GSPP
Good seed and plant practices

Annex 14.1

GSPP Protocol for sampling of seed lots for seed-health testing

Version 2.7

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Annex 14.1 GSPP Protocol for sampling of seed lots for seed-health testing

Goal: Assurance of the effectiveness of GSPP measures, both for small and large seed lots, being grown *Cmm*-free in GSPP certified environments.
This protocol is applicable for both GSPP and non-GSPP starting material (stock seeds), to be introduced in the GSPP system.

1. Introduction:

This sampling protocol is applicable both for small and large seed lots: The size of the seed production determines the number of seeds that needs to be tested.

Compartment-wise testing and smaller samples can be used to generate a reliable sample and test results are generated for this composed test sample.
The seed test on *Cmm* within the GSPP Certification system is a final test in a set of measures. Therefore it should be seen as an insurance of the effectiveness of the hygiene measures as described in the GSPP-protocols for a *Cmm* free plant or seed production.

2. Definitions:

- Test sample: Seed sample used for *Cmm*-test
- Reference sample: Seed sample, which is stored for retesting in case of complaints.
    - The reference sample must be in the same phytosanitary state as the test sample used to declare the batch free from *Cmm*.
    - The reference sample must meet the requirements described in Annex 14.1
- Fruit sampling: Sampling method where fruits are sampled to generate a composed seed lot where test and reference samples are generated from.
- Seed sampling: Sampling method where seeds are collected from one or more separate seed lots to generate a single or composed sample for a test and reference sample.
- Sub lot: Collection of seeds within a production cycle. Can be separate or combined harvests, seed extractions or received seed lots. Separately or mixed.
- Same phytosanitary state: no treatments to the seeds can be applied to the seeds, between the moment of sampling the seed lot for the *Cmm* seed health test and the moment of sampling for the reference sample. Treatments that might influence the (possible) seed health test result of the reference sample can be (but the list is not conclusive) disinfection, priming, coating, pelleting or any other treatment influencing the microbial population on and in the seeds, thereby resulting in a different *Cmm* test result between the original test sample and the reference sample when tested.

3. Sampling protocol for GSPP seeds:

Seeds for the test, and the reference sample, can be collected in two ways: fruit sampling or seed sampling:

1) Fruit sampling:
In order to have a representative sample, collect at least one fruit per harvested plant. Select fruits from the same or clusters above the clusters that will be harvested for seed. Collect these sampled fruits and extract the seeds so that they become one (test) batch. Compose a test sample and reference sample from this seed batch according to the 10-time rule. The minimum number of tested seeds before delivery is 10x the number of plants seeds are harvested of. The result is only valid for the seeds from plants, where sample fruits are collected from. Sample fruits must originate from the same or clusters above the clusters where seeds are harvested from (see Appendix 1). This test result is also only valid for seeds harvested before or at the time of collecting the fruits for fruit sampling. For seeds harvested after this moment of sampling a new seed sample is required.

2) Seed sampling:
Sample seeds from one or multiple seed lots at or after harvest. For seed sampling there are several options depending either on the number of plants the seed lot is harvested from (see 2bi), either on percentage of the seed lot to be tested (see 2bii).

   a) A sufficiently sized test sample for a reliable test can be created in two ways: composed sample or single lot sampling:
      i) Composed sample: Multiple (small) lots can be blended and tested as a composed sample. In case of a breeding program, a sample can be composed out of many single (or multiple) plant(s) seed lots. The result is only valid for the lots represented in the sample.
      ii) Lot sample: Collect seeds per seed lot. The test is only valid for this single seed lot

   b) The number of seeds to be tested can be calculated based on the number of plants or based on the lot weight:
      i) Based on the number of plants the seed lot is harvested from: The number of seeds to be tested is at least 10-times the number of plants the seed lot is received from (see for justification Appendix 2). The result is only valid for the lots represented in the sample.
      ii) Based on a percentage of the seed lot to be tested: The number of seeds to be tested is a percentage from the seed lot. The result is only valid for the lots represented in the sample. The total number of seeds to be tested is at least 0.3% of the total lot size (See various examples in appendix 3).

