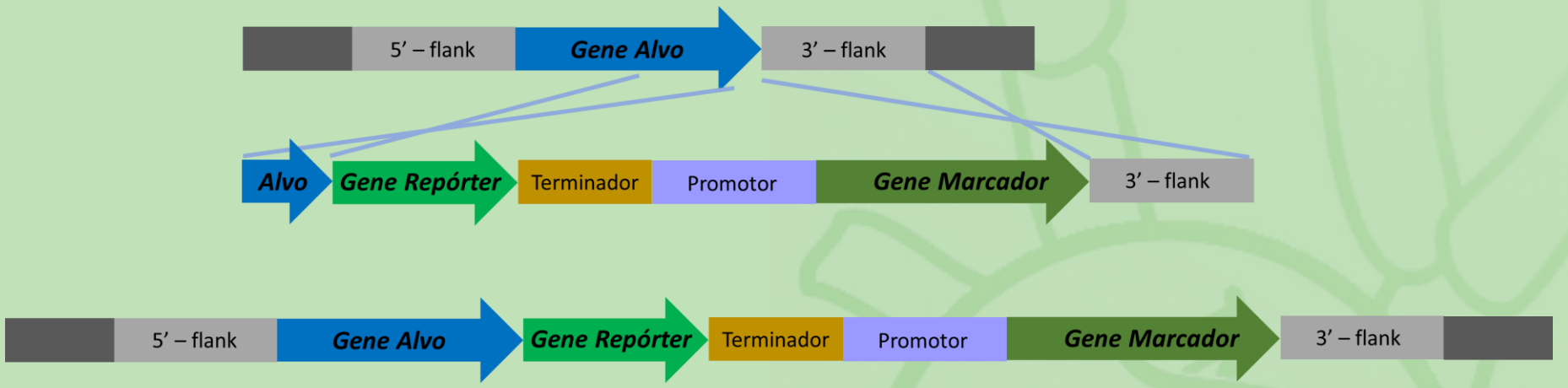
Estudo de Caso: Fusão de um tag ao gene alvo

- Background
  - Isolamento e detecção de proteínas:
    - por peso molecular ou precipitação com anticorpo
    - Necessita um anticorpo específico para a proteína
  - Tags de Epítopo
    - Pequenos peptídeos adicionados a uma proteína de interesse
    - Usados para marcar proteínas que não possuem anticorpo disponível
    - Por serem pequenos, não afetam a função da proteína principal
    - Altamente reconhecidos por anticorpos

Principais Tags				
FLAG	**HA**	**Myc**	**Poli His**	**V5**
DYKDDDDK	YPYDVPDYA	EQKLISEED	HHHHHH	GKPIPNPLLGLDST

Fusão da proteína Histona 3 com HA tag

- Estratégia



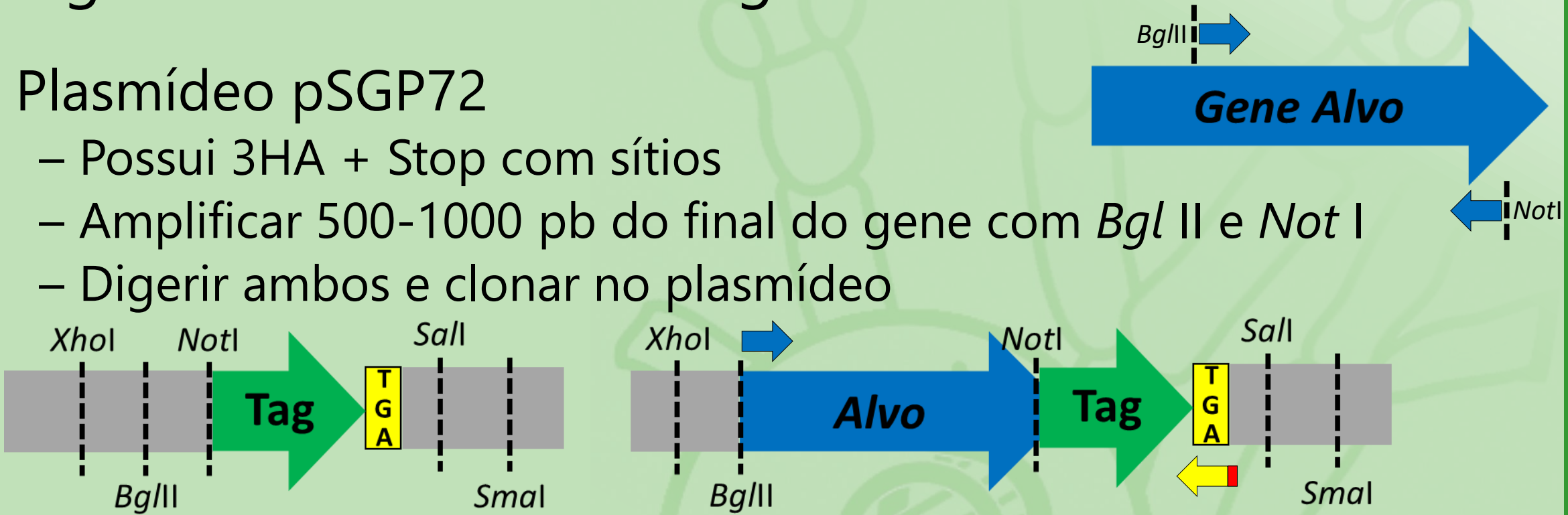
- Preparação
  - Encontrar fonte dos genes/fragmentos acima
    - Genoma do organismo: fonte para o gene alvo
    - Plasmídeos: fonte para o repórter/epítopo, promotor, terminador e gene marcador seletivo
  - Buscar fontes que envolvam o mínimo de etapas

