Bioprotection - enhancing growth and health Research Programme

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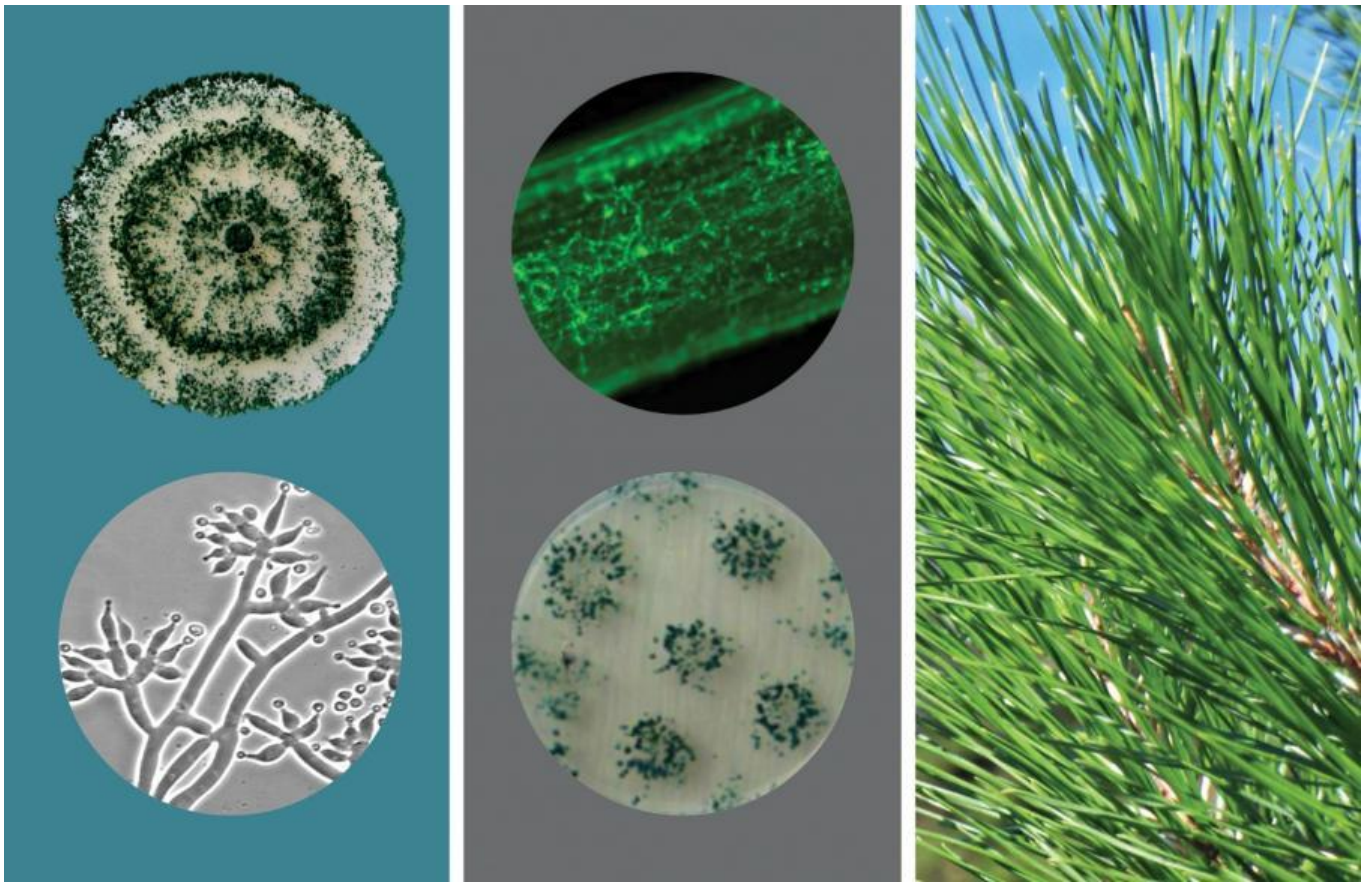
CLIENT REPORT (Confidential)

Assessment of established *Trichoderma* field trials and methodology for scoring disease

Rebecca Ganley



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EXECUTIVE SUMMARY

Report Title: Assessment of established *Trichoderma* field trials and methodology for scoring disease
Author: Rebecca Ganley

The problem

Field trials using seed treated with *Trichoderma* species and strains have been set up on 19 different sites across New Zealand. The suitability of these sites for screening disease needs to be determined and an appropriate methodology to score disease needs to be developed.

This project

The statistical design, replication and host material used for the19 different sites were assessed. Due to the lack of suitable field trials, no methodology for assessing responses across sites or locations could be developed. A methodology for scoring disease in the field was developed.

Key Results

Only 3 field trials out of the 19 established have suitable host material, statistical design, replication to be statistically analysed. Of these trials, one (Cricklewood 2013) is expected to have RNC disease levels suitable for screening and one trial (XPKANG 2013) will be monitored to see if disease levels are sufficient for assessment. The third trial (Pinnacles 2013) is in an area not known to be commonly affected by red needle cast.

