Annual Report

FY 2022-2023

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NATIONAL PLANT PROTECTION CENTRE

DEPARTMENT OF AGRICULTURE

MINISTRY OF AGRICULTURE & LIVESTOCK

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Contents

FOREWORD

The National Plant Protection Centre is pleased to publish the centre's achievements for the fiscal year 2022-2023, a comprehensive reflection of the centre's unwavering commitment to advancing plant protection services and fostering sustainable agricultural practices in Bhutan.

At the heart of our mission is the relentless pursuit of excellence in plant protection. As a leading institute and apex body in this domain, our focus on research, diagnostics, and technology promotion has enabled us to play a pivotal role in effective pest management within the realm of agriculture. Guided by the principles of integrated pest management (IPM), our endeavors are driven by a synergy of knowledge, innovation, and collaborative spirit.

This report encapsulates a year of dedicated efforts from our exceptional team, showcasing the impactful outcomes achieved through the convergence of intellect and hard work. Our six technical programs, backed up by an unwavering administrative team, have worked tirelessly to uphold the values of the Centre in extending invaluable support to our farming community. By mitigating crop loss due to pests, we are making substantial strides towards securing our nation's food and nutrition goals.

The activities undertaken during FY 2022-2023 have been nothing short of remarkable achievement, from enhancing laboratory diagnostics to conducting rigorous field research, from demonstrating cutting-edge technologies to training extension agents, every initiative has been meticulously executed to meet our Centre's objectives. These collective endeavors have significantly contributed to managing and mitigating the impact of critical agricultural pests, including the fall armyworm, citrus HLB, rice blast, chilli blight, early and late blight of potato, apple scab, wheat rust, agricultural weeds, and vertebrate pests.

As you go through the following pages, I trust you will share in our pride and enthusiasm for the strides we have made together. The technical narratives within this report not only capture the essence of our achievements but also underscore our determination to drive positive change that creates a resilient agricultural landscape that is safe for both humans and the environment. All these pursued relentlessly despite the shortcomings in resources both financially and human resources.

I extend my deepest gratitude to our dedicated team, partners, and stakeholders who have been instrumental in our journey thus far. Your unwavering support and collaborative spirit inspire us to continue reaching for new horizons. Together, let us embrace the future with renewed vigor, knowing that our efforts today will shape a more secure and prosperous tomorrow.

Trashidelek!

Yeshey Dema

PROGRAM DIRECTOR

EXECUTIVE SUMMARY

The National Plant Protection Centre (NPPC) is designated as a national referral and coordinating authority for information, policy and activities related to plant protection services. The Centre is also mandated to develop and disseminate integrated pest management (IPM) strategies in managing pests in agricultural and horticultural cropping systems. The NPPC presents its 2022-2023 Annual Report to highlight major activities and achievements for the fiscal year. These activities were planned and implemented by the six technical programs, viz, Entomology, Pathology, Weeds, Vertebrate Pest Management, Pest Surveillance, Plant Protection Product and the National Organic Flagship Program. Some of the major achievements of these programs are highlighted below.

The Entomology program successfully devised field management strategies (variety and insecticide evaluation) against the new and invasive pest, fall armyworm (FAW) through validation of initial findings of previous seasons. Further, NPPC is currently evaluating FAW-tolerant varieties sourced from the International Maize and Wheat Improvement Centre (CIMMYT).

The Pathology program made significant progress in the work with the native *Trichoderma* isolates with results from the greenhouse experiments showing equal effectiveness in suppressing *P. capsici* infection on chili compared to the isolates from Indian and Thailand. Field trails are underway to further optimize the use of this biocontrol agent in Bhutan. The program also developed five disease diagnostic protocols as SOPs. Disease management and diagnostic services were also provided.

The Weeds Science program sourced a plant-based ready-to-use bio-weedicide from India. A multi-location field evaluation trials have shown a positive effect in controlling a range of weed species. Further trials are being planned to obtain robust data set in order to recommend its use in the field. A survey on farmer's perception on resistance of Butachlor to weeds of paddy, an inventory of plants with pesticidal properties in the country, and an inventory of invasive plant species and important agricultural weeds were conducted.

The Pest Surveillance program conducted observation study to confirm the rice blast development and studied field surveillance of rust incidence on a local and an improved wheat variety.

The Vertebrate Pest Management program conducted comparative study of Ultrasonic Monkey Repellent Devices in mitigating crop damages, and Earth Electrodes for Electric Fencing Systems. The National Organic Flagship Program constructed a new biocontrol laboratory at NPPC.

The Plant Protection Product program successfully procured and supplied the required PP products for the 2022-2023season. The Program procured a total quantity of 671.98 MT of Plant Protection Products, including the organic products. Out of this total, approximately 355 MT were supplied to the Dzongkhags in the current fiscal year.

1. Entomology Program

1.1 Field evaluation of African and Bhutanese maize varieties against Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) Larvae

1.1.1 Introduction

The fall armyworm (FAW) is a highly polyphagous pest that damages more than 350 plant species across 76 plant families (Montezano et al., 2018). Maize and sorghum are highly susceptible to FAW damage as these plants are the primary hosts (Casmuz & Juárez, 2010). The average fecundity of one FAW female is 1000 eggs (Murúa et al., 2006) and is capable of flying over 100 km per night (Song et al., 2020). Additionally, FAW does not diapause, enabling it to migrate to new places with suitable environmental conditions(Du Plessis et al., 2020). In Bhutan, FAW was first observed damaging maize in Dabchagang and Pepchu, Guma Gewog and Mendugang in Dzomi Gewog under Punakha Dzongkhag in September 2019 (Mahat et al., 2021). Currently, FAW has been detected in eight Dzongkhags (Chukha, Punakha, Paro, Thimphu, Mongar, Dagana, Wangdiphodrang and Sarpang), primarily damaging maize crops. It is likely that the FAW strain present in these areas is the "corn" strain (C), considering the predominant damage to maize.

Host plant resistance (HPR) an integral part of Integrated Pest Management program (Kumari et al., 2022). HPR involves using resistant cultivars to reduce pest damage (Stout, 2014). Resistant varieties often possess traits such as thicker cuticles, the presence of trichromes and compact stature. Some plants also produce toxic compounds such as alkaloids, phenols, or terpenes that repels insect pests (Divekar et al., 2022). Furthermore, resistant varieties typically yield higher than the susceptible varieties (Smith, 2021). The use of resistant maize varieties can crucial strategy in managing this voracious pest (Prasanna et al., 2018). The International Maize and Wheat Improvement Center (CIMMYT) has recently developed three elite maize hybrids (FAWTH2001, FAWTH2002, and FAWTH2003) that are tolerant to FAW for Eastern and Southern Africa. In Bhutan, the resistance of commonly grown maize varieties such as Shafangma, Bhur Ashom, Yangtsipa, Wengkhar hybrid, and Chaskarpa to FAW has not been assessed, although field reports suggest their susceptibility to FAW damage. The primary objective of the study was to evaluate the resistance of four Bhutanese maize varieties and three African FAW tolerant maize hybrids varieties. Leaf damage by FAW larvae served as an indicator of resistance to FAW in these maize varieties.

1.1.2 Materials and methods

Maize seeds

For this study, the centre used three African FAW tolerant maize hybrids (FAW2001, FAW2002, and FAW2003) and four Bhutanese maize varieties (Yangtsipa, Wengkhar hybrid 1, Bhur Ashom, and Shafangma). The seeds of the three African hybrids were sourced from The International Maize and Wheat Improvement Center (CIMMYT) in Africa, while the seeds of the Bhutanese varieties were sourced from the Agriculture Research and Development Centre, Wengkhar.

Study site

The study was conducted in two terraces at Sonamthang (Kothiline), Samphelling Gewog under Chukha Dzongkhag (26.83095, 89.452383, 352 m) from February to May 2023. The Agriculture Extension officer selected the site based on previous history of FAW damage. The study site falls within a sub-tropical region characterized by hot and humid summer months and warm, dry winter months.

Field preparation, seed sowing and weeding

The land was thoroughly ploughed and divided into 21 plots of 12 m^2 each, with a 60 cm gap between the two plots for ease of movement. Seeds were soaked in water for 24 hours prior to sowing. Plant to plant and row to row distance were maintained at 40cm *40 cm and 50cm* 50cm, respectively. Holes were created using a wooden dribbler, with three seeds in each hole. Seed germination was monitored fortnightly, and plots without germinated seeds were replated. The first weeding occurred three weeks after crop emergence, followed by a second weeding four weeks after the first, depending upon the weed pressure. Thinning was performed at the "knee-high" stage to maintain an optimum plant population of forty plants per plot for leaf damage assessment.

Treatments, Design and Randomization

The trial is arranged in a Randomized Complete Block Design (RCBD) with each variety replicated thrice. Treatment randomization was conducted using STAR (Software Tools for Academics and Researchers).

Data collection: leaf damage

Leaf damage caused by FAW was assessed visually using the Davis scoring scale from 0 to 9 (Davis et al., 1992) with a total of fifteen random plants evaluated per plot (**Table 1**). Leaf damage assessment commenced when the first signs of FAW damage were observed on the maize plants and continued fortnightly until maturity. During each sampling period, two or three leaves emerging from the funnel/whorl were assessed for leaf damage.

Table 1: Davis' 0 to 9 damage scale for the fall armyworm

Scale description	Description
0	No visible leaf damage
1	Only pinhole damage on leaves
2	Pinhole and shot hole damage to leaf
3	Small elongated lesions (0.5–1 cm) on 1–3 leaves
4	Mid-Sized lesions (1 cm–3 cm) on 4–7 leaves
5	Large elongated lesions (>3cm) or small portions eaten on 3–5 leaves
6	Elongated lesions (>30 cm) and large portions eaten on 3–5 leaves
7	Elongated lesions (>30 cm) and 50% of leaf eaten
8	Elongated lesions (30 cm) and large portions have been eaten on 70% of leaves
9	Most leaves with long lesions and complete defoliation were observed

1.1.3 Result and discussion

All evaluated varieties exhibited laef damage scores ranging from 2 to 3 across four observation periods, indicating varying degrees of resistance to FAW infestation (**Table *2***). FAWTH 2001, FAWTH 2003, Wengkhar hybrid, and Shafangma exhibited resistance, while FAWTH 2002 and Yangtsipa displayed partial resistance to FAW. A Davis score of 2 corresponds to damage symptoms characterised by both "pinhole and shot hole" patterns on the leaves. Conversely, a Davis score of 3 is associated with the presence of "small elongated lesions" measuring between 0.5 to 1 cm on the leaves. Additionally, the current study highlights that FAW causes damage at all stages of maize growth, as shown in ***Figure 1***. This findings aligns with the observations made by De Groote *et al*. (2020), who reported that FAW damage occurs at all vegetative stagse of maize plant.

Table 2: Leaf damage severity score of different varieties

Variety	Variety type	Observation				Pooled mean	Pooled mean (round off to 2 decimal place)
		I	II	III	IV		
FAWTH 2001	Hybrid	2.57^{bc}	3.08^{bc}	2.91^{bc}	1.51^{bc}	2.44^{a}	2
FAWTH 2002	Hybrid	3.23^{c}	3.40^{c}	1.71^{c}	1.97^{c}	2.58^{a}	3
FAWTH 2003	Hybrid	2.71^{bc}	1.82^{bc}	2.24^{bc}	1.40^{bc}	2.10^{ab}	2
Yangtsipa	OPV	0.88^{a}	2.08^{a}	3.40^{a}	3.73^{a}	2.50^{a}	3
Wengkhar hybrid	Hybrid	0.60^{a}	0.93^{a}	3.06^{a}	2.60^{a}	1.79^{b}	2
Shafangma	OPV	2.31^{b}	2.33^{b}	2.51^{b}	2.22^{b}	2.34^{ab}	2
P value	-	P<0.001	P<0.001	P<0.001	P<0.001	P< 0.001	-
C.V.%	-	51.64	39.16	23.15	38.15	12.87	-

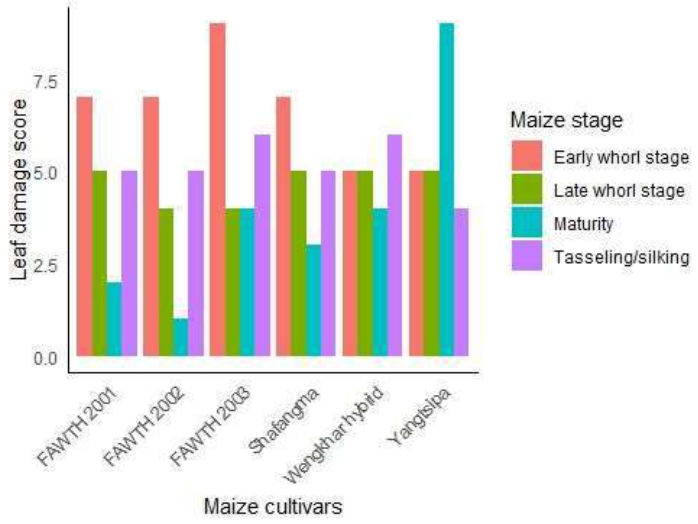

Figure 1 Leaf damage by FAW across different maize stages

1.1.4 Conclusion

The evaluation of various maize varieties showed varying degrees of resilience against FAW infestation. Notably, FAWTH 2001, FAWTH 2003, Wengkhar hybrid, and Shafangma exhibited resistance, while FAWTH 2002 and Yangtsipa displayed a degree of partial

resistance. Furthermore, this study highlights that FAW induced damage occurs throughout all stages of maize plant growth.

1.1.5 References

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1.2 Evaluation of different synthetic and botanical pesticides against fall armyworm *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae)

1.2.1 Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) is a voracious polyphagous pest known to damage over 300 plant species across 76 plant families (Montezano et al., 2018). The higher damage is found in maize and sorghum which are their primary hosts (Casmuz et al., 2010). This invasive pest was first reported in Nigeria in 2016 and subsequently spread to Kenya, Uganda, Rwanda, Tanzania and Ethiopia (Goergen et al., 2016). Similarly, it arrived in the Indian subcontinent in May 2018 and since then has spread across India, Nepal, Bangladesh, Sri Lanka and beyond Southeast Asia (Lamsal et al, 2020).

In Bhutan, FAW was first detected in maize fields in the western part of the country in September 2019 and subsequently was found infesting maize crops in southern parts of the country in December 2019 and April 2020 (Mahat et al., 2021). In Africa, FAW has been reported to cause annual losses ranging from 8.3 MT to 20.6 MT of maize, accounting for approximately 20 per cent of the total production (Day et al., 2017). While there were limited reports of FAW infestation in Bhutan till 2020. However, 2021 witnessed a surge in reports of FAW affecting maize in major maize-growing areas in Bhutan, such as Phuentsholing, Trongsa, Trashigang, Mongar and Zhemgang. The pest's abiity to cover long distances by flying about 100km per night covering about 500 km in a single season (Johnson, 1987) suggests its potential to rqpidly spread and establish in new maize growing areas across Bhutan.

A field study conducted in 2021 indicated that the commonly used insecticide in Bhutan, Cypermethrin 10% EC, was ineffective against FAW. Subsequently, a study in 2022 demonstrated the effectiveness of the new generation insecticides: Chlorantraniliprole 18.5%, Emamectin benzoate 5% SG, Emamectin benzoate bait against FAW. The current study was designed to validate the results of the 2022 study. The expected outcome of this study is to identify and promote the effective insecticide for managing this invasive pest.

1.2.2 Materials and methods

Study site and design

The trial was conducted at Dzomlingthang under Guma Gewog in Punakha Dzongkhag. The trials were laid in a Randomised complete block design (RCBD) with five treatments, each replicated four times. Each plot measured 16 m^2 (e.g., 4m by 4m) to ensure uniform plant number per plot. Maize seeds of Yangtsipa variety were line sown to maintain a consistent plant spacing of 40 cm by 40 cm, with and 50 cm by 50 cm row spacing. A half-metre gap was left between plots to facilitate movement.

Insecticides

Chlorantraniliprole, Emamectin benzoate, Emamectin benzoate bait and neem oil were evaluated at the ratespecified in **Table *3***. Chlorantraniliprole, Emamectin benzoate, and Neem

oil were sprayed using power spray, while Emamectin benzoate bait was manually applied to individual plants. The treatments were administered at 14 days intervals during the early morning.

Table 3: Dilution rates of insecticides used in the trial.

Insecticide	Dilution rate
Chlorantraniliprole 18.5% SC	3ml/10 litres of water
Emamectin benzoate 5% SG	2 gm /10 litres of water
Emamectin benzoate bait	1kg corn flour mixed with 200 gm of Sugar and 2gm of Emamectin benzoate (5-10 gm of the mixture inside the whorl of the plant)
Neem oil (10,000 ppm)	5ml/litre water

Data collection

Fifteen randomly selected plants were used to redord the count of loive larvae and assess leaf damage. These assessments were conducted both prior to and at 7 and 15 days after each application of insecticide. Leaf damage was assessed visually using the Davis scoring scale, which ranges from 1 to 9 (Davis et al., 1992) ***Table 1***. The initial insectide application was carried out upon the detection of FAW in the research plots. During each sampling period, observations were made on two or three leaves emerging from the funnel/whorl for the presence of live larvae and the extend of leaf damage.

1.2.3 Result

Larval population (No of larvae/15 plants)

The distribution of larvae in the research plots was not homogenous before the spraying of insecticides, as shown in ***Table 4***. Following the first spray, plots treated with Chlorantraniliprole 18.5% SC showed the lowest larval count (0.60 larvae/plant), followed by Emamectin benzoate 5% SG (0.72 larvae/plant) and Emamectin benzoate-Corn flour bait (0.86 larvae/plant). However, there were no statistically significant differences observed between these treatments. The non-treated control plots (1.46 larvae/plant) and neem oil treated plots (1.25 larvae/plant) recorded the highest larval counts, but these variations were not statistically significant.

After the second spray, the Chlorantraniliprole 18.5% SC-treated plots showed marginally lower larval counts compared to the non-treated control plots. However, the differences between this treatment and the other treatments were not statistically significant. Following the third spray, larval counts were lower in insecticides treated plots compared to the non-treated control plots, although the difference between the insecticides was not statistically significant. Subsequent to the fourth spray, Chlorantraniliprole 18.5% SC-treated plots (0.01 larvae/plant) had the lowest larval count compared to other treatments.

In summary, when considering the aggregated data from the four spray applications, Chlorantraniliprole 18.5% SC-treated plots consistently showed significantly lower larval counts (0.50 larvae/plant) than neem oil treated plots (1.59 larvae/plant) and non-treated control plots (2.18 larvae/plant). However, no significant difference was observed with Emamectin benzoate 5% SG and Emamectin benzoate-Corn flour bait treated plots.

Table 4: **Effect of insecticides against FAW larvae (pooled over sprays)**

Treatment	Before spray	First spray			Second spray			Third spray			Four spray	Pooled over sprays	Reduction in larval population over control (after four sprayings) (%)
		1 WAS	2 WAS-	Pooled mean	1 WAS	2 WAS	Pooled mean	1WAS	2 WAS	Pooled mean	1 WAS		
Chlorantraniliprole 18.5% SC	0.88[a]	1.05[a]	0.15[a]	0.60[a]	0.28[a]	0.70[a]	0.49	0.11[a]	1.20[a]	0.65[a]	0.01[a]	0.50[a]	95.56
Emamectin benzoate 5% SG	1.03[a]	1.10[a]	0.35[a]	0.72[a]	1.20[a]	0.60[a]	0.9	0.63[a]	1.15[a]	0.89[abc]	0.75[b]	0.82[ab]	81.20
Emamectin benzoate-Corn flour bait	2.76[ab]	0.93[a]	0.80[a]	0.86[a]	4.73[a]	1.16[ab]	2.95	0.13[a]	0.65[a]	0.39[ab]	0.61[b]	1.29[ab]	25.84
Neem oil (1500 ppm)	5.25[b]	0.83[a]	1.66[b]	1.25[b]	4.05[a]	1.91[b]	2.98	1.13[b]	1.03[a]	1.08[abc]	0.55[b]	1.59[b]	36.55
Control	4.80[b]	1.10[a]	1.83[b]	1.46[b]	6.38[a]	1.53[ab]	3.95	2.36[c]	1.53[a]	1.95[c]	0.53[b]	2.18[b]	-
P value	0.001	0.530	0.001	0.0001	0.105	0.014	0.037	0.001	0.84	0.002	0.0001	0.0002	
C.V.%	69.32	11.65	79.26	37.15	76.01	46.95	65.95	106.32	28.63	59.62	53.71	51.34	

Note: Treatment means with the same letter are not significant are not significantly different at $p \geq 0.05$
WAS* week after spraying.

Leaf damage

The distribution of leaf was not uniform across the research plots prior to insecticide spray, ***Table 5***. Folowing the first spray, plots treated with Chlorantraniliprole 18.5% SC exhibited significantly less leaf damage compared to those plots treated with neem oil and the control plots, although no significant difference was observed when compared to plots treated with Emamectin benzoate 5% SG and Emamectin benzoate-Corn flour bait treated plots. After the second spray, Emamectin benzoate-Corn flour bait treated plots exhibited the lowest leaf damage compared to Emamectin benzoate 5% SG, neem oil, and control plots. However, this difference was not statistically significant with Chlorantraniliprole 18.5% SC treated plots. After the third spray, Chlorantraniliprole 18.5% SC and Emamectin benzoate-Corn flour bait recorded the lowest leaf damage compared to Emamectin benzoate 5% SG, neem oil, and control. There was no significant difference in leaf damage after the fourth spray.

Overall, when considering data from all ings, it is evident that Chlorantraniliprole 18.5% SC and Emamectin benzoate-Corn flour bait treatments consistently showed the lowest leaf damage in comparison to other treatments. Control plots and neem oil treated plots consistently had the highest level of leaf damage.

Table 5: Effect of insecticides on leaf damage by FAW (pooled over sprays)

Treatment	Before spray	First spray			Second spray			Third spray			Fourth spray	Pooled over sprays	Reduction in leaf damage over control after four spraying (%)
		1 WAS	2 WAS	Pooled mean	1 WAS	2 WAS	Pooled mean	1WAS	2 WAS	Pooled mean	1 WAS		
Chlorantraniliprole 18.5% SC	1.18^{a}	3.73^{a}	1.28^{a}	2.51^{a}	1.68^{a}	0.72^{a}	1.20^{ab}	0.97^{a}	0.30^{a}	0.63^{a}	0.00^{a}	1.24^{a}	66.14
Emamectin benzoate 5% SG	0.88^{a}	3.75^{a}	1.65^{a}	2.70^{ab}	2.15^{ab}	1.05^{ab}	1.60^{a}	1.53^{a}	0.97^{b}	1.25^{b}	0.03^{a}	1.59^{b}	56.59
Emamectin benzoate-Corn flour bait	0.82^{a}	3.97^{a}	1.67^{a}	2.82^{ab}	1.02^{c}	0.75^{ab}	0.88^{b}	0.90^{a}	0.13^{a}	0.52^{a}	0.02^{a}	1.21^{a}	67.05
Neem oil (1500 ppm)	2.22^{b}	3.75^{a}	2.72^{b}	3.23^{bc}	1.97^{ab}	2.12^{c}	2.04^{c}	3.08^{b}	2.07^{c}	2.58^{d}	0.05^{a}	2.25^{c}	38.59
Control	4.33^{c}	3.73^{a}	4.17^{c}	3.95^{c}	4.67^{d}	4.45^{d}	4.56^{d}	4.78^{c}	3.83^{d}	4.31^{c}	0.02^{a}	3.66^{d}	-
P value	<0.001	0.78	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.785	<0.001	
C.V.%	78.35	2.67	51.13	18.84	60.65	86.86	71.20	73.91	104.72	85.92	81.44	51.51	

Note: Treatment means with the same letter are not significant are not significantly different at $p \geq 0.05$
WAS* week after spraying

1.2.4 Discussion and conclusion

The current study has clearly shown that Chlorantraniliprole 18.5% SC and Emamectin benzoate-Corn flour bait represent the most effective methods for reducing the population of FAW larvae and minimising leaf damage in maize crops. These findings align with the results of previous research studies conducted by (Sangle et al., 2020) and (RK et al., 2020), both of which also substantiated the efficacy of Chlorantraniliprole 18.5% against FAW. Furthermore, the current findings are consistent with the results from 2022, where Chlorantraniliprole 18.5% SC and Emamectin benzoate 5% SC were found to be effective in controlling FAW infestations. However, it is worth noting that the 2022 study did not include the evaluation of Emamectin benzoate-Corn flour bait. Based on the combined evidences stemming from the present study and the 2022 research, it is now strongly recommended to use Chlorantraniliprole 18.5% SC, Emamectin benzoate 5% SC, and Emamectin benzoate-Corn flour bait as a preferred strategy for managing FAW infestations in Bhutan.

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1.3 Monitoring of fall armyworm Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) using sex pheromone traps

1.3.1 Introduction

The fall armyworm *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), originally native to tropical and subtropical regions of the Americas, has rapidly spread to other continents, including African continent (in 2016), and Asia with subsequent reports from the Indian sub-continent, Japan, China, Thailand and the Philippines (Rwomushana, 2023). Its presence in many parts of Asia is particularly conrcerning due to the favourable climatic conditions and abundance of suitable hosts, which suggests the pest may becomic endemic in these regions (Rwomushana, 2023). In Bhutan, this invasive pest was first detected in maize fields at Dabchegang (Punakha) in September 2019, with subsequent infestations reported in other parts of the country (Mahat et al., 2020). Currently, synthetic pesticides are predominantly used to manage using sy this pest in Bhutan, which raises concerns about the potential misuse of pesticides by resource resource-poor farmers.

Accurate information regarding the timing and location of adult pest activity plays a crucial role in establishing an effective early warning system, enabling timely field sampling and control measures (Cruz et al., 2012). Following this initial detection, the FAW has been documented causing significant damage in various maize-growing areas of Bhutan (National Plant Protection Centre [NPPC], 2022). However, there is limited documentation on the economic impact, behavior, and ecology of the FAW in the country.

Pheromone trapping stands as an efficient method for detecting the presence and population growth of FAW in specific areas (Mitchell et al., 1985). These pests release sex pheromones by exposing their last abdominal segments (Cruz-Esteban et al., 2017) which triggers mating behavior in males (Jacobson et al., 1970). Leveraging this sexual communication between male and female mot pheromone can be used for pest management by monitoring, mass trapping, or mating disruption (Carde & Minks, 1995; El-Sayed et al., 2009; Witzgall et al., 2010). Synthetic compounds that mimic natural FAW pheromones, often referred to as "lures," are placed in traps to attract and trap male moths. This method allows for the detection of pest presence or absence in a given area, eliminating the need for time-consuming sampling or unnecessary insecticide application (Cruz et al., 2010). Further, the performance of the insecticide can be enhanced by using the pheromone traps (Cruz et al., 2012), reducing the toxic exposure of the farmers (Hunt et al., 1999) and minimizing environmental damage (Tinoco-Ojanguren & Halperin, 1998).

The main objective of this study is to detect the presence and monitor the population growth of FAW in the four major maize growing regions of Bhutan using sex pheromone traps. This study also evaluates the efficiency of the various commercially available lure for trapping of FAW male moths. The long-term goal of this monitoring initiative is to create a comprehensive map FAW distribution in Bhutan.

1.3.2 Materials and Methods

The study was conducted using six lures ***Figure* 2** at four distinct locations: Khempaithang and Sonamthang in Sampheling Gewog under Chhukha Dzongkhag; Dabchegang in Guma Gewog under Punakha dzongkhag, and Gakidling and Singye gewogs under Sarpang dzongkhag. At Khempaithang, the lures manufactured by Gaiagen Technologies Private Limited (PCI), India was used. For Sonamthang and Dabchegang, the lures manufactured by Pherobank BV, the Netherlands were used. In Sarpang, lures from both Gaiagen Technologies Private Limited, India and Pherobank BV, the Netherlands were used.

Figure 2 Different sex pheromone lures used for the study

Funnel traps were used for trapping and capturing the moths **Figure *3***. These traps were positioned with a 50 m separation from one another within the field, at a height of 1.5m (FAO, 2018). The height of the traps was subsequently adjusted to align with the growth stage of the maize crop, ensuring that the base of the trap always remained above the top of the maize plants. Lures were replaced on a fortnightly basis to maintain their effectiveness, and gloves were used to prevent any contamination of the lures.

Figure 3 Funnel traps used for trapping FAW moths

Data collection

Monitoring activities were initiated in February and continued until the maturity of maize crops cultivated at the selected sites. Every two weeks, data from the traps were collected, focusing on FAW months, FAW-suspected moths, and other species. These collected species were identified and counted at the Entomology Laboratory, with a specific focus on counting

FAW moths. The data collection process used the Epicollect5 application (https://five.epicollect.net/). Subsequently, the data was arranged and analyzed using MS Excel 2019. Mean trap catches for FAW were calculated, and population trends were analyzed, with results presented as means of traps irrespective of differences in lures.

To evaluate the efficiency of the various commercial lures, the means were transformed using $\log_{10}(x+0.5)$ (Yamamura et al., 2006). The transformed data were subjected to ANOVA and post-hoc test Tukey LSD using STAR (Statistical Tool for Agricultural Research, IRRI). The trapping efficiencies of the lures were compared across different months, while the overall trapping efficiency was compared using the pooled means.

1.3.3 Result and discussion

Population trends

The population trends displayed variaitons across different study areas, **Figure *4***. However, it is noteworthy that the moth populations reached their peak around the same time in various study locations. From this study, it can be observed that the peak season for FAW falls in April, regardless of the specific crop stages in these different study locations. The fluctuations in FAW moth populations within a season can be attributed to factors such as the availability of preferred hosts and the local or regional movement of different strains (Meagher et al., 2013). Therefore, further follow-up study maybe necessary to delimit the presence of rice strain in Bhutan, eventhough initial reports categorise FAW in Bhutan as the 'corn-strain' (Mahat et al., 2020).

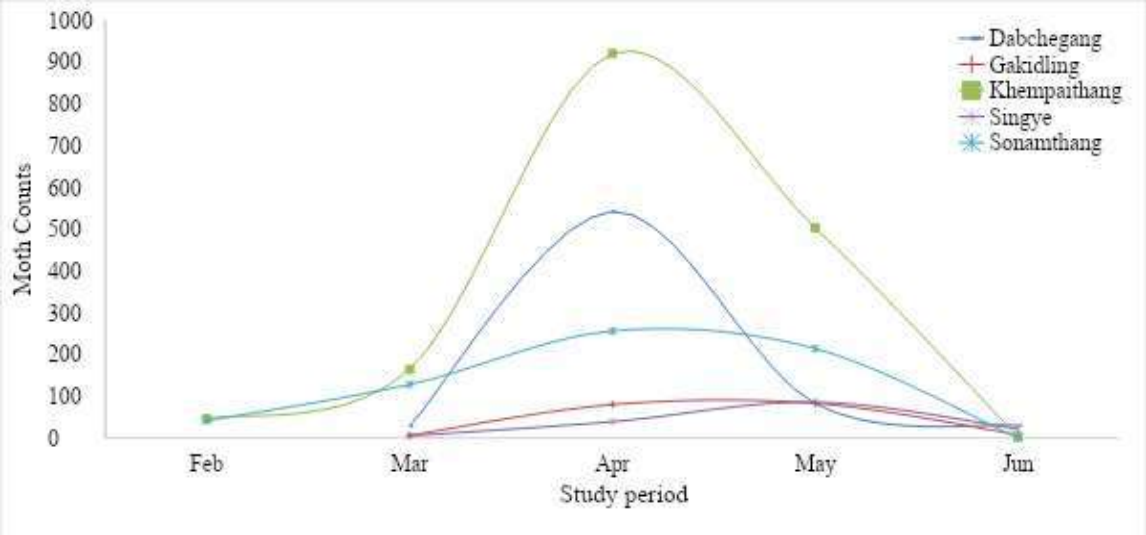

Figure 4 Population trends of fall armyworm in study sites

At Dabchegang, the FAW population experienced an increased towards the end of Ma, with a peak occurring in mid-April, followed by a subsequent decline. It is noteworthy that during the study period, no maize or other known hosts of FAW were cultivated in the study sites.

At Gakidling and Singye gewogs in Sarpang, the FAW population remained relatively low. Maize cultivation was ongoing in both the study sites during the research trial period, with Yangtsipa being the predominant maize variety. The maize growth stage in these areas ranged from 'early whorl' stage to maturity throughout the study.

At Khempaithang and Sonamthang, the FAW moth population exhibited a sharp increase starting in March, peaking in mid-April, and gradually declining thereafter. During the study,

different local cultivars were recorded at different growth stages, spanning from emergence to maturity, with most cultivars maturing in early June.

Effectiveness of the commercial FAW lures

Fall armyworm capture rate

Dabchegang, Punakha

The trapping efficiency of different lures was not exhibiting significant differences for March **Table *6***. Howevr, in April, lure SPFR2115 recorded the highest mean moths per traps (p = 0.0002, CV%, 64.7%) compared to other lures. In May, SPFR2111 caught a significantly higher number of moths (p=0.0406) in comparison to SPFR2114 and SPFR2115, while showing relatively similar results to SPFR2112, and SPFR2113. No significant differences were observed in the moth trapping during June. When evaluating the overall efficiency in Dabchegang, SPFR2111, SPFR2114 and SPFR2115 emerged as the beter performing lures with significant higher moth catches (p=0.0047) **Figure *5***.

Table 6 Table of means, t-test (Tuckey-LSD) for Dabchegang

Lure type	Mean number of moth traps [transformed by $\log_{10}(x+0.5)$]				
	March	April	May	June	Pooled mean
SPFR2111	0.257	0.260^{c}	0.928^{a}	0.149	0.398^{a}
SPFR2112	-0.301	-0.007^{c}	0.245^{ab}	-0.007	-0.017^{b}
SPFR2113	-0.301	0.171^{c}	0.260^{ab}	-0.301	-0.043^{b}
SPFR2114	0.310	0.899^{b}	0.134^{b}	-0.301	0.261^{a}
SPFR2115	-0.301	1.873^{a}	-0.301^{b}	0.290	0.390^{a}
p	0.0776	0.0002	0.0406	0.3261	0.0047
Cv%	-576.06	64.71	185.85	-1368.31	84.57

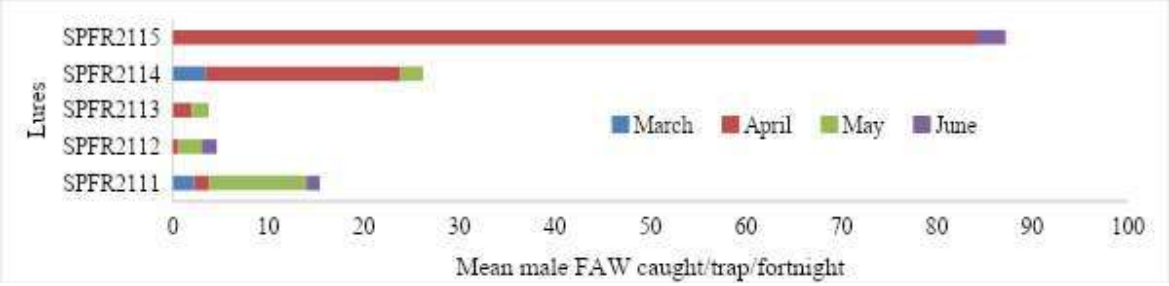

Figure 5 Means of FAW male trapped by different lures at Dabchegang

Gakidling, Sarpang

Similar to the observations in Dabchegang, trap catches were not significantly different in traps with different lures in March **Table *7***. However, in April the PCI lure fixed traps captured the highest number of moths (p=0.0198), while the SPFR2111 had the lowest catch. In April, trap catches in PCI were not significantly different from SPFR2112, SPFR2113, SPFR2114 and SPFR2115. In May, PCI significantly outperformed (p= 0.0286) SPFR2113 in terms of moths captures. No significant difference in trap catches was observed for June. Overall, PCI, SPFR2112, SPFR2113, and SPFR2114 proved to be preferable for Gakidling environment due to significantly higher trap captures throughout the season, **Figure *6***.

Table 7 Table of means and T-test (Tuckey-LSD result) for Gakidling

Lure type	Mean number of moth traps [transformed by $\log_{10}(x+0.5)$]				Pooled mean
	March	April	May	June	
PCI	-0.161	0.690[a]	0.706[a]	-0.110	0.281[a]
SPFR2111	-0.206	-0.021[b]	0.030[ab]	-0.301	-0.125[b]
SPFR2112	-0.110	0.513[ab]	0.405[ab]	-0.301	0.127[ab]
SPFR2113	-0.206	0.488[ab]	-0.037[b]	-0.206	0.010[ab]
SPFR2114	-0.301	0.125[ab]	0.532[ab]	-0.066	0.072[ab]
SPFR2115	-0.301	0.030[ab]	0.214[ab]	-0.161	-0.055[b]
p	0.5340	0.0198	0.0286	0.1930	0.0085
Cv%	-86.30	116.70	118.45	-88.95	300.10

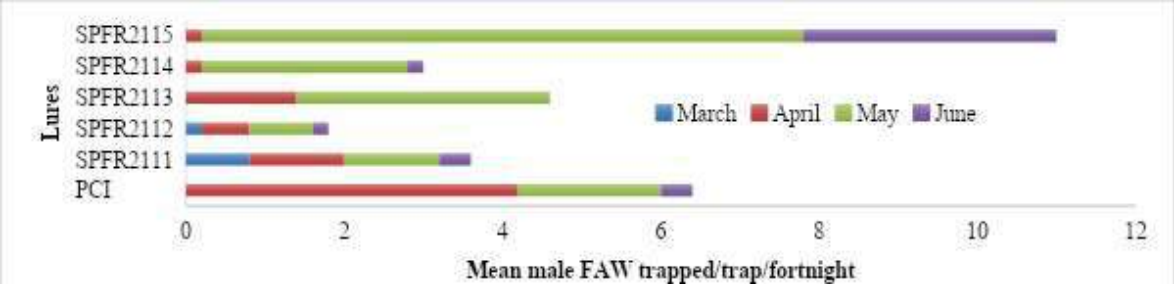

Figure 6 Means of male FAW caught by different lures at Gakidling

Singye, Sarpang

There were no satistically significant differences in the trapping efficiency of the different lures used (p = 0.108) ***Table 8***. During March and May, there were no significant difference in the number of moth captured. However, in April, the PCI lure caught a significantly (p = 0.018) higher number of moths in comparison to SPFR2114 and SPFR2115. Nevertheles, there were so significant differences in trap catches among the SPFR series lures. In June, unlike the initial months, the SPFR2115 caught significantly higher counts of moths (p=0.00090), while no significant differences in trap catches were observed among the remaining lures.