Origem dos fragmentos

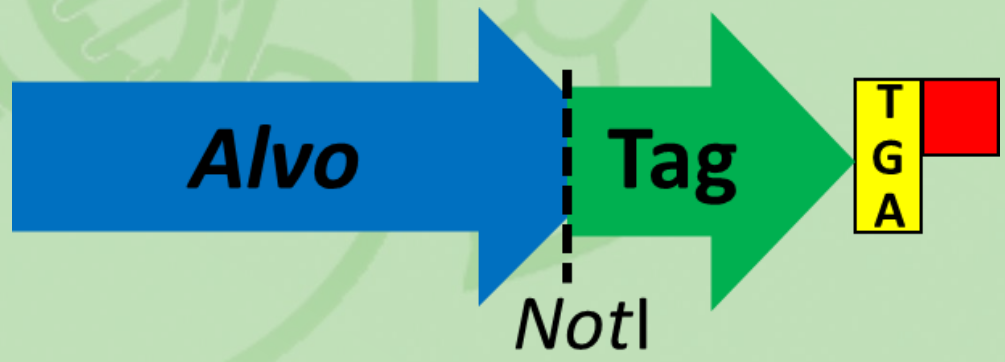
- Genoma do *Colletotrichum graminicola*
  - 500 a 1000 pb do final do gene sem stop códon com sítios de restrição
  - 500 a 1000 pb da região 3'-UTR com cauda no primer F
- Plasmídeo pSGP72
  - 3 cópias do tag HA com stop códon no final
  - Flanqueado por sítios de restrição *in frame* com o gene
- Plasmídeo pSRE47
  - TtrpC + PoliC + *NAT1*

Fragmento 1: Gene com Tag

- Plasmídeo pSGP72
  - Possui 3HA + Stop com sítios
  - Amplificar 500-1000 pb do final do gene com *Bgl* II e *Not* I
  - Digerir ambos e clonar no plasmídeo



- Amplificar fragmento completo
  - Gene (sem stop) + 3HA + stop
  - Adicionar cauda no primer R



Fragmento 1: Gene com Tag

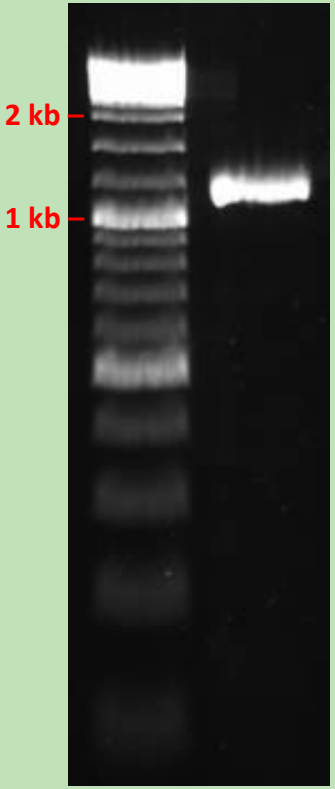
H3 sem stop + sítios

Mix
8.8 água
4 µL Buffer HF (5X)
2 µL dNTPs (10 mM)
2 µL primer F (10 µM)
2 µL primer R (10 µM)
1 µL gDNA (50 ng/µL)
0,2 µL Phusion (2U/µL)