Of the 16 trials that were unsuitable for analysis, 15 were ruled out because the seedlot used for the *Trichoderma* treatments was genetically unrelated to that used for the control in the same field trial. One trial site does not have sufficient replication for statistical analysis to be done.

A methodology for assessing symptoms of red needle cast in the field was developed. This method has been prepared for scoring disease levels in material less than three years old, and will also allow direct comparison to results from artificial inoculation trials.

Implications of Results for Client

The low number of field trials available for assessment affects the analysis of growth and disease symptom responses in the field. Because both disease resistance and growth are heritable traits, the use of appropriate host material is critical. In the trials where inappropriate seedlot material was used statistical analysis cannot be interpreted because one could not distinguish whether any result was due to heritable variation in the host versus a treatment response. The low number of field trials also means that previously planned cross-site-location comparisons of *Trichoderma* treatment performance will no longer be able to be made.

Further Work

The two field trials, Cricklewood 2013 and XPKANG 2013, will be monitored for red needle cast and if disease levels are high enough these sites will be scored.

Assessment of field trials

Nineteen field trials have been established across New Zealand (see Bio-Protection's Forestry Trials Database and Table 1) and were planted with seed that had been pre-treated with mixtures of *Trichoderma* spp., as well as untreated seed to be used as a control. The host material, statistical design, replication used and suitability of the sites for screening disease were assessed and the findings for each trial are summarised in Table 1.

During the course of preparing this report discrepancies between the *Trichoderma* treatments in the field trial documentation and the spreadsheet information were initially found, and duplicate reporting of one field trial was also discovered. These errors have been resolved and corrected in the database. The information used for this report comes from the July 2015 updated version of 'Forestry Trials Database' excel sheet, which is appended to this report.

Host material

Of the 19 field trials, 15 used host material that was not suitable for statistical analysis. For these 15 trials a different seedlot was used for the *Trichoderma* treatments (seedlot 10/216) than for the control (seedlot 13/203). Seedlot10/216 is a stand-selected, open pollinated seedlot sourced from trees that had come from two different stands planted with two different seedlots. The seedlot was sourced from a general planation in the Wairarapa sometime around 2009/10. In comparison, seedlot 13/203 is control pollinated with 48 parents and 56 crosses used to produce this seedlot. The seed was sourced from Seddon Orchard in the South Island in 2012. A parental analysis could determine the amount of shared genetic diversity, however based on the seedlot information it would be surprising if there was any genetic overlap in the treatment and control seedlots used.

As disease resistance and growth are heritable traits, the use of appropriate host material is critical. The use of a seedlot for the control with different genetics from treatments means statistical analysis for either disease resistance or growth cannot be interpreted on these 15 field trials. This is because you could not distinguish whether any result was due to heritable variation in the host versus a treatment response.

For the remaining four field trials, only one seedlot was used for both the treatments and the controls. This was either seedlot 12/207, a control pollinated seedlot that consists of seed from 150 crosses produced from 70 different parents, or seedlot 10/216 (described above). Although the exact parentage of the 10/216 seedlot is not known, it is expected that the genetic diversity of the seed will be very high as it was open pollinated and collected from two stands that had been initially planted from two different seedlots 00/235 (control pollinated; progeny from eight crosses) and 99/387 (control pollinated; progeny from 31 parents). Considering the diversity of both of the 12/207and 10/216 seedlots, and the number of trees used in the field trials, it is highly likely that there is only limited genetic similarity in the material used both within each trial and between trials. This may confound the results for assessment of both disease resistance and growth responses in the field trials.

Statistical design and replication

Of the remaining four field trials, three had statistical design and replication appropriate for statistical analysis. All three trials (XPKANG 2013, Cricklewood 2013 and Pinnacles 2012) had a randomised block design. Two of the field trials had six treatments (including the control) arranged in a three replicate block design. The third field trial had three treatments (including the control) arranged in a five replicate block design.

The replication in the fourth field trial, Wharerata 2012, was not sufficient for any statistical analysis to be performed for either disease resistance or growth responses. Only one treatment and one control were used, although these were replicated in three different areas, the sites

were so far apart they could not be considered replicate blocks but rather need to be considered as three separate field trials with no replication.

Suitability for disease screening

One of the field trials, Cricklewood 2013, is in Gisborne in an area known to have high red needle cast (RNC) and is likely to develop sufficient disease to score for treatment responses. Disease levels of dothistroma needle blight (DNB) in the Gisborne region where these two trials are located is not usually high enough to spray, because of this, these trials are not considered suitable for screening DNB treatment responses.