Table 8: Table of means and T-test (Tuckey-LSD result) for Singye Gewog

Lure type	Mean number of moth traps [transformed by $\log_{10}(x+0.5)$]				Pooled mean
	March	April	May	June	
PCI	-0.301	0.446[a]	0.265	-0.161[b]	0.062
SPFR2111	-0.021	0.154[ab]	0.103	-0.161[b]	0.019
SPFR2112	-0.206	-0.066[ab]	0.030	-0.206[b]	-0.112
SPFR2113	-0.301	0.199[ab]	0.220	-0.301[b]	-0.046
SPFR2114	-0.301	-0.206[b]	0.338	-0.206[b]	-0.093
SPFR2115	-0.301	-0.206[b]	0.723	0.475[a]	0.173
p	0.0918	0.0180	0.2292	0.0009	0.1086
Cv%	-71.00	569.94	158.15	-263.84	38791.22

Sampheling, Chhukha

The trap catches between Ecotech and PCI were not significantly different (p=0.097), eventhough they were subjected to different stages of host.

Species specificity

Dabchegang, Punakha

Proportionately, the total Fall Armyworm counts at Dabchegang were trapped in greater numbers compared to non-target insects. When assessing the effectiveness of different lures for trapping fall armyworms at Dabchegang, it was observed that the SPFR2113 lure performed the best, achieving a capture rate of 100%. Following SPFR2113 lure, SPFR2115, SPFR2114 and SPFR2111rates as shown in **Figure** *7*. On the contrary, the lure SPFR2112 attracted the highest number of non-target insects.

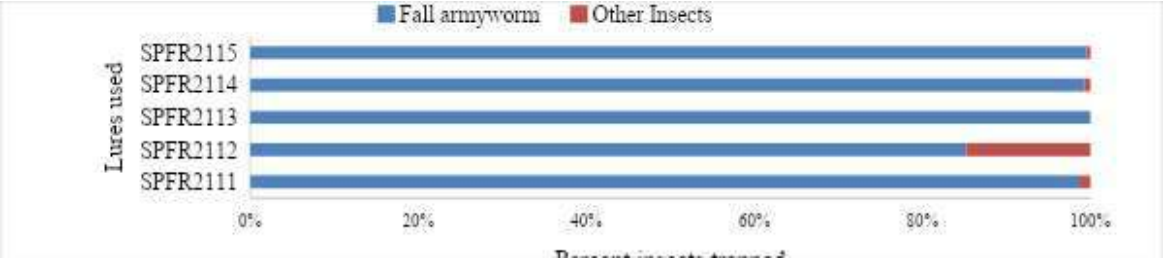

Figure 7 Percent insects trapped by different lures (fall armyworm against other insect species)

Gakidling and Singye, Sarpang

Unlike Dabchegang, the overall count of fall armyworms trapped in Sarppang was lower than that of non-target insects. Among the different lures, SPFR2113 exhibited effectiveness in Sarpang, followed by the highest ratewas the best at Sarpang, followed by SPFR2112 and SPFR2115, as indicated in **Figure** ***8***. However, both the lures SPFR2111 and PCI attracted more non-target insects than fall armyworm moths when compared to other lures.

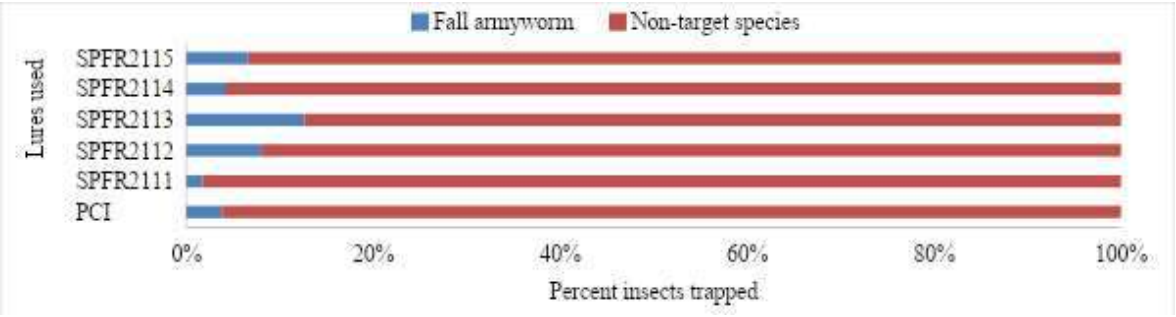

Figure 8 Percent insects trapped by different lures (FAW against other insects)

Sampheling, Chhukha

In Sampheling, the two lures caught proportionately more of fall armyworm than the non-target insects, **Figure** ***9***. The PCI lure performed better in the target specificity at Sampheling.

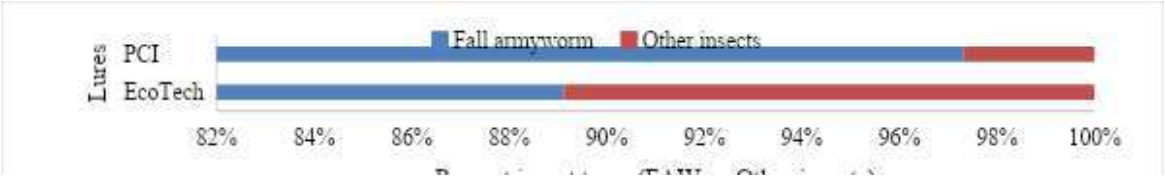

Figure 9 Percent insects caught by different lures at Sampheling (FAW against other insects)

1.3.4 Conclusion

For the current monitoring of fall armyworm, it is advisible to consider the use of SPFR2111, SPFR2114 and SPFR2115, PCI and Ecotech as suitable options. Neverthess, it should be noted that moth counts may not always accurately indicate the actual number of larvae and damage caused by FAWin the field. Therefore, field scouting for FAW larvaeit is important to scout FAW larvae remains a crucial practice. Moreover, it is worth noting that trap catches can be influenced by other abiotic factors such as wind speed, temperature, and relative humidity (Rojas et al., 2004). Conducting further studies to determine the presence of the rice strain of FAW in Bhutan is recommended.

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1.4 Population dynamics of Bactrocera dorsalis, Bactrocera zonata and Bactrocera cucurbitae in fruits and vegetables

1.4.1 Introduction

Fruit flies (Diptera: Tephritidae) comprise approximately 4300 species from around 500 genera (White, 2006) and pose a threat to horticultural crops (Ekesi et al., 2006). They are highly polyphagous with a high fecundity rate, attacking important fruits and vegetables such as mangoes, citrus, peppers, and cucurbits (Ekesi & A., 2011). Fruit flies lay their eggs beneath the surface of fruits and vegetables, and upon hatching, the larvae feed on the fruit pulp. Infested fruits and vegetables become inedible and eventually drop to the ground. Furthermore, infestation also provides opportunities for secondary infections by fungi and bacteria (White & Elson-Harris, 1992).

Understanding the population dynamics of pests is crucial for implementing timely management strategies based on the economic threshold level (Djaman et al., 2019). Population dynamics involves forecasting the abundance and distribution of pests over time, taking into account factors such as reproduction rate, mortality rates, and environmental conditions. Ideally, monitoring fruit flies should start from the fruiting season and continue until harvest, although the precise timing may vary depending on the species of fruit fly and the geographical location. Typically, it is noted that the population of fruit flies rises as fruits ripen (Ganie et al., 2013).

In Bhutan, there has been a notable increase in fruit fly infestations affecting various vegetables such as cucumber, slippery gourd, brinjal, and chilli, as well as fruits including mango, papaya, apple, pear, guava, and peach. Consequently, it is crucial to detect their presence and monitor their activity to develop effective management strategies. The primary objective of this study is to assess the presence of fruit flies (specifically B. *cucurbitae*, B. *dorsalis*, and B. *zonata*) in vegetable fields and orchards, and to monitor their behavior using pheromone traps.

1.4.2 Materials and methods

Monitoring site

Monitoring of fruit flies was conducted in five sites in Thimphu and Wangdiphodrang Dzongkhags from May to July 2023 (see Table 9). Pheromone traps targeting *Bactocera dorsalis* and *Bactocera zonata* were installed in apple and peach orchards in Thimphu. Similarly, mango orchards in Kamichhu and Baychhu had traps set up for the same species. At ARDC Bajo, traps were set up in zucchini, watermelon, and pumpkin fields to monitor *Bactocera cucurbitae*.

Table 9 Monitoring sites

Sl.no	Location	Target crops	No. of Steiner traps
1	NPPC colony, Thimphu	Apple and peach	4
	Semtokha, Thimphu	Apple and peach	6
2	Kamichhu, Wangdiphodrang	Mango	7
	Baychhu, Wangdiphodrang		2
3	ARDC, Bajo, Wangdiphodrang	Zucchini	1
		Watermelon	2
		Pumpkin	1

Traps and lures

A Steiner trap with a wooden lure (manufactured by AMITY BIO-ORGANICS) impregnated with Methyl eugenol and Cue lure was used (refer to Table 10). The Steiner trap has ingress tubes to prevent rain from entering and flies from escaping. It has a mesh halfway across the entry to prevent dead flies from falling out. Additionally, it includes openings at each end of the trap for fly entry and to facilitate the free movement of attractant vapor from the wood block.

Table 10 Lures used for monitoring

Sl.no	Trade Name	Active ingredients	Mode of action	Active Life	Targeted species
1.	*Bactrocera dorsalis* Pheromone Lure	Methyl eugenol	Attractant Pheromone	Up to 3 months (depending on climatic conditions	Male & Female *B. Dorsalis & B. Zonata (Oriental fruit fly)*
2.	*Bactrocera cucurbitaea* Pheromone Lure	Methyl eugenol & Cue lure	Attractant Pheromone	Up to 3 months (depending on climatic conditions)	Male and female *B. Cucurbitaea (melon fruit fly)*

Installation of traps

The traps were positioned in mating sites typically found in the crown of host plants or nearby. Entrances to the traps were cleared of twigs, leaves, and other obstructions to ensure adequate airflow and easy access for the fruit flies. Lures were replaced on a monthly basis. Sterile hand gloves were worn when handling lures to prevent cross-contamination among them.

Data collection

The trapped fruit flies were removed from the traps, counted every two weeks, and identified in the entomology laboratory based on their morphological traits, including wing patterns,

abdomen markings, and scutum coloration (Plant Health Australia. Fruit Fly ID Australia. n.d.).

1.4.3 Result and discussion

Population dynamics of fruit flies at NPPC colony

The population of *B. dorsalis* began to rise during the third week of monitoring, while the population of *B. zonata* remained stable. Overall, both *B. dorsalis* and *B. zonata* exhibited variaitons in their mean numbers per trap across the fruiting stages, with *B. dorsalis* consistently showing higher mean numbers compared to *B. zonata* (see Figure 10).

Figure 10 Population dynamics of fruit flies NPPC colony

Population dynamics of fruit flies at Semtokha

The count of fruit flies began to rise from the third week of monitoring. Initially, both species exhibited low numbers during the flowering stage. Subsequently, during the fruiting stages, the numbers of both species increased, with *B. dorsalis* consistently showing higher average counts compared to *B. zonata* (refer to Figure 11).

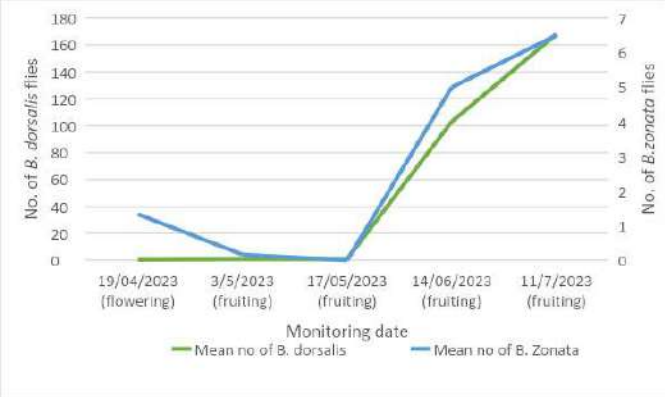

Figure 11 Population dynamics of fruit flies at Semtokha.

Population dynamics of fruit flies at Kamichu and Baychu, Wangduephodrang

In Kamichu, the number of trapped fruit flies began to rise with the onset of the fruiting season. Generally, both *B. dorsalis* and *B. zonata* displayed a comparable trend of increasing average counts during the initial fruiting stage, followed by fluctuations in average counts during the subsequent fruiting stages (see Figure 12). However, at Baychu, there was a substantial increase in the numbers of both *B. dorsalis* and *B. zonata* from the flowering stage to the first fruiting stage, followed by a gradual decline (see Figure 13).

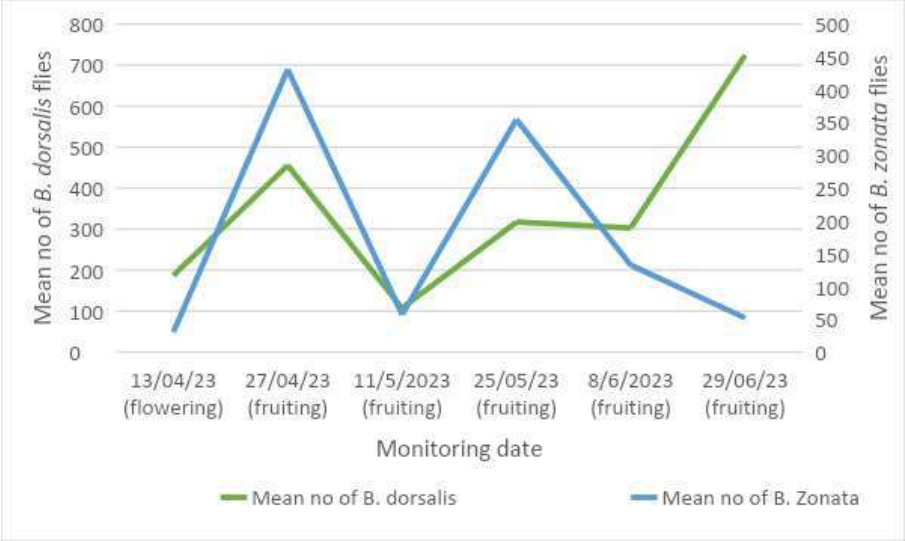

Figure 12 Population dynamics of fruit flies at Kamichu

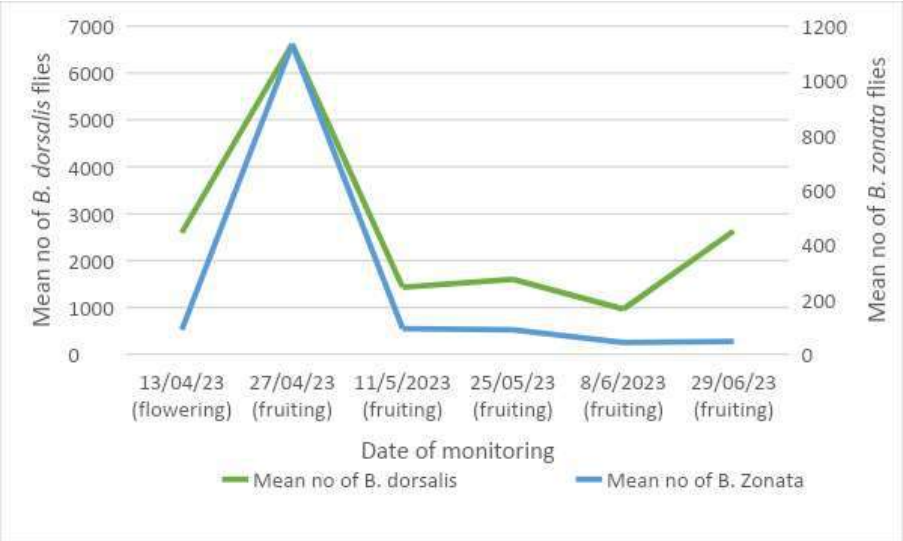

Figure 13 Population dynamics of fruit flies at Baychu

Population dynamics of fruit flies at ARDC, Bajo

In ARDC Bajo, the number of trapped fruit flies peaked during the initial stage of the fruiting season. *Bactrocera dorsalis* showed a consistent trend of decreasing mean counts from the flowering stage to harvesting, with a slight increase observed on the date of harvest (see Figure 14). Bactrocera zonata displayed fluctuating average counts during the fruiting stages, with a notable increase observed on 25/05/23 (fruiting) and a decrease on 8/6/2023 (fruiting). Meanwhile, *Bactrocera cucurbitaea* exhibited a stable mean count during the flowering and first fruiting stages, followed by a slight increase during the second fruiting stage, and a further increase at the time of harvest (see Figure 14).

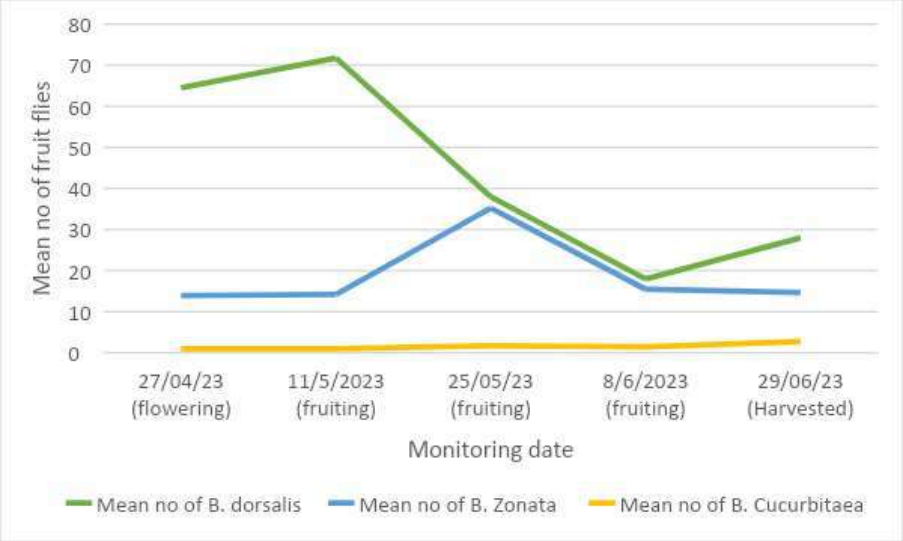

Figure 14 Population dynamics of fruit flies at ARDC Bajo

1.4.4 Conclusion

The study found that fruit fly numbers increased as the fruiting season approached and continued to rise. The increase began in May due to warm and humid conditions, which are favorable for fruit fly survival. Larger fruits also contributed to higher fruit fly numbers as they provided easier oviposition for females. However, the trial is ongoing, and final results will be available upon completion.

Based on the data collected so far, the study shows a consistent rise in fruit fly numbers leading up to and during the fruiting season. This increase commenced in May, attributed to the warm and humid conditions conducive for fruit fly survival. Additionally, larger fruits were found to contribute to increased fruit fly numbers, providing easier oviposition opportunities for females. Howver, it is important to note that the trial is still ongoing, and conclusive results can be made available upon the completion of the trial.

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1.5 Monitoring of leaf and plant hoppers in paddy using light traps

1.5.1 Introduction

More than 100 insect species have been identified as threats to rice crops, affecting them from germination to harvest (Deshwal et al., 2019). Apart from leaf defoliators, plant and leafhoppers pose significant treat by directly damaging plants through sap removal and acting as vectors for diseases such as rice tungro virus, rice ragged stunt, and rice grassy stunt viruses (Sharma et al., 2005). Among the most common leaf and plant hoppers in rice ecosystems are the rice green leafhopper (GLH), *Nephotettix spp*., brown plant hopper (BPH), and white-backed plant hopper (WBPH) (Deshwal et al., 2019). Nymphs and adult hoppers feed on sap from rice leaves and tender plant parts, resulting in the development of yellow patches known as "hopper burn." Additionally, the honeydew produced by hoppers can lead to fungal infections. Numerous studies have indicated that hoppers are migratory insects (Hu et al., 2017; Bao et al., 2016; Otuka, 2013). The migration of hoppers has caused outbreaks in new rice-growing areas (Otuka, 2013). For instance, BPH and WBPH

overwinter in Vietnam and Southern Hainan, migrate northeast in spring to China, Japan, and Korea with southwesterly monsoons, and return south in autumn (Cheng et al., 1979)

Light trap serves as an efficient tool for monitoring insect pests since they are drawn towards light traps (Merckx et al., 2009). It is used to trap nocturnal insects like green leafhopper, brown plant hopper, stem borer, leaf folder and caseworm. Light traps have been used extensively to monitor hoppers activity (Rashid et al., 2022; Mhd bookeri et al., 2021) and predicting hopper burn (Crummay & Atkinson, 1997). It can minimize the damage caused by insect pests without any harmful effects (Sinu et al., 2013) and indicate the maximum activity of these pests (Dadmal & Khadakkar, 2014). Ali et al. (2020) found that light traps captured approximately 94% of insects compared to field sampling, demonstrating that light traps are an effective tool.

In Bhutan, there is a lack of research and knowledge on different hoppers attacking paddy. Therefore, this study was undertaken to identify different types of hoppers and their population dynamics by using light traps in the paddy fields of Chuzergang and Samtenlling Gewogs, Sarpang Dzongkhag with the fund support from the Asian Food and Agricultural Cooperation Initiative (AFACI) Project.

1.5.2 Materials and methods

Monitoring sites and period

Light traps were installed in Dawathang and Karbithang villages under Chuzergang Gewog and ARDC Samtenlling and Samtenlling village under Samtenlling Gewog in Sarpang Dzongkhag. The monitoring commenced from the panicle initiation stage (August) until maturity (November) in all the sites from 2018 to 2022.

Light trap

A light trap made of mild steel, measuring 40 x 40 x 25 cm, equipped with a 100 W incandescent bulb and a funnel length of 30 cm, was used for trapping. Additionally, the trap included a bottom box to accommodate collection trays for trapped hoppers. These traps were positioned in the center of open paddy fields, sheltered to shield them from rain (refer to Figure 15). The light traps were switched on at dawn and turned off at dusk by the respective plant protection officers and farmers.

Figure 15 Light traps for monitoring plant hoppers

Data collection

Weekly collections of adult green leaf hoppers (Nephotettix nigropictus & Nephotettix irescens), brown plant hoppers (Nilaparvata lugens), white backed plant hoppers (Sogatella furcifera), and zigzag plant hoppers (Recilla dorsalis) were gathered and morphologically analyzed in the laboratory by Plant Protection Officers at ARDC Samtenlling. The counting of hoppers commenced one week following the light traps' installation and persisted on a weekly basis until the paddy plants reached maturity

Data analyses

Data analyses were performed using RStudio version 2022.07.2. The relative abundance of hopper species in both the locations were calculated using the formula:

$$Relative\ abundance(\%) = \frac{Total\ number\ of\ individual\ species}{Total\ number\ of\ all\ species} * 100$$

The mean of the total count of hoppers at their respective sites were computed and plotted against the monitoring year to determine the population dynamics of the hoppers.

1.5.3 Result and discussion

Relative abundance of hopper at Chuzergang and Samtenlling

In Chuzergang, the relative abundance of green leaf hoppers (GLH) stood at 66.00%, followed by brown plant hoppers (BPH) at 22.88%, white-backed plant hoppers (WBPH) at 5.07%, and zigzag plant hoppers (ZZPH) at 6.05% (refer to Figure 16). Conversely, at Samtenlling, GLH exhibited the highest relative abundance of 53.20%, trailed by BPH at 21.50%, WBPH at 13.19%, and ZZPH at 12.11% (refer to Figure 16). Notably, the relative abundance of GLH was higher in Chuzergang (66.00%) compared to Samtenlling (53.20%). Moreover, both BPH and ZZPH displayed a similar trend, with higher relative abundances in Chuzergang. Conversely, WBPH exhibited a lower relative abundance in Chuzergang (5.07%) compared to Samtenlling (13.19%).

The dominance of GLH as the primary species in paddy fields in both Samtenlling and Chuzergang is evident. This observation aligns with findings from Bangladesh, where green leafhoppers were similarly noted as the most abundant species in rice fields (Rahman et al., 2017). GLHs are notorious rice pests in Asia, inflicting damage through sap-sucking and transmitting viral diseases like tungro (Rosida et al., 2020). Following GLH, BPH displayed a higher relative abundance compared to ZZPH and WBPH. BPH also causes damage by sap-sucking and transmits viral diseases such as grassy and ragged stunt viruses (Normile, 2008). The excessive use of pesticides to control BPH has led to pesticide resistance in this species (Tanaka et al., 2000).

At Chuzergang, GLH exhibited a higher relative abundance of 66.00%, while BPH accounted for 22.88%, WBPH for 5.07%, and ZZPH for 6.05% **Figure *16***. At Samtenlling, GLH had the highest relative abundance of 53.20%, followed by BPH with 21.50%, WBPH with 13.19%, and ZZPH with 12.11% **Figure *16***. The relative abundance of GLH was higher in

Chuzergang (66.00%) compared to Samtenlling (53.20%). BPH and ZZPH also show a similar trend with higher relative abundances in Chuzergang. In contrast, WBPH has a lower relative abundance in Chuzergang (5.07%) compared to Samtenlling (13.19%).

It is evident that GLH is the most dominant species in paddy fields in Samtenlling and Chuzergang. Similar observation of green leafhopper as the most abundant species in rice fields was recorded in Bangladesh (Rahman et al., 2017). GLHs are one of the most devastating rice pests in Asia causing damage by sucking sap and transmitting virus diseases like tungro (Rosida et al., 2020). Following GLH, the relative abundance of BPH was higher compared to ZZPH and WBPH. BPH causes damage by sucking the sap and transmitting viral diseases such as grassy and ragged stunt viruses (Normile, 2008). Extensive use of pesticides to manage BPH has resulted in BPH resistance to pesticides (Tanaka et al., 2000).

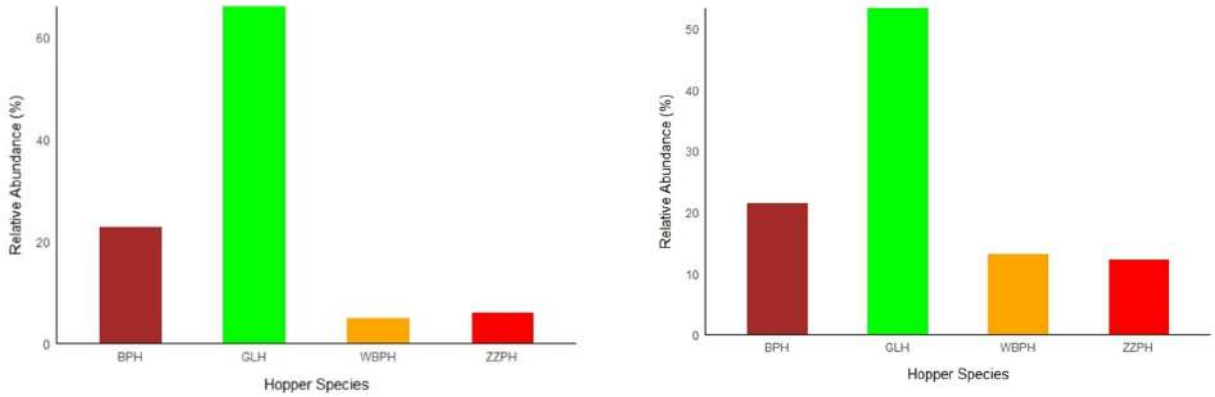

Figure 16 Relative abundance of different hoppers at Chuzergang (left) and Samtenlling (right)

Population dynamics of hoppers

The GLH population increased consistently in both sites, while the other hoppers' population fluctuated during the monitoring years **Figure *17***. This fluctuation in hoppers population could be due to weather and location effects. Rainfall reduces the insect population by damaging their wings and dislodging from the plants (Karthik et al., 2022). Furthermore, heavy downpours wash out eggs, larvae, nymph and small insects (Staley et al., 2007). Madhuri et al. (2017) found that rainfall reduces the GLH population but increases the BPH and WBPH population. Similarly, temperature also affects the fecundity, growth, development and mortality of insects (Ma et al., 2017). Several studies have found that BPH population is positively correlated with temperature (Chaudhary et al., 2014; Krishnaiah et al., 2006). The differences in population of hoppers trapped could be due to light traps. Light trap catch is affected by trap size, height bulb type, location, sampling time (Bowden, 1982).

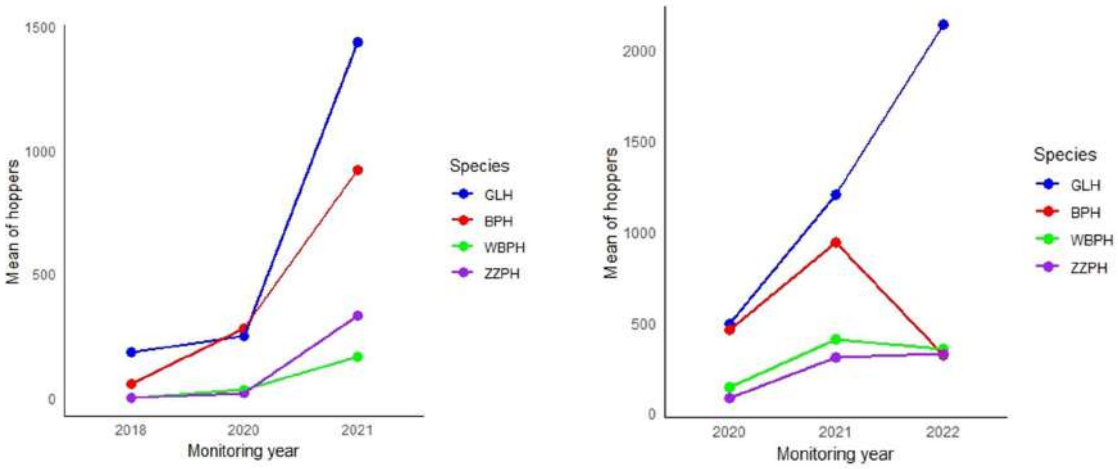

Figure 17 Population dynamics of hoppers over the monitoring years at Chuzergang (left) and Samtenlling (right)

1.5.4 Conclusion

The light trap study concludes that Green leaf hoppers and Brown plant hoppers are the dominant hoppers in Chuzergang and Samtenlling paddy fields. This study provides a foundation for developing effective management strategies, including long-term monitoring, early detection, and intervention measures for GLH and BPH control.

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2. Pathology Program

2.1 Effect of potassium phosphite (phosphonate) against *Phytophthora capsici*

2.1.1 Introduction

Previous studies under field conditions that was initiated in 2020-2021 could not achieve conclusive results due to absence of disease incidence in the fields. Moreover, previous studies also lacked proper control treatments. Therefore, the study was repeated in the FY 2022-2023 to investigate the effect of potassium phosphonate on P. capsici under field and greenhouse conditions.

2.1.2 Materials and methods

Greenhouse experiment

Inoculum preparation: Phytophthora capsici isolate T12A obtained from Thimphu was used in the current greenhouse evaluation. Inoculum of P. capsici was prepared as per Burgess et al. (2008) using wheat and rice hulls at 2:1 ratio. The mixture was soaked in distilled water and incubated at 4°C overnight. Excess water was poured off on the following day and 125 mL of the mixture was transferred to a 250 mL flask. The mixture was then autoclaved two times at 121°C for 15 min and cooled. Agar discs of 4 mm diameter were cut from the margin of three days old P. capsici culture. Ten such agar discs were placed in each flask containing wheat and rice substrate. Flasks were shaken and then incubated at room temperature for two weeks shaking every two days.

Figure 18 (a) Inoculated Substrate incubation (b) Substrate preparation

Seedlings: Local chili variety (ema ‘oru’) were used. Seedlings were raised in nursery trays filled with a potting mixture of leaf litter and garden soil (1:1). Seedlings were transplanted into plastic pots of (12cm diameter X 11cm height) 28 days after sowing. Same potting mixtures was used in the transplantation. Two seedlings were planted in each pot.

Experimental design and treatments: One week after transplantation, chilli seedlings were inoculated with 2% (w/w) pathogen by mixing the inoculum into the potting mix around the seedlings in the pot. Treatments were assigned randomly and applied on the same day of pathogen inoculation **Table *11***. Each treatment was replicated three times and each replicate contained two pots. Pots were arranged in a RCB design on table tops in the greenhouse.

For potassium phosphite treatments, four different concentrations (0.2%, 0.3%, 0.4%, and 0.5%) of potassium phosphite (Skylite Agrochem, Maharashtra, India) were sprayed. Control treatments consisted of plants not inoculated with the pathogen (uninoculated control), uninoculated plants sprayed with water, plants inoculated with pathogen but not receiving any sprays (inoculated control), inoculated plants sprayed with water. Fungicide treatment consisted of plants sprayed with 0.2% ridomil. Subsequent sprays were done at 7, 14, 22, and 29 days after the first spray.

Table 11: Treatments for the greenhouse experiment

Treatment number	Description
Treatment 1	Uninoculated plants
Treatment 2	Uninoculated plants sprayed with water
Treatment 3	Inoculated plants (inoculated control)
Treatment 4	Inoculated plants sprayed with water
Treatment 5	Inoculated plants sprayed with 0.2%
Treatment 6	Inoculated plants sprayed with 0.3%
Treatment 7	Inoculated plants sprayed with 0.4%
Treatment 8	Inoculated plants sprayed with 0.5%
Treatment 9	Inoculated plants sprayed with 0.2% fungicide (ridomil)

Data collection and analyses: Data on plant height and lesion size were collected every week for seven weeks after treatment. Disease severity was calculated following the following formula (Cooke, 1998):

$$\text{Disease severity} = \frac{\text{Length of lesion}}{\text{Total length}} * 100$$

Data on disease severity were arsine transformed. All zero and 100 values were replaced by (1/4n) and (100-(1/4n)) respectively before transformation, where n is the number of units upon which the percentage data was based (Gomez and Gomez, 1984). Transformed data were subjected to a one-way ANOVA using SPSS (version 22, IBM). Untransformed data were presented in the results.

Field experiment

Trial sites: Experimental trial sites were selected in Lingzhipharka and Jamdo, under Meadwang Gewog, Thimphu on 8 June and 9 June 2022, respectively. Data loggers (Tinyt Plus 2) and rain gauge (Rain Collector II, Davis Instruments) were installed in the field to record ambient temperature, relative humidity and rainfall.

Field layout and treatments: At each site, experiment consisted of seven treatments **Table *12*** arranged in a RCB design. Treatments comprised of spraying one or two or four sprays of 0.2% potassium phosphonate, and one to three sprays of water. A control group with no spray was also included. Each treatment was assigned to a plot measuring 1.2 m x 2.8 m. Within each plot, the inner rows containing 10 plants were designated for data collection. Plots were separated by 0.3 m furrows. Each treatment was replicated three times. Field management practices including weeding were done following farmer's practices.

Spray frequency: The first spray, corresponding to two weeks after planting, was done on 14 June and 23 June 2022 at Lingzhipharka and Jamdo respectively. Subsequent sprays in Lingzhipharka were done on 7 and 21 July, and 18 August.

Table 12 Descriptions of treatments

Treatments	Descriptions
1	Untreated control (plant alone)
2	One time water
3	Two times water
4	Three times water
5	One time 0.2% phosphonate
6	Two times 0.2% phosphonate
7	Four times 0.2% phosphonate

2.1.3 Results and discussion

Greenhouse experiment

Disease severity: Inoculated plants showed the highest disease severity during all observation weeks followed by inoculated plants sprayed with water **Figure *19***. Disease severities on these plants ranged from 4.5% to 89% in week 1 and 7 respectively. Plants in all other treatments had disease severities below 10% throughout the observation period **Figure *19***.

Repeated measure ANOVA showed significant differences between treatments **Table *13***. Disease severity was highest (65.85%) in the inoculated plants that did not receive potassium phosphonate or fungicide treatment followed by inoculated plants sprayed with water (49.59%). Disease severities of plants treated with potassium phosphonate or fungicide did not differ significantly from uninoculated plants. Disease severities of plants treated with different concentration of potassium phosphonate and fungicide, and uninoculated plants ranged from 0 % to 3% **Table *13***.

Field experiment

Weather data: In Lingzhipharka, the minimum ambient temperature ranged from 11.6-14°C during the months of June to October 2022. The highest minimum temperature was observed in August. Minimum RH ranged from 48.9-65.7% **Table *14***. The weather data are within the range of favourable conditions for development of *P. capsici*.

Disease severity: Trial at Jamdo was discarded as plants died due to phytotoxicity from herbicides applied by the farmer before chilli transplantation. Information was not disclosed by the farmer prior to establishing the experiment.

Phytophthora capsici infection did not occur during the 2022-2023 season in spite of favourable weather condition for development of the pathogen **Table *13***. Hence, no evaluation of potassium phosphonate could be done. However, effect of potassium phosphite was observed on foliar diseases such as Alternaria leaf spot and *Cercospora* leaf spot. In Lingzhipharka, potassium phosphonate sprays were observed to reduce severity of Alternaria leaf.

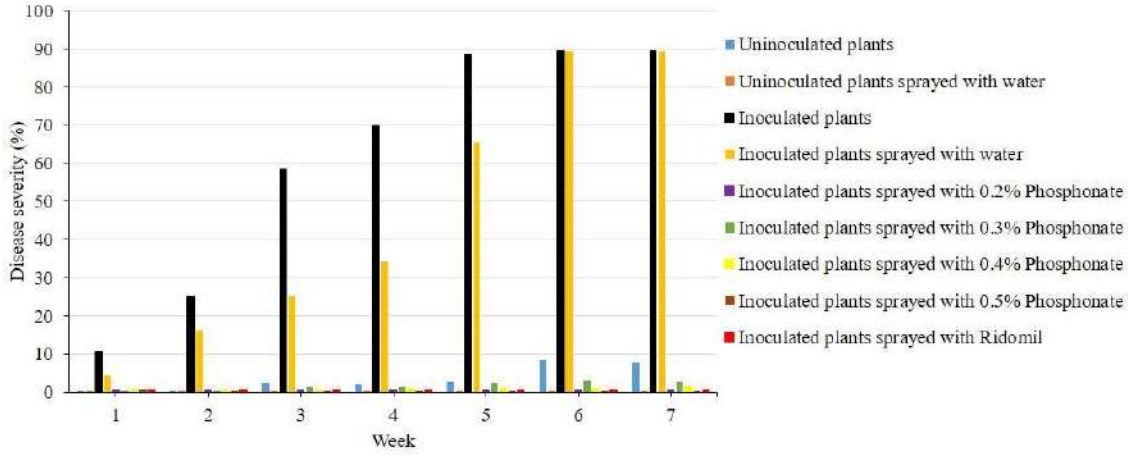

Figure 19 Disease severity of plants treated with different concentration of potassium phosphonate during seven-week period.