Reação
98 °C – 3 min

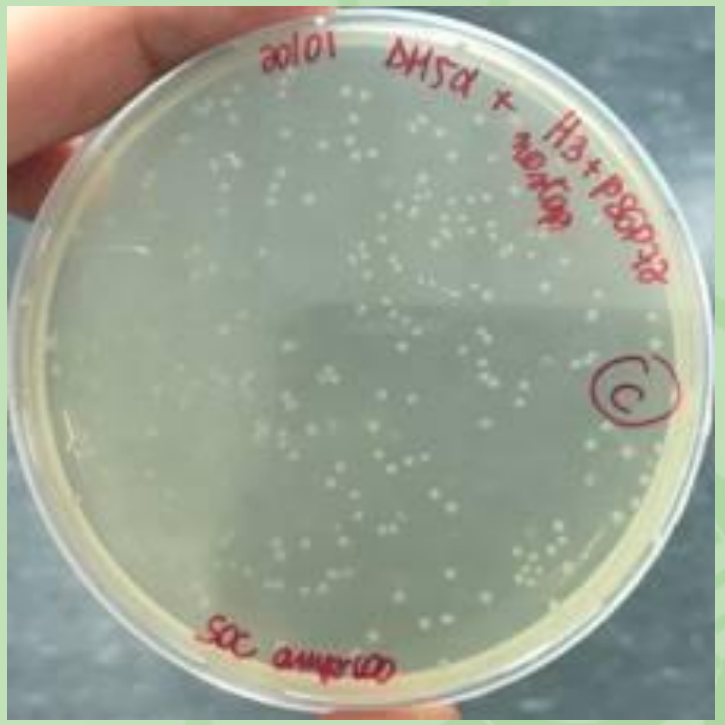
30x { 98 °C – 30 s; 66 °C – 30 s; 72 °C – 30 s }