The XPKANG 2013 field trial is in Kaingaroa, although this region does get RNC, the levels and symptoms in the last few years have been low, and whether the inoculum loading in 2015/16 will be high enough to score treatment responses is not known. This site will be checked for suitability during the RNC season and scored if the levels of disease are considered high enough. Levels of DNB in Kaingaroa may be high enough to score, this will be determined when the trial is checked for RNC. If the expected El Niño episode occurs over summer it is unlikely that DNB will be high enough to score until at least 2017. As Kaingaroa is sprayed with copper for DNB during high impact years, spray history of this field trial needs to be investigated as this could affect RNC and DNB treatment responses. The person responsible for the trial should ask that it be registered and taken off the 2015-16 spray schedule, if this has not already been done.

The Pinnacles 2012 field trial is not in an area where levels of RNC have been high; rather it tends to be affected by physiological needle blight. It is also not considered a high risk area for DNB.

The initial plans to compare disease responses from the *Trichoderma* treatments across sites and locations across New Zealand have been modified. The lack of suitable trial sites to score means there may only be data from one site. If two sites are able to be scored then pairwise comparisons may be performed as both field trials have the same *Trichoderma* treatments and layouts.

Summary

There are three trial sites, of the 19 established, that have suitable host material, statistical design, replication to be statistically analysed. Of these trials, one (Cricklewood 2013) is expected to have RNC disease levels suitable for screening and one trial (XPKANG 2013) will be monitored to see if disease levels are sufficient for assessment. The design layout and replication for these two trials are in the Appendix.

Table 1. Summary of *Trichoderma* treated *Pinus radiata* field trial sites and suitability for screening

Forest plantation area	Company	Locality	Date(s) established	Genotype/seed information		Host material	Statistical design and replication	Disease screening
				Trichoderma	Control			
XP KANG	Timberlands	Kaingaroa	2014	10/216	13/203	Not suitable	N/A	N/A
XP KANG	Timberlands	Kaingaroa	2013	CP 12/207	CP 12/207	Suitable	Suitable	May be suitable for RNC, suitable for DNB
Phoenix_Horohoro	Hancock	Kinleith	2014	10/216	13/203	Not suitable	N/A	N/A
Waituna_Kinleith	Hancock	Kinleith	2014	10/216	13/203	Not suitable	N/A	N/A
MTRN	Hancock	Nelson	2014	10/216	13/203	Not suitable	N/A	N/A
Pearse	Hancock	Nelson	2014	10/216	13/203	Not suitable	N/A	N/A
Otaenga	Hancock	Northland	2014	10/216	13/203	Not suitable	N/A	N/A
Pipiwai	Hancock	Northland	2014	10/216	13/203	Not suitable	N/A	N/A
Wharerata	Juken NZ	Gisborne	2012	SSOP 10/216 (?)	SSOP 10/216 (?)	Suitable	Not suitable	N/A
Cricklewood	Juken NZ	Gisborne	2013	CP 12/207	CP 12/207	Suitable	Suitable	Suitable for RNC
Pinnacles	PF Olsen		2012	SSOP 10/216 (?)	SSOP 10/216 (?)	Suitable	Suitable	Not suitable
Waiau	Ernslaw One	Gisborne (Tokomaru Bay)	2014 – tbc	10/216	13/203	Not suitable	N/A	N/A
Waipaoa	Ernslaw One	Gisborne (Whatatutu)	2014 - tbc	10/216	13/203	Not suitable	N/A	N/A
Karioi	Ernslaw One	Ohakune	2014 - tbc	10/216	13/203	Not suitable	N/A	N/A
Harakeke	Ernslaw One	Wanganui	2014 - tbc	10/216	13/203	Not suitable	N/A	N/A
Ngaruru	Nelson Forests	Nelson	30/05/2014	10/216	13/203	Not suitable	N/A	N/A
Kohatu	Nelson Forests	Nelson	29/05/2014	10/216	13/203	Not suitable	N/A	N/A
Kings Ridge	Nelson Forests	Nelson	3/06/2014	10/216	13/203	Not suitable	N/A	N/A
Lismore	Rayonier	Wanganui	Jul-14	10/216	13/203	Not suitable	N/A	N/A

Trichoderma treatments and trial design

The two field trials that are anticipated to be screened for disease had seed from the same seedlot that was pre-treated with the same five *Trichoderma* treatments (Table 2). The results from these two trials, if both scored, will be able to be compared. If possible, results from the field trials will be compared to data from the lab-based trials.

Table 2. Trichoderma treatments and control used in the Cricklewood 2013 and XPKANG 2013 field trials.

Treatment	*Trichoderma* mixture	Isolate numbers used in mixture
T1	A (PBI)	LU132, LU140, LU584, LU633
T2	no. 11	FCC318, FCC319, FCC320, FCC322, FCC340
T3	no. 6 + 180	FCC13, FCC14, FCC15, FCC16, FCC180
T4	best 2012 trial	FCC362, FCC368, FCC49, FCC55
T5	2nd best 2012 trial	FCC333, FCC327, FCC410, FCC424
T6	control	untreated

Both field trials have the same statistical design and layout (see Appendix). It is unclear how many trees there are for each treatment but it appears there could be between 150-200 trees per treatment. This will need to be clarified when the field trials are visited and this will affect the disease screening method used.