4. Sampling protocol for Non-GSPP starting materials to be introduced into the GSPP system:

Seeds for the test, and the reference sample, can be collected in two ways: fruit sampling or seed sampling:
Two types of starting material can be distinguished:

1. Starting material (seeds) that is produced under own management, and from which detailed production information is known (for example breeding material that is produced at the seed companies themselves).
   The seeds can be tested using all described procedure in section 3.1 (fruit sampling) or 3.2 (seed sampling).

2. Starting material (seeds) lacking detailed production information (e.g. lacking production location, number of plants the batch is produced from or the size of the total seed batch):
   at least 3% of the acquired batch needs to be tested before it can be used as starting material in a GSPP seed production location, for example acquired non-GSPP seeds from other seed companies or gene banks. The result is only valid for the lots represented in the sample.

5. Requirements all materials:

- No visible *Cmm* symptoms found during seed or plant production or symptomatic plants have been tested negative for *Cmm*.
- Testing of tomato seed samples according to the protocol(s) recommended by the GSPP Foundation.
- The seed lot must be thoroughly mixed before sampling to obtain homogeneity.
- A seed lot cannot get the status '*Cmm*-free', when seeds or plants from the same Entity have been tested *Cmm*-positive.

6. Reference sample:

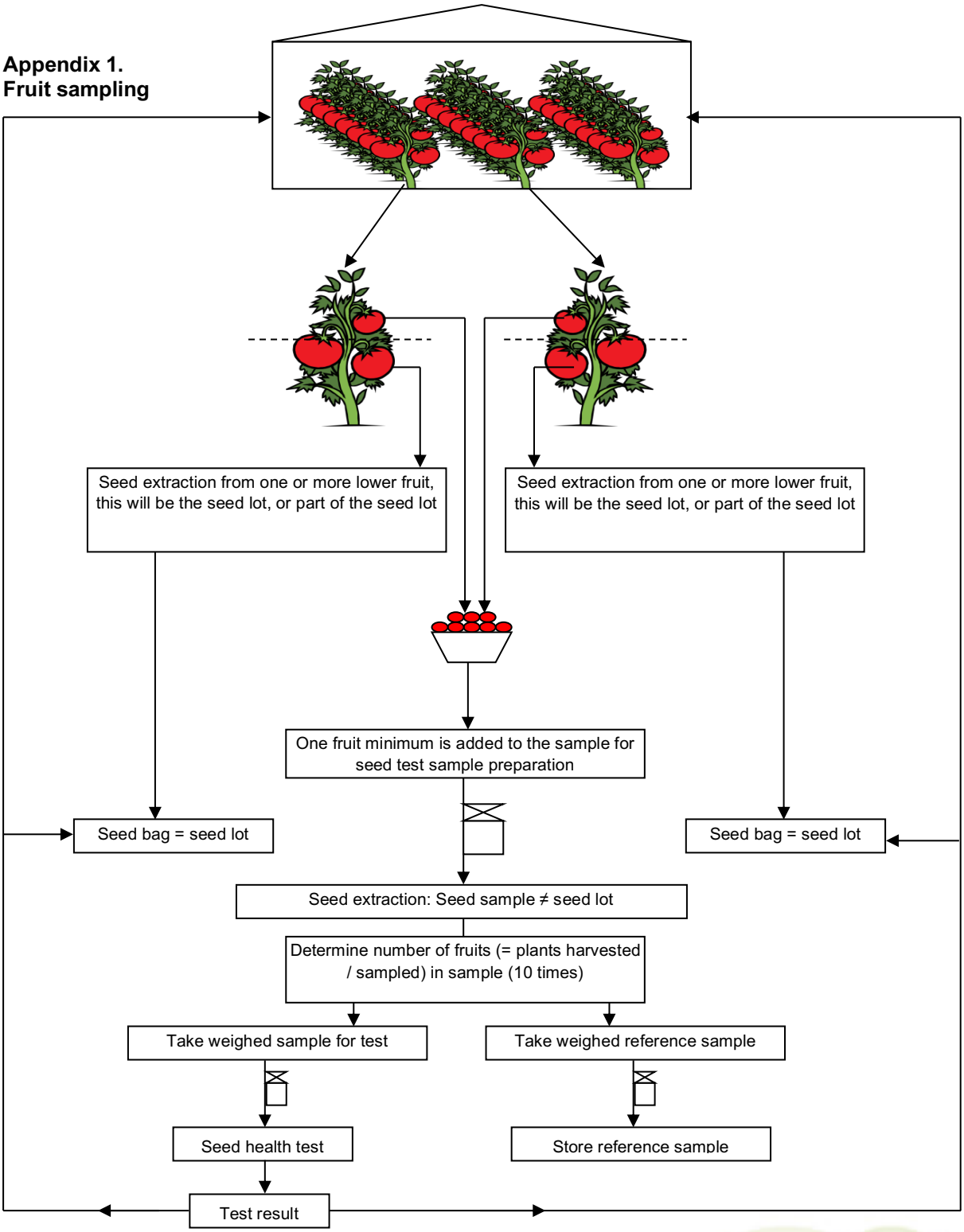
- For all GSPP-seeds produced or used in a GSPP production cycle as starting material a reference sample must be available for retesting.
- Reference samples must be stored to be able to retest the seed lot in case of a dispute. The seed must be stored until at least 2 years after the last sales of that lot or destruction of the lot.
- A reference sample must be in the same phytosanitary state as the test sample used to declare the seed lot free from *Cmm*.
- For reference samples, the same requirements apply as for the test samples.
- Reference samples must meet the minimal seed test sample size requirement (see table of chapter 7 of this annex):
    - Reference samples must be picked at the same time or created later in the production than the test-batches.
    - The reference sample must be picked from the same sample plants as the corresponding test batch is picked from.
- The test result and reference sample is only valid for the lots represented in the test and reference sample.