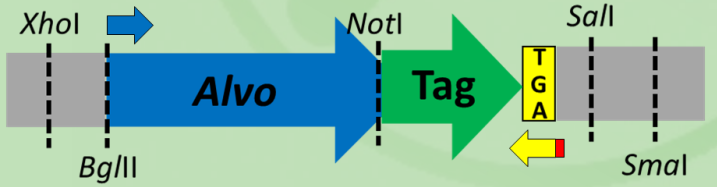
72 °C – 5 min



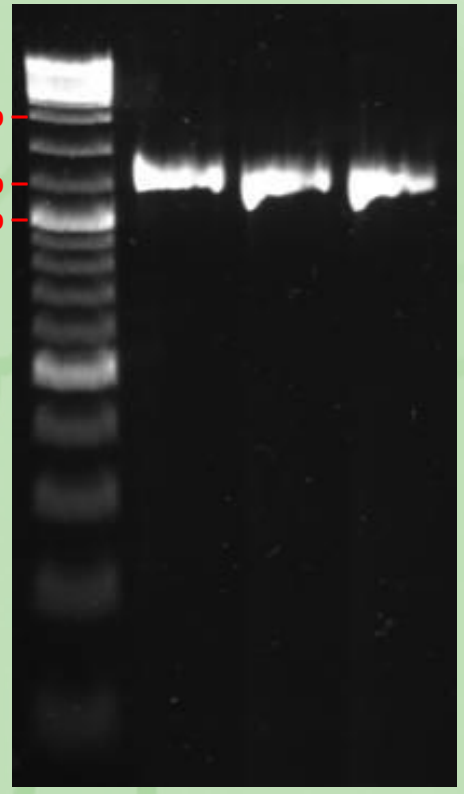
1100 pb + tails

Digestão e clonagem

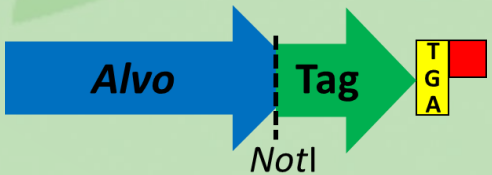




PCR Gene com Tag

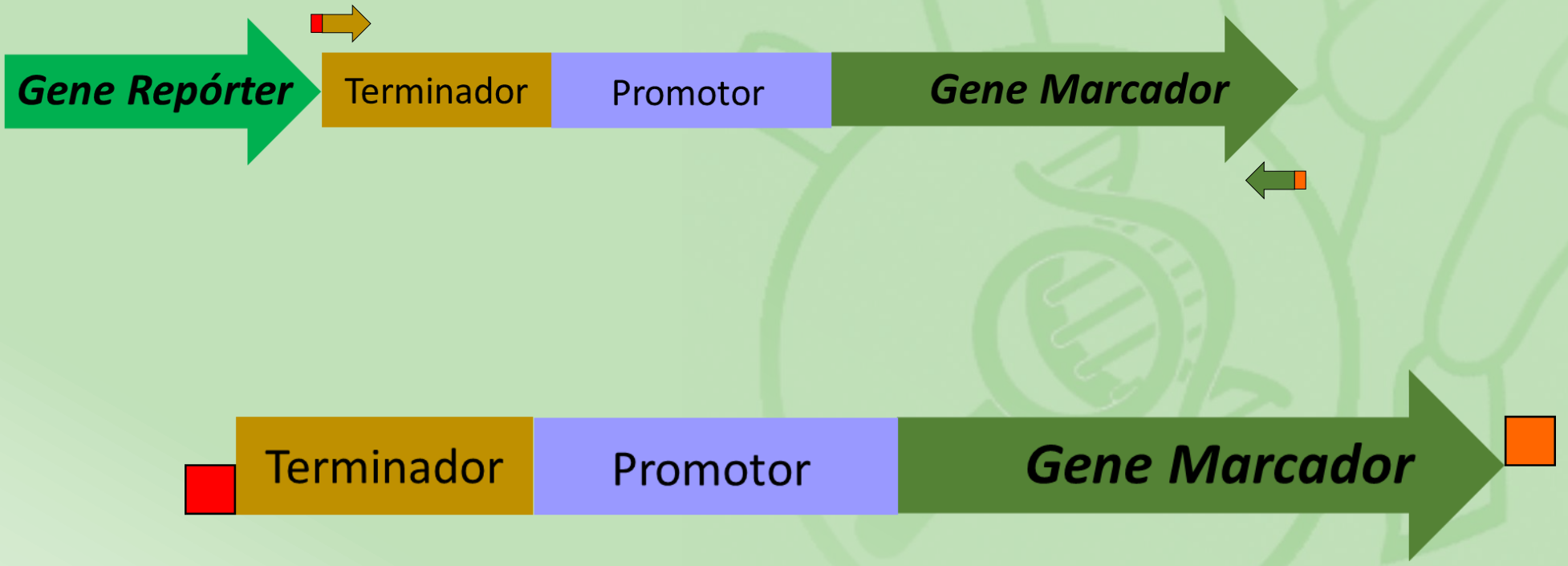


1210 pb + tail



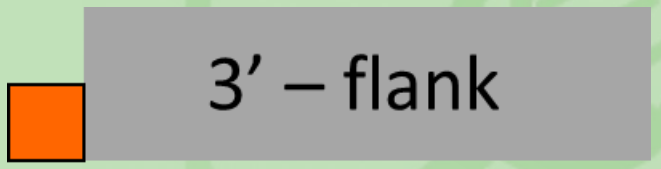
Fragmento 2: Terminador + Marcador Seletivo

- Plasmídeo pSRE47
  - Possui eGFP + TtrpC + PoliC + *NAT1*
  - Amplificar TtrpC + PoliC + *NAT1* com caudas



Fragmento 3: Região 3’-UTR do gene alvo

- Gene alvo
  - 500-1000 pb da região 3’-UTR do gene
  - Amplificar com cauda no primer F

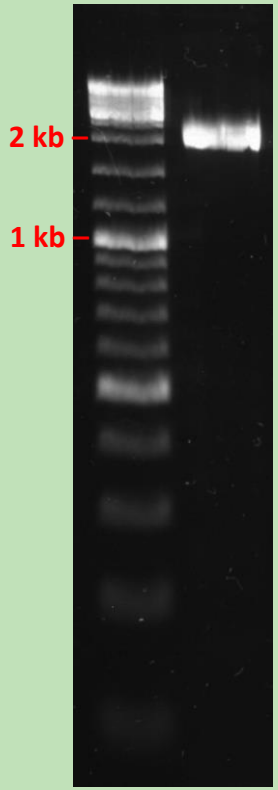


Fragmentos 2 e 3

TtrpC + PoliC + *NAT1*

Mix
8.8 água
4 µL Buffer HF (5X)
2 µL dNTPs (10 mM)
2 µL primer F (10 µM)
2 µL primer R (10 µM)
1 µL plasmid (1 ng/µL)
0,2 µL Phusion (2U/µL)

Reação
98 °C – 3 min
30x { 98 °C – 30 s; 62 °C – 30 s; 72 °C – 30 s }
72 °C – 5 min