Disease screening for red needle cast in field trials

Field disease screening protocols for RNC have been designed and used to screen mature host material, for example, the screening of a genetic trial in Wharerata[1]. In these trials the percentage of crown that was clearly identifiable as being affected by red needle cast, on needles that were alive at that time, was scored and the percentage of crown that was lost overall, where the cause of needle loss was no longer discernible, were also scored. This second assessment included scoring dead needles.

Whilst this method of screening is entirely appropriate for disease screening in mature trees, it is not expected to be a reliable method for screening younger material. In particular this is because it has been acknowledged, based on anecdotal field observations, that although younger material (less than three years old) can become infected, infection is often rare and appears to occur on sites with very high inoculum loading. In infected sites needle loss such as occurs in older material is not common. It also must be recognised that percentage differences in crown loss maybe small and may be difficult to see in younger, smaller trees[2] than in large mature trees. As both field trials suitable for disease screening were established in 2013 the material is all only in its third year of growth.

To ensure that disease screening methods are appropriate for the age of the material examined, and that methods that can be correlated with lab-based disease assessments already completed, the following RNC disease screening protocol for young plants (three years old or younger) with low disease levels have been developed.

On-tree visual assessments

Needle symptoms on a selection of trees will be assessed to confirm the presence of RNC and determine whether there are any symptoms of DNB. Due to the young age of the material, symptoms of cyclaneusma needle cast should not be present. If DNB is present this will be noted, however, it will be difficult to distinguish RNC symptoms from DNB during the entire tree assessments.

All trees in the field trial will be assessed by at least two, but preferably three assessors. The assessors will calibrate disease levels on ten trees. Each assessor will then determine the percentage of infected needles, on one side of each tree, in 5% increments from 0% through to 100% infection for the remainder of trees in the field trial. The same aspect will be assessed for each tree and each assessment will be done independently.

Assessments similar to these have been done with DNB and results (Table 3) have shown that while there was good consistency between assessors and the percentage infection at low infection rates matched well with the actual level of infection, this diverged at higher infection levels. The actual level of infection was determined by scoring the actual number of diseased needles per tree; the assessors in this experiment were all pathologists, with considerable experience in identifying DNB symptoms. This result matches other work that found standard errors were higher in trees estimated to have 40% DNB compared with those with 10% DNB[2]. In general, once the level of infection exceeded 30%, visual assessments overestimated percentage infection. As this could significantly skew results, a second form of assessment will be included and both methods will be used to determine infection levels in the field trials.

[1] Dungey, H S; Low, C B; Stovold, G T (2014) Genetic analysis of red needle cast at Coop road, Wharerata forest. Sidney Output 53809.

[2] van der Pas, J.B.; Kimberley, M.O.; Kershaw, D.J. 1984: Evaluation of the Assessment of Dothistroma Needle Blight in Stands of *Pinus radiata*. New Zealand Journal of Forestry Science 14: 3-13.

Table 3. Visual assessment of the percentage of dothistroma needle blight symptoms compared with actual disease levels in *Pinus radiata*.

	Percentage of crown infected					
Seedling	Assessor 1	Assessor 2	Assessor 3	Assessor 4	Mean	Actual
1	10	7.5	10	10	9.4	14.5
2	10	10	5	5	7.5	6.5
3	20	15	15	20	17.5	14.5
4	35	30	30	25	30.0	29.9
5	45	45	40	45	43.8	35.2
6	60	55	65	75	63.8	41.5
7	70	75	60	70	68.8	36.7
8	80	85	85	80	82.5	57.8

Needle lesion assessments

To help reduce bias from visual screening at higher infection levels and to provide a method where field results can be compared to lab-based trial results, needle lesion assessments will also be conducted.

One branch from each tree will be selected from within an area representative of average RNC symptoms in the crown. The branch will be placed into a plastic bag and stored on ice for assessment in the laboratory. Lesion assessments need to be completed within 36 hours otherwise desiccation could impact results.

As the number of trees present in each trial is not currently known, not all branches collected may be assessed. If possible all branches will be analysed, but this is dependent on the size of the trial. Due to the time constraints discussed above and the time taken to scoring the needles, a limit of 150 branches will be scored. The same selection method for determining which branches will be scored will be used for all treatments. The method used will be determined once the full number of branches collected is known and will be done the laboratory. It will not be based on the disease levels in the branches but on a randomised or replicable (i.e. every second trees is assessed) method.

Once the branches to be assessed have been determined, ten fascicles, with the bases intact, will be harvested from each branch. To select the ten needles to assess, an area on each branch where disease symptoms are present will be located and the needles to be assessed will be randomly selected from this area. The number of needles present per fascicle, the length of each lesion on each individual needle, and the total number of lesions per needle will be recorded. Due to the inherent subjectivity when counting lesions, the number of independent scorers for *P. pluvialis*-infected needles will be limited to ensure consistent lesion scoring. All needles will be scored within an 8 hour time frame, where possible. If scoring cannot be

completed within one day, needles will be scored on the following day and all needle assessments will be completed within 36 hours.