Table 13 Disease severity of chilli seedlings, at different days after treatment (DAT), inoculated with 2% (w/w*) P. capsici* isolate T12A and treated with varying concentrations of potassium phosphonate and fungicide

Treatment	Disease severity (%)*
Inoculated plants (inoculated control)	65.85 a
Inoculated plants sprayed with water	49.59 b
Uninoculated plants	3.02 c
Inoculated plants sprayed with 0.3%	1.19 c
Inoculated plants sprayed with 0.4%	0.21 c
Uninoculated plants sprayed with water	0.00 c
Inoculated plants sprayed with 0.2%	0.00 c
Inoculated plants sprayed with 0.5% Phosphonate	0.00 c
Inoculated plants sprayed with Ridomil	0.00 c
*means with same letter are not significantly diferent at 0.05 level.	

Table 14 Average monthly minimum and maximum temperature and relative humidity (RH at Lingzhipharka

Month	Temperature (°C)		Relative humidity (%)	
	Minimum	Maximum	Minimum	Maximum
June	13.90	24.40	58.70	99.90
July	13.50	26.50	48.90	100.00
August	14.00	26.20	50.80	100.00
September	12.70	23.40	55.90	100.00
October	11.60	21.20	65.60	100.00

Discussion

Negligible disease severities were observed on plants inoculated with P. capsici and treated either with varying concentration of potassium phosphonate or fungicide (Ridomil). Results of the present greenhouse study are consistent with previous studies conducted in NPPC and other studies that showed the inhibitory effect of phosphonate on various plant pathogens.

Reduction in severity of Alternaria bight was reported by Sharma et al. (2019) in sunflower. Phosphonate works by inducing systemic acquired resistance in plants which activates their defense mechanisms against pathogens (Smillie et al., 1989).

2.1.4 Conclusion

The results of the past and current greenhouse study indicate that disease suppression by using 0.2% of potassium phosphonate is not different from 0.5%. Hence, 0.2% of potassium phosphite can be used against P. capsici.

The absence of *P. capsici* incidences in the last two seasons of chilli indicate shift in pathogen dynamics. Phytophthora blight of chili may not be the major problem in Jamdo and Lingzhipharka and probably in other chilli growing areas. Hence, the causal agent(s) of wilting in chilli needs to be re-investigated. References

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2.2 Bioassays on native Trichoderma sp. against *Phytophthora capsici* under field condition

2.2.1 Introduction

The National Plant Protection Centre (NPPC) had initiated evaluation of native strains of Trichoderma sp. for managing *Phytophthora* blight starting 2018. Series of laboratory and greenhouse studies were conducted in the past years. Evaluation under field condition was initiated in 2021. However, due to absence of disease incidence, evaluation on the efficacy of Trichoderma under field condition could not be achieved. Hence, the study was repeated

in 2022-2023 for field evaluation of *Trichoderma* spp. against *Phytophthora* blight and leaf spot diseases. Greenhouse experiment was repeated to validate the findings of the past two studies.

2.2.2 Materials and methods

Greenhouse experiment

Isolates of Trichoderma sp.: Three types of Trichoderma sp. were used: (1) Trichoderma isolate Tc7A; (2) Trichoderma isolate from Pest Control India (PCI), henceforth called as Trichoderma PCI, and (3) Trichoderma isolate from Thailand, henceforth called as Trichoderma Thailand.

Preparation of Trichoderma sp. Inoculum: Inoculum of Trichoderma sp. was prepared based on the methods by Smith et al. (1990). Rice husk and saw dust were mixed at 1:1 ratio. Then 120g of the mix was placed in a 250mL flask and 65mL water was added. The substrate was autoclaved two times at 121°C for 15 min each time, and cooled. Then the substrate was inoculated with 10 discs (4 mm diameter) obtained from a 7 days old culture of Trichoderma sp. The inoculated substrate was incubated at room temperature (~22 °C) for 3 weeks, shaking every two days starting at four days after incubation. Rice husk and saw dust mix substrate without any inoculum was incubated as controls.

Preparation of pathogen inoculum: Pathogen inoculum preparation was adapted from Burgess et al. (2008). A substrate mixture of wheat and rice husk mixed at 2:1(v/v) was placed into a 250 mL Erlenmeyer flask filling up to 150 mL level. The substrate was moistened by adding 65 mL of distilled water and sterilized twice by autoclaving at 121°C for 15 minutes. Once the substrate has cooled, 10 mycelial discs of 4 mm diameter taken from the margin of actively growing 7 days old culture of P. capsici and placed in the substrate and mixed well. The inoculated substrate was incubated at room temperature (~22 °C) for 3 weeks, shaking every two days beginning at 4 days after incubation.

Chilli seedling: Local chilli (‘ema oru’) were raised in seedling trays filled with potting mix comprising of 1:1 ratio of top soil and leaf litter. Seeds were treated with Mixol (8% Metalaxyl and 84% Mancozeb) before sowing. Seedlings were transplanted at 45 days after sowing. Plastic pots of 1L size with 12.5 cm top diameter and 11cm height were used for transplanting. Three seedlings were planted in one pot. Treatments were applied one week after transplanting.

Experimental design and treatments: The experiment consisted of 11 treatments with five replications each arranged in a completely randomized design (CRD) **Table *15***. One pot represented one replicate.

Pathogen inoculum was applied by mixing 2% (w/w) of P. capsici in the substrate around the seedlings in the pot. Trichoderma treatments were applied by mixing 100g of Trichoderma around the seedlings in the pot. For fungicide treatment, 0.2% Mixol was sprayed on respective seedlings. Control treatments consisted of (1) pathogen inoculated seedlings without treatment, henceforth called as untreated control, (2) seedlings planted in potting mix treated with the respective Trichoderma isolates, henceforth called as Trichoderma controls,

(3) seedlings planted in sterile potting mix, henceforth called as potting mix control (4) seedlings planted in sterile substrate, henceforth called as substrate control, and (5) seedlings planted in non-inoculated potting mix and sprayed with fungicide, henceforth called as fungicide control.

Each pot was placed on a wide glass petri dish to prevent cross contamination. Pots were watered with 50mL of sterile distilled water on the day of establishment and continued every other day till the experiment was terminated. One week after inoculation, pots were flooded to induce infection.

Table 15 : List of *Trichoderma* isolates, *P. capsici*, fungicide and their combinations applied as treatment

Treatment	Description
Trichoderma Tc7A + PC T12Aa	Trichoderma Tc7A treated
Trichoderma PCI + PC T12Aa	Trichoderma PCI treated
Trichoderma Thailand + PC T12Aa	Trichoderma Thailand treated
Fungicide + PC T12Aa	Fungicide treated
PC T12Aa only	Untreated control
Trichoderma Tc7A only	Trichoderma Tc7A control
Trichoderma PCI only	Trichoderma PCI control
Trichoderma Thailand only	Trichoderma Thailand control
Mixol only	Fungicide control
Potting mix only	Potting mix control
Substrate only	Substrate control

Data collection: Data on plant height, length of lesion on the plant and number of leaves were collected every week for seven weeks after the appearance of first infection. Data on dry weight was collected at the end of the experiment. Disease severity percentage was calculated using the following formula (Cooke 1998):

$$\text{Percent disease severity}(\%) = \frac{\text{Lesion length}}{\text{Plant height}} * 100$$

Disease severity data were arc sine transformed before analysis. Before transformation, all zero and 100 values were replaced by 1/4(n) and [100 -1/4(n)] where n is the number of units upon which the percentage data was based (Gomez and Gomez, 1984). The transformed data was subjected to repeated measure ANOVA (SPSS version 23). was examined and Greenhouse-Geisser correction was applied if the assumption of sphericity was not met. Untransformed data were presented in the results. Non-transformed data are represented.

Field Evaluation

Site selection: Two sites were selected under Maedwang Gewog, Thimphu Dzongkhag: (1) One site was in Siluna, Jiminang Chiwog (27. 41638 N and 89. 55361 E, 2472 MASL). The area has a gentle slope facing east and soil type of loamy and beneath the apple orchard; (2) The other site was in Jamdo, Khasadrapchu Chiwog (27.3888N and 89.57556 E, 2180 MASL). Jamdo area is plain with sandy loam soil.

Isolates of Trichoderma sp.: Three types of Trichoderma sp. were used: (1) Trichoderma isolate Tc7A, (2) Trichoderma isolate from Pest Control India (PCI), henceforth called as Trichoderma PCI, (3) and Trichoderma isolate from Thailand, known as Trichoderma Thailand. Inoculum of Trichoderma sp. was prepared as in the greenhouse experiment.

Treatments and experiment layout: Experiment in each site comprised of five treatments with three replications arranged in RCB design. Each treatment was assigned to a plot size of 1.2m x 2.8m. Plots were separated by 0.3 m wide furrows. Treatments consisted of (1) application of Trichoderma isolate Tc7A; (2) application of Trichoderma PCI; (3) application of Trichoderma Thailand; (4) Substrate alone (un-inoculated substrate); and (5) no treatment. Trichoderma treatments were applied by mixing 2800 g of each Trichoderma isolate **Figure *20*** in the respective plot before transplanting. For controls, substrate without Trichoderma was used. Non-treated control did not contain any treatment. No artificial pathogen inoculum was applied.

Figure 20 Isolate of *Trichoderma* Tc7A (native) incubated in laboratory for 3 weeks and trial site after inoculation and transplanting chilli (right) at site 1, Siluna, Jiminang.

In each plot, 45 days old greenhouse raised chili ('ema oru') seedlings were transplanted with a planting distance of 30 x 30 cm. The inner rows with containing 10 seedlings were tagged for data collection. Plants were irrigated (manually) and managed according to farmer's practice.

Data collection: Data on air temperature and relative humidity were collected using tinytags (PLUS 2-TGP-4500, Omni Instrument Australia Pty Ltd) set to record every hour. Rainfall data was collected using a rain gauge with tipping bucket (Rain Collector II, Davis Instruments).

Plants were observed for disease development every week from June to October 2022. Incidence of Cercospora leaf spot and Alternaria leaf spot were also recorded. Symptomatic plant parts were collected and examined in the laboratory using the methods by Drenth and Sendall (2001) for confirmation. Yield data was collected at the end of the season.

2.2.3 Results

Greenhouse experiment

No disease symptom was exhibited until the seedlings were flooded one week after inoculation. Symptom appeared on untreated control plants one day after flooding while treated plants took up to 5 – 7 days to show first symptom.

Untreated control (pathogen only) showed the highest disease severity ranging from 20% in the 1st week to 100% 7th week while all other controls showed disease severity less than 5% throughout the experiment period **Table *15***. Plants treated with Trichoderma isolates showed lower disease severity than untreated control. However, disease severity of these plants

reached 90% by the 7th week. Disease severity of plants treated with fungicides remained below 4% **Figure *21***.

A repeated measures analysis of variance (ANOVA) with Greenhouse-Geisser correction revealed a significant mean difference on disease severity between different treatments [F (3.085, 154) = 20.897, p = 0.000]. Post hoc tests using Tukey HSD indicated significant differences in disease severities of plants inoculated with pathogen alone (untreated control) and other treatments **Table *16***. Disease severity was highest (75.53%) in plants inoculated with pathogen alone (untreated control) followed by inoculated plants treated with Trichoderma Thailand (61.89%) and Trichoderma PCI (58.72%). These were not significantly different from each other. Among the treated plants, plants treated with Trichoderma isolate Tc7A showed the lowest disease severity (48.28%) followed by inoculated plants treated with the fungicide ridomil (50.90%). However, disease severities of plants treated with Trichoderma isolates or fungicides were not significantly different. Plants in the control treatments showed 0% to 1.19% disease severity **Table *16***.

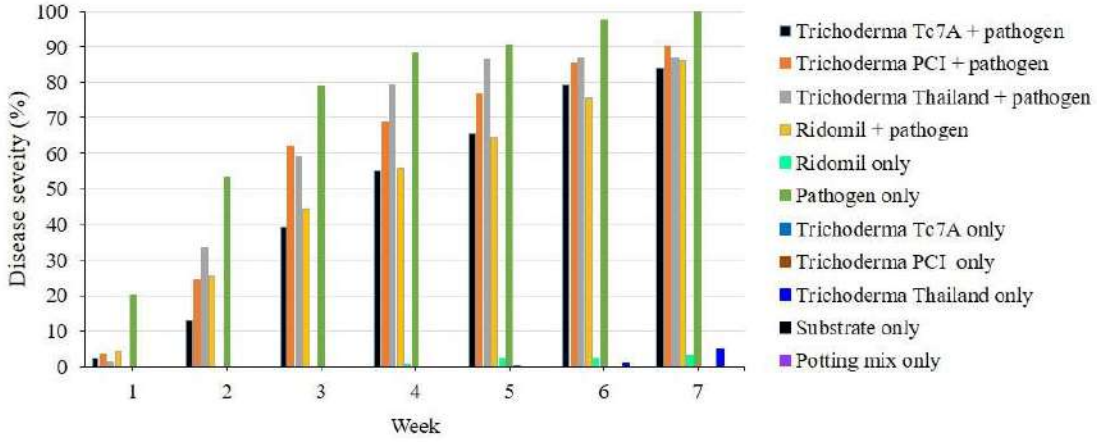

Figure 21 Disease severity progression of plants treated with different isolates of *Trichoderma* and fungicide (ridomil) during seven weeks period.

Table 16 Mean diseases severity (%) of plants with different treatments

Treatment	**Mean disease severity (%)***
Pathogen alone	75.53 a
Trichoderma Thailand + Pathogen	61.89 ab
Trichoderma PCI + Pathogen	58.72 ab
Ridomil + Pathogen	50.90 b
Trichoderma isolate Tc7A + Pathogen	48.28 b
Ridomil alone	1.19 c
Trichoderma Thailand alone	0.81 c
Trichoderma isolate Tc7A alone	0.03 c
Substrate alone	0.00 c
Potting mix alone	0.00 c
Trichoderma PCI alone	0.00 c
* means with same letter are not significantly different at 0.05 level	

Field experiment

Weather: Weather data shown in **Table *17*** indicate that Jamdo received less rainfall than Siluna. The monthly average rainfall ranged from 0.04 to 0.14mm and 0.03 to 0.19mm in Jamdo and Siluna respectively (Figure 13). Highest rainfall of 0.14mm and 0.19mm was observed in July for Jamdo and Siluna respectively.

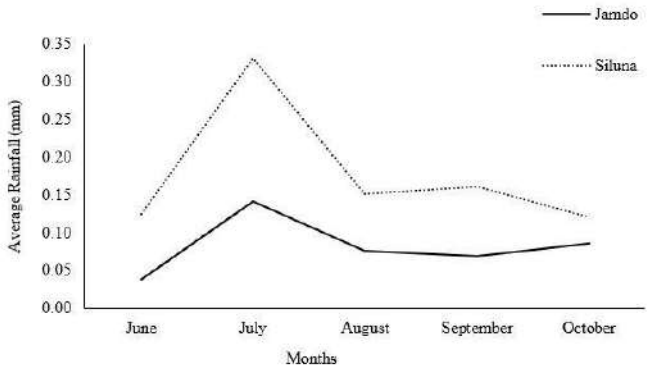

Figure 13: Monthly average rainfall in Jamdo and Siluna from June to October 2022.

Table 17 Monthly average minimum and maximum temperature and relative humidity at Jamdo and Siluna from June to October 2022

Month	Temperature (°C)				Relative Humidity (%)			
	Minimum		Maximum		Minimum		Maximum	
	Jamdo	Siluna	Jamdo	Siluna	Jamdo	Siluna	Jamdo	Siluna
June	15.4	14.1	27.5	23.5	48.4	58.6	98.2	99.8
July	15.2	13.7	29.6	26.9	45.9	48.1	100.0	100.0
August	15.4	14.1	29.3	26.7	48.4	51.8	100.0	100.0
September	13.8	12.7	27.8	23.5	47.8	56.5	100.0	100.0
October	12.1	11.3	26.1	21.4	51.2	66.7	100.0	100.0

Jamdo is relatively warmer and less humid than Siluna **Table *17*** minimum ambient temperature ranged from 11.4 to 14.1°C in Siluna compared to 12.1 to 15.4 °C in Jamdo. Temperature was highest in July for both Jam and Siluna with maximum temperature of 26.9°C and 29.6 °C for Siluna and Jamdo respectively. Minimum relative humidity (RH) ranged from 48.1 - 66.7% in Siluna and 45.9 to 51.2% in Jamdo **Table *17***.

Yield Data: Yield data was collected in October. In both sites, highest yield was obtained from the plots treated with substrate alone **Table *18***. However, yield differences were not significantly different from each other in both sites.

Disease severity: In both sites, *Phytophthora* blight symptoms were not observed. Low severity of bacterial fruit rot (confirmed through laboratory examination), Cercospora leaf spots (CLS) and Alternaria leaf spot (ALS) were common during the cropping season at both sites. At Siluna, high severity of Alternaria leaf spots was observed during the fourth week. Disease severity of 6.68% of Alternaria leaf spot was observed in the control treatment where plants did not receive Trichoderma treatment. Plants in the plot treated with Trichoderma

Thailand showed 2.62% disease severity. However, diseases severity was not significantly different from each treatment throughout the readings **Figure *22***.

Table 18 Average yield (kg) of chili from plots treated with different *Trichoderma* isolates

Treatments	Siluna*	Jamdo*
Trichoderma Tc7A	0.39[a]	0.23[b]
Trichoderma PCI	0.57[a]	0.22[b]
Trichoderma Thai	0.36[a]	0.18[b]
Substrate alone	0.64[a]	0.29[b]
Control	0.50[a]	0.24[b]

**Means followed by same letters are not significantly difference at 0.05 level*

Similar trend was observed in Jamdo. Severity of Alternaria leaf spot not significantly different between the treatments **Figure *23***.

In both the sites Cercospora leaf spot severity was high during the initial two weeks (after transplanting) but declined in the later weeks. There was no significant differences in the severity of Cercospora leaf spot between the treatments on all observation dates.

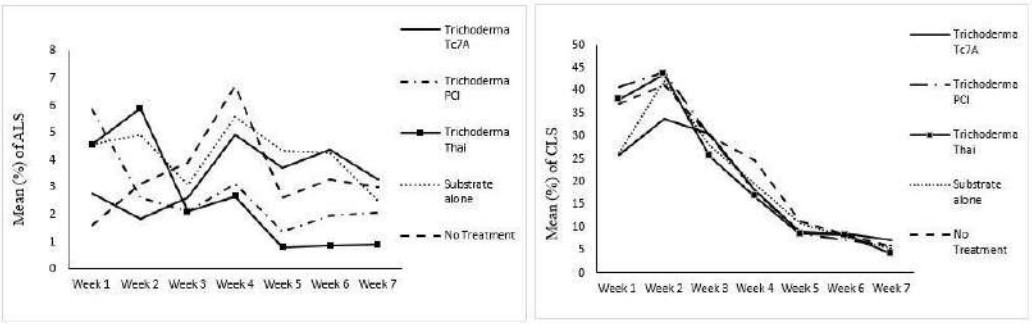

Figure 22 Mean percent of disease severity of *Alternaria* leaf spots (left) and *Cercospora* leaf spots spot (right) in Siluna.

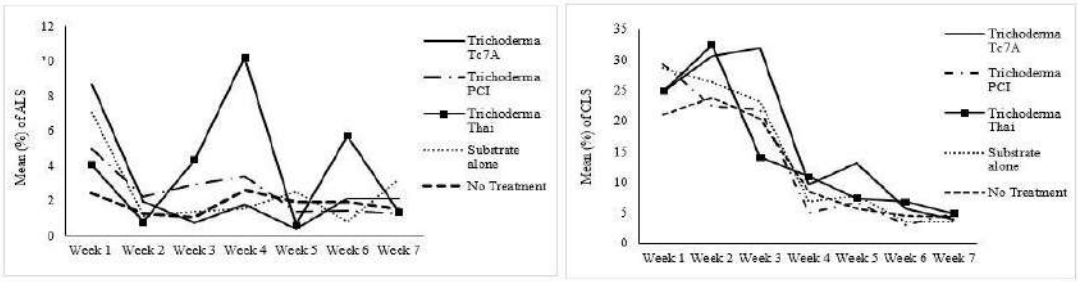

Figure 23 Mean percent of disease severity of *Alternaria* leaf spot (left) and *Cercospora* leaf spots (right) in Jamdo.

2.2.4 Discussion and conclusion

Trichoderma isolates showed suppression of Phytophthora capsici under greenhouse condition in the initial weeks. Overall, plants treated with the native Trichoderma isolate Tc7A exhibiting lowest disease severity among the treatments which was significantly different from the plants in the untreated control indicates its potential as biocontrol agent. Disease severity of 50.9% at the end of the week in inoculated plants treated with fungicide indicates high virulence of the pathogen which could be due to high inoculum rate in the experiment. The current greenhouse experiment indicates that even with high inoculum rate, Trichoderma isolates provide suppression of the disease. Studies conducted in NPPC lacked evaluation of different rate of inoculum (e.g., spore counts). Further studies on spore quantification is required.

Phytophthora blight did not occur in the fields at Jamdo and Siluna in the FY 2022 – 2023 though weather conditions during the evaluation period was within the range of favourable conditions for development of Phytophthora capsici. Due to the absence of P. capsici incidence, evaluation of Trichoderma isolates under field condition remains inconclusive. However, based on the greenhouse evaluation, it is safe to say that use of Trichoderma isolates should provide disease suppression even in the fields. Amending soil with Trichoderma isolates did not seem to provide any protection against foliar diseases. Further studies are required.

2.2.5 References

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2.3 Data collection for disease forecasting system in Apple Scab and Potato Late Blight

2.3.1 Introduction

This activity was initiated in April 2020. The objective of this activity is to generate baseline data over the years on these two diseases to enable development of disease forecasting specific to conditions of Bhutan. Development of reliable disease forecasting requires data from several years. Hence, this activity is being continued.

2.3.2 Materials and methods

Data on potato late blight severity was collected from Desiree and Yusi Maap varieties in the NCOA- Yusipang trial plots following the Henfling, 1987 rating scale as in **Table *19***. Data on apple scab were collected from 10 varieties present in the germplasm block in NCOA-Yusipang: Well Spur, Oregon Spur, Red Spur, Red Chief, Red Free, Top Red, Vance delicious, Shin Kalu, Crimson Golden and Braeburn. From each tree, four branches/twigs each with 100 leaves were identified for data collection.

Currently, the NPPC is using an IPM module (UC Davis) to forecast apple scab and potato late blight based on leaf and soil moisture, and ambient temperature data recorded by a Weather station (Vantage Pro2, DAVIS Instruments, USA) in NCOA Yusipang and auto-fed to the IPM module.

Table 19 Key for assessing the severity of late blight under field conditions, taken from Henfling, 1987

Score	*Phytophthora infestans* (%)		Symptoms
	Average	Boundaries	
1	0		*P. infestans* not observed
2	2.5	Trace < 5	*P. infestans* present. Maximum 10 injuries per plant.
3	10	5 < 15	Plants seem to be healthy, but injuries can be easily observed. There are no more than 20 affected leaves.
4	25	15< 35	*P. infestans* is easily observed on the plants. About 25% of the leaf area is affected by injuries.
5	50	35< 65	Plants look green, but each one is affected by the pathogen, lower leaves are necrotic. About 50% of the leaf area is destroyed.
6	75	65< 85	Plants look green with brown spots. About 75% of the leaf area is affected. Leaves in the middle of the plant are destroyed.
7	90	85< 95	Only upper leaves are green. Most of leaves are affected and many stems have external injuries.
8	97.5	95< 100	Plants look brown, few upper leaves are green and most of the stems are hardly affected or dead.
9	100		Leaves and stems are destroyed.

2.3.3 Results and discussion

Potato late blight: Infection in both Desiree and Yusipang started by May. Desiree was observed to be very susceptible to late blight with 100% plant death by end of July 2022. The damage in the infected Desiree plants varied from 2 to 9 scale of Henfling (1987) in July **Figure *24***. However, Yusi Maap remained moderately resistant to late blight throughout the growing season.

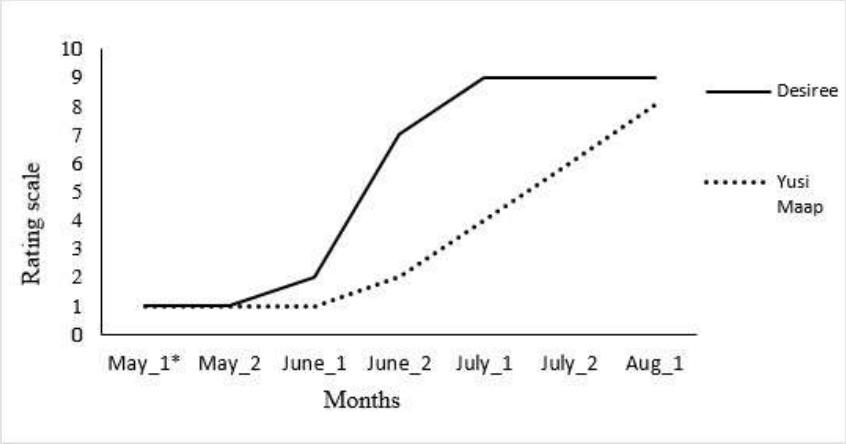

Figure 24 Late blight disease progression at Yusipang from May to August 2022

Apple scab: Initial symptom of apple scab characterized by oily blotched leaves was observed by 1st week of May 2022. In the beginning, the symptom was limited to a few leaves of Top red variety with low incidence. However, later in the growing season, other varieties like Oregon Spur, Vance delicious and Red spur were most infected by the apple scab but were moderately resistant **Figure *25***. Other diseases like rust and powdery mildew dominate during the season. Powdery mildew was usually found on young flushes in a few trees while rust infected the later stage of tree phenology.

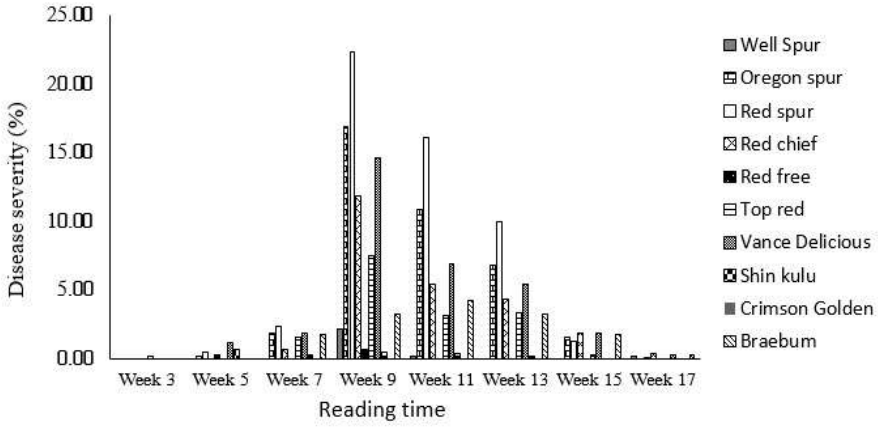

Figure 25 Initial symptom of apple scab (3rd week reading time corresponds to 1st week of May).

Result based on apple scab ascospores maturity model shows that there is high risk for apple scab development from May to August, in Yusipang area. Potato late blight was found to be highly prevalent from June to August based on disease incidence and severity data.

2.3.4 Conclusion

Based on accumulated data from, both late blight and apple scab start showing symptoms in the fields by May indicating infection taking place way ahead of this. This is useful for management of apple scab.

Desiree is vulnerable to late blight while Yusi Maap showed good resistance. Any fungicide spray against late blight may be scheduled by mid-April as disease symptoms are observed in first week of May.

2.3.5 References

Henfling, J. W. (1987). Late blight of potato (Vol. 4). International Potato Center. Lima, Peru.

2.4 HLB testing of citrus samples

2.4.1 Introduction

Huanglongbing (HLB) is a disastrous disease of citrus that has no cure. Testing of citrus germplasms in the repository is necessary to maintain disease free citrus germplasms. A total of 385 citrus samples received from the National Citrus repository from 12 – 28 December 2022 were tested using real-time PCR. Samples were collected and processed according to established protocols, including DNA extraction, PCR amplification, and detection using specific primers and probes.

2.4.2 Materials and methods

Sampling method: Samples were collected in two separate batches to minimize the risk of sample deterioration during transportation and storage. The first batch of samples was

collected from each plant, consisting of a total of seven samples, comprising six leaf samples and one bark sample. The leaf samples were taken from different parts of the tree to ensure representativeness, and the number of leaves sampled varied according to leaf size, with approximately 10-16 leaves per sample. The bark samples consisted of 10-12 bark strips of ~1-2 x 3-5cm, taken from the main trunk of the tree.

Each sample was labelled with detailed information, including the name of the block and its location within the repository, the variety name, and the plant number assigned by the National Plant Protection Centre (NPPC). Based on the assessments of previous samples and the HLB test results, the number of samples per plant was reduced for the subsequent sample collection. From each plant, on one leaf sample and one bark sample were collected.

Sample detail: In this study, a targeted sampling approach was used to retest specific citrus varieties that was recommended for retest in the 5th and 6th batch tests. Samples were collected from previously tested 44 plants that represented 40 varieties. New samples were collected from 117 plants representing 56 varieties.

Testing method: Samples were tested using real-time PCR using the following primers and probes (Li et al., 2006):

HLBas 5'- GTCGAGCGCGTATGCAA-3'
HLBr: 5'- CTACCTTTTTCTACGGGATAACGC-3'
HLB probe: 5'- AGACGGGTGAGTAACGCG-3'
COXf: 5'-GGTATGCCACGTCGCATTCCAGA
COXr: GAATGCCCTTAGCAGTTTTGGC - 3'
COX probe: 5'- ATCCAGATGCTTACGCTGG-3'

Real-time PCR result interpretation: A threshold value of 36 was used to interpret the test results. A Ct value of zero or more than 36 ((Ct= 0 or Ct > 36) indicates that the pathogen is 'not detected'. A Ct value greater than zero but less than or equal to 36 (Ct < 0 or Ct ≤ 36) is considered 'positive'. The results of all samples obtained from a plant determined whether further testing was required or not. All plants were tested twice a year before being confirmed and recommended for mass production or destruction.

Plants were cleared for mass propagation if all of their samples showed Ct values of zero in two consecutive tests. Conversely, plants were rejected for mass production if all of their samples showed Ct values of 'positive' in two consecutive tests. Plants whose test results deviated from this range were recommended for re-sampling and re-testing. If the test results were contradictory (samples with Ct in the positive range in one test and negative range in the second test or vice versa), then re-sampling and re-testing was recommended. If some samples still produced Ct in the positive range in the following (third) test, then the plants were recommended to be destroyed.

2.4.3 Results

Based on the current results (7th batch test), 20 plant varieties were cleared for mass propagation. Plant Q9 (Panbang tshalu) was recommended to be destroyed **Table *20***. For all the other remaining plants, further testing is required for confirmation ***Annexure 1***.

Table 20 Varieties cleared for propagation and to be destroyed. Repository ID given in parenthesis.

Varieties cleared for propagation			
Sl. No.	**Variety (Repository ID)**	**Sl. No.**	
1.	Cara Cara (79)	11.	Car Cara (F97)
2.	Sasaki (93)	12.	120 yrs Pemagatsho Mandarin IN23 (IN23)
3.	Junar (118)	13.	Pemagatsho Kapur (IN22(a))
4.	Ohtsu (4)	14.	Namchala Kalajambir (IN22)
5.	Aoshima (137)	15.	Ohtsu (F105)
6.	Semjong Lime (195)	16.	Pelrithang Kalijambir (IN30)
7.	Bears Lime (220)	17.	Fortunella (378)
8.	Sartshang Kapur (IN25)	18.	Fortunella (F93)
9.	Chubu Mandarin (IN26(a))	19.	Gomdar Mandarin (IN27(a))
10.	Baleygang Phokshat (IN27)	20	Frost Ureka (IN25(a))
Variety to be destroyed			
1.	Drukjeep Tshalu	Q10	

2.5 Development of SoP for plant disease diagnosis

2.5.1 Diagnostic Protocol for 'Candidatus Liberibacter' species

Disease

Huanglongbing (HLB) is the official common name. Other common names are citrus greening, yellow shoot and many more in earlier literatures.

Hosts

The disease affects Citrus and some genera in Rutaceae.

Taxonomy information

Name: '*Candidatus* Liberibacter *africanus'* Garnier et al. 2000; Synonym: '*Candidatus* Liberibacter *africanum*' Jagoueix et al., 1994.
Name: '*Candidatus* Liberibacter *americanus'* Texeira et al. 2005; Synonym: '*Candidatus* Liberibacter *americanum'* Jagoueix et al., 1994.
Name: '*Candidatus* Liberibacter *asiaticus'* Garnier et al. 2000; Synonym: '*Candidatus* Liberibacter *asiaticum'* Jagoueix et al., 1994.

Taxonomic position

Domain – Eukaryota
Kingdom – Bacteria
Phylum – Proteobacteria
Class – Alpha-Proteobacteria
Order – Rhizobiales
Family – Phyllobacteriaceae
Genus – Liberibacter
Species – '*Candidatus* Liberibacter *africanus'*; '*Candidatus* Liberibacter *americanus'*'*Candidatus* Liberibacter *asiaticus'*;

Distribution

'*Candidatus* Liberibacter *africanus'* (CLaf) – present in Asia and Africa (Bové 2006).
'*Candidatus* Liberibacter *americanus'* (CLam) – present in Brazil, South America (Bové 2006).
'Candidatus Liberibacter asiaticus' (CLas) – present in Asia, Africa, Oceania, North and South America (Bové 2006).

Vectors

'*Candidatus* Liberibacter *africanus'* – vectored by *Trioza erytreae* (Bové 2006).
'*Candidatus* Liberibacter *americanus'* – vectored by *Diaphorina citri* (Bové 2006).
'*Candidatus* Liberibacter *asiaticus'* – vectored by *Diaphorina citri* (Bové 2006).

Detection

Symptoms

Symptoms of HLB are not specific and may be confused with nutrient deficiency and symptoms due to other factors like water logging, root or trunk rots, root or trunk boring insects etc.

Some typical symptoms associated with the HLB pathogens are yellow shoots and blotchy mottle symptoms **Figure *26***. Trees show yellow shoots due to irregular distribution of the bacterium in the infected tree. Leaves on infected branches will show blotchy mottle and some may show nutritional deficiencies e.g., zinc. Fruits may develop colour inversion where colouring start from the peduncle end in contrast to the styler end colouring first. Fruits may taste bitter.

Figure 26 Huanglongbing symptoms; (left) yellow shoots; (right) blotchy mottle on mandarin leaves

Detection using molecular methods

Electron microscopes and bioassays using indicator plants were common methods of detection before molecular methods were developed. In the recent years, molecular tools such as polymerase chain reaction (PCR) provide the most reliable and confirmatory tests for

detecting HLB pathogens. Both conventional and real-time PCRs are applicable for detection of the HLB pathogens in both plant and vectors. Primers and probes are given in **Table *21*.**

Sample collection

Plant sample

Methods are developed based on literatures as well personal experience.

Materials required for sampling:

- Plastic bags or zip lock bags
- Stapler and pins
- Pencil
- Labelling paper (made from A4)
- Knife or a leather punch (of ~6-8mm in diameter)
- 5 mL vials with screw caps
- 80% ethanol
- Bleach
- Cool box or locally available materials

Leaf samples

- For symptomatic mature trees of about ≥ 10 years old if yellowing branches are present, symptomatic leaf samples should be collected from the yellowing branches. Two leaf samples and one bark per tree should be collected.
- Look for symptomatic branches e.g., yellow branches. Within the symptomatic branch, look for mottled leaves, yellow leaves, leaves with yellowing veins and/or corky veins, small upright leaves, leathery leaves, nutrient deficient leaves e.g., Zn deficiency.
- From each symptomatic branch collect at least 10-12 symptomatic leaves (with petioles) of about 10cm long per sample. Note: if leaves are of small size then increase the number of leaves up to 15-20.
- From each tree, include one bark samples comprising of young twigs and main trunk (refer below for bark sampling).
- For asymptomatic trees, collect two leaf samples from around the canopy of the tree, and one bark sample.
- Do not collect samples from yellowing branch due to physical damage as in the case of trunk borer infestation or other causes.
- Place each sample in a plastic bag with labels (written with pencil on plain paper).

Bark samples

- Use a leather puncher or a sharp knife
- Take at least 6 pieces of bark per sample from a branch (~6-8mm in diameter if using puncher or length if using a knife) OR young twigs of about 10-15 cm with green

barks. Collect at least 4-6 sections of young twigs per sample. If symptomatic branch is present, collect barks from such branch and also from the main trunk.

- For barks from the main trunk, collect around 6 pieces above the soil.
- Place the composite samples in a plastic bag and label.
- Clean the tools with bleach before using one the next tree (e.g., Robin fabric whitener): take about 150 mL bleach in a 250 mL container and immersing the entire tip which cuts through bark into the liquid, then wipe with clean cotton or tissue paper.
- Clean and oil the tools with sewing machine oil after use.

Vector/psyllid sample

Sampling adult psyllids:

Adults are present throughout the year if high population occurs during flush period.

- Tap branches onto a bowl or tray.
- Suck the falling adults using an insect aspirator (or pooter). Some bigger nymphs may also fall during tapping and the nymphs can be collected as well.
- Alternately, adults can be collected directly from the trees using a manual or battery-operated pooter.
- Collect at least 20-30 adults per site e.g., orchard or nursery.
- Transfer psyllids (both adults and nymphs collected by tapping) into tubes with 80% ethanol
- Label each tube: date, location name and GPS coordinates, host plant name/variety, psyllid species, psyllid stage.

Sampling nymphs:

Nymphs of Diaphorina species can be brushed off from the shoots or twigs and place in vials containing 80% ethanol.