7. Summary table for GSPP seeds:

Lot size (number of seeds)	Lot size (weight)	Number of plants lot is harvested from	Minimum sample size (number of seeds)
3.000	10g	1	10
10.000	30 g	3	30
100.000	300 g	30	300
1.000.000	3 Kg	300	3.000
3.300.000	10 Kg	1.000	10.000
10.000.000	30 Kg	3.000	30.000
33.000.000	100 Kg	10.000	100.000

Summary table for the minimal sample size for GSPP seeds

Appendix 1.
Fruit sampling



Appendix 2. Seed sampling examples

i) The sample can be taken from:
   (1) The last single sub lot prior to delivery when the seed sub lot is not mixed before sampling with previous sub lots. The minimum number of tested seeds before delivery is 10x the number of plants seeds are harvested of (see example 1).
   (2) The seed samples can be sampled equally divided over, or with emphasis on the last sub lots during the production cycle prior to delivery including the final sub lot of seeds that will be delivered from, when the seed sub lots are not mixed before sampling with previous sub lots. The minimum number of tested seeds before delivery is 10x the number of plants seeds are harvested of (see example 2).
   (3) The total lot (anywhere during the production cycle), when multiple sub lots are mixed before sampling with previous sub lots. The minimum number of tested seeds before delivery is 10x the number of plants (see example 3).