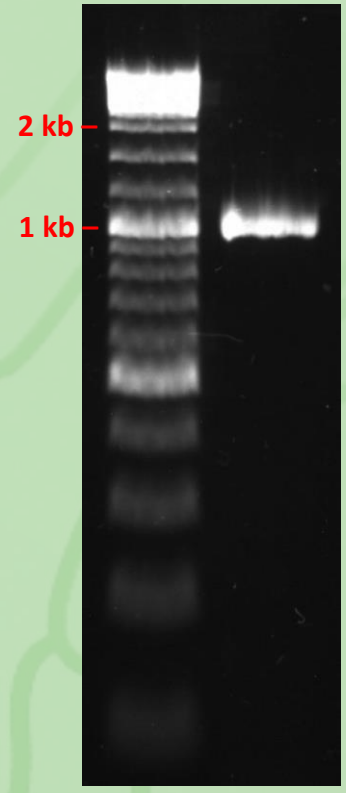


2070 pb + tails

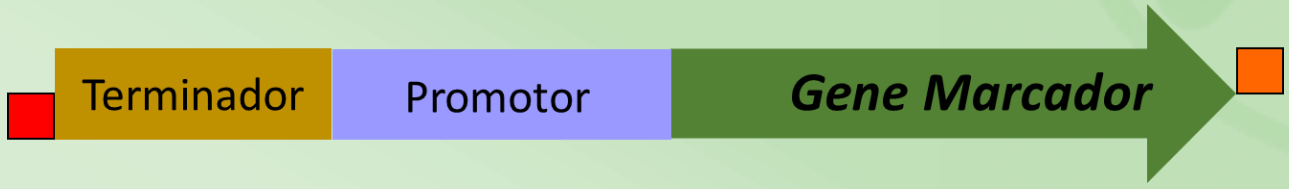
H3 – 3’-flank

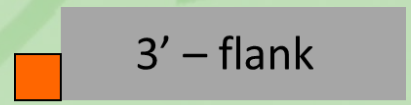
Mix
8.8 água
4 µL Buffer HF (5X)
2 µL dNTPs (10 mM)
2 µL primer F (10 µM)
2 µL primer R (10 µM)
1 µL gDNA (50 ng/µL)
0,2 µL Phusion (2U/µL)

Reação
98 °C – 3 min
30x { 98 °C – 30 s; 62 °C – 30 s; 72 °C – 30 s }
72 °C – 5 min



964 pb + tail





Double-Joint PCR, Nested and Cloning in pJET1.2

	Tamanho	Proporção	DJ	Final	Máx. 1000 ng	Concentração	Volume
Fragmento 1	1230 pb	1,25	1	1,25	125 ng	74 ng/µL	1,7 µL
Fragmento 2	2100 pb	2,1	3	6,3	630 ng	81 ng/µL	7,8 µL
Fragmento 3	984 pb	1	1	1	100 ng	48 ng/µL	2,1 µL

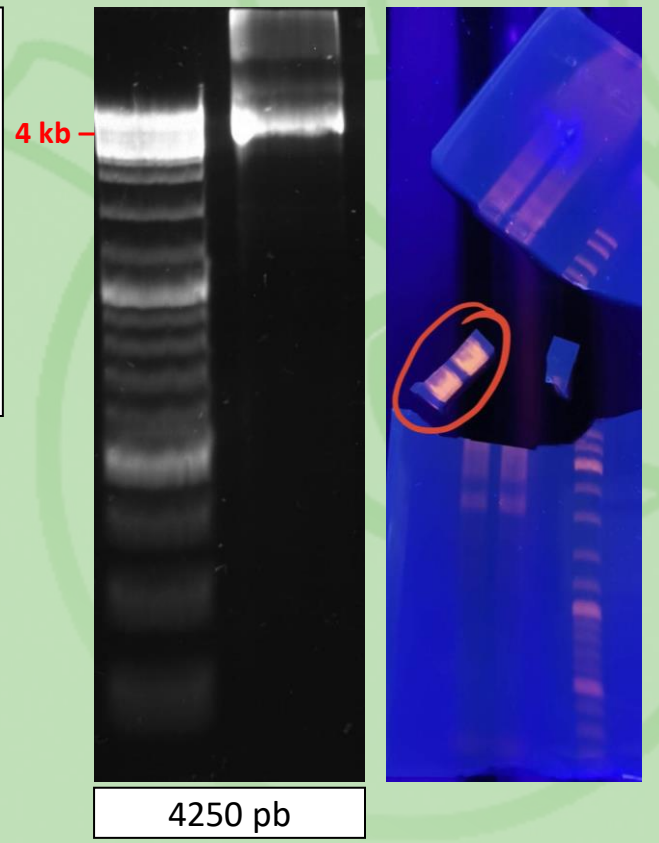
Mix (50 µL)
23,9 água
10 µL Buffer HF (5X)
4 µL dNTPs (10 mM)
1,7 µL Frag. 1 (74 ng/µL)
7,8 µL Frag. 2 (81 ng/µL)
2,1 µL Frag. 3 (48 ng/µL)
0,5 µL Phusion (2U/µL)