Nymphs of the citrus green psyllid, *Cacopsylla heterogena* can be collected as follows:

- Nymphs develop inside the folds of young tender leaves called ‘pouch galls’.
- Look for trees with pouch galls on young shoots.
- Collect the pouch galls that have nymphs inside (bigger nymphs are desirable, refer pictures). Place all the pouch galls with nymphs straight into a 5 mL tube and pour 80% ethanol.
- Keep samples collected from different trees separately and label accordingly if infection on individual tree is to be tested. Label each tube: date, tree number (as per the germplasm map), host plant name/variety.

Sample care and submission to NPPC

- If leaves are wet from rain or dew then wipe dry with tissue paper before packing in the plastic bags.
- Keep all samples cool after collection, preferably in the fridge at 4°C (not the freezer) till transportation.

- Prepare a sample list containing sample details according to the labels (for plant parts and psyllids).
- Dispatch samples to NPPC within 1-2 days of collection along with the list. Transport in cool boxes.

Labelling samples

- Labels should include: date of collection, location name & GPS coordinates, species and variety name for plant sample and in case of psyllid, species name and variety of the host plant where psyllids are collected from, and collector name.
- Labels can be hand written using pencil or typed (LaserJet print).

Once sample reach the laboratory

- Open package in the extraction room.
- Verify samples according to the label and sample list.
- Record in the sample register and assign laboratory number.
- Store samples at 4°C during verification and extraction.
- Process DNA extraction.

DNA extraction

Although several methods of DNA extractions are available, in NPPC we use the CTAB method because it is a cheap method that can yield good quality DNA. Many commercial DNA extraction kits which provide more rapid extraction protocols are available from many manufactures e.g., Sigma, Bioline and Qiagen and can substitute the current method of CTAB. The CTAB protocol detailed below is applicable for both plant and psyllid samples. If using a DNA extraction kit, extraction procedures must follow the manufacture's instruction or with modifications if required. DNA extraction kits are more convenient and may be safer than using chloroform to separate organic soluble from DNA but they are expensive.

Some of the DNA extraction kits that can be used are:

- ISOLATE II Plant DNA kit and ISOLATE II Genomic DNA kit from Bioline (used for psyllids). Kits from BIOLINE are cheaper and give similar DNA quality as to using more expensive kits.
- For psyllids, REDExtract-N-Amp™ Tissue (or plant) PCR Kit from Sigma works very well and provides a rapid DNA extraction protocol for psyllids.
- DNeasy Plant Pro and Plant kits from Qiagen.

DNA extraction from plant samples using CTAB (modified Doyle and Doyle, 1990)

- Label all tubes and plastic pouch before starting extraction: two sets of 2 mL tubes and one set of 1.5 mL tube. Turn on the water bath with the temperature set at 65°C.
- Wash samples thoroughly (or if using barks: place the barks in a petri dish with distilled water, clean the barks by scrapping off all dead tissues on the outside very

lightly with a scalpel, then rinse with distilled water two times). Include an extraction control comprising of a disease-free plant (same species if available if not any other plant).

- Blot dry with tissue paper/paper towel.
- Using disposable blades, cut mid ribs (or cut the barks) and transfer (0.5 g) in the extraction pouch.
- Add 3–3.5 mL of CTAB (add 20 µL of β-mercapto ethanol per 10 mL of CTAB just before use to each pouch. S
- Grind the samples with the homogenizer at 3000 rpm until no tissue are visible. Homogenization can also be done using Fastprep instrument.
    - If using FastPrep, grind plant tissue using liquid nitrogen before placing CTAB: place 0.5g tissue in 2mL tube (special tube with screw cap), place sterile 6 mm diameter beating beads, dip tubes in liquid nitrogen for 10 sec then place frozen tubes in the FastPrep instrument and secure well. Pulse following FastPrep protocol. Once tissue tis ground to powder, add CTAB filling the tube to 1.9 mL. Then proceed to step 8 below.
- Transfer all extracts into a 2 mL tube with a pastuer pipette.
- Place all the tubes in a floater and incubate in the water bath for 30 min at 65°C (shake the tubes by inverting the tubes gently after 15 min).
- Centrifuge for 5 min at 3000 rpm (~8000 x g) and 20°C.
- Transfer 1 mL of the supernatant into another set of 2 mL tubes.
- Add 1 mL of chloroform: Isoamyl alcohol (24/1) to each tube. Shake by inverting the tubes a few times.
- Centrifuge at 20°C and 14000 rpm (~16100 x g) for 5 min.
- Transfer ~0.850 to 0.9 mL (usually it is easier to transfer 850 µL without sucking the lower phase of the liquid) of the supernatant to 1.5 mL tube. Take care not to take in any chloroform from the bottom – if this happens, put back everything in the original tube and centrifuge again.
- Add 0.54 mL (540 µL) of Isopropanol to each tube. (Isopropanol should be stored at −20°C).
- Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
- Centrifuge at 4°C and 14000 rpm for 20 min.
- Discard the supernatant.
- Re-suspend the pellet in 1mL of 70% ethanol.
- Vortex slightly/mix by hand for few seconds by inverting.
- Centrifuge at 20°C and 14000 rpm for 5 min.
- Discard the supernatant.
- Re-suspend the pellet in 95% ethanol.
- Vortex slightly or hand mix by inverting.
- Centrifuge at 20°C and 14000 rpm for 5 min.

- Discard the supernatant. Carefully siphon off the remaining supernatant with a smaller calibrated pipette (20-200 µL). Discard tip for each sample.
- Leave open the tubes for 30 min to 45 min on bench top.
- Re-suspend the pellet in 80–100 µL of PCR water (or sterile distilled water) or in 1x TE buffer for long term storage or if transporting.
- Store DNA at −20°C till further use.

DNA extraction from psyllids using CTAB

- Although single adult psyllid has been used to test for HLB pathogen, in our protocol we use groups of five psyllids (adults or 4–5th instar nymphs) for DNA extraction. If fewer psyllids are available, then use whatever number (> 1) is available but decrease the DNA elution volume to 20 µL.
- For psyllids preserved in ethanol, dry the psyllid completely to let the ethanol evaporate. This can be achieved by placing the psyllids on clean paper towel using a fine tip brush. Then pick the desired number e.g., five adult psyllids, into each 1.5 mL tubes. Leave the tube open (in the laminar hood) for 10 min before adding extraction buffer. Include extraction control using a disease free psyllid if available or another insect that is not a vector of CLaf/CLam/CLas, e.g., house fly.
- In each 1.5 mL tube containing psyllids, add 300 µL CTAB buffer and crush with a sterile plastic pestle.
- Once the psyllids are macerated, incubate for 30 minutes at 65°C (shake the tubes by inverting the tubes gently after 15 min).
- Centrifuge for 5 minutes at 3000 rpm (~8000 x g) at 20°C.
- Transfer 150 µL of the supernatant into another set of 2 mL tubes.
- Add 150 µL of chloroform: Isoamyl alcohol (24/1) to each tube. Shake by inverting the tubes a few times.
- Centrifuge at 20°C and 14000 rpm (~16100 x g) for 5 min.
- Transfer 120 µL of the supernatant to 1.5 mL tube. Take care not to take in any chloroform from the bottom- if this happens, put back everything in the original tube and centrifuge again.
- Add 80 µL of cold Isopropanol to each tube. (Isopropanol should be stored at −20°C).
- Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
- Centrifuge at 4°C and 14000 rpm for 20 min.
- Discard the supernatant.
- Re-suspend the pellet in 200 µL of 70% ethanol.
- Vortex slightly/mix by hand for few seconds by inverting.
- Centrifuge at 20°C and 14000 rpm for 5 min.
- Discard the supernatant.
- Re-suspend the pellet in 200 µL 95% ethanol.
- Vortex slightly or hand mix by inverting.
- Centrifuge at 20°C and 14000 rpm for 5 min.

- Discard the supernatant. Carefully siphon off the remaining supernatant with a smaller calibrated pipette (20–200 µL). Discard tip for each sample.
- Leave open the tubes for 30 min to 45 min on bench top.
- Re-suspend the pellet in 30 µL of PCR water (or sterile distilled water) or in 1x TE buffer for long term storage.
- Store DNA at −20°C.

DNA extraction from psyllids using REDExtract-N-Amp™ Tissue (or plant) PCR Kit from Sigma

The protocol using this kit is modified as below depending on the number of psyllids used per extraction.

- Set water bath to 95°C before proceeding
- Place psyllids in 1.5 mL tube as described in the CTAB method.
- For extraction from single adult psyllid, add 10 µL of the extraction buffer and crush the psyllid with blunt 20–200 µL pipette tip (flame the tip and press against the cap of the sample tube to make it blunt).
- Incubate at 95°C for 10 min.
- Remove from the water bath and add 10 µL dilution buffer.
- Store the extract at −20°C till further use.
- For extractions from 2–3 adults add 15 µL of each of extraction and dilution buffer.
- For extraction from five adults, add 25 µL of each buffer.
- For nymphs, use 10 µL and 15 µL of each buffer for 2–3 and five nymphs respectively.

Conventional PCR (cPCR)

Primers OI1/OA1/OI2C **Table *21*Table *22*** detect '*Candidatus* Liberibacter *asiaticus'* and '*Candidatus* Liberibacter *africanus'* and gives an amplification band of 1160 bp. Distinction between the two species requires digestion of the amplicon with restriction endonuclease XbaI. ''*Candidatus* Liberibacter *asiaticus'* produces two bands, 640 bp and 520 bp while '*Candidatus* Liberibacter *africanus'* produces three bands (520 bp, 506 bp, 130 bp).

Primers A2/J5 detects beta-operon region of '*Candidatus* Liberibacter *africanus'* and '*Candidatus* Liberibacter *asiaticus'*. Band size for '*Candidatus* Liberibacter *africanus'* is 669 bp and for '*Candidatus* Liberibacter *asiaticus* is 703 bp **Table *21*Table *23***

Primers GB1 and GB3 detects 16S rDNA of '*Candidatus* Liberibacter *americanus'* but not that of '*Candidatus* Liberibacter *asiaticus* or '*Candidatus* Liberibacter *africanus'* **Table *21*Table *24*** Duplex PCR using primers GB1/GB3 and A2/J5 is used for detection of '*Candidatus* Liberibacter *africanus'*, '*Candidatus* Liberibacter *americanus'* and '*Candidatus* Liberibacter *asiaticus'* **Table *21***, ***Table 25***.

Table 21 Primers for conventional cPCR to detect HLB pathogen, 'Candidatus Liberibacter sp.'

Primer name	Primer sequence (5' to 3')	Target gene	Reference
Forward: OI1	5'GCG CGT ATG CAA TAC GAG CGG CA 3'	16S rDNA	Jagoueix et al. 1996)
Forward: OA1	5′-GCG CGT ATT TTA TAC GAG CGG CA-3′		
Reverse: OI2C	5' GCC TCG CGA CTT CGC AAC CCA T 3'		
Forward: A2	5′- TAT AAA GGT TGA CCT TTC GAG TTT -3′	rplA/rplJ	Hocquellet et al. 1999
Reverse: J5	5′- ACA AAA GCA GAA ATA GCA CGA ACA A -3′		
Forward: GB1	5′-AAG TCG AGC GAG TAC GCA AGT ACT-3′	16S rDNA	Teixeira et al. (2005)
Reverse: GB3	5′-CCA ACT TAA TGA TGG CAA ATA TAG-3′		

Table 22 cPCR reaction mix preparation for OI1/OIA/OI2C

Reagents	µL/reaction	Final concentration	PCR condition
PCR water	12.8 µL		Initial denaturation at 94°C for 2 min;
10x PCR mix (without MgCl2)	2.5 µL	1x	35 cycles of: Denaturation at 92°C for 60 sec; Annealing & extension at 72°C for 90 sec;
MgCl2 (25mM)	2.5 µL	2.5 mM	
dNTP Mix (10mM each)	0.6 µL	0.24 mM each	
OI1 (10µM)	1.0 µL	0.4 µM	
OIA (10µM)	1.0 µL	0.4 µM	Final extension at 72°C for 10 min;
OI2C (10µM)	1.0 µL	0.4 µM	Hold at 10°C
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/µL	
DNA template	2 µL		
Total reaction volume	25 µL		

Table 23 PCR mix preparation for A2/J5

Reagents	µL/reaction	Final concentration
PCR water	13.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
A2 (10µM)	1.0 µL	0.4 µM
J5 (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/ µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR conditions for A2/J5

Initial denaturation at 94°C for 2 min; 35 cycles each of: Denaturation at 92°C for 20 sec; Annealing at 62°C for 20 sec; Elongation at 72°C for 45 sec followed by a final extension at 72°C for 10 min (one cycle only) and hold at 10°C.

Table 24 PCR reaction mix for GB1 and GB3

Reagents	µL/reaction	Final concentration
PCR water	13.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
GB1 (10µM)	1.0 µL	0.4 µM
GB3 (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5 U/µl)	0.125	0.025 U/µL
DNA template	2 µL	
Total reaction volume	25 µL	

Table 25 : Duplex of A2/J5 and GB1/GB3 (EPPO, 2021)

Reagents	µL/reaction	Final concentration
PCR water	10.375 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25 mM)	2 µL	2 mM
dNTP Mix (10 mM each)	0.5 µL	0.2 mM each
GB1 (20 µM)	1.25 µL	1µM
GB3 (20 µM)	1.25 µL	1µM
A2 (20 µM)	1.25 µL	1µM
J5 (20 µM)	1.25 µL	1µM
Taq (DNA polymerase (5U/µl)	0.125	0.625 U or 0.025 U/ µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR condition for duplex PCR of GB1/GB3 and A2/J5 (EPPO, 2021)

Initial denaturation at 96°C for 5 min followed by 35 cycles of: denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec and 72°C for 10 min; Final extension at 72°C for 10 min and cool/hold at 4°C.

Control for cPCR

- No Template Control (NTC) is a negative control that uses PCR water (no DNA template) with the PCR mix.
- Extraction control (EC) is a negative control consisting of plant/ insect known to be free of the liberibacters.
- Positive control (PC) is a known positive DNA of the specific species.

Table 26 Primers and probes based on 16S rDNA for detection of the three 'Candidatus Liberibacter' species (Li et al., 2006) in plant and psyllid samples

Primer/Probe name	Primer sequence (5' to 3')	Reference
Forward: HLBaf	5′-CGA GCG CGT ATT TTA TAC GAG CG-3′	Li et al., 2006
Forward: HLBam	5′-GAG CGA GTA CGC AAG TAC TAG-3′	
Forward: HLBas	5′-TCG AGC GCG TAT GCG AAT ACG-3′	Duan et al., 2009
Forward: CLas-4G	5′-AGT CGA GCG CGT ATG CGA AT-3′	Bao et al., 2020
Reverse primer: HLBr	5′-GCG TTA TCC CGT AGA AAA AGG TAG-3′	Li et al., 2006
probe: HLBp	5′-FAM-AGA CGG GTG AGT AAC GCG-BHQ1-3′	Li et al., 2006
Forward primer: COXf	5′-GTA TGC CAC GTC GCA TTC CAG A-3′	
Reverse: COXr	5′-GCC AAA ACT GCT AAG GGC ATT C-3′	
Probe: COXp	5′-TET-CAG ATG CTT ACG CTG-BHQ1-3′	
For *Diaphorina citri* use the following primer and probe instead of COX		
Forward primer : DCF	5′-TGG TGT AGA TGG TTG TGA TCT GAT GTG-3′	Manjunath et al., 2008
Reverse primer: DCR	: 5′-ACC GTT CCA CGA CGG TGA-3′	
Probe: DCP	5′-HEX-TGT GGG CGA GGC TAC AGA AC-BHQ1-3′	
For *Cacopsylla heterogena* use the following primer and probe instead of the primer and probe for *D. citri*.		
Forward primer: WGCaF	5'-GCA CTC AAG GAC CGG TTT GAC GG -3'	Om, 2017
Reverse primer: WGCaR	5'-ACT GCC GCG CAC ATT CCC CTC -3'	
Prober: WGp	5'-TET - TTA CTG ACC ATG ACT CTG GAC GC BHQ2-3'	Li et al., 2008

Result interpretation of cPCR

- A test result is positive if expected band size for the specific species are obtained:
- 1160 bp for CLaf or CLas using OI1/OIA/OI2C;
- 669 bp for CLaf and 703 bp for CLas using A2/J5;
- 027 bp for CLam, 669 bp for CLaf, 703 bp for CLas in the duplex PCR using GB1/GB3 and A2/J5.
- All negative controls do not produce bands
- Positive control shows the expected band size for the specific species: 1160 bp for CLaf or CLas using OI1/OIA/OI2C; 669 bp for CLaf and 703 bp for CLas using A2/J5; and 1027 bp for CLam, 669 bp for CLaf, 703 bp for CLas in the duplex PCR using GB1/GB3 and A2/J5.
- If any of the controls deviate from the above, then the tests should be repeated. Re-sampling may be required under certain circumstances.

Real-time PCR

Real-time PCR uses primers and probes developed by Li et al. (2006) for plant samples and Manjunath et al. 2008 for *D. citri* samples. An alternate primer for D. citri wingless gene developed by Li et al. 2008 can also be used **Table *26***. A reverse primer and a probe common

to all the three species are used. Internal control is provided by the plant DNA which is detected by the primer and probe for cytochrome oxidase (COX) gene. For psyllids, internal controls target the wingless gene of psyllids.

Preparation of PCR reaction mix

PCR reaction mix using the above primers and probes are given in ***Table 27***.

Table 27 PCR reaction mix for detection of all three 'Canditatus Liberibacter' species

Reagents	µL/reaction	Final concentration	Remarks
PCR water	1.5 µL		Leave out forward primers of HLBaf and HLBam if routine work/detection for CLas is done; Replace HLBas with CLas-4G forward primer in case low titre of bacterium is suspected.
10x PCR mix (without MgCl2)	1.3 µL	1x	
MgCl2 (50 mM)	1.5 µL	6 mM	
dNTP Mix (10 mM each)	0.3 µL	0.24 mM each	
Primer Mix of HLBaf, HLBam, HLBas, HLBr (2 µM)*	1.5 µL	240 nM	
HLBp (1 µM)	1.9 µL	150 nM	
Primer Mix of COXf/COXr or DCF/DCR or WGCaF/WGCaR (2 µM)*	1.5 µL	240 nM	
COXp or DCP or WgP (1 µM)	1.9 µL	150 nM	
Taq (DNA polymerase (5 U/µl)	0.125 µL	0.025 U/µL	
DNA template	1 µL		
Total reaction volume	µL		

**see primer dilution*

Real-time PCR conditions

Pre-incubation at 50oC for 2 min followed by initial denaturation at 95oC for 10 min and 40 cycles of: denaturation at 95oC for 30 sec, followed by annealing and extension at 58oC for 40 sec.

Real-time PCR result interpretation

Test is valid only if:

- The positive control (PC) shows exponential amplification curve.
- Non template control (NTC) does not show an amplification curve or amplification curve is below the defined Ct value.
- The internal control e.g., COX/DC/WG should show amplification curves for COX or DC or WG depending on the sample type (COX for plant, DC for D. citri, WG for C. heterogena).
- A sample is positive if the sample shows an exponential amplification curve or has an amplification curve above the Ct value, provided all of the above conditions are met.
- If any of the above conditions are not met, the test should be repeated.

- If a sample does not show an amplification curve for HLB it could mean, provided all above conditions are met, that the DNA of the pathogen is not present in that sample and the results is noted as 'Not detected' instead of saying negative.

Sequencing should be performed using A2/J5 or any other cPCR primers to confirm the identification of bacteria species if samples are tested for detection of CLaf and CLam as these are not detected in Bhutan till date. Include all three species if other species are being tested for acquisition and/or transmission of the CLaf, CLam and CLas.

2.5.2 Diagnostic Protocol for *Pyricularia oryzae* Cavara 1892

Disease
Rice blast

Hosts
Hosts of *P. oryza*e include species of cereals (rice - Oryza sativa, African rice - Oryza glaberrima, perennial wild rice - *Oryza longistaminata*, finger millet - *Eleusine coracana*, fox tail millet - *Setaria italic*, fonio millet - *Digitaria exilis*, barley - *Hordeum vulgare*, Italian rye grass - *Lolium multiflorum*, common millet - *Panicum miliaceum*, wheat - *Triticum aestivum*, maize - *Zea mays*) and grasses (armgrass millet - *Brachiara distachya*, crabgrass - *Digitaria sanguinalis*, crowfoot grass or goosegrass - *Eleusine indica*, barnyard grass - *Echinochloa crus-galli*, cutgrass - *Leersia hexandra*, torpedo grass - *Panicum repens*, itchgrass - *Rottboelia exaltata* etc.

Taxonomic information

Name: *Pyricularia oryza*e Cavara 1892 (anamorph or asexual stage; teleomorph or sexual stage: *Magnaporthe oryzae* Couch (2002).

Taxonomic position:

Domain – Eukaryota
Kingdom – Fungi
Phylum – Ascomycota
Class –Sordariomycetes
Order – Magnaporthales
Family – Pyriculariaceae
Genus – *Pyricularia*
Species – *Pyricularia oryzae* (EPPO 2023).

Detection

Symptom

Pyricularia oryzae infects almost all the parts of rice plant: leaf (leaf blast), leaf collar (collar blast), culm, culm nodes (node blast), panicle nodes (neck blast or neck rot), and panicle

(panicle blast). The pathogen can infect rice plant at any growth stage. Infection appear as lesion or spots.

Leaf blast: Initial symptom on leaf appears as white to grey-green lesions with green borders. Lesions become elliptical or diamond shaped with whitish to grey centres and brown to necrotic borders as they become old **Figure *27***. Lesions can coalesce and become large and may kill the entire leaf.

Node infection: Dry dark-brown discolouration appears at the node of blast infected culm (Figure 19). The culm breaks off from the node killing the entire rice plant. When infection of node occurs at the junction of leaf blade and leaf sheath it is called collar rot, and when infection at the base of the panicle (the junction of stem and the seed head) it called neck blast. Infection on the neck causes unfilled grain which appears as white head a condition known as blanking **Figure *27***.

Panicle blast: Gray-brown necrotic lesion appears on the panicles which also infects, spikes, spikelet, and panicle branches. The panicle appears as brown or dark head. As the infection matures, the panicles branches break at the lesion.

Confusion with other diseases and disorders

Brown spot

Initial symptoms of rice blast on leaves can be easily confused with brown spot symptoms. However, the basic difference is that the leaf blast lesions are spindle shaped with pointed ends whereas the brown spot lesions are round to oval in shape, brown in colour with yellow halo around the lesion (Figure 20). Brown spot is caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker (teleomorph- *Cochliobolus miyabeanus*). Conidia of B. oryzae are 63-153 x 14-22 µm in size, curved with numerous septa **Figure *28***.

Figure 27 Rice blast: (left) spindle shaped lesion on leaves; (center) node blast; (right) neck blast with white head/panicle (photo: N.Om).

Rice stem borer

Rice stem borer infest rice plants at any growth stage. Larvae of stem borer burrow inside the culm and causes dead heart during vegetative stage and whitehead during reproductive stage. Whitehead symptom produced by neck rot can be easily confused with rice stem borer infestation. However, whiteheads caused by stem borer can be easily pulled off from the plant

whereas whiteheads due blast cannot. Moreover, signs of stem borer such as pupae, holes and frass may also be also visible.

Figure 28 Brown spot: (left) oval shaped brown spots with yellow or necrotic borders; (right) conidia of Bipolaris sp. (Photo: N. Om).

Identification

Identification can be done both through morphological and molecular methods.

Morphological methods

Inducing sporulation on plant tissue

- Place fresh sample showing initial symptom of blast in a moist chamber (plastic bag or petri plate lined with wet tissue or blotting paper).
- Incubate for 28°C for 24 - 48 hrs. Incubate under light if sporulation fails. Try incubation on artificial media.
- Examine for sporulation: take a mass of mycelium and place on a glass slide loaded with water or cotton blue; observe under 10x or higher for the typical pyriform conidia of *P. oryzae*.

Inducing sporulation on media

Sporulation P. oryzae can be isolated on water agar or Potato Dextrose Agar (PDA) which can be sub-cultured on PDA. Other media for sporulation of P. oryzae includes cornmeal-rice straw agar, oatmeal agar and cornmeal agar.

Morphological characteristics

Conidia (spores) solitary, pyriform (pear-shaped) to obclavate with 2 septa, 25-33.5 x 7-11 µm, each conidium bears a basal appendage called hilum which is 0.5–1 x 1–2µm. Conidiophores septated and brown **Figure *29***. Variation in conidia shapes have been reported ranging from oval, short pyriform, pyriform and long pyriform **Figure *30***.

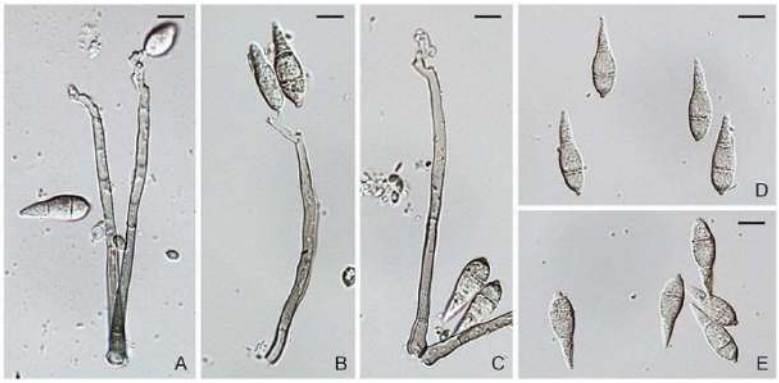

Figure 29 Conidiophores and conidia of P. oryzae (A-E) (Luo and Zhang, 2022)

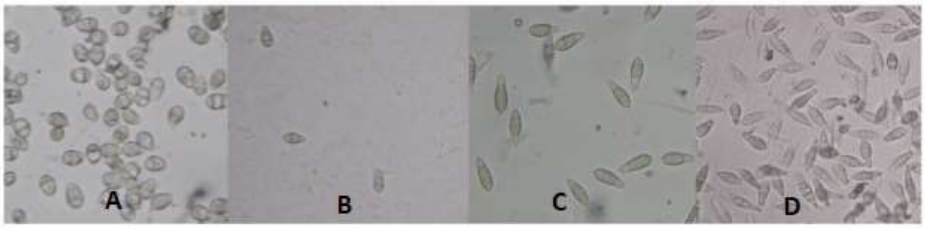

Figure 30 Variation in conidia shapes of P. oryzae: (A) oval; (B) short pyriform; (C) pyriform; (D) long pyriform (Longya et al., 2020).

Molecular methods

Laboratory requirements

- 2.0, 20, 200, and 1000 µL sterile pipette tips (prefer barrier tips)
- Microcentrifuge
- Microcentrifuge tubes 1.5 and 2.0 mL.
- Benchtop vortexer.
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator; Camera/gel documentation system
- PCR reagents
- Running buffer, 0.5 - 1x TBE (see NPPC protocol on buffer preparation)
- Agarose
- DNA ladder
- DNA staining agent (e.g., GelRed, avoid use of ethidium bromide) & Gel loading dye

DNA extraction

Refer specific DNA extraction methods provided by the manufacturer if using a commercial extraction kit (e.g., Bioline, Puregene, Qiagen, Sigma, etc.). Refer NPPC protocol on DNA extraction from fungi and fungi-like organism method.

Prepare PCR mix reaction as per the protocol received with the DNA Taq DNA polymerase or use the protocol in **Table *28***.

Use the DNA to amplify actin, beta-tubulin and calmodulin gene regions using the primer pairs and PCR conditions in **Table *28***. Run the PCR product in a TBE gel. After the gel electrophoresis, PCR product should produce a band at 320bp, 550bp and 500bp for actin, beta-tubulin and calmodulin gene region respectively. The PCR products should be purified using PCR Purification Kit by following manufacturer's protocol. The purified products should submitted to DNA sequencing facilities (Macrogen Inc. or others). The DNA sequence data is BLAST analyzed in NCBI and compared with reference sequence data listed in **Table *29*** for confirmation.

Table 28 : PCR mix preparation

Reagents	µl/reaction	Final concentration
PCR water	17.3	
10x PCR mix (without MgCl2)	2.5µl	1x (2mm MgCl2)
MgCl2 (25mM)	0.5	
dNTP Mix (10mM each)	0.6 µl	0.24mMeach
Primer 1 (10µM)	1µl	0.4µM
Primer 2 (10µM)	1µl	0.4µM
Taq (DNA polymerase (5µ/µl)	0.125µl	0.025U/µl
DNA template	2 µl	
Total reaction volume	25 µl	

Table 29 Primer pair for amplification of different gene region (Couch and Kohn, 2002).

Locus	Amplicon	Primer pair	Primer Sequence
Actin	320bp	ACT-512F	5'-ATGTGCAAGGCCGGTTTCGC-3'
		ACT-783R	5'-TACGAGTGCCTTCTGGCCCAT-3'
Beta-tubulin	550bp	Bt1a	5'-TTCCCCCGTCTCCACTTCTTCATG-3'
		Bt1b	5'-GACGAGATCGTTCATGTTGAACTC-3'
Calmodulin	500bp	CAL-228F	5'-GAGTTCAAGGAGGCCTTCTCCC-3'
		CAL-737R	5'-CATCTTTCTGGCCATCATGG-3'
PCR conditions: Initial denaturation: 1 cycle at 98°C for 30 sec; 30 cycles of: denaturtion at 98°C for 10 sec; annealing at 60°C for 30 sec; extension at 72°C for 30 sec; Final extension at72°C for 5 min; Hold at 10°C			

2.5.3 Diagnostic Protocol for Phytophthora capsici Leonian (1992)

Disease
Common name is *Phytophthora blight*. In Bhutan, it is referred to as *chilli blight*.

Hosts

Phytophthora capsici indeed poses a significant threat to various plants and not just chilli peppers. Though primarily thought to affecting chilli, Phytophthora capsici has a wide host range its ability to affect plants in the Solanaceae, Cucurbitaceae, and Fabaceae (Lamour et al. 2012) families makes it a concern to agriculture. The widespread distribution of this pathogen (Erwin and Ribeiro, 1996; Sun et al, 2008) further emphasises its importance in agricultural management.

Taxonomic information

Name: *Phytophthora capsici* Leonian (1922)

Taxonomic position:
Domain – Eukaryota
Kingdom – Chromista
Phylum – Oomycota
Class –Oomycetes
Order – Peronosporales
Family – Peronosporaceae
Genus – *Phytophthora*
Species – *Phytophthora capsici*

Detection

Detection of the pathogen can be based on symptoms, morphological and molecular characters.

Symptoms

The pathogen can infect all parts of the plant with infections occurring both in the nursery and in transplanted fields. Affected areas exhibit dark brown lesions on stems, branches, crown, and roots (Figure 31). This infection leads to tissue rotting and subsequent wilting of the plants (Figure 31). In nursery settings, infection leads to damping-off, causing patches of dead seedlings. Infected plant parts exhibit water-soaked and dark brown lesions. Symptoms on leaves begin as light green and progress to yellowish as the disease advances. Leaf spots may start from the leaf margin that is either in contact with the soil or due to spores splashed from soil or from the nearby infected tissues. On fruits or chili pods, water-soaked lesions develop, resulting in softening of the fruits. Infected fruits may also develop a white powdery growth.

Morphology

Several morphological characteristics are used in identifying *P. capsici*. Similar to other *Phytophthora* species, the morphological characters used in identifying include sporangium shape, size, length-to-width ratio, papillation, and caducity, as well as the shape of sporangia.

Under warm and wet conditions, infected tissues produce lemon-shaped sporangia. On culture media, sporangia production must be induced by incubating cultures submerged in sterile distilled water exposed to light.

P. capsici primarily exists in its asexual stage, where asexual reproduction produces sporangia which are microscopic sac-like structures that house spores known as zoospores. Zoospores, being flagellated, exhibit motility in the presence of free moisture. Zoospore release from the sporangia can be induced (in the laboratory) by incubating cultures at low temperature, typically around 4°C for 10-15 min.

Under specific conditions, such as the presence of both mating types A1 and A2, sexual spores or oospores may also be observed. This occurs either on infected tissues or under laboratory conditions where the mating types have contacted each other. Oospores, being sexual resting spores, have the capability to persist in soil for extended periods, sometimes many years and germinate forming mycelia and sporangia under favorable, wet conditions.

Figure 31 Symptoms of *Phytophthora* blight: dark brown lesions on stem, crown and root, and wilting of plants.

Isolation

Phytophthora capsici can be isolated from various sources including soil, irrigation water, and plant tissues. Isolating the pathogen from infected plant tissue is the simplest method, while isolating it from soil and water involves baiting the pathogens using materials such as rhododendron leaves or susceptible plants like eggplants, tomatoes, and chili pods.

Isolation and establishment of pure culture of *P. capsici* require selective media. Selective media such as V8, CA and MEA media are used. At the NPPC, P. capsici from plant tissue has been isolated using carrot agar (CA). Isolation can be initiated with direct culture on media or transfer from moist chamber incubation. Moist chamber incubation is useful for initiating sporulation.

To isolate and establish a pure culture of *P. capsici*, selective media are necessary. Media like V8, CA, and MEA are commonly utilized for this purpose. In the NPPC laboratory, *P. capsici* has been successfully isolated from plant tissue using carrot agar (CA). Isolation procedures can begin either with direct culture on media or by transferring from moist chamber incubation, where the latter method is particularly useful for inducing sporulation.

Isolation of Phytophthora capsici from plant tissue using selective carrot agar (CA) media

- Using a kitchen grinder, grind 200g of washed carrot slices in warm water, just enough to cover the carrot slices.
- Filter the ground carrot through four layers of cheese cloth (a thin, guazy cotton fabric with a loose weave) to obtain the carrot juice.
- Place 15g of agar in a 2L flask.
- Add the carrot juice to the flask with agar. Adjust the final volume of liquid to 1L with distilled or RO water and mixed well
- Autoclave at 121°C for 15 min.
- Cool the autoclaved media to 50-60°C.
- Add antibiotics to suppress growth of bacteria and other fungi to the cooled media.
- Then pour into sterile petri plates inside a laminar flow. Let the plates dry in the laminar flow for ~30 min. Wrap the plates using plastic bags or cling wrap and store in the fridge till further use
- Alternately, autoclaved media can be stored on the shelves in the flask for pouring later.
- Note: media amended with antibiotics (or fungicides) must be used within 2-3 weeks.
- In NPPC, nystatin is the only antibiotic (antifungal) used.
- Prepare antibiotic stock as by dissolving 500mg (powder or tablet) in 5ml of sterile distilled/RO water that give a stock concentration of 100mg/ml. Then add 0.25ml of the stock solution to 1L media to achieve a final concentration of 25 µg/ml in 1L media (Drenth and Sendall, 2001).

Direct incubation in moist chamber

- Wash the plant tissue (e.g., leaves or fruits or roots or whole plant if seedling) in running water. Rinse with sterile distilled water.
- Flame sterilises all tools e.g., forceps, loops, needles.
- Cut ~5 – 10 cm length of the plant tissue, or whole leaf for leaf samples, at the edge of diseased part including a few cm of the tissue.
- Surface sterilise tissue with 1% bleach (sodium hypochlorite) or 70% ethanol by dipping the tissue for 30-60 secs. Whole tissue must be covered in ethanol. Rinse away ethanol with sterile distilled water 2-3 times. Drain off excess water from the samples and blot dry using sterile paper towel.
- Place plant tissue in a petri dish lined with wet paper towel/filter paper or filled with shallow distilled water.
- Incubate under light at 18-25°C for 24-48 hrs.
- Examine for sporangia production and if present, then transfer the infected part onto a selective media.

Plating infected plant tissues on selective media

- Wash soil and other dirt off the sample in running water.
- Cut small section of the plant sample (whether leaf or stem or root), of around 0.5-1cm^2 in size, from the edge of the diseased area. Sample should include both diseased and the adjacent healthy tissue. If it is a stem or root sample of >1 cm diameter, it may be desirable to use the outer layer or bark only.
- Sterilize all tools and plant material as above (Section B).
- Blot dry plant tissues after rinsing.
- Transfer to selective media CA. Usually four pieces of 0.5-1cm2 samples (from step 2) are plated onto each plate at equidistance. Press the tissue into the medium to ensure good contact between the tissue and the antibiotics in the medium that will supress bacterial growth.
- Incubate at 28°C in the dark (use an incubator) and observe every day for growth of non-septate hyphae. If other fungi are observed to grow, then transfer plugs of agar from active region, making sure that the plug contains only one type of growth, to another plate with selective media. Once a pure culture is obtained. Observe for hyaline non-septate hyphae.

Inducing sporangia formation in plates

- From the pure culture of an isolate which is < 7 days old, transfer single agar plug to petri plates with basal media of CA or CMA or V8 juice. Incubate at 28°C. Examine cultures daily.
- When growth is about half of the petri plate, transfer 6-10 agar plugs into sterile empty petri plate.
- Flood the petri plate with sterile distilled water covering the surface of the agar plugs.
- Incubate at room temperature (~20-25°C) under continuous light. Examine after 12-24 hrs for mycelial growth and sporangia formation under a dissecting microscope.
- If sporangia are not formed after 24-48 hrs. It is probably not Phytophthora.
- If difficult to see under a dissecting scope, take a small mycelial growth on a glass slide and observe under higher magnification using a compound microscope. Sporangia of Phytophthora are lemon-shaped. Sporangia of Pythium are globose and more irregular.
- Once sporangia formation is confirmed, proceed for characterisation and identification of the isolate.

Identification of isolates

Once an isolate is established to be that of Phytophthora, proceed to identification of the species based on morphology and molecular characteristics. Morphological characters include:

Colony morphology on standard media e.g., PDA.

Sporangial morphology*:*

- Sporangiophore characters

- Hyphal swelling
- Oospores
- Lack of chlamydospores

Note: Culture for identification and subsequent work such as pathogenicity and inoculum production must preferably be obtained from single hyphal tip, or single spore. Culture obtained in such manner should be incubated at the optimum temperature (~28°C) on basal medium such CA or CMA or V8 juice but not PDA. Sporangia production and other work should follow.

Colony morphology:

- Whether mycelium is on the surface (appressed) or aerial, submerged.
- Coenocytic hyphae, branch at nearly right angles and sometimes constricted at the base.
- Hyphal swelling.
- Chlamydospores are not common in *P. capsici*.