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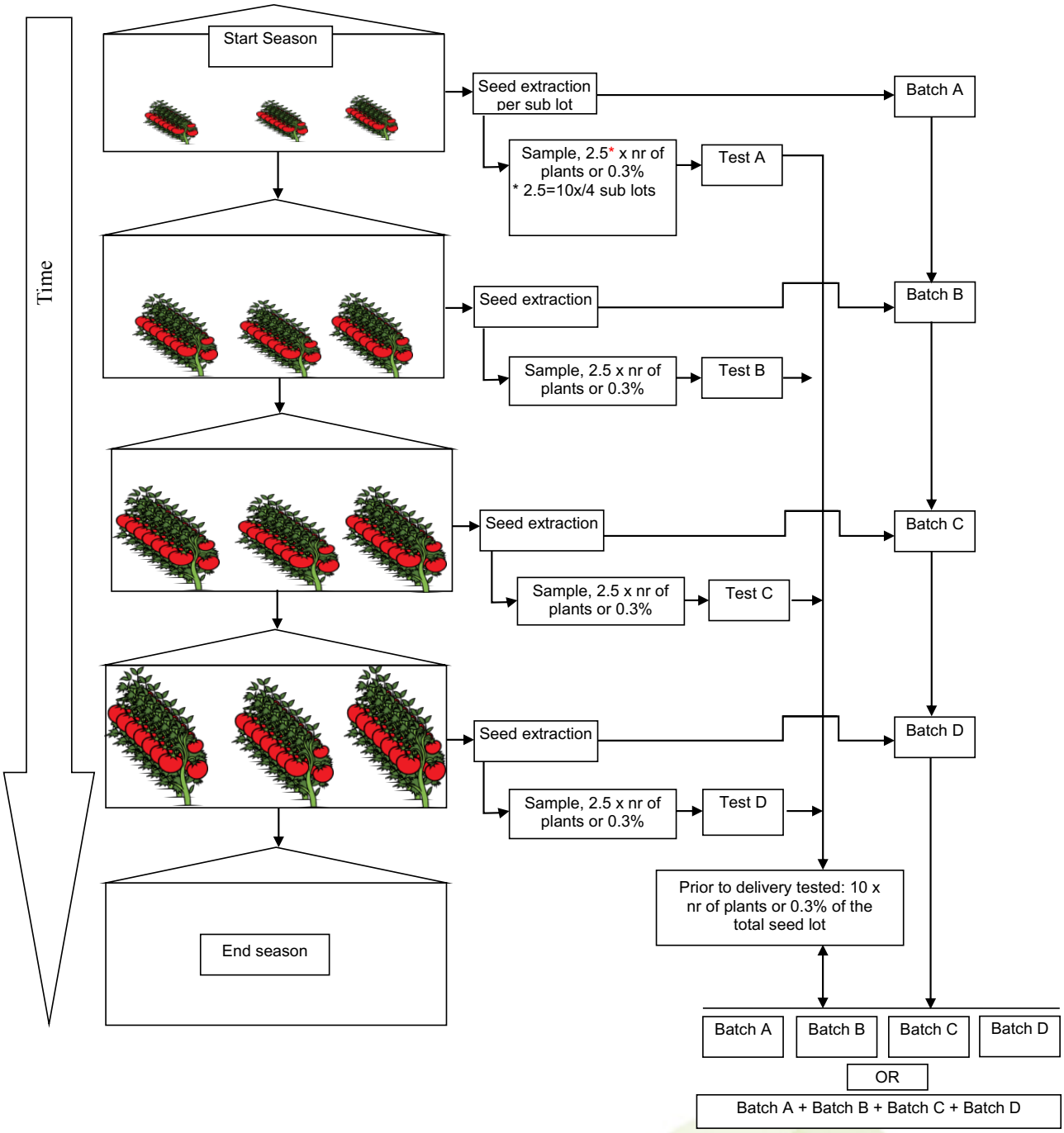
Appendix 2: Examples for sampling of seed lot sizes