Reação
98 °C – 5 min
30x { 98 °C – 30 s; 62 °C – 2 min; 72 °C – 2 min }
72 °C – 10 min

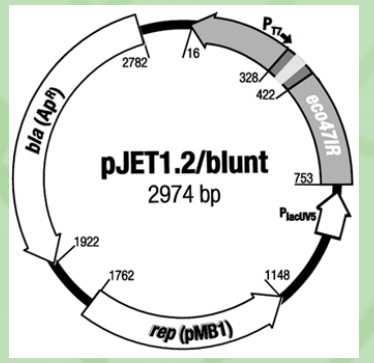
Purificado

Mix
8.8 água
4 µL Buffer HF (5X)
2 µL dNTPs (10 mM)
2 µL primer F (10 µM)
2 µL primer R (10 µM)
1 µL plasmid (1 ng/µL)
0,2 µL Phusion (2U/µL)

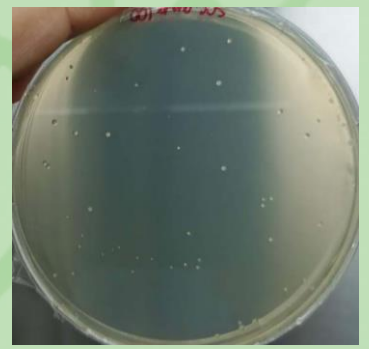
Reação
98 °C – 3 min
30x { 98 °C – 30 s; 62 °C – 30 s; 72 °C – 30 s }
72 °C – 5 min

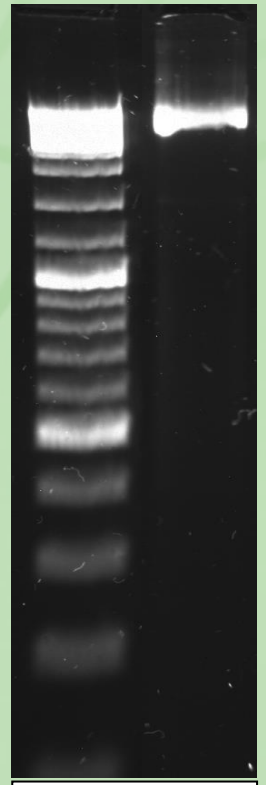


4250 pb



Clonado




4250 pb

Transformação de Protoplastos de *C. graminicola*

TRANSFORMATION OF *Colletotrichum graminicola* PROTOPLASTS

1. Wash spores from OMA plate with 1 mL sterile solution (water, 0.02% tween 20 or 0.8% NaCl) and inoculate 100 mL YES medium in 250-300 flasks with aluminum foil covers. Incubate for 3-4 days at 23 °C with slow rotation (80-90 rpm).

Flasks and medium previously autoclaved. The fungus sporulates better in small OMA plates, sealed with micropore and kept under constant light. If no longer using the plate, wash directly with YES medium until the solution is dark orange. During incubation, falcate conidia (macrospores) germinate into mycelial masses, which grow and sporulate inside the medium, producing oval or long conidia (microconidia). Make daily slides to observe the point where most of the spores are the oval type. Start the protocol early morning in that day. Alternatively to spores, inoculate 4 squares of mycelium in each flask (5 x 5 mm), peeling the top layer with a sterile metal picker.

2. Filtrate the culture in two layers of Myracloth, previously autoclaved inside a glass funnel. Collect the filtrate in 50 mL falcon tubes.

Place 2 layers of myracloth inside a funnel, autoclave inside an Erlenmeyer flask and dry completely in the warm chamber. Pour the whole cultivate inside the funnel under sterile bench and make slides to confirm that only spores were collected. Alternatively use filter paper 28 µm or Whatman paper n.1.

3. Centrifuge the tubes at 2000g for 10 minutes RT to collect conidia. Discard the supernatant.

Spores will form a pellet on the bottom of the tube, which should have enough material to guarantee a good transformation yield (2-3 mm pellet). Carefully pour out the supernatant, and if the pellet is too small, mix spores from 2 or more tubes in the next step (avoid using too many spores, or the buffer won't act properly).

4. Resuspend spores in 10 mL Protoplastization Buffer and incubate in shaker at 30 °C with 40-50 rpm rotation for 2-3 h.

Carefully pipette 10 mL of Protoplastization buffer into the falcon tube with the spores pellet and lye the tubes on the shaker, fixing with a tape. The rotation speed should form only a smooth "wave" that touches the lid and bottom of the tube.

5. Check after 2 hours for the presence of protoplasts (spheroplasts).

It is important to observe a reasonable amount of protoplasts, as spherical cells that lost their oval shape in the absence of part of cell wall. The more protoplasts in relation to presence of still oval spores, the better, but they should not be over digested, assuming a rather big size and so not being able to regenerate.