Sporangial morphology (Drenth and Sendall, 2001; Abad *et al.*, 2023)

- Sporangia shapes are ovoid, obivoid, ellipsoid ***(Figure 32)*.**
- Dimension of sporangium (length to breadth ratio). Length and breadth of at least 25 sporangia must be measured to confirm the shape.
- Sporangiophore bears the sporangia. Sporangiophores are unbranched or irregularly branched occasionally simple sympodia (**Figure *33*).**
- Hyphal swellings occasionally produced in aqueous cultures; Lacks chlamydospores.
- Presence of an apical thickening of the sporangium called papilla (nipple). *P. capsici* has sporangia that are papillated or semi- papillated, sometimes with 2-3 apices (**Figure *33*).**
- Presence or absence of caducity (shedding of sporangium at maturity). Crush the agar or mycelial growth taken from agar in Section C step 7 to dislodge sporangia and observe if the sporangia get detached from sporangiophores.
- Length of the pedicel on the shed sporangia: short (< 5um), intermediate (5-10um) or long (> 10um) **Figure *33***. The pedicel (sporangial stalk) is the hyphal strand left behind on the sporangium when it is detached from the sporangiophore. Pedicels of at least 25 sporangia must be measured to get the average length.

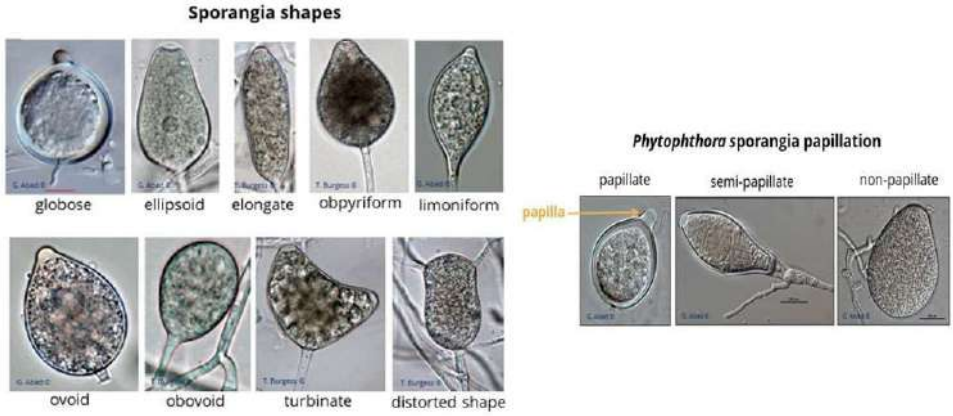

Figure 32 Shape and papillation of sporangia of Phytophthora (images from Abad et al. 2023).

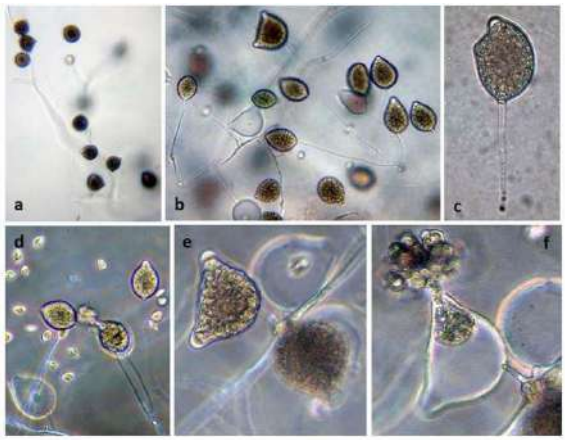

Figure 33 Phytophthora capsici: (a) sporangia originated in unbranched and simple sympodial sporangiophores, (b) sporangia originated in unbranched and irregular branched sporangiophores, (c) semipaillate ellipsoid caducous sporangium with long pedicel, (d-f) zoospores produced from semipapillated or bipapillate sporangia (images and description from Abad et al. 2023).

Morphology of sexual spores

- Male structure: antheridium
- Female structure: Oogonia
- Spores formed as a result of fusion of male and female are called oospores.
- Requires mating types A and B to confirm the mating type of the isolate as well as to produce oospores. Paring of the target isolate with a known mating type will help determine the mating type of the isolate.
- Oospores production will take long, around 30 days.

Staining of sporangia/oospores/any structure

For observation under higher magnification using a compound microscope, take agar plugs containing mycelia and gently place them onto a glass slide. Then, carefully crush the agar plugs to spread the mycelia evenly across the slide. You can choose to stain the sample with lacto-phenol blue, which helps in highlighting the structures for better visibility OR fix and stain sporangia as below.

Prepare a solution of 0.05-0.1% of acid fushsin in 85% lactic acid (full strength).

- Immerse agar plugs (containing mycelia/sporangia/sexual structures) in this solution overnight.
- Mount mycelium mat in clear lactoglycerol for microscopic examination
- Measure at least 25 structures of each type (whether sporangia for size & shape or pedicel length OR oospores).

Once stained, cover the slide with a coverslip and ensure there are no air bubbles. The prepared slide can then be observed under higher magnification using a compound microscope for detailed examination of the fungal structures.

Molecular

Molecular identification is done using ITS rDNA and COI genes from DNA of culture samples. Primers and PCR mix preparation for conventional PCR are given in ***Table 30*** and **Table *31*** respectively.

Table 30 Primers for conventional PCR for detection of *P. capsici*

Primer name	Primer sequence (5' to 3')	Target gene &	Reference
Forward: ITS5	5' – GAAGTAAAAGTCGTAACAAGG – 3'	ITS rDNA	White et al. 1990.
Reverse: ITS4	5' – TCCTCCGCTTATTGATATGC – 3'		
Forward: PC1	5'– GTCTTGTACCCTATCATGGCG – 3'	ITS rDNA (region 1)	Zhang et al. 2006
Reverse: PC2	5'– CGCCACAGCAGGAAAAGCATT– 3'		
Forward: COIF-1	5' – TCAWCWMGATGGCTTTTTTCAAC – 3'	Cytochrome oxidase I	Robideau et al 2011
Reverse: COIR-1	5' – RRHWACKTGACTDATRATACCAAA – 3'		

Table 31 cPCR mix preparation

Reagents	µL/reaction	Final concentration
PCR water	12.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
Forward primer (10µM)	1.0 µL	0.4 µM
Reverse primer (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR conditions (Ristaino et al. 1998, White et al. 1990, Zhang et al. 2006):

For ITS5/ITS4: The thermal cycling parameters as outlined below is followed: were initial denaturation at 96°C for 2 min followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min.

ITS5/IT4 gives a PCR product size of 899 bp.

For PC1/PC2: Initial denaturation of one cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec; 70°C for 30 sec and 72°C for 30 sec. A 7-min final extension at 72°C. Hold at 4-10°C.

PC1/PC2 gives a band of 560 bp.

Refer molecular identification of Phytophthora online at https://idtools.org/phytophthora/index.cfm?pageID=1877.

The website provides information on morphology with images and detailed SoPs from isolation to culture storage, and molecular methods and protocols required for conducting molecular identification including sequencing analysis using ITS and COI regions. The website has links for factsheet on over 100 species of Phytophthora.

2.5.4 Diagnostic Protocol for Venturia inaequalis (Cooke) Winter

Disease

Apple scab

Hosts

Though *Venturia inaequalis* primarily infects cultivated apple varieties, it can also infect other wild relatives of apple like crab apple, hawtorn (*Crataegus spp*.), firethorn (*Pyracantha spp*.), mountain ash (*Sorbus spp*.), *Coteneaster spp,* Vibutnum and loquat (*Eriobotrya japonica*) (CABI, 2021).

Taxonomic information

Domain – Eukaryota
Kingdom – Fungi
Phylum – Ascomycota
Class – Dothideomycetes
Order – Venturiales
Family – Venturiaceae
Genus – Venturia
Species – Venturia inaequalis

Distribution

Apple scab is present in many countries of Africa, Asia, Europe, North America, Oceania and South America.

Detection

Symptoms

Apple scab can affect leaves, petioles, sepals, blossom, fruits and pedicel.

Foliar symptoms, as depicted in Figure 34, present as lesions on leaves, which may occur individually or scattered across the blade. These lesions have the potential to coalesce, cover and expan on either side of the leaf surface. Initially, lesions emerge on the underside of the leaf during bud break, appearing as light green compared to the healthy surface of the leaf. As the infection progresses, the lesions transition to an olive-green hue and acquire a velvety texture. Some lesions may darken to black without distinct margins. Upon closer inspection under a 10x lens, dark diffused fungal growth resembling mats are visible.

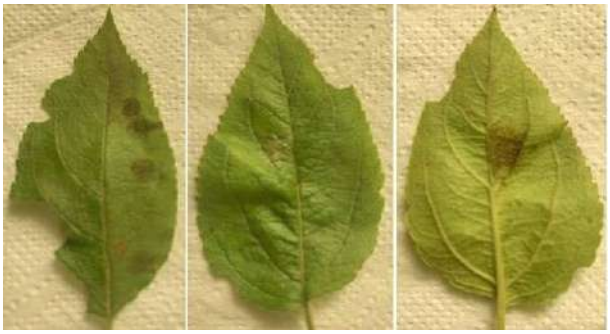

Figure 34 Dark velvety lesions on leaf: (Leaf and middle) – lesion on upper side leaf surface; (right) – lesion on underside leaf surface

Fruit symptoms, as illustrated in Figure 35, are particularly susceptible in young apples, which are highly susceptible to infection, resulting in the formation of larger lesions. Initially, fruit lesions are typically very small. However, compared to lesions on leaves, they exhibit slower growth, darker pigmentation, and more defined borders.

As lesions mature, they develop a bare, brown, and corky appearance at their centers. Cracking and deformation of the fruit are common occurrences. Dropping of infected fruits

may also occur when the infected pedicel is girdled. In cases where infection occurs late in the season, lesions may be as small as pin-heads and may remain invisible until storage.

Shoot and twig symptoms: Symptols include the formation of pustules on young shoots, appearing as light brown blister-like swellings resembling scabs on bud scales, petioles, and fruits in storage. As the infection progresses, these blisters develop further, taking on an olive-colored hue as the fungus grows and ruptures the cuticle, forming a whitish ring around the pustules.

Additionally, twigs and shoots affected by the infection display symptoms of cankers and dieback. Consequently, the overall health of the trees is compromised, leading to stunted growth and weakened vitality, ultimately resulting in reduced fruit production.

Figure 35 Scab lesions on fruits (Photos: N.Om & S. Chophel)

Identification

Confirmation of the disease involves examining plant samples in the laboratory, a process that can be achieved using both morphological and molecular techniques.

Morphological techniques

Collection of samples

- Collect infected samples e.g., leaf or fruits
- Place samples in paper bags with labels: Location name & GPS coordinates; Variety name; date of collection; collector's name.
- Wrap the paper bags in polythene and keep at 4°C till further use.

Materials required

- Leaf samples with visible symptoms of apple scab
- Microscope
- Scalpel (fine tip) or Razor blade
- Inoculating needles
- 70% ethanol
- Petri dishes
- Potato Dextrose Agar (PDA) or other suitable fungal growth medium
- Incubator

Visual inspection of samples

- Examine samples under the stereomicroscope and look for presence of dark mat of mycelium. If leaf samples, it is better to examine both upper and lower surfaces as mycelium growth on lower leaf surface is quite common. The mycelium mat contains numerous scab spores (conidia).
- Scrape of small section of the mycelium mat with help of scalpel, razor blade or inoculating needle.
- Place a small drop of cotton blue on a microscope slide and place the sample on the slide, cover with a cover slip and observe under a compound microscope
- Typical conidia of apple scab pathogen are single celled, uninucleate, and narrower at one end than other like a bowling pin ***Figure 36***.
- Mass of conidia appears dark or brown than single conidia. Size range between 6 and 12 µmin width, and 12 and 22 µmin length. Conidia are produced by special hyphae called conidiophores (Gauthier, 2018).

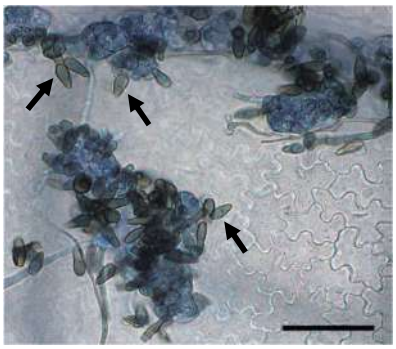

Figure 36 Conidia of *V. inaequalis* (Bowen 2011)

Isolation and sporulation

Moist chamber method: Sample can also be incubated in moist chamber to induce sporulation for direct examination of samples:

- Line a petri dish with blotting paper/filter paper (plastic bags or plastic containers can be substituted for petri dish especially if the samples are bulky e.g., fruits or tubers).
- Add a few drops of sterile distilled water using a dropper or a wash bottle to moisten the filter paper. Alternately, filter paper can be dipped in sterile water before placing in the petri dish; tip off excess water from the petri dish. Blotting /filter paper should be moist but not overflowing with water.
- Place two or more sterile wood tooth-picks on the moist filter paper.
- Place sample (e.g., leaf with lesions) on the tooth-picks.
- Incubate at 28°C for up to a week.
- Check for sporulation within every 24 hr.
- Typical conidia will be formed if the infection is due to apple scab pathogen.

Isolation on artificial media

- Flame scalpels, forceps and inoculating needles to sterilize before use.
- Cut small section of the plant sample (whether leaf or fruit), of around 0.5-1cm2 in size, from the edge of the diseased area. Sample should include both diseased and the adjacent healthy tissue.
- Surface sterilize sample with 1% bleach (sodium hypochlorite) or 70% ethanol by dipping the tissue for 30-60 secs. Whole tissue must be covered in bleach or ethanol. Rinse two to three times with sterile distilled water. Drain off excess water from the samples and blot dry using sterile paper towel.
- Transfer to PDA. Usually four pieces of 0.5-1cm2 samples (from step 2) are plated onto each plate at equidistance. Press the tissue into the medium to ensure good contact between the tissue and the antibiotics in the medium that will suppress bacterial growth.
- Incubate at 28°C for up to 7 days. Subculture on PDA and incubate 28°C for 7-10 days.
- Apple scab caused by fungus *Venturia inaequalis* will appear as dark fuzzy colonies on the plate.
- Prepare slides and examine under microscope to check for conidia of *V. inaequalis.*
- For long term storage and for use in future, mix spores in 10% glycerol in preservation vials and store cultures at low temperature (−20 to −80°C).

Molecular method

Conventional polymerase chain reaction (PCR) and real-time PCR using TaqMan probes are described here for detection of *V. inaequalis*. Confirmation must be done through sequencing. Primers for cPCR are provided in **Table *32***. Use at least two out of the six primer sets. Reaction mix preparation is provided in **Table *33*.**

Table 32 Primers for detection of *V. inaequalis* using cPCR

Primer	Sequence	Target gene	Reference
Forward: ITS5	5' – GGAAGTAAAAGTCGTAACAAGG – 3'	Internal transcribed region	White et al. 1990; (use either ITS5 or ITS 1 as the forward primer with ITS4 as reverse).
Forward: ITS1	5' – TCCG TAGGTGAACCTGCGG – 3'		
Reverse: ITS4	5' – TCCTCCGCTTATTGATATGC – 3'		
Forward: EFCF1	5' – AGTGCGGTGGTATCGACAAG – 3'	*Elongation factor alpha 1 (EF-1α)*	EPPO Bulletin 2016
Reverse: EFCF2	5' – TGCTCACGGGTCTGGCCAT – 3'		
Forward: ELF1	5' – CGAGAAGTTCGAGAAGGT – 3'		Prencipe et al. 2020
Reverse: ELF2	5' – CCAATGACGGTGACATAG – 3'		
Forward: TUB2Fd	5' – GTBCACCTYCARACCGGYCARTG – 3'	*Nuclear beta-tublin (TUB2)*	Groenewald et al. 2013
Reverse:TUB2Rd	5' – CCRGAYTGRCCRAARACRAAGTTGTC – 3'		
Forward: CAL-228F	5' – GAGTTCAAGGAGGCCTTCTCCC – 3'	*Nuclear calmodulin (CALM)*	Carbone & Kohn 1999
Reverse: CAL -737R	5' – CATCTTTCTGGCCATCATGG – 3'		
Forward: ACT-512F	5' – ATGTGCAAGGCCGGTTTCGC – 3'	*Nuclear actin ACT*	Carbone & Kohn 1999
Reverse:ACT-783	5' – TACGAGTCCTTCTGGCCCAT – 3'		

Note: Same primer set used for sequencing unless stated.

Fungal DNA extraction

Laboratory requirements

- 2.0, 20, 200, and 1000 µL sterile pipette tips (prefer barrier tips)
- Micro centrifuge
- Micro centrifuge tubes 1.5 and 2.0 mL.
- Benchtop vortexer.
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and power pack
- UV transilluminator; Camera/gel documentation system
- PCR reagents
- Running buffer, 0.5 - 1x TBE (see NPPC protocol on buffer preparation)
- Agarose
- DNA ladder
- DNA staining agent (e.g., GelRed, avoid use of ethidium bromide) & Gel loading dye

DNA extraction from plant tissue

Refer NPPC protocol on DNA extraction from plant tissue.

DNA extraction from pure culture

- Culture sample on PDA for 12 – 15 days. Obtain pure culture by single spore culturing.
- Slice off ~2 cm2 piece of agar of the pure culture.
- Place the mycelial piece in 2 mL tube (screw cap tubes are required if using FastPrep).
- Grind the sample using sterile micro pestle or any available grinding instrument e.g., using beating beads in FastPrep.
- Refer NPPC protocol on DNA extraction from fungi and fungi-like organism method for CTAB method of DNA extraction OR the manufacture's instruction for commercial kits.

PCR reaction mix preparation

Table 33 cPCR mix preparation

Reagents	µL/reaction	Final concentration
PCR water	13.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
Forward primer (10µM)	1.0 µL	0.4 µM
Reverse primer (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/ µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR conditions

For ITS5/ITS4: Initial denaturation at 95 °C for 5 min, followed by 30 cycles of: denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min 40 sec, and one cycle of final extension at 72°C for 10 min. hold at 4-10°C. DNA band size (on gel and sequencing) is 550 – 1700 bp.

For ITS1/ITS4: Initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing temperature of 54 °C for 1 min and a final extension step at 72 °C for 1 min. Hold at 4-10 °C.

For elongation factor alpha 1 (EF-1a):

EFCF1/EFCF2: Initial denaturation at 95 °C for 5 min, followed by 40 cycles of: denaturation at 94 °C for 30 sec, annealing at 52°C for 30 sec, extension at 72 °C for 30 sec, and one cycle of final extension at 72 °C for 10 min. Hold at 4-10 °C. DNA band size (on gel and sequencing) is about 680 bp. Same primer pair used for sequencing.

ELF1/ELF2: Initial denaturing step of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 sec, 58 °C for 50 sec, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Hold at Hold at 4-10 °C.

TEF1 is a protein coding region and thus standard code should be applied for translation.

For TUB2 using primer TUB2Fd/TUB2Rd: Initial denaturation at 95 °C for 5 min, followed by 40 cycles of: denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 30 sec, and one cycle of final extension at 72 °C for 10 min. Hold at 4-10 °C. DNA band size (on gel and sequencing) is about 450 bp. Same primer pair used for sequencing. TUB2 is a protein coding region and thus standard code should be applied for translation.

For ***CALM*** using primer **CAL-228F/CAL737R**: Initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec, and one cycle of final extension at 72°C for 10 min. Hold at 4-10°C. DNA band size (on gel and sequencing) is about 520 bp. Same primer pair used for sequencing. ***CALM*** is a protein coding region and thus standard code should be applied for translation.

For nuclear actin (*ACT*) gene using primer ACT-512F/ACT-783: Initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 30 sec, and one cycle of final extension at 72°C for 10 min. Hold at 4-10°C. DNA band size (on gel and sequencing) is about 290 bp. Same primer pair used for sequencing. ***ACT*** is a protein coding region and thus standard code should be applied for translation.

For ***COI*** gene using primer **OomACT-512F/ACT-783**: Initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 30 sec, and one cycle of final extension at 72°C for 10 min. Hold at 4-10°C. DNA band size (on gel and sequencing) is about 290 bp. Same primer pair used for sequencing. ACT is a protein coding region and thus standard code should be applied for translation.

Primers and probes based on translation elongation factor1 alpha (EF1-α) for real-time PCR are provided in **Table *34*** and PCR reaction mix preparation in **Table *35*.**

Fungal DNA (for real-time PCR)

DNA extraction method in the previous section can also be applied for extracting DNA for real-time PCR. Alternately, isolate on malt extract agar (MEA) and obtain pure culture on MEA and incubate for 30 days at 20 °C in the dark. Use at least 100 mg of fresh weight mycelium for DNA extraction, follow the rest of the DNA extraction as in CTAB method or manufacturer's instruction.

Real-time PCR condition

Initial denaturation at 95 °C for 10 min followed by 40 cycles of 57 °C for 1 min and 95 °C for 15 sec.

Table 34 Primers and probes for detection of V. inaequalis from fungal spores (Prencipe et al. 2020).

Primer/Probe Name	Sequences (5' to 3')	Target gene	Reference
Forward primer: F1	5' – C ACTTCCCCGCTATTCACGT – 3'	Elongation factor 1	Prencipe et al. 2020
Reverse primer: R11	5' – GCAATCGTTAGCATCGTCATAGTG – 3'		
Probe: Ven1	[FAM]– CTCAAGGCAGCCCAACTTTCTCCGGT–[BHQ1]		

Table 35 Real-time PCR mix preparation

Reagents	µL/reaction	Final concentration
PCR water	14.78 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
Forward primer ELF1 (3µM)	0.4 µL	3 µM
Reverse primer ELF2 (3µM)	0.4 µL	3 µM
Ven1 probe (5µM)	0.2 µL	
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/ µL
DNA template	1 µL	
Total reaction volume	20 µL	

2.5.5 Diagnostic Protocol for *Ralstonia solanacearum* species complex (Smith) Yabuuchi, Kosako, Yano, Hotta & Nichiuchi

Ralstonia solanacearum species complex (Smith) Yabuuchi, Kosako, Yano, Hotta & Nichiuchi

Disease

Bacterial wilt of potato or brown rot of potato; moko disease of banana and many more.

Hosts

Ralstonia solanacearum species complex has a wide host range that includes many cultivated and wild plants, and ornamentals and weed species. Important hosts are aubergine (*Solanacearum melongena*) banana and plantain (*Musa spp*.), and chilli (*Capsicum annuum*), potato (*Solanacearum tuberosum*) and tomato (*S. lycospersicum*).

Taxonomic information

Name: *Ralstonia solanacearum* (Smith) Yabuuchi et al. emend. Safni et al.; Synonym: *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al.; *Pseudomonas solanacearum* (Smith 1896) Smith 1914; *Xanthomonas solanacearum* (Smith) Dowson 1943); *Erwinia solanacearum* (Smith) Holland 1920 and many more.

Phylotype: Phylotype II

Name: *Ralstonia psuedosolanacearum* Safni, Cleenwerck, deVos, Fegan, Sly & Kappler; Synonym: *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al.; *Pseudomonas solanacearum* (Smith 1896) Smith 1914; *Xanthomonas solanacearum* (Smith) Dowson 1943); *Erwinia solanacearum* (Smith) Holland 1920 and many more.

Phylotype: Phylotypes I and III

Name: *Ralstonia syzygii* (Roberts et al. 1990) Vaneechoutte et al. 2004 emend. Safni et al., 2014; Synonym: *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al.; *Pseudomonas solanacearum* (Smith 1896) Smith 1914; *Xanthomonas solanacearum* (Smith) Dowson 1943); *Erwinia solanacearum* (Smith) Holland 1920 and many more.

Phylotype: Phylotype IV

Taxonomic position (EPPO 2023)
Domain – Eukaryota
Kingdom – Bacteria
Phylum – Proteobacteria
Class – Betaproteobacteria
Order – Burkholderiales
Family – Burkholderiaceae
Genus – *Ralstonia*
Species – *Ralstonia solanacearum*, *Ralstonia pseudosolanacearum*, *Ralstonia syzygii*

Detection
Symptom

The bacterium causes rapid wilting of leaves and stems. Plants turn yellow and necrotic as the disease progresses. Brown discolouration of stems above the soil line may become visible. Slimy yellowish bacterial ooze is visible on cut surfaces of the plant.

In potato, oozes may be visible on the eyes and stolons. A brownish ring is visible along the vascular ring when tubers are cut where bacterial ooze appear **Figure *37***.

Bacterial streaming test

Place a cut stem or tuber in water and observe for a milky thread-like bacterial ooze streaming from the cut plant into the water. **Caution:** do not mistake the cloudy exudates released immediately when a cut potato tuber is placed in water for bacterial ooze– this is just starch particle being released, wait for a few a sec to 1 min to observe the milky thread-like bacterial ooze.

Bacterial streaming test is however not confirmatory test.

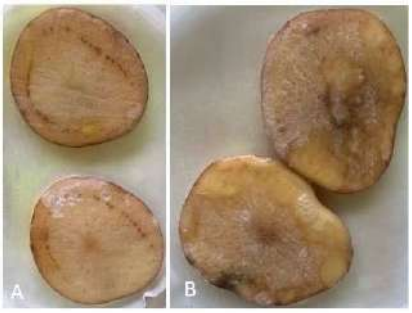

Figure 37 Symptoms on cut tubers: (A) brown discoloration; (B) yellowish bacterial ooze (photo: N.Om).

Immunofluorescence test

Specific antibodies are available to detect the bacteria. The following field test kits and immunofluorescence (EPPO 2022):

- Pocket diagnostic (PD51119) with monoclonal antibody 9Y against *R. solanacearum* (Phylotype II).
- ImmunoStrip (ISK 33900) with monoclonal antibody Ps- 1 against *R. pseudosolanacearum*.
- Loewe Biochemica, Sauerlach, DE (07356).
- Plant Print Diagnostics S. L., Valencia, ES (1546-H/IVIA).
- Primer Diagnostics, Wageningen, NL (B-RsoI_C).

Identification

Identification can be done both through morphological and molecular methods.

Morphological methods

Isolation from symptomatic plant samples

- Take a section of symptomatic tissue (discolored tissue but not totally rotten or decayed) or take the bacterial ooze and suspend in a small volume e.g., 5 mL of sterile distilled or RO water for 5-10 min. Plant tissue may be mashed before placing in the water.
- Place 50-100 µL of the bacterial suspension and spread it on general nutrient agar such as potato dextrose agar (PDA), nutrient (NA), yeast peptone glucose agar

(YPGA), sucrose peptone agar (SPA) or on semi-selective mSMSA/Sequeira medium (Table 1).
- Incubate the plates at 28C for 2-6 days.
- Pick single colony and subculture on nutrient agar or semi-selective media for storage or further identification work using microscopy and molecular methods.

Sampling and isolation from asymptomatic plant samples

Refer EPPO (2022) 'PM 7/21 (3) *Ralstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (*Ralstonia solanacearum* species complex)'.

Colony characteristics

- On general nutrient media, colonies of virulent strains develop as flat, irregular and fluidal colonies which are cream to white in colour. Small non-fluidal colonies are that of non-virulent strains.
- On mSMSA/Sequeria medium, virulent forms produce colonies with blood red whorls while those of non-virulent are small, round and non-fluidal and are entirely deep red in colour.

Molecular methods

Several molecular methods involving conventional PCR tests, TaqMan real-time PCR, and loop-mediated isothermal amplification (LAMP) are available. DNA sequencing is performed for accurate identification of phylotypes and sequevars using 16S rDNA, egl, mutS and hrB barcodes.

Laboratory requirements

- 2.0, 20, 200, and 1000 µL sterile pipette tips (prefer barrier tips)
- Micro centrifuge
- Micro centrifuge tubes 1.5 and 2.0 mL
- Benchtop vortexer
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and power pack
- UV transilluminator; Camera/gel documentation system
- PCR reagents
- Running buffer, 0.5 - 1x TBE (see NPPC protocol on buffer preparation)
- Agarose
- DNA ladder
- DNA staining agent (e.g., GelRed, avoid use of ethidium bromide) & Gel loading dye.

DNA extraction from plant tissue

Refer specific DNA extraction methods provided by the manufacturer if using a commercial extraction kit (e.g., Bioline, Puregene, Qiagen, Sigma, etc.). Refer NPPC protocol on DNA extraction from plant tissue.

DNA extraction from colonies

- Follow manufacturer's protocol if using commercial kits
- Isolate sample on culture media and incubate for up to 7 days.
- Once colonies are formed, suspend one colony in 100 µL of PCR water.
- Heat at 95°C or 100°C for a minimum of 10 min. A freezing step before heating may be performed.
- Store lysate at -20°C till further use.

For more details refer EPPO (2022) 'PM 7/21 (3) Ralstonia solanacearum, R. pseudosolanacearum and R. syzygii (Ralstonia solanacearum species complex)'.

Conventional PCR

Prepare PCR mix reaction as per the protocol received with the DNA Taq DNA polymerase or use the protocol in **Table *37***.

Use the primer pairs and PCR conditions in **Table *38***. Run the PCR product in a TBE gel. After the gel electrophoresis, PCR product should produce a band at 320bp, 550bp and 500bp for actin, beta-tubulin and calmodulin gene region respectively. The PCR products should be purified using PCR Purification Kit by following manufacturer's protocol. The purified products should be submitted to DNA sequencing facilities (Macrogen Inc. or others). The DNA sequence data is BLAST analysed in NCBI and compared with reference sequence data listed in **Table *38*** for confirmation.

Table 36 Artificial media (EPPO, 2022).

Generic media			
Nutrient Agar (NA)		**Sucrose peptone agar (SPA)**	
Peptone	5.0 g	Sucrose	20.0 g
Yeast extract	3.0 g	Peptone	5.0 g
NaCl	0.5 g	K_2HPO_4	7.0 g
Microbiological grade agar	15.0 g	Microbiological grade agar	12.0 g
Distilled water	1.0 L	Distilled water	1.0 L
Yeast peptone glucose agar			
Yeast extract	5.0 g		
Bacto peptone	5.0 g		
Glucose	10.0 g		
Microbiological grade agar	15.0g		
Distilled water	1.0 L		
Adjust pH to 7.2			
Semi-selective media			
Modified semi-selective medium from South Africa (mSMSA)			
Bacto peptone	10.0 g	Autoclave and cool to 45-50°C and add the following:	
Glycerol	5 mL	2,3,5-triphenyl-2H-tetrazolium chloride	0.050 g
Bacto Agar	15.0 g	Crystal violet	0.005 g
Casamino acid	1.0 g	Chloramphenicol (water soluble)	0.005 g
Yeast extract	1.0g	Penicllin G (benzylpenicillin sodium salt)	825 U
Deionized distilled water	1 L	Polymyxin B (sulphate salt)	600 000 U
		Bacitracin	1250 U
Final pH should be around 6.5			

Table 37 PCR mix preparation

Reagents	**µl/reaction**	**Final concentration**
PCR water	12.375	
10x PCR mix (without MgCl2)	2.5µl	1x (2mm MgCl2)
MgCl2 (25mM)	0.5	
dNTP Mix (10mM each)	0.125 µL	0.1 mMeach
RS-1-F (10µM)	2 µL	0.8 µM
RS-1-R (10µM) or RS-3-R	2 µL	0.8 µM
NS-5-F (10µM)	0.15 µL	0.06 µM
NS-6-R(10µM)	0.15 µL	0.06 µM
Taq (DNA polymerase (5µ/µl)	0.2 µL	1 U
DNA template	5 µL	
Total reaction volume	25 µL	

Note: if BSA is used adjust the volume of PCR water to obtain the final volume of 25 µL

Table 38 Primer pair for conventional PCR to detect *R. solanacearum* and *R. pseudosolanacearum*

Species/strain	Amplicon	Primer pair	Primer Sequence
R. Solanacearum (Phylotype II)	718 bp	RS-1-F	5′-ACTAACGAAGCAGAGATGCATTA-3′
		RS-1-R	5′-CCCAGTCACGGCAGAGACT-3′
R. pseudosolanacearum (Phylotype I)	716 bp	RS-1-F	5′-ACTAACGAAGCAGAGATGCATTA-3′
		RS-3-R	5′-TTCACGGCAAGATCGCTC-3′
Both. Use as internal control	310 bp	NS-5-F	5′-AACTTAAAGGAATTGACGGAAG-3
		NS-6-R	5′-GCATCACAGACCTGTTATTGCCTC-3′.

Multiplex the above primers. Include PCR mix control and water control, and positive control if available. Save positive DNA samples for use as positive control in future tests.

PCR conditions for both sets of primers:

PCR conditions: an initial 5-min incubation at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C. Hold at 10°C.

PCR result interpretation:

- The test is considered positive if a band of 718 bp (RS-1-F and RS-1-R), 716 bp (RS-1-F and RS-3-R) and 310 bp (NS-5-F and NS-6-R) is visualized.
- The test is considered negative if no band or a band of a different size than expected is visualized.
- Repeat the tests if any of the controls did not worked.

Real-time PCR

Use the real-time reaction mix in

Table* 39** and primer pairs in **Table *40 to conduct real-time PCR.

Table 39 : Real-time PCR mix preparation

Reagents	µL/reaction	Final concentration
PCR water	12. 875	
10x PCR buffer (without MgCl2)	2.5 µL	1x (2mm MgCl2)
MgCl2 (25mM)	3.5 µL	3.5 mM
dNTP Mix (10mM each)	2 µL	0.2 mM each
Forward primer: RS-I-F, (10µM)	0.25 µL	0.3 µM
Forward primer: B2-I-F,	0.25 µL	0.3 µM
Forward primer: COX-F	0.25 µL	0.3 µM
Reverse primer: RS-I-R (10µM)	0.25 µL	0.3 µM
Reverse primer: B2-II-R (10µM)	0.25 µL	0.3 µM
Reverse primer: COX-R (10µM)	0.25 µL	0.3 µM
Probe: RS-P	0.125 µL	0.1 µM
Probe: B2-P	0.125 µL	0.1 µM
Probe: COX-P)	0.125 µL	0.1 µM
Probe: RSP-55T	0.125 µL	0.1 µM
Taq (DNA polymerase (5µ/µl)	0.125 µL	1 U
DNA template	2 µL	
Total reaction volume	25 µL	

Table 40 Primers and probes for TaqMan real-time PCR

Species/strain	Primer pair	Primer Sequence
The primer probes target specific 16S rRNA gene within R. solanacearum, R. pseudosolanacearum and R. syzygii strains.	RS-I-F	5′-GCATGCCTTACACATGCAAGTC-3′
	RS-II-R	5′-GGCACGTTCCGATGTATTACTCA-3′
	B2-I-F	5′-TGGCGCACTGCACTCAAC-3′
	B2-II-R	5′-AATCACATGCAATTCGCCTACG-3′
	COX-F	5′-CGTCGCATTCCAGATTATCCA-3′
	COX-R	5′-CAACTACGGATATATAAGAGCCAAAACTG-3′ (COX F/R and COX P target plant cytochrome oxidase if plants samples are used).
Improve specificity with the following		
RS-I-FmAK		5′-CATGCCTTACACATGCAAGTC-3′
RS-II-RmAK		5′-CACGTTCCGATGTATTACTCA-3′
RS-P (probe)		5′-[FAM]-AGCTTGCTACCTGCCGGCGAGTG-[TAMRA]-3″
B2-P (probe)		5′-[VIC]-TTCAAGCCGAACACCTGCTGCAAG-[TAMRA]-3′
COX-P (probe)		5′-[VIC]-TGCTTACGCTGGATGGAATGCCCT-[TAMRA]-3′
RSP-55T* (probe)		5′-[FAM]-AGCTTGCTACCTGCCGG-[NFQ-MGB]-3'

**modification of the RS-P probe. This probe help in reducing false-positive results.*

PCR controls must be used:

- Positive isolation control (PIC) – which is disease plant (if available) should be used.
- Negative isolation control (NIC) which is the buffer 50mM phosphate buffer, or a disease free plant extract used for isolation of bacterial from plant tissue.
- Positive control (PC) which is positive DNA extract
- Negative control or non-template control (NTC) – which usually PCR water instead of DNA,

Result interpretation

- The PIC and PC, should show exponential amplification curves for both primers and probe channels.
- The NIC and NTC should have any amplification curves for both primer and probe channels.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

2.6 Other activities

2.6.1 Demonstration on use of *Trichoderma* spp. and potassium phosphite against soil borne pathogen

2.6.1.1 Introduction

Chili holds significant cultural and economic importance in Bhutan, being a key ingredient in Bhutanese cuisine. However, its production faces substantial challenges due to chili blight, a common disease caused by the fungus *Phytophthora capsici*. This disease can infect various parts of the plant, leading to symptoms such as wilting, root and collar rot, dark brown lesions on stems, and greyish-brown or water-soaked lesions on leaves. First observed in Punakha in 1995, chili blight has since been reported in other districts including Wangdi, Thimphu, Paro, and Trashiyangtse.

To manage chili blight, various cultural practices have been recommended and practiced, including crop rotation, wider planting distances, and the use of biological control agents (BCAs) such as *Trichoderma* species. The National Plant Protection Centre has taken initiatives to isolate native strains of *Trichoderma* sp. for managing potato diseases. Following extensive laboratory, greenhouse, and field bioassays, the strain Tc7A was identified as the most promising isolate. In the financial year 2022 to 2023, field demonstrations were conducted in Gemina under Maedwang gewog, Thimphu Dzongkhag to showcase the application of Trichoderma (Tc7A) in combination with potassium phosphite for managing chili blight.

2.6.1.2 Materials and Methods

Site selection

Field demonstration was conducted at Siluna Maedwang Gewog, Thimphu Dzongkhag.

Isolates of Trichoderma sp.

Native isolate *Trichoderma* Tc7A, isolated from soil sample collected from Thimphu was used.

Preparation of Trichoderma sp. inoculum

The inoculum of *Trichoderma* sp. was prepared following the methods outlined by Smith et al. (1990). Double-sterilized rice husk (960 grams per 350 ml) was inoculated with 80 discs (4 mm in diameter) from a 7-day-old culture of *Trichoderma* sp. The inoculated substrate was then incubated at room temperature (approximately 22°C) for four weeks. To ensure uniform colonization, the substrate was shaken every two days, starting four days after the initiation of incubation. Once the substrate was fully colonized by *Trichoderma*, it was transported to the field for demonstration purposes.