	Example 1				Example 1				Example 3			
	Plants											
	1,000				Delivery at sub lot 4				Total sub lots			
Sub lot	seeds received	10-time		Sub lot	seeds received	3%	10-time		Sub lot	seeds received	3%	10-time
1	300,000	0		1	300,000	0	0		1	300,000		
2	300,000	0		2	300,000	0	0		2	300,000		
3	300,000	0		3	300,000	0	0		3	300,000		
4	300,000	0		4	300,000	9,000	10,000		4	300,000		
5	300,000	0		5	300,000	-	-		5	300,000	9,000	10,000
6	300,000	0		6	300,000	-	-		6	300,000		
7	300,000	0		7	300,000	-	-		7	300,000		
8	300,000	0		8	300,000	-	-		8	300,000		
9	300,000	0		9	300,000	-	-		9	300,000		
10	300,000	0		10	300,000	-	-		10	300,000		
11	300,000	10,000		11	300,000	-	-		11	300,000		
	3,300,000				3,300,000					3,300,000		
Total sample taken		10,000				9,000	10,000				9,000	10,000

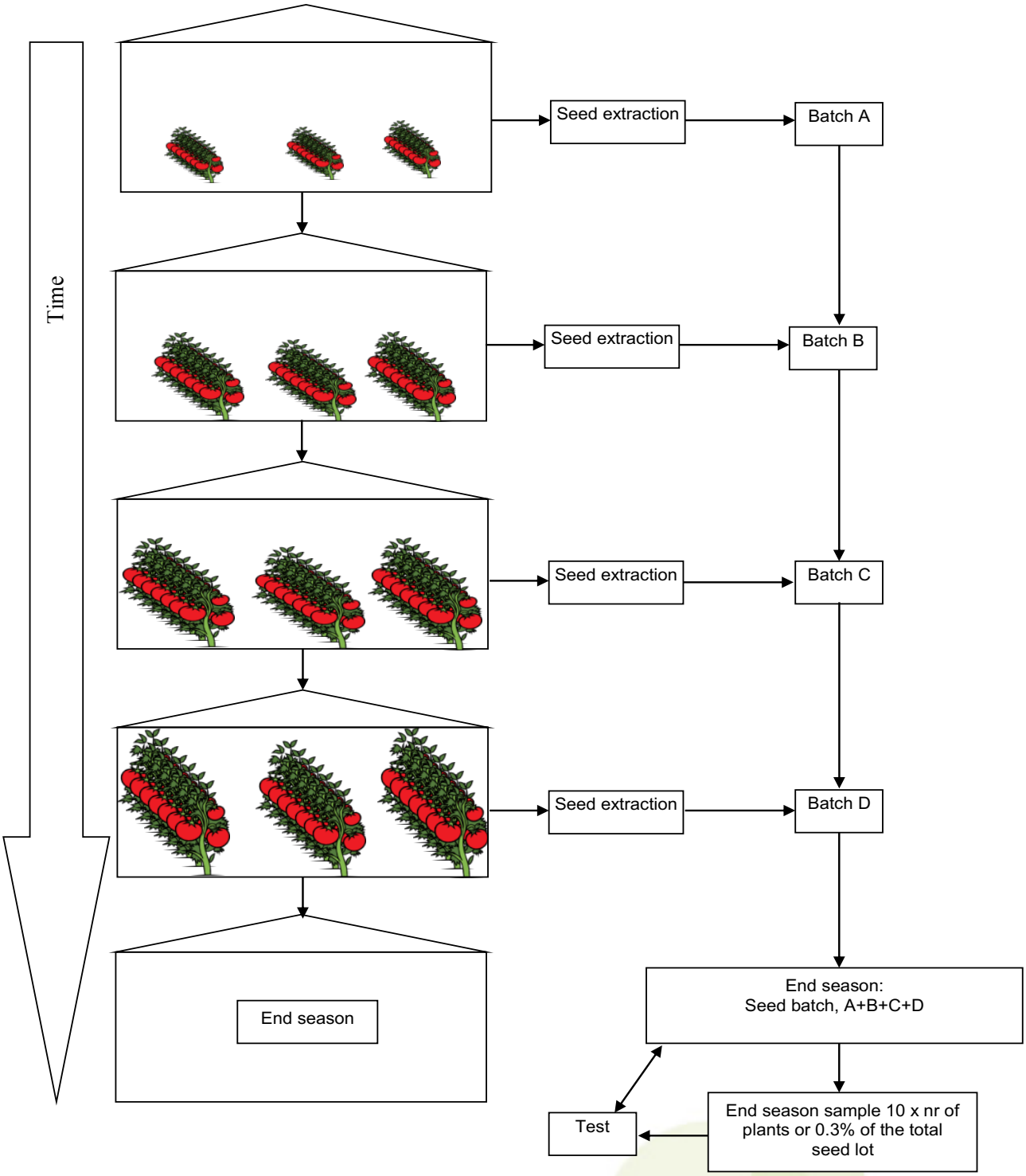
Example 2

	Plants	Delivery after final receiving, equally divided over 11 sub lots			Plants	Delivery after receiving 4, equally divided over 4 sub lots			Plants	Delivery after receiving 6, equally divided over 6 sub lots			Plants	Delivery after receiving 4 and 11, equally divided over 4 and 11 sub lots	
	1,000				1,000				1,000				1,000		
Sub lot	seeds received	0.3%	10-time	Sub lot	seeds received	0.3%	10-time	Sub lot	seeds received	0.3%	10-time	Sub lot	seeds received	0.3%	10-time
1	300,000	900	909	1	300,000	2,475	2,500	1	300,000	1,650	1,667	1	300,000	2,475	2,500
2	300,000	900	909	2	300,000	2,475	2,500	2	300,000	1,650	1,667	2	300,000	2,475	2,500
3	300,000	900	909	3	300,000	2,475	2,500	3	300,000	1,650	1,667	3	300,000	2,475	2,500
4	300,000	900	909	4	300,000	2,475	2,500	4	300,000	1,650	1,667	4	300,000	2,475	2,500
5	300,000	900	909	5	300,000	-	-	5	300,000	1,650	1,667	5	300,000	900	909
6	300,000	900	909	6	300,000	-	-	6	300,000	1,650	1,667	6	300,000	900	909