Disease confirmation

To confirm the disease symptom expression observed has been caused by the causal agent of that disease, needles will be assessed for pathogen presence. For RNC symptoms, needles will be surface sterilised with 70% ethanol for 30 sec, and rinsed twice with sterile distilled water. After blotting dry, the needle sections were plated onto PARP agar and incubated at 17°C for 5-7 days before assessment. Needles from at least two trees per treatment per replicate will be assessed.

Data preparation and analysis

The average lesion length per fascicle will be calculated by computing the average per 10 fascicles per tree. In order to analyse the data as a proportion of fascicles with lesions, a binary response variable will first be created by calculating the number of fascicles with lesions per tree. This information will be used to construct a two-column matrix holding the number of 'successes' (fascicles with lesions) and the number of 'failures' (fascicles without lesions) as response variable for a binomial regression model. For an analysis of the percentage of affected fascicles, any fascicle with at least one needle showing lesion development will be considered to have lesions. The data will be analysed for significant differences in disease symptom response between the *Trichoderma* treatments and the controls, using both average lesion length and number of lesions per fascicle. The high level of genetic diversity of the seedlot used will be considered when interpreting the results.

Results from the branch assessments will be compared with results obtained for the whole tree evaluations, in particular for entire tree assessments showing disease levels above 30%.

Where possible, results from the field trials will be compared to lab-base trial results to evaluate the performance of the *Trichoderma* treatments.

Conclusions

Review of the *Trichoderma* treatment field trials that have been established across New Zealand haven shown that three of the trials have suitable design and replication for statistical analysis. Of these three sites, one is in an area that has a high incidence of red needle cast and depending on disease levels in 2016, a second site may also be suitable for disease assessments.

The small number of field trials available for assessment means that previously planned cross-site-location comparisons of *Trichoderma* treatment performance will no longer be able to be made. However, if both sites are scored then pairwise comparisons could be made as both field trials have the same *Trichoderma* treatments and layouts.

Methodology for assessing symptoms of red needle cast in both trials are provided. These methods have been prepared for scoring disease levels in material less than three years old.

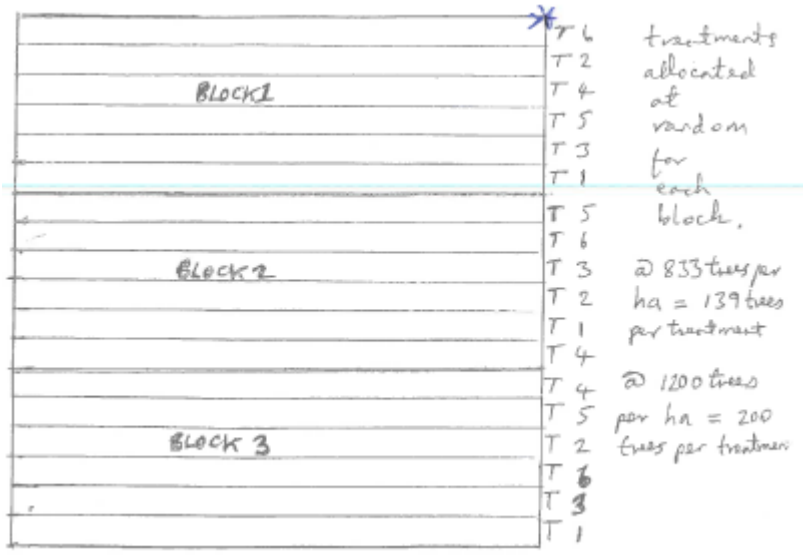
Acknowledgements

Biometricians Mark Kimberly and Martin Bader (Scion) provided statistical advice on the field trial layouts and analysis. Forest pathologist Lindsay Bulman (Scion) provided advice on field trials assessments and design. Robert Hill (Bio-Protection, Lincoln) provided the Forestry Trials Database. Wei-Young Wang (PF Olsen) and the Radiata Pine Breeding Company provided information on the seedlots used in the field trials.