Participants

Thirteen farmers from Siluna, Maedwang Gewog, Thimphu Dzongkhag, along with the Agriculture Extension officer of Jeminang, participated in the field demonstration. During the demonstration, participants received a briefing on chilli diseases and the advantages of utilizing soil-borne beneficial organisms like *Trichoderma* sp., alongside cultural practices

for blight management. Land selection criteria and methods for seedling selection were also included in the discussion.

Bed Preparation

Bed preparation was demonstrated by constructing an elevated bed measuring 30 cm in height, 1.2 m in width, and 2.8 m in length.

Incorporation of Inoculum

The method of adding Trichoderma inoculum into the soil was demonstrated by spreading and mixing 2800g of Trichoderma inoculum in the soil before transplanting the seedlings (**Figure *38***).

Transplanting seedlings

Chili seedlings were sown in soil enriched with Trichoderma, following the recommended planting distance of 30 cm. The importance of maintaining wider planting distances was emphasized during the demonstration. This practice facilitates better air circulation and allows ample space for optimal plant growth.

Figure 38 Field demonstration on amendment of Trichoderma in the soil

Potassium phosphonate as alternative to synthetic fungicide

Use of potassium phosphonate against plant pathogens was introduced to the participants. Results of trials conducted by NPPC were also shared with the participants.

2.7 Ad hoc activities of Pathology Program

2.7.1 Laboratory Diagnostic Report of problem associated with Adzuki beans

The Pathology Program team collected bean samples as per Ms. Kinley Wangmo's request during the team's visit to ARDC-Yusipang to meet with the Agro-met focal on August 8, 2022.

Fields Observation:

- Leaf lesions are straw coloured in the centers and have concentric rings **Figure *39***
- Severely affected plants appeared blighted.

Laboratory examination and observations: Dark brown raised spots (fruiting bodies) were observed forming concentric rings within the lesions (Figure 1). Numerous conidia that are

oblong to cylindrical, hyaline, with rounded tips were observed. Conidia were mostly aseptate, though a few uniseptate were also observed **Figure *39***.

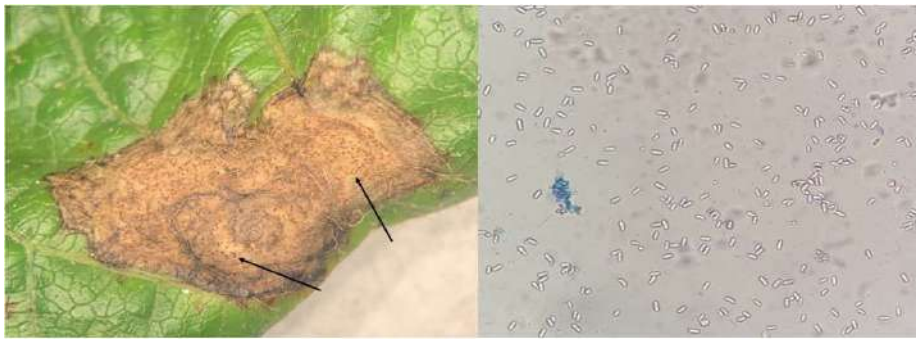

Figure 39 Leaf lesion: (Left) Lesion with concentric rings. Arrows indicate fruiting bodies spots; (Right) Conidia (450x

Inference: based on symptoms and conidia morphology, the problem in Adzuki beans is likely to be Ascochyta blight.

Recommendations: The following cultural practices can be effective in managing this disease:

- Use tolerant varieties.
- Use disease free seed. If certified disease-free seeds are not available. Use treated seed to avoid introducing the fungus to the field
- Destroy or incorporate infested plant residue thoroughly to hasten decomposition and to minimize the possibility of spore production and dissemination.
- Follow a crop rotation schedule for a minimum of two, ideally three years.
- Determine an appropriate planting time to avoid main monsoon season.
- Modify planting distance with wide row and plant spacing**.**

3. Weed Program

3.1 Efficacy of Bio-weedicide in controlling agricultural weeds

3.1.1 Introduction

Weeds are plants that grow alongside cultivated crops, but they are unwanted because they compete with crops for nutrients, water, sunlight, and space, potentially reducing crop productivity (Monteiro & Santos, 2022). They cause the most yield loss in crops, accounting for 32%, compared to 18% for animal pests and 15% for pathogens (Oerke & Dehne, 2004). Additionally, some weeds can act as alternative hosts for plant diseases and insect pests, adding costs and reducing the quality and value of the produce.

Many farmers underestimate the importance of weed management because the negative impacts of weeds, such as competition for nutrients, water, sunlight, and space, are not always

obvious. Unlike other pests, many weed species are typically present in an area, allowing another weed to quickly replace the one that's controlled (Bailey, 2014).

In Bhutan, farmers traditionally relied on farm labor exchange and manual hand-weeding for weed management. However, due to the increasing shortage and rising wage rates of farm labor, farmers have had to turn to chemical weed management methods. This shift contradicts the country's national organic agriculture policy, which promotes chemical-free farming practices. Therefore, exploring non-chemical methods for weed control has become essential to align with Bhutan's organic agriculture goals and objectives. This emphasizes the importance of integrated weed management to address weed issues while maintaining organic farming principles.

A promising and cost-effective alternative to conventional weed management systems is the use of bio-weedicide. However, introduction of any new plant protection product in the country must be evaluated first for its efficacy for target weeds and its phytotoxicity to standing crops. This includes assessing any undesirable effects on crops and their products, such as phytotoxicity, yield, quality, and impacts on beneficial insects (FAO, 2006).

The inhibitory effects of plant extracts used in bio-weedicides can vary depending on the concentrations of the bio products used (Bordin et al., 2021), necessitating confirmatory tests to verify the claims on the product label (Kalamarakis & Markellou, 2007). The main objective of the current study is to evaluate the efficacy of a non-selective bio-weedicide, Niramoy Natural Weedicide, for managing agricultural weeds.

3.1.2 Materials and methods

Study site and period

Trials were carried out in three distinct regions, viz. Khariphu in Mewang Gewog under Thimphu dzongkhag; ARDC Samtenling under Sarpang dzongkhag, where an open field without any crops was used and in potato field in Phobjikha under Wangdue dzongkhag. The research was conducted in August and September 2022, a time characterized by high weed growth in agricultural fields. By selecting this specific time frame, the trials aimed to capture the prevalent weed species and their behaviour under challenging conditions.

Bio-weedicide

The Niramoy Natural Weedicide is a plant-based ready-to-use bio-weedicide developed by Barnalee Bio Agrotek, located at Nagaon, Assam, India. The active ingredients are derived from indigenous cow urine (70%), Crepe-ginger (6%), Neetle leaf (6%), Elegan tape vine (6%), Prickly iveys (6%).

Experimental design and Treatments

The trial was laid out in a single factor Randomized Complete Block Design (RCBD) with four treatments with each treatment replicated thrice. Treatments and dilution rate as follows:

Treatment 1: Niramoy with 5ml/L of water
Treatment 2: Niramoy with 7ml/L of water
Treatment 3: Niramoy with 10ml/L of water (company recommended rate)
Treatment 4: Control (no spray)

Data Collection

The individual weed species, and their densities (counted the number of individuals of a particular weed species in the respective plot) were recorded prior to the application of the spray. Subsequently, the observations were made at five-day intervals, starting from the fifth day after the application and continued until the 20th day, to assess the effect of the treatment on weed density.

The application of Niramoy took place one day after the experiment as the experiment was conducted in a field with a high weed infestation. At the time of application, most of the weeds were in the 5-6 leaf stages, covering both the vegetative and flowering stages. To ensure standardized application, uniform measuring equipment such as ½ and 1-litre bottles, and a knapsack sprayer were used across all treatments.

Plant injury observations data were collected at five-day intervals from the fifth day until the 20th day after the treatment application. The plant injury observations were recorded using a scale ranging from 1 to 5, with corresponding descriptions: 1 = No effect; 2 = Light symptoms (minor chlorosis); 3 = Medium symptoms (moderate chlorosis/leaf curling); 4 = Heavy damage (severe chlorosis/dead leaves); 5 = Complete kill (plant dead) **Table *41***. This systematic approach allowed the evaluation of the spray's impact on the plants over time, capturing the progression of any observed injuries from minor symptoms to severe damage or complete plant death.

Table 41 Injury rating table (adapted from EU; Hasan et al. 2021; Jiddimani et al., 2017)

Scale description	Description of main categories	Detailed description
1	Tolerant	Slight to some strains of weed loss (No effect)
2	Moderately tolerant	Injury more pronounced but not persistent (Light symptoms)
3	Moderately susceptible	Some weeds survive (Moderate chlorosis, leaf curling)
4	Susceptible	Few weeds survive (Heavy damage)
5	Highly susceptible	Very few weeds survive or complete destruction of weeds (Complete kill)

Data analysis

All data analyses were performed using Microsoft Excel. The mean total count of the weed species with the highest recorded number was computed and plotted against the data collection intervals (days after spraying) to assess the product's impact on weed density. Similarly, the mean weed injury rating were computed and plotted against the data collection intervals to determine the phytotoxicity of the product on the weeds.

3.1.3 Results and discussion

ARDC Samtenling in Sarpang

Ten weed species were recorded at the trial site, including both broadleaf and grassy weeds. The broadleaf weeds identified were *Ageratum conyzoides*, *Spermacoce latifolia*, *Ludwigia spp., Alternanthera sessilis*, *Commelina communis*, *Crassocephalum crepidioides*, and *Sida acuta*. The grassy weeds found were *Digitaria ciliaris*, *Cynadon dactylon* and *Eleusine*

indica. All the recorded species are typically dryland weeds, *Cynadon dactylon* is also found to thrive in wetland.

The calculated mean weed count showed a reduction in the numbers of *Ageratum conyzoides* during all the successive data collection after spraying across all treatments except the control **Figure *40***. Specifically, treatment 3 (10 ml/L of water) exhibited a significant decline in *Ageratum conyzoides* numbers by the 5th day after spraying, with counts reaching zero at 10 days after spray (DAS), and subsequently showing absence at the 15 and 20 DAS. Likewise, treatment 1 (5 ml/L of water) and treatment 2 (7 ml/L of water) showed a decline in weed counts but not as pronounced as observed in treatment 3. Unlike treatment 3, where the weed count reached zero at 10 DAS, treatments 1 and 2 exhibited a reduction that remained consistent at 15 DAS and 20 DAS, without reaching zero counts.

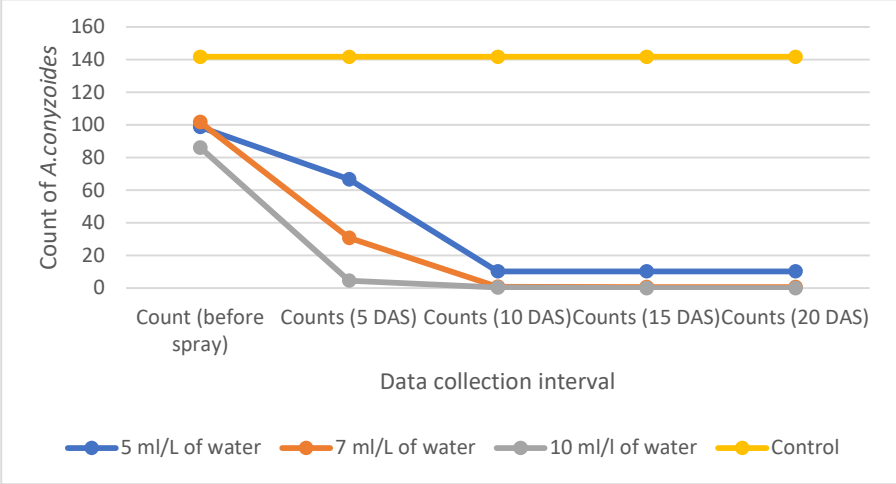

Figure 40 *Ageratum conyzoides* count at different intervals

The mean weed injury rating revealed a rating of 3, indicating moderate chlorosis and leaf curling across all treatments at 5 DAS. This injury observation persisted consistently throughout the data collection period for treatment 1. However, in treatment 2, from 10 DAS onward, the weed exhibited severe chlorosis and substantial plant damage (rating of 4). Whereas all the *Ageratum conyzoides* was completely killed (rating of 5) by 10 DAS in treatment 3 and not a single *Ageratum conyzoides* was found until the conclusion of the data collection period **Figure *41***.

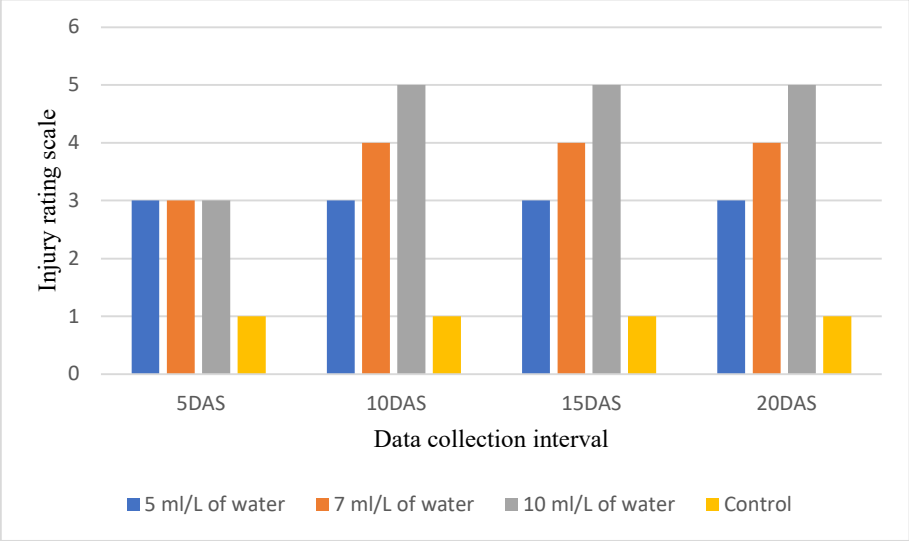

Figure 41 Phytotoxicity (injury observations) of Ageratum conyzoides at different intervals

**Injury rating scale description*
1-Tolerant (No effect)
2-Moderately tolerant (light symptoms)
3-Moderately susceptible (Moderate chlorosis, leaf curling)
4-Susceptible (Heavy damage)
5-Highly susceptible (Complete kill)

Before Spray (No phytotoxicity)

Phytotoxicity effect, 5 DAS

Phytotoxicity effect 10 DAS

Phytotoxicity effect 20 DAS

Phobjikha in Wangdue

From the experimental trial plots, seven broadleaf weed species were recorded including *Galinsoga quadriradiata*, *Galinsoga parviflora*, *Galium aparine*, *Persicaria nepalensis*, *Dysphania Spp*., *Clinopodium umbrosum* and *Capsella bursa-pastoris*. The only grass weed recorded was Poa annua. Weed species like *Galinsoga quadriradiata*, *Galinsoga parviflora* and *Persicaria nepalensis* are the most prevalent weeds in dryland crops.

Across all treatments except the control treatment, the calculated mean weed count showed a reduction in the numbers of *Galium aparine* during all the successive data collection after spraying. **Figure *42***. The number of *Galium aparine* consistently decreased from 5 DAS to 20 DAS in treatment 1. However, the count did not reach zero by the end of the observation period whereas treatment 2 and treatment 3 recorded decline in the number from 5 DAS to 15 DAS, and then the count reached zero at 20 DAS. These treatments seemed to have a faster impact on reducing the weed population compared to treatment 1.

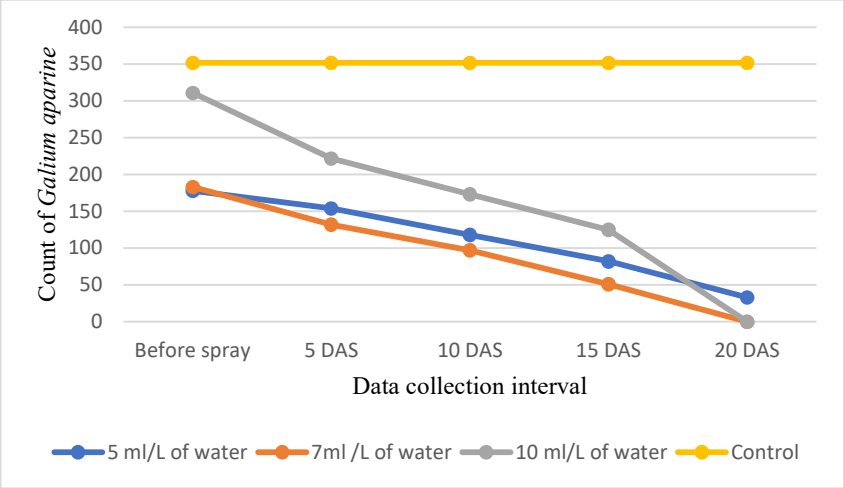

Figure 42 *Galium aparine* count at different intervals

The mean weed injury rating indicated a score of 2, reflecting light injury symptoms in treatment 1 at both 5 and 10 DAS. The condition then worsened, with moderate chlorosis and leaf curling observed, resulting in a score of 3 at 15 DAS. By 20 DAS, the weeds displayed severe chlorosis and significant plant damage, reaching a rating of 4. In contrast, treatments 2 and 3 recorded an injury rating of 3 at both 5 and 10 DAS, which escalated to a rating of 4 at 15 DAS. By the time they reached 20 DAS, all Galium aparine had been completely eradicated, resulting in a rating of 5. ***Figure 43***.

Treatments 2 and 3 demonstrated a more significant impact on weed control, as evidenced by their faster progression to higher injury ratings and the eventual complete eradication of the weed population. This indicates that using higher dosages of herbicide, specifically at concentrations of 7 ml/L and 10 ml/L of water, is more effective than the lower concentration of 5 ml/L of water.

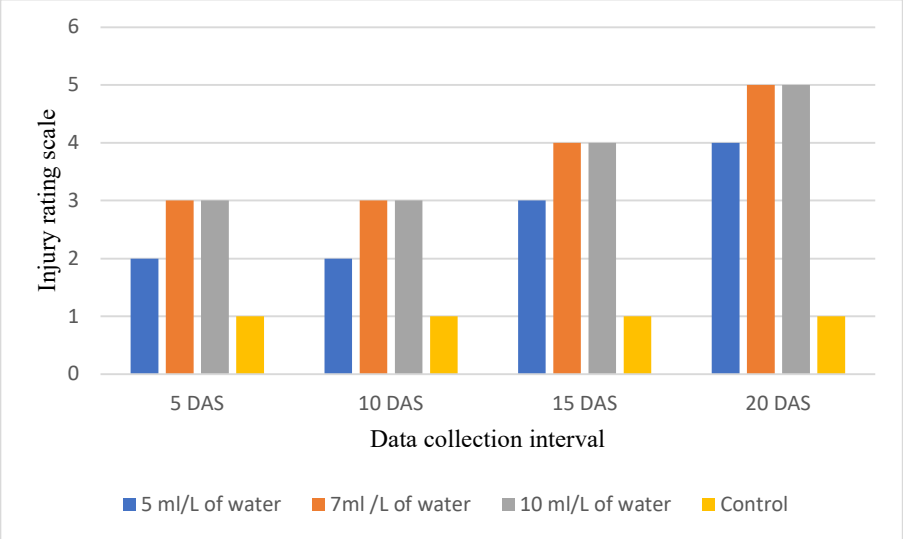

Figure 43 Phytotoxicity (injury observations) of *Galium aparine* at different intervals at Khariphu in Mewang gewog, Thimphu.

There were seven different weed species recorded from the trial site at Khariphu comprising of five broadleaf weeds and two grassy weeds. The identified broadleaf weeds are *Artemisia campristris, Verbascum thapsus, Taraxicum officinalis, Sonchus arvensis and Vicia hirusita. The grassy weeds recorded were Imperata cylindrica* and *Digitaria ciliaris*. Among these species, *Imperata cylindrica* exhibited high densities in all trial plots, followed by *Artemisia campristris*.

Following the application of various concentrations of Niramoy, the response of *Imperata cylindrica* differed among the treatments. In Treatment 1, there was no immediate decrease in the number of *Imperata cylindrica* at 5 DAS. However, a reduction in the weed's population was observed starting from 10 DAS, and this trend continued with a slight decline until 20 DAS. For Treatment 2, there was an initial decline in the number from 5 DAS. This reduction continued gradually, leading to a moderate decrease by the time 20 DAS was reached. In a similar manner, Treatment 3 showed a decrease in the weed count until 15 DAS. By the 20th day, the weed count reached zero, indicating complete eradication of the *Imperata cylindrica* population, **Figure *44***. These finding suggest that Treatment 3 at concentration of 10 ml/L of water (company recommended dosage) had the most effective control over the grassy weed *Imperata cylindrica*. Treatment 2 also resulted in notable suppression of the weed population but not as extensive as in Treatment 3.

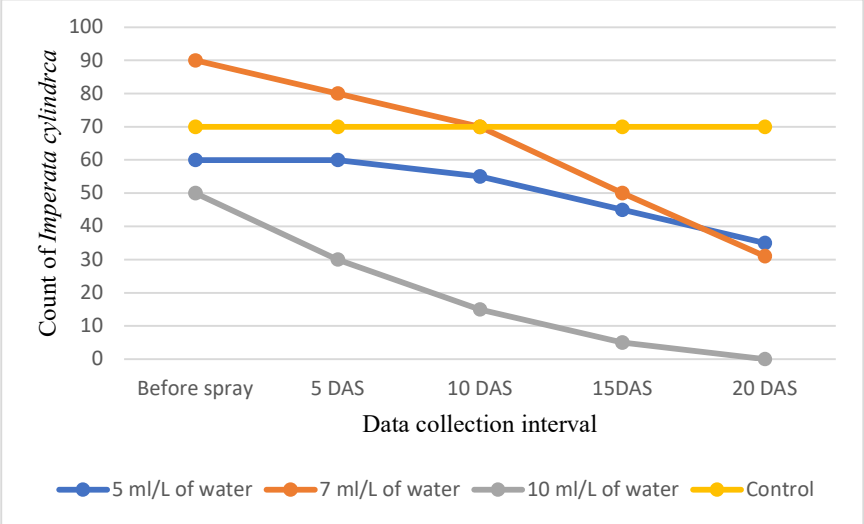

Figure 44 *Imperata cylindrica* count at different intervals

The mean injury rating scale initially showed no injury to the weed at 5DAS in treatment 1. However, there was a gradual progression towards displaying light injury symptoms by 10 DAS and from 15 DAS onward until 20 DAS, the weed exhibited a moderate chlorosis. In treatment 2, the weed showed a rating of 3 (moderate chlorosis and leaf curling) at both 5 and 10 DAS. Then the symptoms advanced to a rating of 4 (severe chlorosis) at 15 DAS and 20 DAS. The *Imperata cylindrica* in treatment 3 also started showing moderate chlorosis and leaf curling at 5 DAS and reached at rating 4 indicated by severe chlorosis and dead leaves at both 10 and 15 DAS. By 20th day, the weed was completely killed **Figure *45***.

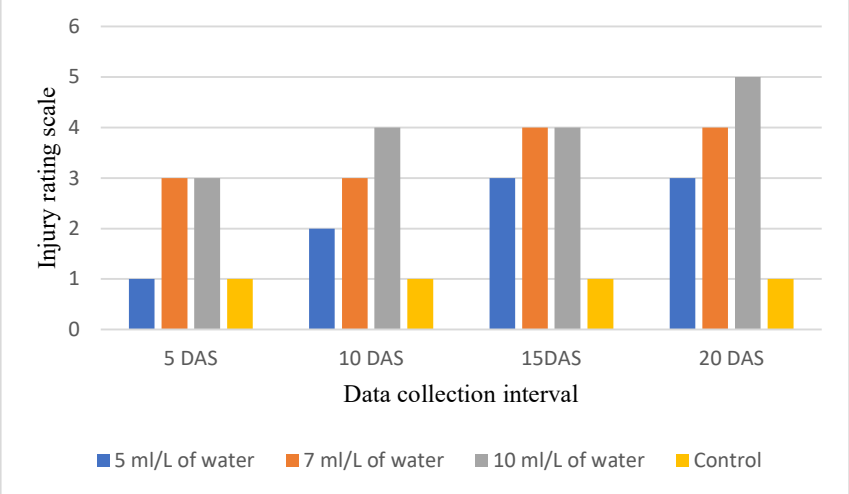

Figure 45 Phytotoxicity (injury observations) of *Imperata cylindrica* at different intervals

The combined results from the three trial sites indicate that all three treatments with varying dosages resulted in a decrease in the populations of broadleaved weeds, including *Ageratum conyzoides* and *Galium aparine*, over time. These reductions were accompanied by different injury observations at different time intervals. However, only treatment 2 and treatment 3 proved to be effective in completely eradicating the weeds from the plots by the 20th day after spraying. This suggests that these two treatments exhibited stronger weed control

capabilities compared to treatment 1. When considering grassy weeds like *Imperata cylindrica*, the results indicated that although all three treatments led to a decrease in the weed count, only treatment 3, which involved a dosage of 10 ml/L of water, was successful in entirely eradicating the weed population.

The findings provide valuable insights into the effective dosages required for weed control. It's evident that a dosage of 7 ml/L appears to be effective in eliminating more delicate and less resilient broadleaved weeds. On the other hand, the hardy and tougher weed species like *Imperata cylindrica* seem to demand a higher dosage of 10 ml/L for successful eradication.

The findings provide important insights into the optimal dosages needed for effective weed control. It is clear that a dosage of 7 ml/L is effective in eliminating more sensitive and less resilient broadleaf weeds. Conversely, the hardy and tougher weed species, such as *Imperata cylindrica*, appear to require a higher dosage of 10 ml/L for successful eradication.

3.1.4 Conclusion

The trials conducted in different regions of country, including the Western, Central, and Southern regions, demonstrated the effectiveness of Niramoy across diverse agro-ecological zones and climatic conditions. The results showcase its efficacy in controlling various weed species, providing a promising option for farmers in their weed management strategies. Overall, these reports confirm the effectiveness of Niramoy Natural Weedicide in controlling weeds, regardless of the weed species and densities. Further research and field trials are recommended to validate these findings and explore the potential of Niramoy as a cost-effective and environmentally friendly weed control solution for farmers. Though promoted in India as bio-herbicide, its active ingredients and other chemical impurities needs to be validated through laboratory testing before this product can be promoted in the fields.

3.1.5 References

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3.2 Baseline survey to determine the resistance of weeds to Butachlor *5G*

3.2.1 Introduction

Rice is one of the important cereal crops in Bhutan and it is largely cultivated for domestic consumption. Rice is grown in all agro-ecological zones of Bhutan, across all 20 Dzongkhags, except the alpine zone in the north. As per the 2021 data, the area under rice production was 24055 acres with a total production of 40508 MT (NSB-ASR, 2021). However, the production of paddy is observed to decrease gradually due to urbanization and rapid socio-economic development (NSB-ASR, 2021). The major challenges in rice produciton are labour shortage, lack of irrigation and weed management. Weed management has emerged as a significant challenge in rice production in Bhutan due to labor shortages and limited irrigation facility.

Weeds are identified as a major biological constraint hindering the achievement of optimal rice productivity in major rice producing countries of South Asia (Rao & Matsumoto (Eds.), 2017). In Bhutan, rice yield losses of up to 50% due to weeds have been reported from lowland rice production (Karma & Ghimiray, 2006). The Royal Government of Bhutan introduced Butachlor *5G* in granular formulation in the 1980s to manage grass and sedge weeds in paddy fields. For over 2-3 decades, paddy growers in different parts of the country have been using only Butachlor *5G* leading to a gradual increase in demand despite a decrease in the cultivated area.

Continuous use of a single herbicide can cause herbicide resistance in weeds (Cobb, 2022). Butachlor *5G*, a pre-emergence selective herbicide under the Chloroacetanilide group, is used in Bhutan to control annual grasses and sedges such as *Echinochloa spp*., *Paspalum distichum*, *Cynodon dactylon*, *Cyperus iria*, *Cyperus difformis*, *Schoenoplectus juncoides*, and *Fimbristylis littoralis* in paddy fields.

Despite the widespread use of Butachlor *5G* in Bhutanese paddy fields for decades, there is a lack of research on the development of resistance in paddy weeds. In an attempt to address this knowledge gap, this study is aimed to gather farmers' perspectives towards the probable resistance developemnt in paddy weeds.

3.2.2 Materials & Method

Study site

The study was carried out in four rice-growing areas in Punakha, Wangduephodrang, Paro, and Thimphu. A total of 17 gewogs were selected for the study from these four dzongkhags using the Probability Cluster Sampling. ***Table 42***.

Table 42 Gewogs selected for the study under each dzongkhag

Dzongkhag	Gewogs
Wangdue Phodrang	Bjena, Rubesa, Kazhi, Ngyisho, Thoedtsho, Nahi, Gasetsogom, Gasetsowom
Punakha	Barp, Kabesa, Shelngana, Dzomi
Paro	Tsento, Dopshari, Lungye, Lamgong
Thimphu	Maedwang

Butachlor *5G* has been widely used by paddy growers in these areas to control weeds in the paddy fields for over two decades, and its application and demand from the fields have been increasing over the years.

Sample size

The Probability Cluster Sampling method was used to sample a representative farmer for the interview. A total of 190 farmers were randomly sampled from the 17 gewogs under four dzongkhags for the interview **Table *43***.

Table 43 Number of samples and gewogs by dzongkhags

Dzongkhag	Gewogs (No.)	Number of households (No.)
Punakha	4	50
Paro	4	50
Thimphu	1	40
Wangduephodrang	8	50
Total	17	190

Data collection

Farmers' perceptions on the development of weed resistance to Butachlor *5G* were collected using a semi-structured questionnaire from August to October 2022.

Data analysis

The collected data were analysed using Microsoft Excel and the Statistical Package for Social Sciences (SPSS) version 22. Descriptive statistics, frequency analysis, and graphs were used to determine the weed management practices currently practiced by the farmers and their perception of weed resistance development to Butachlor *5G.* Correlation analyses were also conducted to examine the relationship between the dosage of Butachlor *5G* application and the farmers' perception on the development of resistance in weeds.

3.2.3 Result & Discussion

Demography

The demographic details of the respondents, including their gender and age are as presented in **Table *44***. The results indicate that of the total respondents (n=190), 61.6% (n=117) were female while 38.4% (n=73) were male. The average age of the respondents was 50.9 years, with the youngest respondent being 24 years old and the oldest respondent being 84.

The household size, agricultural experience, and education background of the respondents are as presented in **Table *45***. The results show that the maximum household size was 8 persons per household, while the minimum was 1. The average household size was 3 people, and only family members who permanently stay at home were included in the count, with the exclusion of school-going children.

Most of the respondents had no formal education, accounting for 63.2% (n=120) of the total respondents. Of the total respondents, 20% (n=38) received non-formal education, while 16.8% (n=32) had attended formal education in schools. The survey result showed that most of the respondents had extensive agricultural experience, with 95.3% (n=181) having more than 5 years of experience. Only a small percentage, 4.7% (n=9), have been involved in agriculture farming for less than 5 years.

Table 44 Age of respondent and household size

		Age	HH_Size
N	Valid	190	190
Mean		50.8947	3.56
Median		52.0000	3.00
Minimum		24.00	1
Maximum		85.00	8

Table 45 Gender, Education background and Agriculture experience

Gender	Number (n)	Percentage (%)
Male	73	38.4
Female	117	61.6
Education background		
Uneducated	120	63.2
Non-formal	38	20
Educated	32	16.8
Agriculture experience		
< 5 years	9	4.7
>5 years	181	95.3

Land holding

According to the survey, the households owned a total of 269.88 acres of wetland, of which 238.38 acres (88.4%) were cultivated and 31.5 acres (11.6%) were left fallow. The average landholding for wetland and dryland was 1.42 acres and 0.42 acres, respectively, while the maximum landholding was 6 acres for wetland and 4.67 acres for dryland. The respondents

reported various reason for keeping their land fallow. The primary reason for leaving the land fallow was the shortage of irrigation water (n=27), followed by labor shortage (n=8), wildlife problem, and fragmented land (n=6) and (n=2), respectively **Figure *46***.

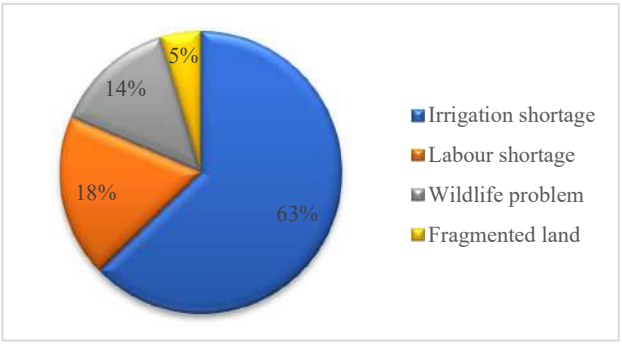

Figure 46 Reasons for keeping the land fallow

Important weeds in Paddy

The weed species *Potamogeton disctinctus*, also known as shochum in Bhutan was the most recorded species. Out of the 190 farmers surveyed, 176 reported finding *P. distinctus* in their paddy fields, and it was also found to be the most dominant weed **Figure *47*** . The second most recorded weed species was *Schoenoplectus juncoides* (n=172), followed by *Pontederia vaginalis* (n=138), *Acmella uliginosa* (n=114), *Cyperus difformis* (n=106), *Echinochloa crusgalli* (n=89), *Cynodon dactylon* (n=72), and *Bidens tripartita* (n=68). The least common weed species was *Alternanthera sessilis* (n=8). Thirty-two respondents reported the presence of other weed species, including *Fibristylis spp*., *Lemna minor*, *Eriocoulon spp*., *Echinochloa colona*, and *Cyperus rotundus*.

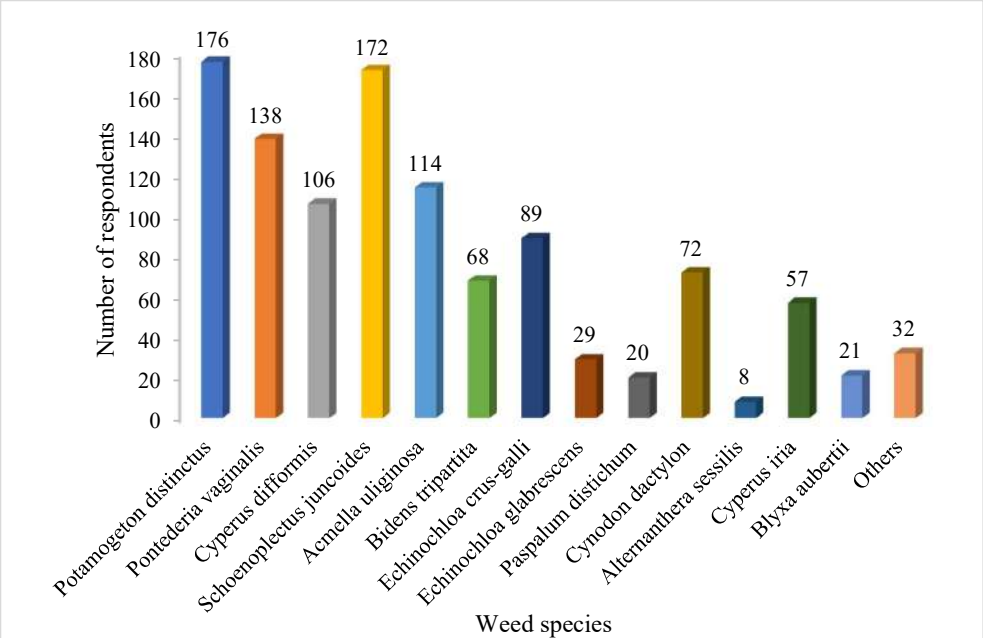

Figure 47 Ranking of important weed species in paddy

Weed management practice.

Farmers in the study used various methods to manage weeds in their paddy fields. The majority (93%, n=177) practiced an integrated weed management approach that involved hand weeding, cultural methods such as good tillage and ploughing in winter, and herbicide application, **Figure *48***. A small proportion (6%, n=12) used a combination of manual hand weeding and herbicide, while only 1% relied solely on manual hand weeding, which is considered a sound management practice despite its laborious and challenging nature.

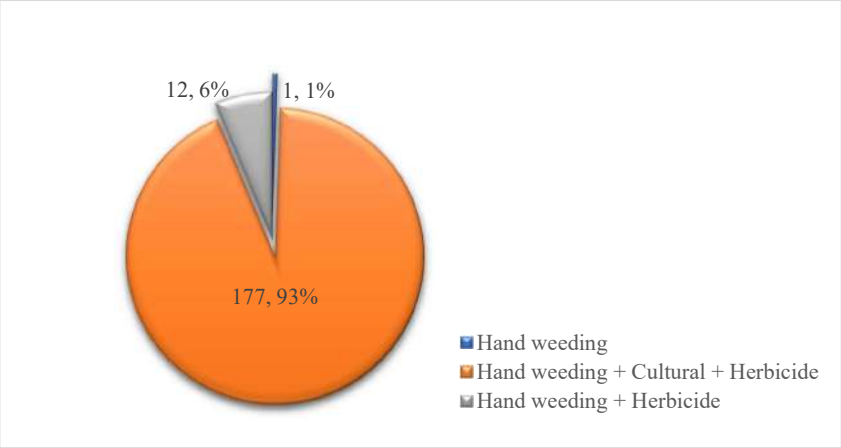

Figure 48 Types of weed management practiced

The farmers use different herbicides to manage weeds in their paddy fields **Figure *49***. Among the three herbicides commonly used, Butachlor *5G* and Ethoxysulfuron 15WDG are used by 168 farmers (88.4%). The survey found that 19 farmers (10%) use only Butachlor 5G, while one farmer in Shelgana under Punakha dzongkhag, uses only Ethoxysulfuron 15WDG. Two farmers in Rubesa under Wangdue Dzongkhag use all three herbicides (Butachlor 5G, Ethoxysulfuron 15WDG, and Glyphosate 41SL) to manage weeds.