6. Centrifuge at 800 g for 10 min at 4 °C to collect spheroplasts and discard the supernatant.

Spheroplasts are sensitive, so if possible, reduce the acceleration and deceleration of the centrifuge, to prevent abrupt movements. Take the tubes from the cold centrifuge directly to ice, in holes previously made with empty tubes to avoid friction. Discard the supernatant turning the tubes slowly.

7. Ressuspend in 10 ml of STC buffer, centrifuge again (800 g, 4 °C, 10 min), discard the supernatant and resuspend in 1 ml of STC.

Pipette 10 mL STC solution and carefully mixed to dissolve the pellet and wash away residues of the protoplastization buffer. After centrifugation, pour out the supernatant and resuspend the pellet in 1 mL of the same STC buffer. In this step it's possible to combine protoplasts from more tubes, washing them with the same 1 mL solution. One final tube is enough for each cassette transformation.

8. Aliquot the 1 mL suspension with protoplasts into 4 x 250 µL in 15 mL falcon tubes on ice, add 1-5 µg of DNA (purified cassette) in only two tubes, pipetting slowly to mix well and incubate on ice for 30 min.

Place four 15 mL falcon tubes on ice and make 250 µL aliquots of the last 1 mL solution.
These tubes will be divided into:
a) DNA + fungicide (transformation tube, will be poured later in fungicide plates to recover only resistant colonies that harbour the cassette).
b) DNA no fungicide (control of viability of protoplasts, later pouring in plates with no fungicide, can be skipped to save DNA).
c) no DNA + fungicide (fungicide control, no colonies should grow in these plates, to confirm that the fungicide is correctly inhibiting non mutants).
d) no DNA no fungicide (control of viability of all cells, where all spores can germinate and grow, confirming that the cells resisted to the protocol).
To avoid spending a lot of DNA with a control, it's possible to skip the tube "b" and make two tubes "a", increasing the final transformation yield. Tubes "c" and "d" are enough to attest if the cells are viable and didn't die in the protocol and whether the fungicide is working properly in inhibiting non-transformed cells.
The tube(s) with DNA should not more than 50 µL of purified deletion cassette. If more DNA is necessary, concentrate the DNA in a speed vac but don't use more than 50 µL. Alternatively to 250 µL of spores, 100 µL spores aliquot can be used with maximum of 25 µL DNA.

9. Add 1 ml of PEG buffer, mix well by slowly pipetting and incubate RT for 20 minutes.

After the ice incubation, add 1 mL PEG solution to each of the four tubes. Since the PEG is viscous, it should be mixed well by pipetting, but slowly to avoid pressure in the protoplasts.

10. Complete the falcon tube until 10-12 mL with Regeneration Medium (0.6% agar) still liquid and warm (~45 °C), immediately mixing the solution with 4-5 inversions, and pouring on top of the solid Regeneration Medium plates (1.5 % agar, with or without fungicide, according to each treatment). Wait to cool down, seal with parafilm and incubate at 23 °C until the first colonies appear (4-5 days).

Prepare RM in both 0.6% and 1.5% agar concentrations.
With the RM 1.5%, prepare plates with and without 400 µg/mL fungicide, according to each treatment. Pour around 25 mL medium in each ø10-11cm plate.
Keep the RM 0.6% in the 60 °C chamber until minutes before use, wait till the temperature drops to around 45-50 °C (still hot but possible to hold in the hands without burning). After the 20 min incubation, complete the falcon tubes till 10-12 mL with warm RM 0.6% and immediately close the lid, invert the tubes 4-5 times flicking the bottom to disperse the dense PEG solution (but avoiding bubbles), and immediately pour the homogeneous solution on top of the RM 1.5% plate, spreading equally across the lower medium. Make the same for all the four tubes before the RM 0.6% temperature gets too low, which solidifies during mixing, forming clumps. The RM 0.6% should not contain fungicide, since the lower RM 1.5% medium contains a higher concentration which will diffuse to the top RM 0.6% medium.

11. Transfer colonies to PDA plates (12 or 24 wells) with 100 µg/ml fungicide and incubate for around 7 days.

Use 12/24-well plates containing PDA with fungicide to transfer the first colonies and let them grow until you can remove a small fragment and transfer to OMA plates without fungicide. Transfer as much colonies as possible by cutting a small fragment of the top agar around each colony and transferring to the new medium. Observe the colonies that appear at later times because some mutants might grow slower. The first colonies usually appear after 4-5 days, as white stars sometimes better visualized against the light.

12. Transfer colonies to OMA plates without fungicide for regular growth and sporulation, sealing with micropore and keeping at 23 °C for around 7 days.