7	300,000	900	909	7	300,000	-	-	7	300,000	-	-	7	300,000	900	909
8	300,000	900	909	8	300,000	-	-	8	300,000	-	-	8	300,000	900	909
9	300,000	900	909	9	300,000	-	-	9	300,000	-	-	9	300,000	900	909
10	300,000	900	909	10	300,000	-	-	10	300,000	-	-	10	300,000	900	909
11	300,000	900	909	11	300,000	-	-	11	300,000	-	-	11	300,000	900	909
	3,300,000				3,300,000				3,300,000				3,300,000		
Total sample taken		9,900	10,000	Total sample taken		9,900	10,000	Total sample taken		9,900	10,000	Total sample taken		16,200	16,363

Appendix 2: Pictures for Example 2



Appendix 2:
Pictures for Example 3.



Appendix 3. Seed sampling

The sample can be taken from:

(1) The last single sub lot prior to delivery when multiple seed sub lots are not mixed before sampling with previous sub lots. A sample of at least 0.3% of the total lot size must be taken on the last harvested sub lot.

(2) The seeds can be sampled equally divided over, or with emphasis on the last sub lots during the production cycle prior to delivery including the final sub lot of seeds that will be delivered from, when the seed sub lots are not mixed before sampling with previous sub lots. A minimum of 0.3% of the (expected) total seed lot from the production must be tested before delivery. (see Appendix 2. examples 2. See for justification Appendix 4). This corresponds to app. 10x the number of plants the seeds are harvested from [a)].

(3) The total lot (anywhere during the production cycle), when multiple sub lots are mixed before sampling with previous sub lots. A minimum of 0.3% of the (expected) total seed lot from the production must be tested. (see Appendix 2. example 3. See for justification Appendix 1). This corresponds to app. 10x the number of plants the seeds are harvested from.