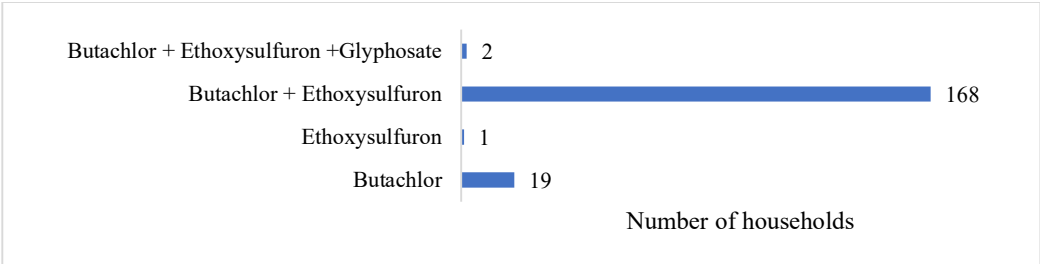

Figure 49 Types of herbicides used

Butachlor usage

According to the survey results, Butachlor 5G was used in the field for a maximum of 40 years and a minimum of 3 years. The average duration of its usage was 21 years. However, despite its widespread use, majority of the farmers (n=105) were not aware of the actual dosage recommended (1 bag of 10 kg Butachlor 5G per 4 Langdo[1] wetland) by the National Plant Protection Centre (NPPC) while only 85 respondents were ware of the recommended dosage. About 83% of the respondents (n=158), applied butachlor more than the recommended dosage while only 14 (7.3%) used the recommended dosage, and 9 (4.7%) used lower than the recommended dosage ***Figure 50***.

All the respondents reported applying butachlor once during the paddy season, with 147 applying it within 2-5 days after transplantation (DAT), and 26 applying it on the same day of transplantation. However, 5 respondents applied it before transplantation, and 12 farmers applied it within 5 to 15 days after transplantation.

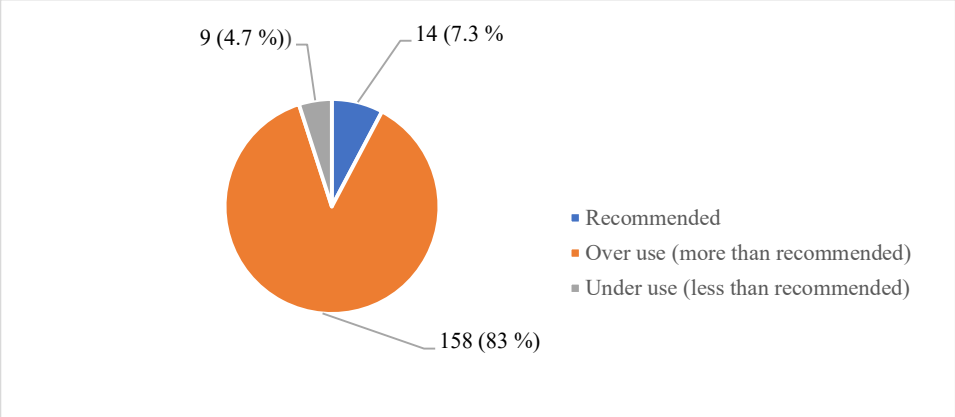

Figure 50 Dosage of butachlor used by farmers.

Of the 190 respondents, 157 reported an increase in the dosage of Butachlor 5G by 30-40% from the recommended amount over the years while 14 respondents reported using a constant amount each year, and 9 had reduced their application rate by switching to Ethoxysulfuron as an alternative to control broadleaved weeds.

Of the total respondents, 86.85% (n=165) reported a decrease in the effectiveness of Butachlor over the years, while 11.05% (n=21) said it remained the same, and none reported an increase in effectiveness. While the 2.10% (n=4) were unsure about its effectiveness.

Despite the declining effectiveness of Butachlor 5G, 138 respondents said they would not be able to grow paddy without it, while 45 respondents believed they could cultivate paddy without its application. Seven respondents were unsure.

A total of 118 respondents (62.10%) felt it was time to replace Butachlor 5G with other effective herbicides, while approximately 58 respondents (30.53%) believed that there was no need for a replacement. Additionally, 14 respondents (7.37%) were uncertain about whether it should be replaced.

[1] 4 langdo =1 acre (wetland)

Farmers' perception on butachlor resistant

Out of the 190 farmers surveyed, 50.53% (n=96) indicated that the weeds might had developed resistance to Butachlor, while 33.16% (n=63) reported that the weeds had not developed resistance. However, 16.31% (n=31) were uncertain about the potential development of resistance to Butachlor 5G. **Figure *51***.

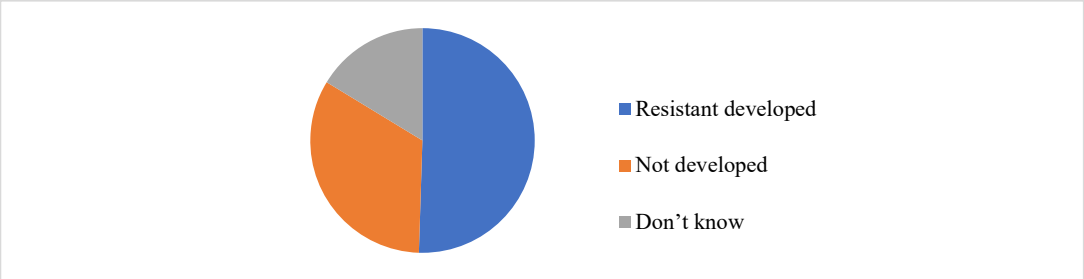

Figure 51 Farmers perception on resistant development of butachlor to weeds of paddy

To assess the relationship between changes in dosage (increase or decrease) and perceived resistance development, the Pearson correlation coefficient was computed. The results indicated a strong positive correlation between the two variables, r (188) = .23, p = .002. The findings indicates that, from the farmers' perspective, an increase in dosage is associated with a corresponding increase in the perception of resistance development.

3.2.4 Conclusion

The present study reveals that farmers use Butachlor in higher doses than recommended dose, primarily to control grasses and sedges in paddy fields, as well as broadleaf weeds like *Monochoria vaginalis* and *Potamogeton distinctus* (locally known as shochum).

According to both the survey report and farmer reports, the most common weed in paddy fields was *Potamogeton distinctus*, with *Schoenoplectus juncoides*, *Monochoria vaginalis*, and *Acmella uliginosa* uliginosa being the next most prevalent species.

The study could indicate that these weeds may have developed resistance to Butachlor 5G, which could explain the increase in dosage applied by the farmers. However, additional research is needed to confirm these findings through a more comprehensive, research-based study.

3.2.5 References

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3.3 An inventory of plants with pesticidal properties

3.3.1 Introduction

Bhutan is a small, landlocked kingdom characterized by rugged mountainous terrain in the Eastern Himalayas. The country has committed to various international agreements aimed at protecting its rich biodiversity and reducing greenhouse gas emissions (Gross National Happiness Commission [GNHC], 2011). Bhutan's reliance on a predominantly natural agricultural system presents significant opportunities for promoting and sustaining organic agriculture, which can help lower greenhouse gas emissions and positively influence climate change. However, the primary challenge in advancing organic agriculture in Bhutan lies in managing pests, diseases, and weeds. Research and development of organic plant protection strategies are still in their infancy, and information regarding indigenous plant protection practices utilized by farmers remains poorly documented.

Pesticidal plants, commonly referred to as botanical pesticides, are naturally occurring pesticides derived from plants (Anjarwalle et al., 2016). These pesticides have been used for centuries as a traditional method of pest control, leveraging the plants' innate defense mechanisms that have evolved over millions of years to fend off herbivores. Plants with pesticidal properties produce chemical compounds that repel insects and inhibit their feeding. While pesticidal plants have been extensively studied and documented in other countries, this area remains largely unexplored and not documented in Bhutan.

The National Plant Protection Centre currently depends on synthetic pesticides and imported bio-pesticides for agricultural pest management. However, these alternatives are costly and pose toxicity risks to both humans and the environment. The lack of information on local pesticidal plants hinders the development of locally produced bio-pesticides. By documenting botanical species with pesticidal properties, it would be possible to create a variety of organic bio-pesticides for effective pest and disease management in agriculture. Therefore, the primary goal of this study is to inventorize and document plants with pesticidal properties within the country.

3.3.2 Materials and Methods

Study site and period

The inventory was conducted in four eastern dzongkhags (Lhuentse, Mongar, Trashiyangtse and Trashigang) covering 26 gewogs as shown in table below (**Table *46***) from May 16 to June 14, 2023

Table 46 Gewogs covered during the inventory in four dzongkhags

Dzongkhag	Gewog
Lhuentse	Maenbi, Minjey, Khoma, Tsaengkhar

Monggar	Kengkhar, Saling, Mongar, Chhaling, Drepoong, Ngatshang, Chaksakhar, Drametse, Narang
Trashi Yangtse	Boomdeling, Tongmajangsa, Khamdang, Toedtsho, Yalang
Trashigang	Yangnyer, Khaling, Kanglung, Bartsham, Shongphu, Radhi, Merak, Pjongmaed

Data collection

Data collection included the survey date, administrative location (including site/village, gewog, and dzongkhag), geocoordinates, as well as the scientific, common, and local names of plants. Additionally, information on their abundance, habitats, and growth stages at the time of the survey was gathered. Surveys were conducted in various areas, including the periphery of forests, to ensure comprehensive information on a diverse range of botanical species. All plants with pesticidal properties were documented.

Survey Equipment

- Global Position System (GPS) device fwas used for geocoordinate recording and location tracking.
- Digital phones with the Epicollect5 app were employed for data recording and uploading which eased and streamlined the process.
- Digital cameras captured plant parts and their habitats for the documentation.
- Tools such as secateurs, scissors, and knives were used to collect plant samples.
- We utilized herbarium pressing boards for preserving samples for future reference and taxonomic studies, Figure 52.

Figure 52 Data collection of plants with pesticidal properties

3.3.3 Results and discussion

During the survey conducted across 26 gewogs in four eastern dzongkhags (Mongar, Lhuentse, Trashigang, and Trashiyangtse), a total of 18 plants with pesticidal properties were

recorded, with four species belonging to the Asteraceae family. ***Table 47***. The Asteraceae family, also known as the daisy family, is one of the largest plant families. Two species were identified under the Rutaceae family. The remaining 11 botanical plants were distributed among various individual botanical families. Each of these plants belonged to a different family, showcasing the diverse range of plants with pesticidal properties in the surveyed area.

Table 47 List of botanicals with pesticidal properties

Sl. No.	Scientific Name	Common Name	Family	Part used
1	*Acorus calamus*	Sweet flag	Acoraceae	Leaves & Rhizome
2	*Ageratum conyzoides*	Billygoat-weed	Asteraceae	Leaves & Flower
3	*Ageratina adenophora*	Crofton weed	Asteraceae	Leaves and Stem
5	*Allium Sativa*	Garlic	Amaryllidaceae	Clove
6	*Artemisia annuna*	Sweet wormwood	Asteraceae	Leaves & Stem
7	*Cannabis sativa*	Hemp	Cannabaceae	Leaves
8	*Chromolaena odorata*	Siam weeds	Asteraceae	Leaves
9	*Cymbopogon bhutanicus*	Lemongrass	Poaceae	Leaves
10	*Guajava psidium*	Common guava	Myrtaceae	Leaves & Fruit
11	*Jatropha curcas*	Purging nut	Euphorbiaceae	Leaves & Seed
12	*Justicia adhatoda*	Malabar nut	Acanthaceae	Leaves
13	*Juniperus recurva*	Drooping juniper	Cupressaceae	Leaves
14	*Lantana camara*	Common lantana	Verbenaceae	Leaves & Fruit
15	*Melia azedarach*	Chinaberry	Meliaceae	Leaves & Fruit
16	*Murraya koenigii*	Curry tree	Rutaceae	Leaves
17	*Vitex negundo*	Chinese chaste tree	Lamiaceae	Leaves
18	*Zanthoxylum armatum*	Winged prickly ash	Rutaceae	Leaves & Fruit

3.3.4 Conclusion

The inventory conducted in four eastern dzongkhags provided preliminary insights into the presence of plants with pesticidal properties. A total of 18 plants were identified, belonging to different families, including Asteraceae and Rutaceae. The findings highlight the rich diversity of plants with pesticidal properties in these Dzongkhags. The inventory serves as a valuable starting point for establishing baseline information on plants with pesticidal properties.

3.3.5 References

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3.4 An Inventory of agricultural weeds and invasive plants (Preliminary report)

3.4.1 Introduction

Bhutan though primarily an agrarian society, it is closely linked to its natural environment. The population relies more heavily on natural resources for sustainable agricultural production than on conventional agricultural systems. The country's rich biodiversity provides essential resources such as timber, fuel, food, and manure, while also offering habitats for numerous microorganisms, insects, birds, and animals vital for a healthy ecosystem. However, the increasing presence of invasive weeds and alien plants poses significant threats to crop production, native plant diversity, and the overall environment. Over the years, many invasive species are suspected to have crossed over the borders and colonization by these invasive species in the country has increased significantly.

To understand the adverse effects of invasive plants on both humans and the environment, it is essential to implement timely and effective management measures. However, in the absence of sufficient knowledge and reliable data on invasive weeds and plants in the country, development of effective management strategies or techniques is not possible. Therefore, a fundamental aspect of a strategic management approach involves inventorying and mapping the distribution of invasive plant species. The purpose of this inventory is to document and map the presence of weeds and invasive plant species in the country, which will help in formulating appropriate management strategies

3.4.2 Materials and Methods

Study site and period

The inventory was conducted from April 18 to May 3, 2023, in Sarpang and Chhukha dzongkhags, and from May 16 to June 14, 2023, in four eastern dzongkhags (Lhuentse, Mongar, Trashiyangtse, and Trashigang), covering a total of 40 gewogs, **Table *48***.

Table 48 Gewogs covered during the inventory in six dzongkhags

Dzongkhag	Gewog
Sarpang	Singye, Gakidling, Dekiling, Shompangkha, Samtenling, Gelephu, Serzhong, Chuzergang, Umling, Tareythang
Chhukha	Samphelling, Loggichina, Phuntsholing, Geling, Bongo
Lhuentse	Menbi, Khoma, Menji, Tshenkhar
Monggar	Kengkhar, Saling, Chhali, Drepong, Ngatshang, Chaskhar, Drametse, Narang.

Trashi Yangtse	Boomdeling, Tongmajangsa, Khamdang, Toedtsho, Yalang
Trashigang	Yangnyer, Khaling, Kanglung, Bartsham, Shongphu, Radhi, Merak, Pjongmaed

The selection of gewogs was basedon several factors, including the size of the area, agricultural activities, and the porosity of both internal and international borders. This aproach enabled the team to cover a diverse range of ecosystems and habitats where invasive plant species are likely to thrive.

Data Collection

The team used a systematic approach to collect data on the distribution, abundance, and ecological impacts of invasive plant species in each of the surveyed gewogs. During the survey, the date, administrative location (including site/village, gewog, and dzongkhag), geocoordinates, as well as the scientific, common, and local names of the weeds were recorded. Additionally, information on their abundance, habitats, growth stages, and invasiveness status for each invasive species was documented using smartphones, ***Figure 53***.

Line transects were used in various land types, including agricultural land, roadsides, settlement areas, and the periphery of forests, to gather comprehensive information on diverse plant species. Data were collected using a 4x4 m quadrant at each point, with 50 meters between points in agricultural fields and an open distance for roadsides and forest periphery, depending on the presence of invasive plants, ***Figure 54***. The length of each transect was determined by the number of points included. All invasive plants and weeds within each quadrant were recorded in terms of density: low (1-20%), medium (20-60%), and high (60-100%) coverage.

Figure 53 Inventorying the invasive plants & agricultural weeds

Figure 54 Collection of weed samples

Survey Equipment

- Global Position System (GPS) device was used for geocoordinate recording and location tracking.
- Digital phones with the Epicollect5 app were employed for data recording and uploading which eased and streamlined the process.
- Digital cameras captured plant parts and their habitats for the documentation.
- Tools such as secateurs, scissors, and knives were used to collect plant samples.
- Herbarium pressing boards were used for collecting and preserving samples for future reference and taxonomic studies

During the survey we used Global Position System (GPS) for recording the geocoordinate and digital phones with the Epicollect5 application for recording the data and uploading the data. Digital camera was also used for capturing the parts of plants and capturing the locations and habitats. The secateurs, scissors, knife/blade to cut the plant sample; set of herbariums pressing board for collecting and pressing the plant samples for future reference and for the taxonomic studies. In cases where new or unfamiliar species are encountered, herbarium collections were taken for taxonomic identification and future reference **Figure *54***. To ensure comprehensive documentation, photos of various parts of the plants was captured for digital documentation.

During the survey, a Global Positioning System (GPS) was used to record geocoordinates, while digital phones equipped with the Epicollect5 application facilitated data recording and uploading. A digital camera was used to capture images of plant parts as well as their locations and habitats. Tools such as secateurs, scissors, and a knife/blade were used to

collect plant samples, and a set of herbaria pressing boards was used for pressing these samples for future reference and taxonomic studies. In cases where new or unfamiliar species were observed, herbarium collections were made for taxonomic identification and future reference, ***Figure 54***. To ensure thorough documentation, photographs of various plant parts were taken for digital records.

3.4.3 Result and discussion

During the field survey, a total of 161 species belonging to 39 distinct families were recorded and documented. The family with the highest number of recorded species was Asteraceae (Compositae) which included 36 species, followed by Poaceae (Gramineae) with 15 species **Figure *55***. Among the surveyed families, 23 had more than one recorded species, while the following 16 families each had only one recorded species: *Apocynaceae, Apiaceae, Araceae, Araliaceae, Asparagaceae, Cactaceae, Cannabaceae, Dennstaedtiaceae, Equisetaceae, Geraniaceae, Juncaceae, Mazaceae, Nyctaginaceae, Portulaceae, Ranunculaceae,* and *Sauruaceae.*

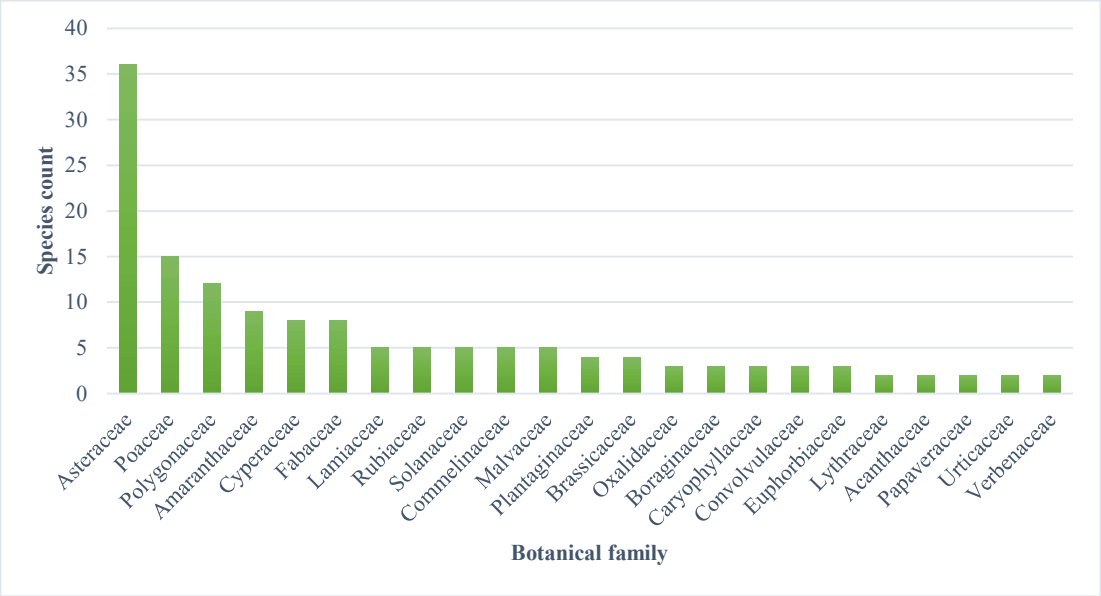

Figure 55 Number of species recorded from 23 different Botanical families.

(n=108) (Families having more than one species)

During the inventory, 45 invasive plants **Table *49*** and 116 agriculture weed species **Table *50*** belonging to 21 different families, and their characteristics were thoroughly examined. These invasive plants have shown a remarkable ability to establish themselves in various habitats, ranging from barren land to roadsides, fallow lands, forests, and the periphery of agricultural fields. Their capacity to colonize such diverse environments indicates their adaptability and resilience. Unlike native plants that are typically adapted to specific environmental conditions, invasive plants possess traits that allow them to thrive in a wide range of ecosystems. This adaptability enables them to spread rapidly and outcompete native vegetation.

Table 49 Invasive species recorded with botanical family.

Sl.no	Invasive species	Family
1	*Agave americana*	Asparagaceae
2	*Acmella uliginosa*	Asteraceae
3	*Ageratina Adenophora*	Asteraceae
4	*Ageratum conyzoides*	Asteraceae
5	*Alternanthera pungens*	Amaranthaceae
6	*Alternanthera sessilis*	Amaranthaceae
7	*Amaranthus spinosus*	Amaranthaceae
8	*Argyreia argentea*	Convolvulaceae
9	*Argemone Mexicana*	Papaveraceae
10	*Axonopus compressus*	Poaceae
11	*Bidens pilosa*	Asteraceae
12	*Cannabis sativa*	Cannabaceae
13	*Chromolaena ordorata*	Asteraceae
14	*Commelina benghalensis*	Commelinaceae
15	*Conyza bonariensis*	Asteraceae
16	*Conyza canadensis*	Asteraceae
17	*Conyza floribunda*	Asteraceae
18	*Cosmos bipinnatus*	Asteraceae
19	*Crassocephalum crepidioides*	Asteraceae
20	*Cuscuta campestris*	Convolvulaceae
21	*Datura stramonium*	Solanaceae
22	*Drymaria cordata*	Caryophyllaceae
23	*Galinsoga parviflora*	Asteraceae
24	*Houttuynia cordata*	Saururaceae
25	*Hypoestes phyllotachya*	Acanthaceae
26	*Hyptis suaveolens*	Lamiaceae
27	*Imperata cylindrica*	Poaceae
28	*Ipomoea hederifolia*	Convolvulaceae
29	*Lantana camara*	Verbenaceae
30	*Lepidium virginicum*	Brassicaceae
31	*Lotus corniculatus*	Fabaceae
32	*Mikania micrantha*	Asteraceae
33	*Mimosa pudica*	Fabaceae
34	*Opuntia monacantha*	Cactaceae
35	*Oxalis latifolia*	Oxalidaceae
36	*Parthenium hysterophorus*	Asteraceae
37	*Paspalum distichum*	Poaceae
38	*Pennisetum cladestinum*	Poaceae
39	*Ricinus communis*	Euphorbiaceae
40	*Scoparia dulcis*	Plantaginaceae
41	*Sida acuta*	Malvaceae
42	*Stellaria media*	Caryophyllaceae
43	*Tithonia diversifolia*	Asteraceae
44	*Trifolium repens*	Fabaceae
45	*Xanthium indicum*	Asteraceae

Table 50 List of Agricultural weeds recorded.

Sl.no	Agricultural weeds (Non-invasive species)	Family
1	*Ageratum houstonianum*	Asteraceae
2	*Amaranthus hybridus*	Amaranthaceae
3	*Amaranthus lividus*	Amaranthaceae
4	*Amaranthus viridis*	Amaranthaceae
5	*Anaphalis margaritacea*	Asteraceae
6	*Arachis duranensis*	Fabaceae
7	*Artemisia campestris*	Asteraceae
8	*Arthraxon hispidus*	Poaceae
9	*Arthraxon quartinianus*	Poaceae
10	*Bidens bipinnata*	Asteraceae
11	*Bidens tripartita*	Asteraceae
12	*Boerhavia diffusa*	Nyctaginaceae
13	*Capsella bursa-pastoris*	Brassicaceae
14	*Centella asiatica*	Apiaceae
15	*Chenopodium album*	Amaranthaceae
16	*Clinopodium umbrosum*	Lamiaceae
17	*Colocasia*	Araceae
18	*Commelina diffusa*	Commelinaceae
19	*Commelina hasskarlii*	Commelinaceae
20	*Commelina maculata*	Commelinaceae
21	*Cuphea carthagenensis*	Lythraceae
22	*Cyanotis vaga*	Commelinaceae
23	*Cyanthillium cinereum*	Asteraceae
24	*Cymbopogon*	Poaceae
25	*Cynodon dactylon*	Poaceae
26	*Cynoglossum furcatum*	Boraginaceae
27	*Cynoglossum lanceolatum*	Boraginaceae
28	*Cyperus cyperoides*	Cyperaceae
29	*Cyperus facus*	Cyperaceae
30	*Cyperus iria*	Cyperaceae
31	*Cyperus kyllingia*	Cyperaceae
32	*Cyperus rotundus*	Cyperaceae
33	*Cyperus tenuispica*	Cyperaceae
34	*Dichrocephala integrifolia*	Asteraceae
35	*Digitaria ciliaris*	Poaceae
36	*Digitaria sanguinalis*	Poaceae
37	*Dysphania ambrosiodes*	Amaranthaceae
38	*Dysphania pumilio*	Amaranthaceae
39	*Echinochloa colona*	Poaceae
40	*Eclipta alba*	Asteraceae
41	*Eleusine indica*	Poaceae
42	*Equisetum diffusum*	Equisetaceae
43	*Emilia sonchifolia*	Asteraceae
44	*Euphorbia hirta*	Euphorbiaceae

45	*Fagopyrum dibotrys*	Polygonaceae
46	*Fagopyrum gracilipes*	Polygonaceae
47	*Fumaria officinalis*	Papaveraceae
48	*Galinsoga quadriradiata*	Asteraceae
49	*Galium aparine*	Rubiaceae
50	*Geranium nepalense*	Geraniaceae
51	*Heliotropiums indicum*	Boraginaceae
52	*Hydrocotyle*	Araliaceae
53	*Juncus prismatocarpus*	Juncaceae
54	*Kyllinga squamulata*	Cyperaceae
55	*Lamium amplexicaule*	Lamiaceae
56	*Leucas aspera*	Lamiaceae
57	*Leucas ciliata*	Lamiaceae
58	*Mazus delavayi*	Mazaceae
59	*Malva verticillata L*	Malvaceae
60	*Nasturtium officinale*	Brassicaceae
61	*Oxalis corniculata*	Oxalidaceae
62	*Oxalis debilis*	Oxalidaceae
63	*Parochetus communis*	Fabaceae
64	*Peristrophe*	Acanthaceae
65	*Persicaria bistorta*	Polygonaceae
66	*Persicaria capitata*	Polygonaceae
67	*Persicaria chinensis*	Polygonaceae
68	*Persicaria hydropiper*	Polygonaceae
69	*Persicaria nepalensis*	Polygonaceae
70	*Persicaria pubescence*	Polygonaceae
71	*Persicaria runcinata*	Polygonaceae
72	*Physalis divaricata*	Solanaceae
73	*Plantago major*	Plantaginaceae
74	*Poa annua*	Poaceae
75	*Polygonum aviculare*	Polygonaceae
76	*Portulaca oleraceae*	Portulacaceae
77	*Pouzolzia hirta*	Urticaceae
78	*Pouzolzia zeylanica*	Urticaceae
79	*Pseudognaphalium affine*	Asteraceae
80	*Pseudognaphalium pensylvanicum*	Asteraceae
81	*Pteridium aquilinum*	Dennstaedtiaceae
82	*Ranunculus chinensis*	Ranunculaceae
83	*Richardia scabra*	Rubiaceae
84	*Rotala indica*	Lythraceae
85	*Rumex acetocella*	Polygonaceae
86	*Rumex nepalensis*	Polygonaceae

87	*Schoenoplectus juncoides*	Cyperaceae
88	*Senna obtusifolia*	Fabaceae
89	*Setaria palmifolia*	Poaceae
90	*Setaria pumila*	Poaceae
91	*Sida rhombifolia*	Malvaceae
92	*Siegesbeckia orientalis*	Asteraceae
93	*Solanum khasinum*	Solanaceae
94	*Solanum nigrum*	Solanaceae
95	*Solanum torvum*	Solanaceae
96	*Sonchus arvensis*	Asteraceae
97	*Sonchus asper*	Asteraceae
98	*Sonchus olaraceae*	Asteraceae
99	*Spergula arvensis*	Asteraceae
100	*Speranskia turberculata*	Euphorbiaceae
101	*Spermacoce latifolia*	Rubiaceae
102	*Spermacoce ocymoides*	Rubiaceae
103	*Stellaria vestita*	Caryophyllaceae
104	*Stylosanthes biflora*	Fabaceae
105	*Synedrella nodiflora*	Asteraceae
106	*Tabernaemontana divaricata*	Apocynaceae
107	*Taraxacum officinale*	Asteraceae
108	*Thlaspi arvense*	Brassicaceae
109	*Tridax procumbens*	Asteraceae
110	*Trifolium dubium*	Fabaceae
111	*Triumfetta rhomboidea*	Malvaceae
112	*Urena lobata*	Malvaceae
113	*Verbana bracteata*	Verbenaceae
114	*Veronica arvensis*	Plantaginaceae
115	*Veronica persica*	Plantaginaceae
116	*Youngia japonica*	Asteraceae

3.4.4 Conclusion

An inventory of agricultural weeds and invasive plants conducted across six dzongkhags (Sarpang, Chhukha, Lhuentse, Monggar, Trashiyangtse, and Trashigang) has provided valuable insights into the presence and characteristics of invasive plant species as well as important agricultural weeds. A total of 162 species belonging to 39 families were recorded and documented, with Asteraceae and Poaceae emerging as the most prominent families.

These invasive plants have demonstrated their ability to colonize diverse habitats, from barren land to roadsides, fallow lands, forests, and agricultural fields' peripheries. Their adaptability and resilience contribute to their rapid spread and out competition of native vegetation. This poses significant threats to crop production, native plant diversity, and the environment. Therefore, it is crucial to develop appropriate management strategies to mitigate the adverse impacts of these invasive plants. The inventory serves as a foundation

for understanding the distribution, abundance, and ecological impacts of invasive plants and agricultural weeds in the surveyed areas, paving the way for the development of effective management techniques and conservation efforts to protect Bhutan's natural resources and agricultural systems.

3.4.5 References

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4. Pest Surveillance Program

4.1 Rice Blast Disease Scoring on IR-64 rice variety

4.1.1 Introduction

Rice blast disease, caused by the fungus *Magnaporthe oryzea*, is prevalent in flooded and shaded areas of rice fields. The disease tends to spread rapidly when susceptible rice varieties are grown extensively in monoculture and when the environmental conditions are conducive for its development (NPPC: Pest of Bhutan website). It is a prevalent disease in rice fields that are flooded and shaded, which creates optimal conditions for the pathogen to thrive. The disease tends to spread rapidly under the following conditions:

- When rice varieties that are susceptible to the disease are grown extensively in a monoculture system, the disease can spread more easily.
- The development and spread of rice blast is favourable when the environmental conditions are conducive, such as high humidity, moderate temperatures, and prolonged periods of leaf wetness.

In summary, rice blast disease thrives in flooded and shaded rice fields, especially when susceptible rice varieties are grown in monoculture and the environmental conditions support the disease's growth and propagation. Shaded conditions often result in higher humidity levels and reduced airflow, which further increases the likelihood of infection. These factors can lead to early epidemics, particularly in areas near tree lines or other structures that cast shade.

The last significant rice blast epidemic in Bhutan occurred during the 1995-1996 rice growing seasons, as documented by Ghimiray et al. (2013). Following this outbreak, numerous blast-resistant rice varieties were introduced. One such variety, IR-64, was introduced in 1988 to manage rice blast in Bhutan (Ghimiray et al., 2013). Despite its initial effectiveness, there have been unconfirmed reports of IR-64 losing its resistance, leading to substantial losses for farmers. Moreover, farmers in the Punakha and Lobesa regions have noted variations among the IR-64 varieties they cultivate, with differences observed in panicle characteristics and

plant height. This study aimed to confirm the development and severity of rice blast on IR-64 varieties grown by farmers and agricultural research centers.

4.1.2 Materials and Methods

Study site

The study was conducted in the rice blast hotspot areas of Sibjikha and Tshokorna, located within the Barp Gewog under the Punakha Dzongkhag. Seeds of the IR-64 rice variety were sourced from two different sources:

- The Agricultural Research and Development Centre (ARDC) in Bajo, which provided the pureline variety.
- Local farmers in the same locality, who provided the local variety.

Separate plots were established for each seed lot in both Sibjikha and Tshokorna, where paddy nurseries were established. The seeds were sown on April 25 in Sibjikha and on May 7 in Tshokorna. In Tshokorna, farmers treated both seed lots with the fungicide tricyclazole by soaking them for three hours prior to sowing.

The seedlings were transplanted 60 days and 50 dyas after sowing in Sibjikha and Tshokorna respectively. At the time of transplantation, the seedlings were approximately 15-25 cm tall in Sibjikha and 25-30 cm tall in Thokorna**.**

Data collection

For each rice variety, 50 randomly selected plants (hills) were tagged in each of the study sites at Sibjikha and Tshokorna.

The disease severity was monitored on a weekly basis from the identified and tagged plants/hills of paddy for each rice variety. The disease scoring was done using a guide adapted from the International Rice Research Institute (IRRI), as shown in Table 51.

The data collection took place from 15th July 2022, to 30th September 2022, covering the period when the plants had reached the tillering stage in both the study sites, ***Figure 56.***

Table 51 Disease scoring guide

Blast Scoring Guide

Table 1: Description of SES Scale (IRRI, 2002) for blast disease scoring

0-9	Scale	Disease severity
0	No lesion observed.	Immune
1	Small brown specks of pin point size (smaller than 0.5 mm in diameter)	Resistant
2	Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin. Lesions are mostly found on the lower leaves.	Moderately Resistant
3	Lesion types same as in 2 with 1-3 mm in diameter, but significant number of lesions on the upper leaves.	Moderately Resistant
4	Typical spindle shaped susceptible blast lesions, 3 mm or longer infecting less than 4% of leaf area.	Moderately Susceptible
5	Typical susceptible blast lesions of 3 mm or longer infecting 4- 10% of the leaf area.	Moderately Susceptible
6	Typical susceptible blast lesions of 3 mm or longer infecting 11-25% of the leaf area.	Susceptible
7	Typical susceptible blast lesions of 3 mm or longer infecting 26-50% of the leaf area.	Susceptible
8	Typical susceptible blast lesions of 3 mm or longer infecting 51-75% of the leaf area many leaves are dead.	Highly Susceptible
9	Typical susceptible blast lesions of 3 mm or longer infecting more than 75% leaf area affected	Highly Susceptible

Figure 56 Observation plots in Sebjikha, Baap, Punakha

4.1.3 Results and discussion

Sebjikha

The rice blast disease affected both the pure line and local rice varieties, exhibiting a similar trend throughout the crop growing stages, as shown in Figure 57. Throughout the study period, the pure line variety showed higher disease severity, with scores ranging from 2.5 to 5 in comparion to the local varieties. The local varieties had lower disease scores, ranging from 1.1 to 3.8; thus, suggesting that while both the pure line and local rice varieties were

susceptible to the rice blast disease, the pure line variety experienced more severe disease symptoms and higher disease scores across the monitored growth stages.

The local rice variety exhibited resistant to moderately resistant reaction types throughout the study period, with disease scores ranging from 1 to less than 4. In contrast, the pure line variety initially displayed a resistant to moderately resistant reaction type, with lesion scores varying from 1 to less than 4. However, by mid-September, the disease severity increased to a moderately susceptible level, with a score of 5. This shift in susceptibility may be attributed to the environmental conditions in the area, which were characterized by high humidity and temperature, favoring the development of blast disease.

Towards the end of September, the pure line variety's susceptibility gradually declined to a moderately resistant type. The blast symptoms on the leaves of both rice varieties disappeared as the crop matured. However, the blast disease did not affect any nodes or panicles, which could have caused chaffy grains and reduction in yield. Though the disease affected the leaves, it did not significantly affect the nodes and panicles, which could have caused chaffy grains hence the reduction in yield.

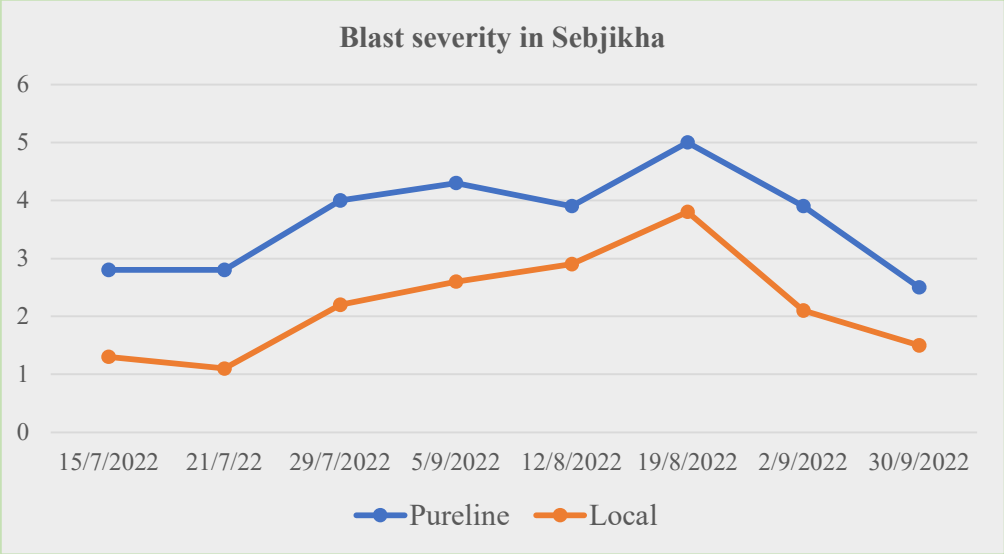

Figure 57 IR-64 from both the source showing their severity against blast disease in Sebjikha

(0: Immune, 1: Resistant, 2-3: Moderately Resistant, 4-5: Moderately Susceptible, 6-7: Susceptible, 8-9: Highly Susceptible)

Tshokorna

The blast disease affected only the pure line variety which showed moderately susceptible to susceptible reaction type of symptoms **Figure *58***. Both the variety did not show any signs of blast until the first week of July. After that pure line started to show few blast lesions of moderately resistant reaction type until mid-August. However, by the first week of September

the susceptible lesions were recorded on the leaves of few pants (Figure 48) and its susceptibility symptoms disappeared from the following week and by the end of September, no blast diseases were observed in both the varieties.