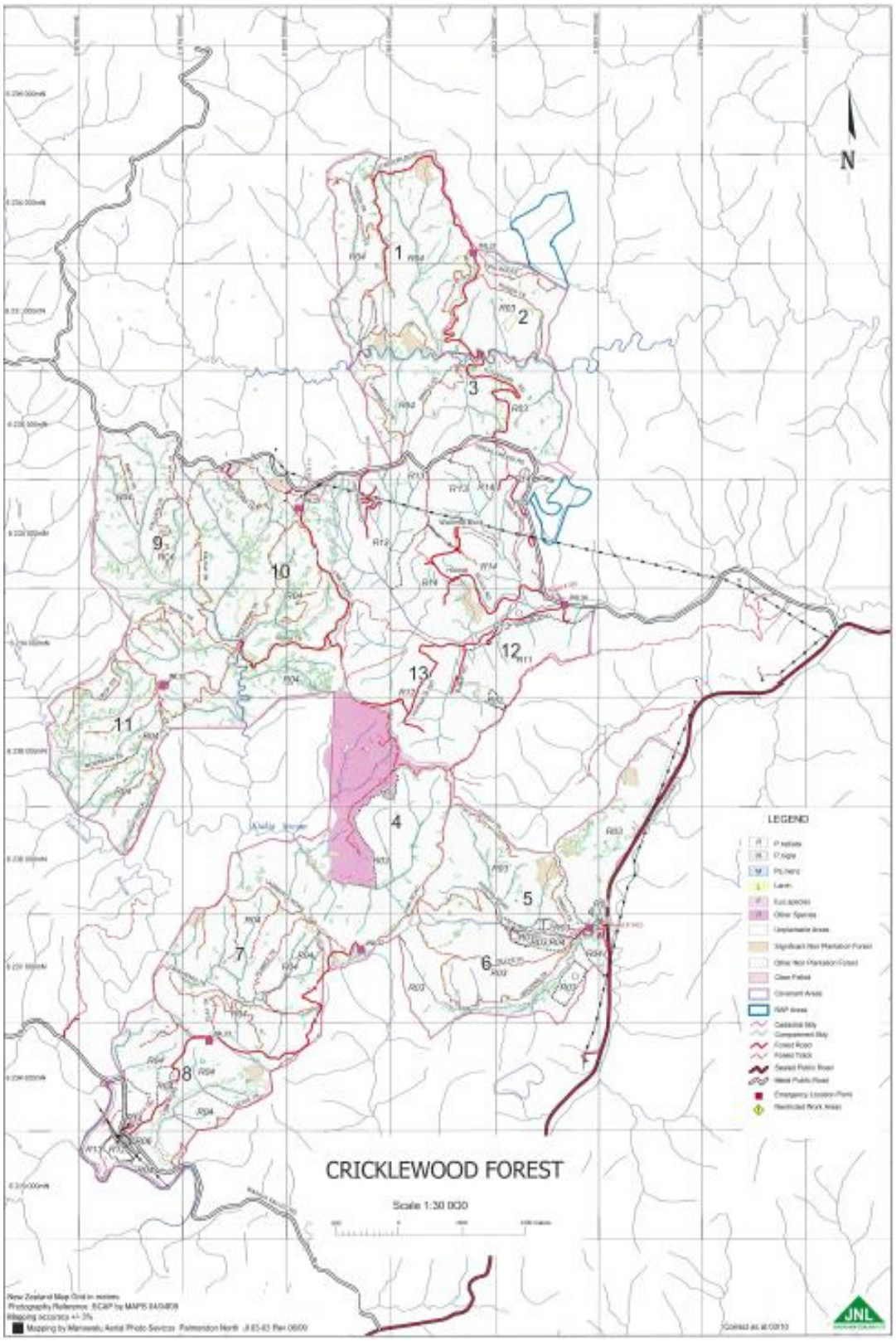
Appendix

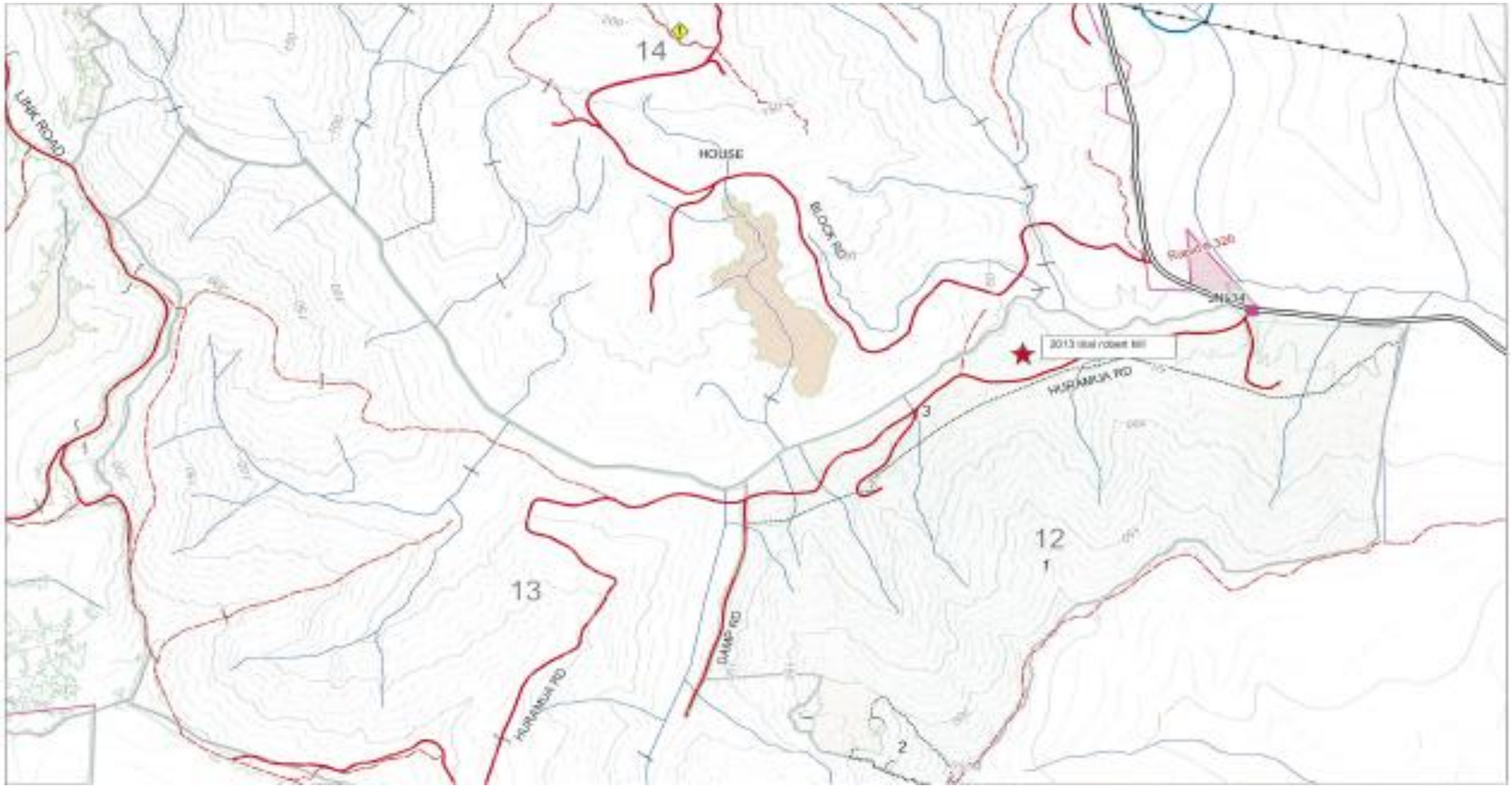
Trial design

This design was used for both trials, Cricklewood 2013 and XPKANG 2013, which will be scored for disease symptom responses.