In this step, the mutants will grow and produce spores to be used for purification of colonies. Small plates or 12-24 wells plates with generous amount of OMA are recommended.

13. Wash conidia with 10 µl of sterile water until light orange, dilute in 1 mL water in 1.5 mL epp tube, dilute 1-2 µl of this suspension in 50 µl water and spread in plates containing selective medium (PDA + fungicide 100 µg/ml).

It is possible to try different concentrations, as long as the final concentration is low enough to form distinct colonies on the plate, ensuring that the purified colonies came from germination of a single spore. Colonies not well purified might lead to mixed results in further experiments, due to the mix of different strains. To easy the process, prepare tubes with 1 mL water or 0.02% Tween 20, take 10 µL out of these tubes, wash the plate and return to the tube. Then homogenize, apply 2 µL of this suspension on the back of the plate lid, add 50 µL water/tween, mix and apply on the plate in use, spreading with a glass spreader.

14. Pick isolated colonies from each strain and transfer to new selective plates (PDA + fungicide 100 µg/ml) – *master plate*

These master plates will be the source of pure transformed strains, so transfer isolated colonies from the previous step into 12/24-well selective plates. After the colonies growth, they can be streaked in OMA plates and the master plate stored in 4 °C for short time (1-3 months) while the strains are characterized and selected.

15. Peak these pure strains into new small OMA plates without fungicide or 12-well plates for sporulation.

Spores from pure colonies will be used for: a) preparing long term stocks in glycerol to store in -80 °C freezer and b) prepare liquid cultures for DNA extraction and PCR/Southern Blot for characterization of strains.

16. Make long-term stocks by washing plates with water, tween or NaCl solution until dark orange, mix 500 µL of the suspension with 500 µL of glycerol 25% (filter sterile), keep on ice for 1h, freezer -20 °C for 1h and then to freezer -80 °C.

Well prepared -80 °C stocks can be preserved for many years. The recommended procedure is to cool down the temperature 1-10 °C per minute until it reaches -80 °C. An easy way to achieve that is placing the tubes inside a Styrofoam box with thick walls (2-3 cm) inside the -80 °C for 2h before transferring to regular storage boxes.

Overview

CgWT in OMA (15 days) → mycelial fragments transferred to YES medium (4 days) → Filtrate in miracloth → Protoplastization of oval spores → Transformation Protocol → First Colonies from RM plates into PDA + fungicide → back to OMA for sporulation → wash spores and spread in PDA + fungicide → isolated colonies back to PDA + fungicide (master plate) → Back to OMA for sporulation of purified colonies → use spores to make -80 °C stocks and DNA extraction

MEDIA and REAGENTS

YES MEDIUM (1 liter)
171,15 g sucrose (500mM)
1 g Yeast extract (1g/l)
Mix in 500 mL distilled water, complete to 1 L and autoclave.

PROTOPLASTIZATION SOLUTION (22 ml)
22 ml NaCl 0,7M (autoclaved)
440 mg lysis enzyme from *Trichoderma harzianum*
22 µL β-mercaptoethanol
22 µL ampicillin 100 mg/ml.
Mix in sterile bench in a sterile 50 mL falcon right before using it.
The β-mercapthoethanol should be added inside the air vent hood. The enzyme is weighed outside the sterile bench, but the Ampicilin helps avoiding contaminations
22 mL can be used for 2 protoplastization tubes with 10 mL each

STC (100 ml)
18.217 g Sorbitol (1 M)
0.5549 g $CaCl_2$ (50 mM)
1 mL 1M Tris-HCl pH8.0 (10 mM)
Distilled Water to 100 mL
Filter sterilize (0,45 µm) and store at RT.

PEG SOLUTION (100 ml)
40 g PEG4000 (40%)
1 mL 1M Tris-HCl pH 8 (10 mM)
4.4731 g KCl (0.6 M)
0.5549 g $CaCl_2$ (50 mM)
Distilled water to 100 ml
Filter sterilize (0,45 µm) and store at RT. (Hard to filtrate due to viscosity)

REGENERATION MEDIUM (400 mL)
137,2 g Sucrose (1 M)
0,4 g Yeast extract (0,1 %)
0,4 g Casein Hydrolysate (0,1 %)
Distilled Water to 400 ml
Mix well and divide into two fractions:
→ 100 ml: add 0,6 g (0,6 %) agar
→ 300 ml: add 4,5 g (1,5 %) agar
Autoclave, keep the 0.6% at 60 °C chamber before use (or melt in the microwave in the transformation day) and pour plates with 1.5% with and without fungicide (400 µg/mL).