Appendix 4. Justification seed sampling based on the 10-times rule

- The calculation of the ten-time rule is based on a binomial distribution (see e.g. Gu and Novak, 2004[1]).
- General formula $P = 1 - (1-r)^n$, P = probability of detection, r = infected seeds / healthy seeds + infected seeds, n = test sample size.
- The sample size is based on the number of plants a seed lot is produced from.
- Assuming there are 1000 plants used for seed production.
- Each plant will yield 33 fruits containing 100 seeds each, is 3300 seeds per plant.
- TSW is 3 gr = 9.9 gr per plant.
- One plant yields 10 gr seeds = 3.300 seeds
- This production will yield 1000 plants x 9.9 gr per plant = 9.9 kg.
- An infected plant is present from the start of the season onwards resulting in 1.650 infected seeds.
- The infection rate is: 1.650 infected seeds / 3.298.350 healthy seeds + 1.650 infected seeds = 0.0005
- Based on the 10x-rule, the required sample size is 10 x 1000 seeds = 10.000 seeds.
- $P = 1-(1-(1.650/3.300.000))^{10.000} = 99{,}3\%$
- This corresponds to: 10.000 / 3.300.000 = 0.3% of the total seed lot that needs to be tested.
- Plant number does not influence on the probability of detection, however, the moment of infection does.

Assume 3000 plants:

Same conditions as above.
1.650 infected seeds produced, in total 9.900.000 seeds produced,
Based on the 10x rule = 30.000 seeds to be tested.
$P = 1-(1-(1.650/9.900.000))^{30.000} = 99.3\%$
30.000 / 9.900.000 = 0.3% of the total seed lot produced.

Assume 15 plants:
Same conditions as above.
1.650 infected seeds produced, in total 49.500 seeds produced,
Based on the 10x rule = 150 seeds to be tested.
$P = 1-(1-(1.650/49.500))^{150} = 99.5\%$
150/49.500 seeds = 0.3% of the total seed lot produced.

Consequences of sampling seed lots based on the 10-time rules of harvested seeds at different harvest times and justification of the 3 % sample size.
Sample test seeds per sub lot:

- A total of 1000 plants is harvested from
- Three fruits x 100 seeds per fruit x 999 = 299.700 seeds per harvest
- One infected plant yields 150 infected seeds.
- Assume seeds are tested at a single harvest.
    - Then : $P = 1-(1-150/299.700+300)^{10000} = 100\%$ (probability of detection)
    - The actual necessary sample size to achieve P = 0.99 is:
    - N = log(0.01)/ log(1-150/299.700+300) = 9208 seeds = 3,0% of the total seed lot should be tested
    - 9208 seeds from 1000 plants = 9.2 seeds per plant to be tested.

[1] (ref. Gu and Novak, 2004, Am. J. Trop Med Hyg. 71:636-638).

The probability of detection of an infected seed is minimally 99% based on the following assumptions
The calculations are based on the assumptions, and that "the number of seeds per plant" does influence the percentage that needs to be tested mentioned under ii). The calculation based on number of plants per seed lot (i) is not influenced by the number of seeds per plant. Therefore, the calculation based on the number of plants can be used as a backup, especially when the "amount of seeds per plant" is significantly less than indicated in the assumptions.

- Each fruit contains 100 seeds.
- Each plant produces 10 grams of seeds during its production cycle, TSW = 3, is 3300 seeds per plant.
- At each picking moment, 3 fruits per plant are picked.
- The complete production cycle is eleven pickings, is 33 fruits.
- In case of *Cmm* infection the pathogen is distributed homogenously throughout the whole plant and fruit.
- Fifty percent of the seeds in an infected fruit are contaminated (50 healthy seeds + 50 infected seeds).
- The infection establishes at the start of the seed harvest season and from there on all fruits on a single plant become contaminated.
- Infections become better detectable in time, e.g. the disease spreads homogenously within and between plants. As a result, later set fruits have a higher probability of detection compared to earlier set fruit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