Field trial Cricklewood 2013

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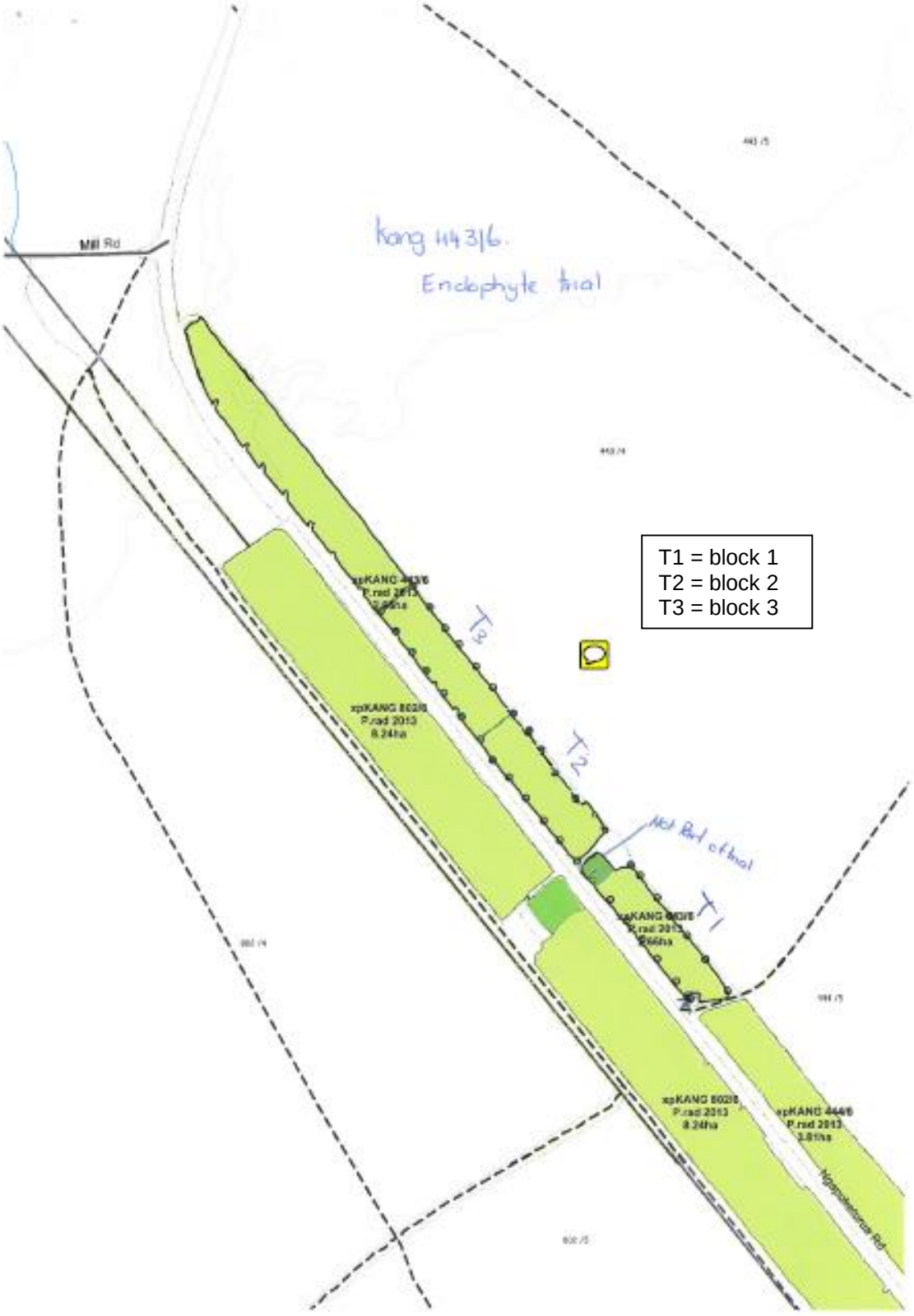
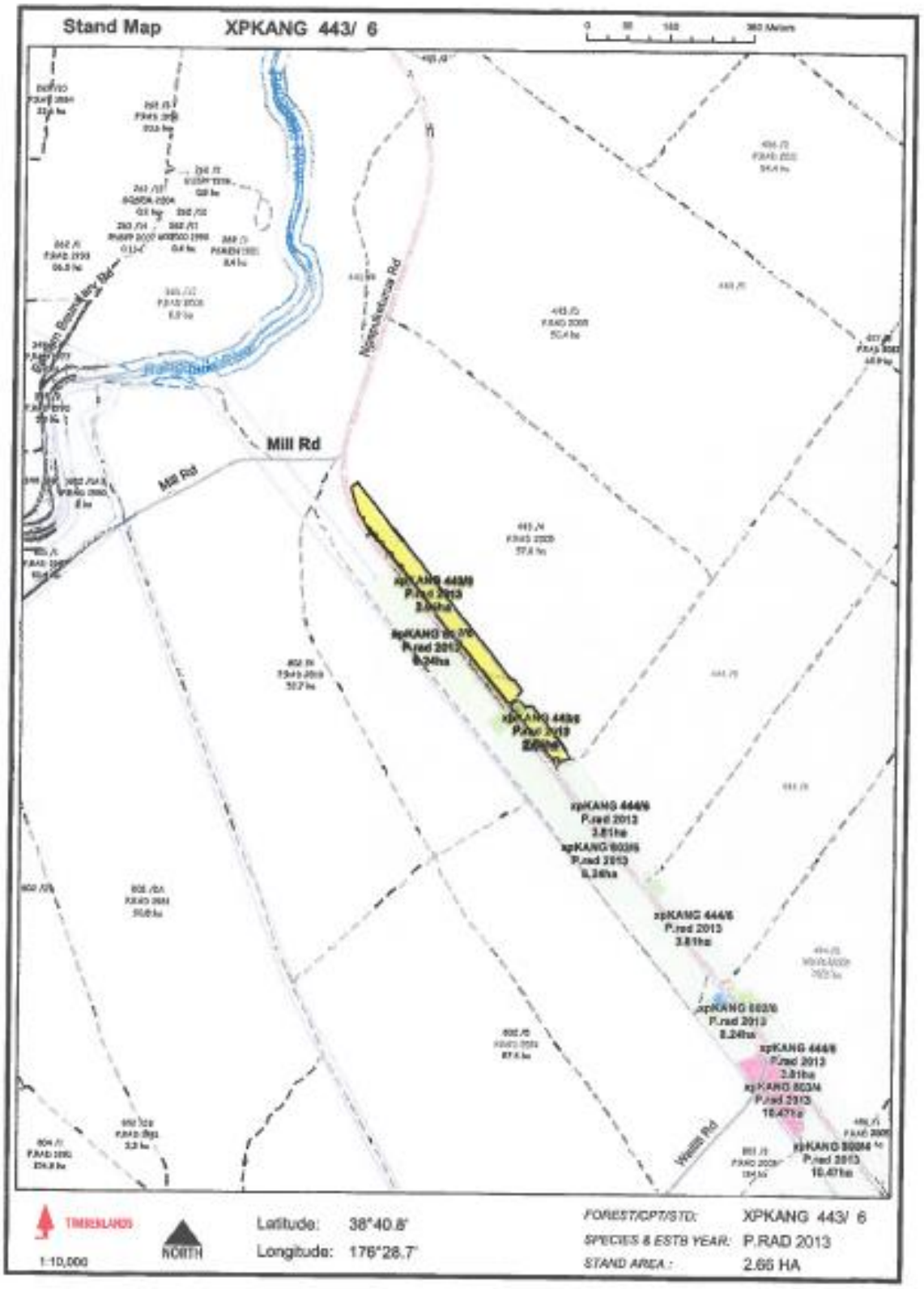
Field trial XPKANG 2013





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