The rice blast disease primarily affected the pure line variety, which exhibited moderately susceptible to susceptible reaction types, as shown in Figure 58. Though both the varieties did not show any signs of blast disease until the first week of July, the pure line variety started to display few blast lesions with a moderately resistant reaction type post first week of July, which persisted until mid-August. However, by the first week of September, susceptible lesions were recorded on the leaves of a few plants, ***Figure 56***. Interestingly, the susceptibility symptoms disappeared from the pure line variety in the following week, and by the end of September, no blast disease symptoms were observed in both the pure line and the local varieties.

Thus, indicating that the pure line variety was more susceptible to the rice blast disease, initially displaying moderately resistant symptoms that later progressed to a more severe susceptible stage before eventually disappearing by the end of the crop season. In contrast, the local variety remained largely unaffected by the disease throughout the study period.

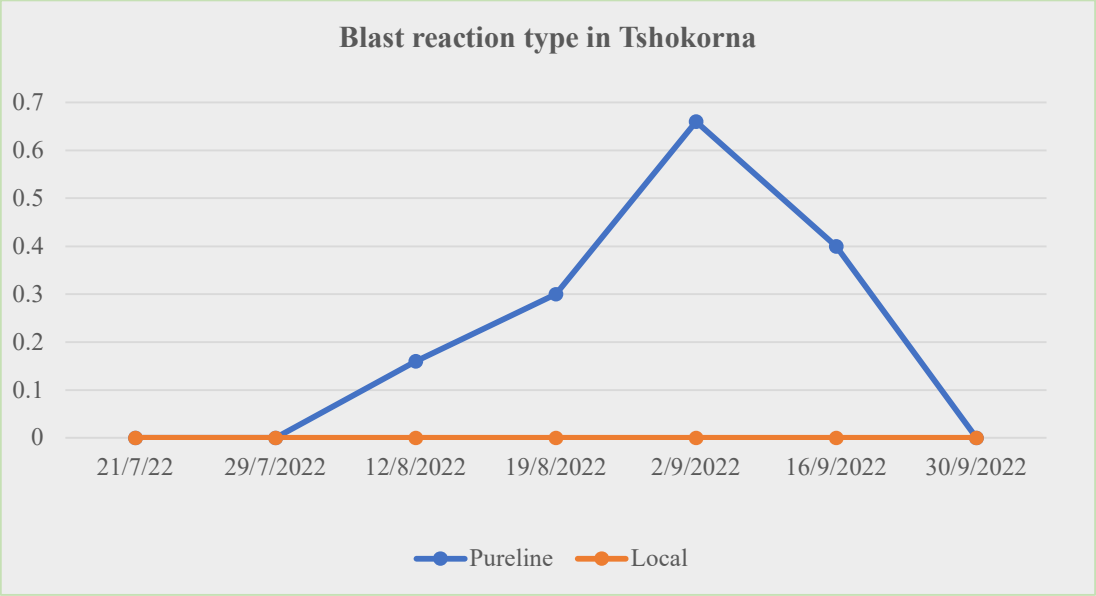

Figure 58 IR-64 from both the source showing their severity against blast disease

(0: Immune, 1: R, 2-3: MR, 4-5: MS, 6-7: S, 8-9: HS)

4.1.4 Conclusion

The varieties from both the two seed sources showed moderately resistant to moderately susceptible reaction type with pure line variety exhibiting higher susceptibility compared to the local ones. Though blast symptoms were recorded at the early stages on its leaves, the disease pressure declined considerable as the crop matured without having any impact on its

yield. However, the further in-depth and well-planned research needs to be conducted to confirm the results.

The rice varieties sourced from both ARDC Bajo (pure line) and local farmers (local) demonstrated a variety of reaction types, ranging from moderately resistant to moderately susceptible. The pure line variety exhibited higher susceptibility than the local varieties. Although blast symptoms were observed on the leaves of the pure line variety in the early growth stages, the disease pressure decreased considerably as the crop matured, ultimately having no effect on the final yield.

The findings indicate that the pure line variety is more susceptible to rice blast than the local varieties; however, the disease did not impact crop yield. To confirm these results, further in-depth and well-structured research is necessary. While this study offers preliminary insights, additional investigations are essential to establish more definitive conclusions regarding the resistance levels and the disease's effects on various rice varieties. Therefore, conducting comprehensive studies to validate these observations is crucial for providing stronger, evidence-based recommendations for managing rice blast in the region.

4.1.5 References

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4.2 Wheat rust monitoring survey

4.2.2 Introduction

Wheat rust surveys are conducted as part of the global wheat rust monitoring system to regularly monitor rust outbreaks and the potential development of new rust races in the region. Wheat rust surveys for the fiscal year 2022-2023 were conducted in the major wheat-growing areas of the Haa and Paro dzongkhags from 9th to 11th May 2023.

4.2.3 Materials and Methods

Data collection

In each gewog, major wheat fields were randomly selected for the survey. Within each field, a diagonal pattern was used to observe plants at every 10-15 steps. Information on location, variety name, crop stage, rust type, rust incidence, rust severity, reaction type and the presence of alternate hosts were recorded. The survey data was manually recorded on printed forms using the format of the SAARC (South Asian Association for Regional Cooperation) toolbox application.

The survey data was previously recorded using the SAARC rust surveillance toolbox application and uploaded to the global wheat network server when internet connectivity was available. However, since the conclusion of the Delivering Genetic Gains in Wheat (DGGW) project in 2021, the subscription for the rust toolbox has expired. This has limited access to

the application and its functionalities, which are crucial for ongoing rust surveillance and management efforts in wheat production.

4.2.4 Results and discussion

Wheat and barley are important cereal crops grown in the Haa and Paro Dzongkhags. Local wheat varieties such as Yue Kaa, Bhoe Kaa, and Gudhum, along with Drukbi Kaa, are the primary types cultivated in both Haa and Paro Dzongkhags. The Bumthang Kaa Drukchu variety was observed in a single location in Balamna, Samar Gewog under Haa Dzongkhag. In terms of barley cultivation, the variety known as Gurum is also grown in both Haa and Paro.

Surveys in Haa were conducted in Samar, Bji, and Katso Gewogs from May 9 to 10, 2023, during which wheat and barley crops were at the booting to milk stage. In Balamna under Samar Gewog, a low incidence and severity of yellow rust with moderately resistant reaction type was observed on Bumthang Kaa Drukchu, ***Table 52*****.** No rust was detected in any other fields across Haa during this survey period.

In Paro, surveys were conducted on May 9, 2023, in Naja Gewog and on May 11, 2023, in Tsento and Lango Gewogs. The local wheat varieties Yue Kaa, ***Figure 59***, and Bhoe Kaa/Gudhum were cultivated, along with a local barley variety known as Gurum, ***Figure 60***. During this period, the crops were at the booting to milk stages. A low incidence and severity of yellow rust were recorded on Yue Kaa in Susuna (Naja Gewog) with a moderately susceptible reaction type, while a moderately resistant reaction type was observed in Drugyel (Tsento Gewog), ***Table 52***. A moderate incidence of loose smut was found on barley (Gurum) in Naja Gewog. Moderate to high levels of powdery mildew affected both wheat and barley across all sites in Naja Gewog. In addition, a high incidence of loose smut was recorded on Yue Kaa in Drugyel (Tsento gewog), and a high incidence of white tips of ear was observed at one site where Yue Kaa was cultivated in Chunji under Tsento Gewog.

Table 52 Wheat rust prevalence in the Haa and Paro dzongkhags in May 2023 (L-Low, M-moderate, H-High; R-Resistant, MR-Moderately Resistant, MS-Moderately Susceptible, S-Susceptible)

Sl. No.	Location	Observation date	Variety	Crop stage	Rust type	Inci-dence (L/M/H)	Severity (L/M/H)	Reaction (R/MR/MS/S)	Other diseases observed
1	Naja, Paro	9/5/2023	Yue Kaa	Flowering	Yellow rust	Low	low	MS	Moderate incidence of powdery mildew
			Bhoe Kaa/Gudhum	Booting to heading	-	-	-	-	High incidence of powdery mildew
			Gurum (barley)	Flowering	-	-	-	-	Moderate incidence of powdery mildew and loose smut
2	Samar, Haa	9/5/2023	Bumthang Kaa Drukchu	Heading	Yellow rust	low	low	MR	-
3	Bji, Haa	10/5/2023	Local (Bhoe kaa and Yue Kaa)	Heading	-	-	-	-	-
4	Katso, Haa	10/5/2023	Local (Bhoe Kaa and Drubi Kaa)	Heading	-	-	-	-	-

5	Tsento, Paro	11/5/2023	Local (Bhoe Kaa)	Flowering	-	-	-	-	-
			Local (Yue Kaa)	Flowering to milk	Yellow rust	low	low	MR	Incidence of loose smut is quite high especially in Drugyel area on Yue Kaa and high incidence of white tips of ear/awn on Yue Kaa in Chunji in Tsento.
6	Lango, Paro	11/5/2023	Local (Gudhum)	Flowering	-	-	-	-	-

Figure 59 Yue Kaa (a); Bhoe Kaa or Gurum (b); purple auricle and nodes of Bhoe Kaa (c & d).

Figure 60 Yellow rust symptoms on wheat leaves, Paro

4.2.5 Conclusion

Wheat growers in the Haa and Paro valleys primarily cultivated local varieties of wheat and barley. At the time of the study, the crops were at the booting to milk stages depending on the specific varieties and their locations. In Paro, low incidences of yellow rust were recorded at one site each in Naja and Tsento Gewogs. In Haa, yellow rust was observed only on the Bumthang Kaa Drukchu variety in Samar Gewog. However, no instances of brown rust were recorded across any of the surveyed sites.

5. Plant Protection Product Program

5.1 Plant Protection Product Procurement and Supply

The Plant Protection Product Program is tasked with managing the procurement and supply of quality Plant Protection Products, overseeing activities such as indent collection from dzongkhags, procurement processes, and distribution of pesticides to ensure effective pest management. In the fiscal year 2022-2023, the program successfully procured a total of 671.98 MT of Plant Protection Products, with approximately 355 MT distributed to the dzongkhags. The demand for these products varied significantly, with herbicides being the most demanded product, accounting for 93% of the total demand. Non-toxic products made up 2.78%, followed by fungicides at 2.5%, insecticides at 1.4%, and rodenticides at a mere 0.01%. Various types of herbicides, insecticides, and fungicides were procured and distributed to different dzongkhags, as illustrated in accompanying figures **Figure *62***, **Figure *63***, and **Figure *64*** .

The Program remains committed to efficiently meeting the pest management needs of the agricultural sector through its procurement and supply efforts.

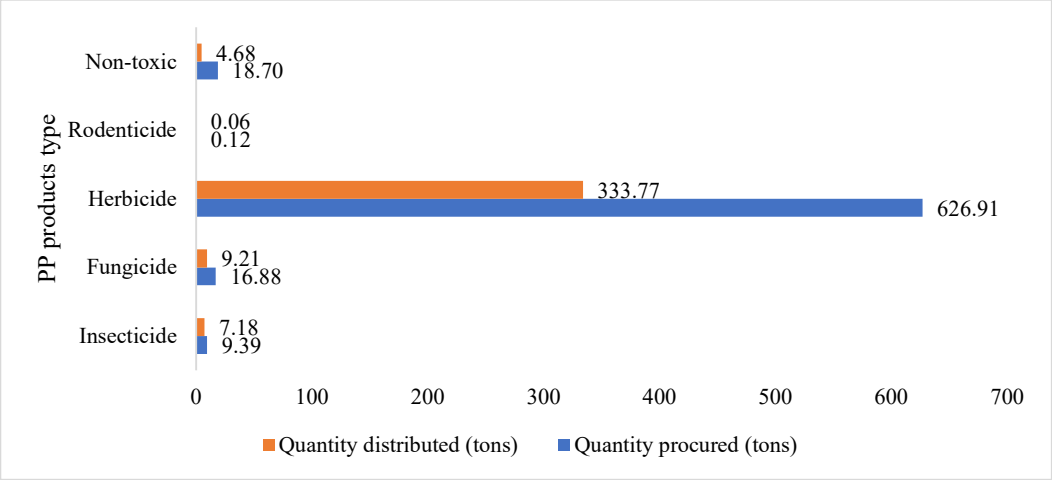

Figure 61 Outlook on Pesticide procurement and supply

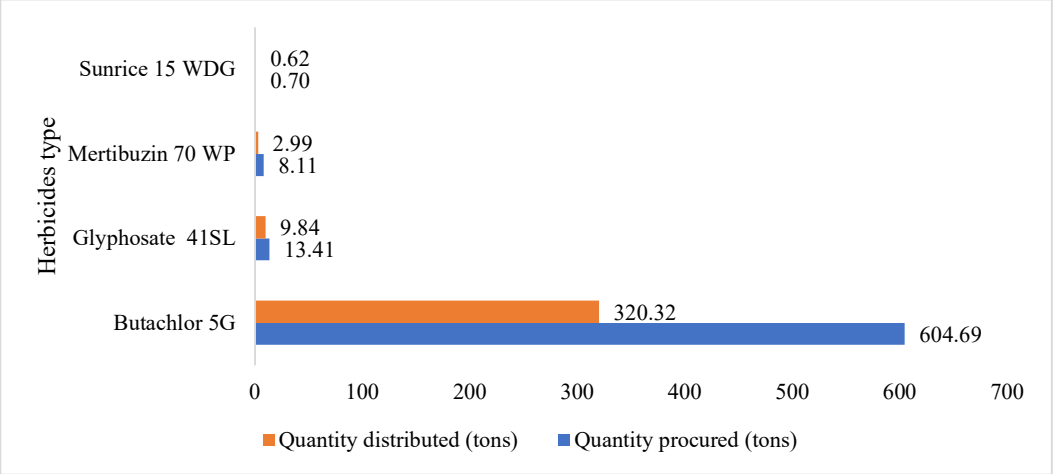

Figure 62 Herbicides procured and supplied

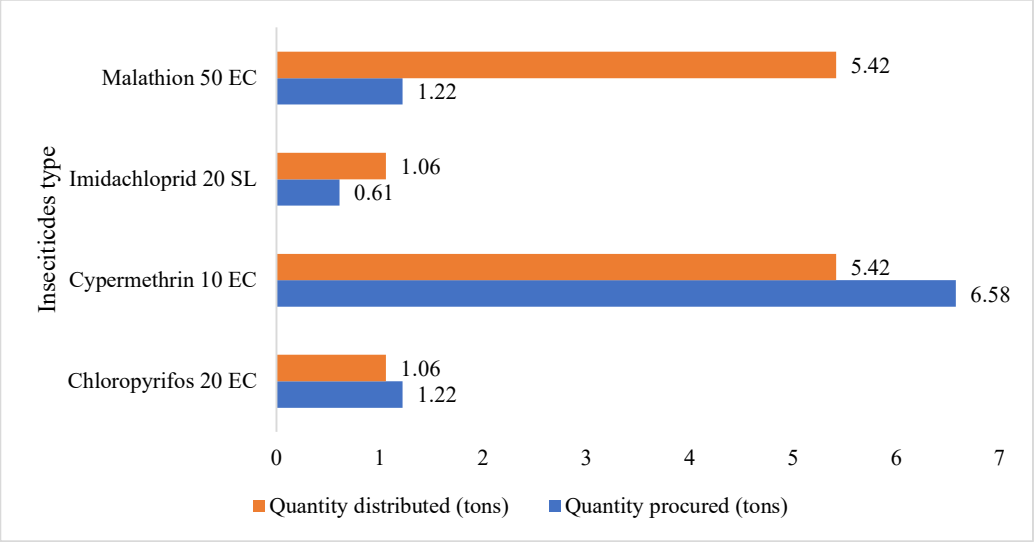

Figure 63 Insecticides procured and supplied

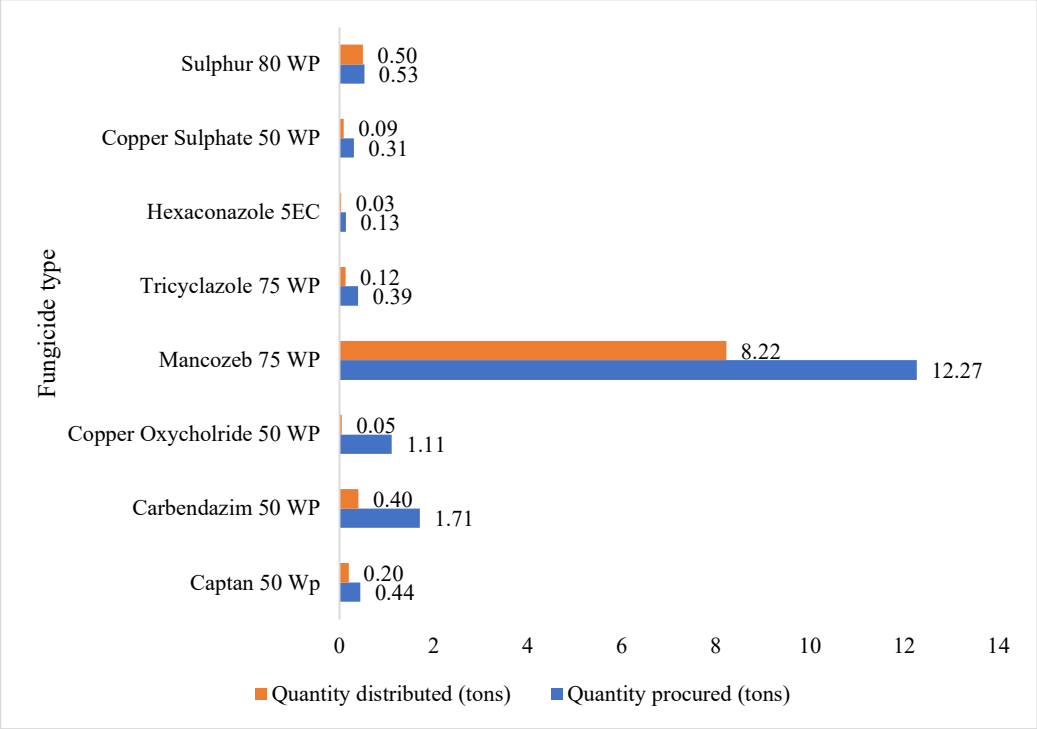

Figure 64 Types of fungicide procured and supplied

6. National Organic Flagship Program

6.1 Construction of biocontrol laboratory

On June 19, 2023, the esteemed Secretary of the Ministry of Agriculture and Livestock officially opened the National Plant Protection Referral Laboratory. This newly established facility, funded by the National Organic Flagship Program (NOFP), was constructed at an estimated cost of Nu. 35.499 million. The laboratory represents a significant milestone in enhancing plant protection research, studies, and diagnostics. Its primary objective is to tackle emerging challenges in plant health and reduce risks associated with pests, aligning with international standards.

The laboratory offers specialized facilities for research in essential areas of plant protection, including entomology, pathology, and weed science. By integrating these critical disciplines within a single facility, the laboratory seeks to promote collaboration and innovation in addressing plant protection issues and advancing Integrated Pest Management (IPM) practices. The center is enthusiastic about the potential this laboratory holds and anticipates making significant contributions to plant protection research and services.

7. Vertebrate Pest Management Program

7.1 Comparative Analysis of Earth Electrode Resistance and Fence Voltage in Electric Fencing Systems: A Study in Sekeyna and Damji, Bhutan

7.1.1 Introduction

The effectiveness of electric fencing in protecting agricultural fields from animal intrusion is significantly influenced by the quality and strength of the earth electrode system. In Bhutan, since the introduction of electric fences in 2013, notable benefits have been observed. However, challenges arise due to the variability of soil types across different agro-ecological zones and the diverse materials used for earth electrodes, which can affect voltage consistency and the longevity of the fences. This study amied to explore several key aspects:

- Earth Resistivity - Investigating the resistivity of different earth electrodes.
- Minimum Electrode Requirement- Determining the minimum number of electrodes necessary to achieve a soil resistance of 10 ohms.
- Shelf-Life Evaluation- Examining the durability of various earthing electrodes in different agro-ecological zones.
- Efficiency Comparison- Comparing the effectiveness of vertical versus horizontal electrodes.

These investigations are crucial for optimizing electric fencing systems in Bhutan's diverse agricultural landscapes.

The study involved field measurements to assess the performance of various types of earth electrodes, specifically GI, copper, and earth slabs. The findings of this research are aimed at optimizing the design and maintenance of electric fencing systems to improve their effectiveness in preventing animal intrusion. To meet the study's objectives, data on earth resistivity for different electrodes was collected using standardized techniques, including the Wenner four-point method. Systematic testing and analysis conducted to determine the minimum number of electrodes needed to achieve a target soil resistance of 10 ohms. Additionally, the effective shelf-life of the various earthing electrodes was evaluated over time across different regions, taking into account factors such as soil type, moisture content, and environmental conditions.

A comparative analysis was performed to evaluate the efficiency of vertical versus horizontal electrodes in maintaining an optimal voltage level along the fence line. The results of this research provide valuable insights for optimizing the selection and deployment strategies of earth electrodes in Bhutan's agricultural fields. These findings will contribute to improving the effectiveness and durability of electric fences, ultimately boosting agricultural productivity by reducing crop damage caused by animals.

7.1.2 Materials and Methods

Study site

The study was conducted in Sekeyna (Thimphu) and Damji (Gasa), representing distinct agro-ecological zones in Bhutan. These locations were chosen due to their differing soil types and environmental conditions.

Experimental Design

The study followed a Randomized Complete Block Design with four treatments (T1= GI rod, T2= GI plate, T3= Copper plate, T4= GEE slab) with each treatment replicated thrice. The pit dimensions were standardized at 1x1x1m (length, breadth, height) as per Penjor *et al*. Four types of earth electrodes were used: GI rod, GI plate, copper plate, and GEE slab, **Figure *65***. The dimensions of the electrodes were standardized as per the protocol. To improve conductivity, backfill materials such as lump charcoal and common salt were incorporated.

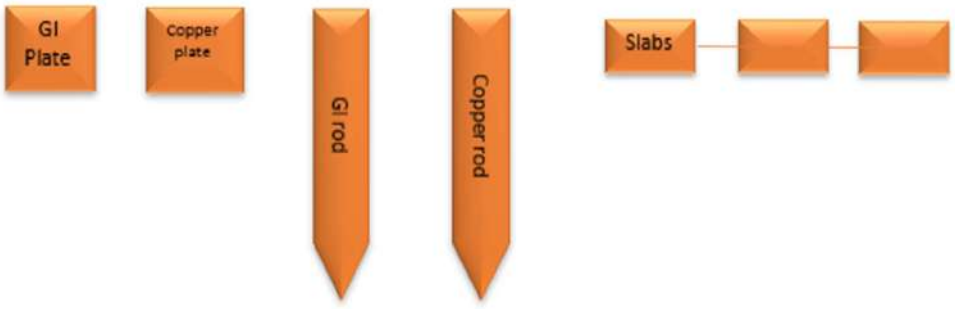

Figure 65 Four types of earth electrode used in the study

Data Collection

Earth resistance readings were taken for each type of earth electrode at both Sekeyna and Damji sites. Fence voltage measurements were collected following the specified format. To gather data, the voltage of the fence was measured by connecting the grounding point of the fence to each of the specified treatments.

7.1.3 Result and discussion

Earth Resistance:

The resistance values showed variations among the different electrode types. At the Sekeyna site, the average resistance readings were as follows: T1 (GI rod) at 17.78 ohms, T2 (GI plate) at 25.06 ohms, T3 (copper plate) at 20.17 ohms, and T4 (GEE slab) at 25.54 ohms. In contrast, at the Damji site, the average resistance values were recorded as 14.95 ohms for T1, 17.70 ohms for T2, 19.53 ohms for T3, and 26.50 ohms for T4.

When comparing the two sites, it is evident that earth resistances vary significantly. Sekeyna consistently displayed higher resistance values than Damji across all electrode types. This difference may be linked to differences in soil types and agro-ecological conditions at the two locations. The composition and moisture content of the soil are critical factors influencing earth resistance. Further investigations and soil analysis would be necessary to understand the specific factors contributing to these variations.

Fence Voltage

The fence voltage measurements provide insights into the effectiveness of the earth electrodes in maintaining a sufficient voltage to deter animals. In Sekeyna, the average fence voltage for T1, T2, and T3 were 7.57 kV, 6.52 kV, and 7.02 kV, respectively. In Damji, the corresponding average values were 8.02 kV, 7.77 kV, and 8.08 kV. These results indicate that the fence voltages remained within the desired range of 3 kV throughout the study period.

Comparing the fence voltage between the electrode types, it can be observed that GI Rod consistently provided the highest fence voltage in both Sekeyna and Damji. GI Plate and Copper Plate exhibited slightly lower fence voltages, while GEE slab generally had the lowest voltage readings. These differences can be attributed to variations in the conductivity and resistance properties of the different electrode materials.

Overall, the study suggests that the choice of earth electrode type can significantly impact the earth resistance and resulting fence voltage. GI rod consistently demonstrated better performance, providing lower resistance and higher fence voltages compared to the other electrode types. However, further investigations are necessary to evaluate the long-term stability and durability of the different electrodes under varying soil and climatic conditions.

7.1.4 Conclusion

This study provides valuable insights into the earth electrode resistance and fence voltage in electric fencing systems. The results highlight the variations among different earth electrode types and their implications for system performance. By considering the unique agro-ecological characteristics of specific locations, optimized earth electrode configurations can be recommended to enhance the efficiency and longevity of electric fencing systems in Bhutan.

7.2 Comparative Study of Ultrasonic Monkey Repellent Efficacy in Mitigating Crop Damage: Case Study in Punakha Kabisa, Gasa Damji, and Thimphu Sekeyna, Bhutan

7.2.1 Introduction

Monkeys can pose significant challenges in agricultural settings, leading to crop damage and disruptions in various environments such as factories and warehouses. Traditional control methods, including physical barriers and harmful deterrents, often raise ethical concerns and may not be effective in the long term. The advent of ultrasonic monkey repellent devices presents a promising alternative that is both humane and non-invasive. This study aims to assess the effectiveness of an ultrasonic monkey repellent device that uses sonic impact to deter monkeys from specific targeted locations.

The study will focus on evaluating the deterrent effectiveness of the device, which uses variable frequency technology to establish an uncomfortable zone for monkeys. By combining sonic and ultrasonic frequencies, the device creates a distressing environment that discourages monkeys from entering the farms. The use of variable frequency modulation is designed to prevent monkeys from developing immunity, thereby improving the long-term effectiveness of the repellent.

To evaluate the device's efficacy, a series of controlled experiments were conducted. The ultrasonic monkey repellent device was installed in strategically chosen areas, and its performance was assessed based on the observed behavior of monkeys in response to the emitted sonic and ultrasound signals.

Key parameters such as the distance of effective deterrence, duration of monkey avoidance, and overall success rate was measured and analyzed. Behavioral observations, including monkeys' proximity to the protected area, time spent in the vicinity, and frequency of approach attempts, will provide insights into the device's ability to create a non-comfort zone

for the monkeys. Moreover, the study considered the safety aspect of the device, ensuring that the emitted frequencies do not cause discomfort or harm to humans within the vicinity. The frequency range used by the device has been carefully selected to minimize disturbances to humans while maintaining effectiveness against monkeys

7.2.2 Materials and Methods

Study Site
The study sites (Kabisa, Damji, and Sekeyna) were selected based on farmers' reports and verified by local government officials and gewog RNR officials.

Device
The device emits high-intensity sound waves that range from 10 KHz to 65 KHz, frequencies that are inaudible to humans and most household pets, making it particularly effective against pests, Figure 66. These penetrating sounds significantly impact the neural systems of pests, causing discomfort and unease, which leads to abnormal behaviors such as agitation and avoidance. Importantly, the device's frequency modulation occurs at a rate of 1 to 60 times per second, preventing pests, including monkeys, from becoming accustomed to the emitted waves, thereby enhancing its effectiveness. Operating on electronic principles, it offers a hygienic and cost-effective alternative to traditional methods without disrupting electronic systems. Additionally, its automatic frequency modulation and sweeping functionality across the 10 KHz to 65 KHz range further strengthen its ability to deter pests effectively.

Figure 66 Ultrasonic monkey repellent device

Data collection
Data collection was carried out by agricultural extension officers in collaboration with farmers, following a biannual consultation process based on a predefined format. This method was designed to obtain thorough and consistent information. The agricultural extension officers interacted directly with the farmers to gather their insights and experiences. ***Figure 67***.

Figure 67 Demonstration of the working of the ultrasonic monkey repellent device and data collection

7.2.3 Result and Discussion

The data gathered from the monitoring sites showed varying results regarding the effectiveness of the ultrasonic monkey repellent, ***Table 53***. In Kabisa, monkeys were largely unaffected by the repellent, as evidenced by their entry into the fields and subsequent crop damage. In Damji, the repellent exhibited some effectiveness, leading to a reduction in crop damage incidents. However, in Sekeyna, the device proved to be more effective, successfully deterring monkeys and significantly minimizing crop damage.

Table 53 Efficacy of Ultrasonic monkey repellent in different monitoring sites

Monitoring date	Monitoring site	Pest	Entered field (Yes/No)	Crop & stage	Scared Away (SA)/Not Scared (NS) None (N)	Remarks
1/1/2022	Kabisa, Punakha	Monkey	Yes	Vegetable	NS	Monkey didn't get scared
1/6/2022	Kabisa, Punakha	Monkey	Yes	Paddy	SA	Monkey didn't get scared
1/12/2022	Kabisa, Punakha	Monkey	Yes	Vegetable	NS	Monkey didn't get scared
1/6/2023	Kabisa, Punakha	Monkey	Yes	Paddy	NS	Monkey didn't get scared
1/1/2022	Damji, Gasa	Monkey	Yes	Vegetable	SA	Monkey got scared
1/6/2022	Damji, Gasa	Monkey	Yes	Paddy	NS	Monkey didn't get scared
1/12/2022	Damji, Gasa	Monkey	Yes	Vegetable	NS	Monkey didn't get scared
1/6/2023	Damji, Gasa	Monkey	Yes	Paddy	NS	Monkey didn't get scared
1/1/2022	Sekeyna, Thimphu	Monkey	Yes	Vegetable	SA	Monkey got scared
1/6/2022	Sekeyna, Thimphu	Monkey	Yes	Paddy	SA	Monkey didn't get scared

1/12/2022	Sekeyna, Thimphu	Monkey	Yes	Vegetable	NS	Monkey didn't get scared
1/6/2023	Sekeyna, Thimphu	Monkey	Yes	Orchard	NS	Monkey didn't get scared

Initially, the data from Punakha and Kabisa indicated that the ultrasonic monkey repellent had variable effectiveness in deterring monkeys. For vegetable crops, the repellent did not significantly deter the monkeys, as reflected by the "Not Scared (NS)" designation. In contrast, for paddy crops, the repellent successfully frightened the monkeys away, as indicated by the "Scared Away (SA)" label. This suggests that the effectiveness of the repellent may depend on the specific type of crop involved.

In Damji, the repellent exhibited mixed effectiveness. It effectively deterred monkeys from vegetable crops, as indicated by the "Scared Away (SA)" label, but had little impact on repelling them from paddy crops, as shown by the "Not Scared (NS)" designation. This variability in effectiveness may be influenced by factors such as the proximity of the study site to monkey habitats or the behavioral patterns of the local monkey population.

In summary, the results suggest that the effectiveness of the ultrasonic monkey repellent in reducing crop damage differs across various study sites and crop types. The repellent demonstrated promising outcomes in certain instances, successfully deterring monkeys and minimizing crop damage. However, in other situations, its effectiveness was limited, indicating a need for further research and refinement of the repellent technology.

7.2.4 Conclusion

The findings of this comparative study indicate that the effectiveness of ultrasonic monkey repellents in reducing crop damage varies across different regions in Bhutan. While the repellent showed limited effectiveness in Punakha Kabisa, it proved to be more effective in Gasa Damji and Thimphu Sekeyna. These results highlight the importance of conducting site-specific evaluations and ongoing monitoring to customize repellent strategies for particular areas. Additionally, further research and development are essential to optimize the technology and improve its efficacy in mitigating crop damage caused by monkey intrusions in Bhutan

Annexure 1: Results of test conducted on citrus samples from the National Citrus Repository from 12- 28 December, 2022.

Sl.No.	Citrus type	Repository ID	Lab ID	Test batch No.	Recommendation
1	Cara Cara	79	1-22 to 7-22	7th	Clear for Propagation
			176-21 to 182-21 & 411-22 to 417-2	6th	
2	Clementine	87	8-22 to 14-22	7th	Re-test
			39-21 to 45-21 & 341-22 to 347-22	6th	
3	Sasaki	93	15-22 to 21-22	6th	Clear for Propagation
			149-21 to 154-21 & 355-22 to 361-22	7th	
4	Dekoponkan	104	22-22 to 28-22	6th	Retest
			169-21 to 175-21 & 439-22 to 445-22	7th	
5	Junar	118	29-22 to 35-22	6th	Clear for Propagation
			32-21 to 38-21 & 383-22 to 389-22	7th	
6	Ohtsu 4	120	36-22 to 42-22	6th	Clear for Propagation
			155-21 to 161-21 & 425-22 to 431-22	7th	
7	Aoshima	137	43-22 to 49-22	6th	Clear for Propagation
			162-21 to 168-21 & 432-22 to 438-22	7th	
8		195	50-22 to 56-22	6th	Clear for Propagation

	Semjong Lime		142-21 to 148-21 & 397-22 to 403-22	7th	
9	Bearss Lime	220	57-22 to 63-22	6th	Clear for Propagation
			135-21 to 141-21 & 307-22 to 313-22	7th	
10	Pomelo	360	64-22 to 70-22	6th	Retest
			197-21 to 203-21 & 362-22 to 368-22	7th	
11	Bearss Lime	379	71-22 to 77-22	6th	Retest
			190-21 to 196-21 & 225-22 to 231-22	7th	
12	Local Khengkhar	F35	78-22 to 84-22	6th	Retest
			53-21 to 58-21 & 376-22 to 382-22	7th	
13	Pemaling Mandarin	IN24	85-22 to 87-22	6th	Retest
			87-21 to 93-21 & 267-22 to 273-22	7th	
14	Sartshang Kapur	IN25	88-22 to 90-22	6th	Clear for Propagation
			80-21 to 86-21 & 404-22 to 410-22	7th	
15	Chubu Mandarin	IN26(a)	91-22 to 93-22	6th	Clear for Propagation
			122-21 to 128-21 & 390-22 to 396-22	7th	
16	Baleygang Phokshay	IN27	94-22 to 96-22	6th	Clear for Propagation
17	Pemagatsho Mandarin	IN28(a)	97-22 to 99-22	6th	Retest
			115-21 to 121-21 & 369-22 to 375-22	7th	

18	Tama Mandarin	IN28	100-22 to 102-22	6th	Retest
			108-21 to 114-21 & 293-22 to 299-22	7th	
19	Cara Cara	F97	103-22 to 105-22	7th	Clear for Propagation
			18-21 to 24-21 & 253-22 to 259-22	6th	
20	120 yrs Pemagatsho Mandarin IN23	IN23	106-22 to 108-22	7th	Clear for Propagation
			73-21 to 79-21 & 260-22 to 266-22	6th	
21	Pemagatsho Kapur	IN22(a)	109-22 to 111-22	7th	Clear for Propagation
			94-21 to 100-21 & 286-22 to 292-22	6th	
22	Namchala Kalajambir	IN22	112-22 to 114-22	7th	Clear for Propagation
			66-21 to 72-21 & 446-22 to 452-22	6th	
23	Ohtsu	F105	115-22 to 117-22	7th	Clear for Propagation
			204-21 to 210-21 & 232-22 to 238-22	6th	
24	Thambir Tshalu	Q16	118-22 to 120-22	7th	Retest
25	Tachog Tshalu	Q17	121-22 to 123-22	7th	Retest
26	Kichu Tshalu	Q4	124-22 to 126-22	7th	Retest
27	Tsunokari	Q5	127-22 to 130-22	7th	Retest

28	Washington Navel	Q6	130-22 to 132-22	7th	Retest
29	Pelrithang Tshalu	Q11	133-22 to 135-22	7th	Retest
30	Solong Shing, Pemagatsho	Q8	136-22 to 138-22	7th	Retest
31	Drukjeep Tshalu	Q10	139-22 to 141-22	7th	Destroy
32	Pelrithang Kalijambir	IN30	142-22 to 144-22	7th	Clear for Propagation
33			25-21 to 31-21 & 246-22 to 252-22	6th	
34	Lemon Eira	Q1	145-22 to 147-22	7th	Retest
35	Kinnow	Q2	148-22 to 150-22	7th	Retest
36	Orong Tshalu	Q12	151-22 to 153-22	7th	Retest
37	Marangdut Japanese Tshalu	Q13	154-22 to 156-22	7th	Retest
38	Marangdut Lemon	Q14	157-22 to 159-22	7th	Retest
39	Fortunella	378	160-22 to 162-22	7th	Clear for Propagation
			183-21 to 189-21 & 279-22 to 285-22	6th	

40	Fortunella	F93	164-22 to 165	6th	Clear for Propagation
			211-21 to 217-21 & 319-22 to 325-22		
41	Ohtsu	F99	166-22 to 168-22	7th	Retest
			218-21 to 224-21 & 239-22 to 245-22	6th	
42	Thambir Lime	Q15	169-22 to 171-22	7th	Retest
43	Gomdar Mandarin	IN27(a)	172-22 to 174-22	7th	Clear for Propagation
44	Frost Ureka	IN25(a)	175-22 to 177-22	7th	Clear for Propagation
45	Sershong Kumquate	Q7	178-22 to 180-22	7th	Retest
46	Mineola	Q3	181-22 to 183-22	7th	Retest
47	Panbang Tshalu	Q9	184-22 to 186-22	7th	Destroy
48	Goling Pomelo	F1	187-22 to 188-22	7th	Retest
49	Berti Citron	F2	189-22	7th	Retest
50	Goling Humpang	F3	191-22	7th	Retest
51	Zurphey Citron	F4	193-22 to 196-22	7th	Retest

52	Berti Pomelo	F6	197-22 to 198-22	7th	Retest
53	Berti Lime	F7	199-22 to 200	7th	Retest
54	Trongpam Lime	F8	201-22 to 202-22	7th	Retest
55	Goling Humpang	F9	203-22 to 204-22	7th	Retest
56	Berti Citron	F10	205-22 to 206-22	7th	Retest
57	Tshanglajong Citron	F11	207-22 to 208-22	7th	Retest
58	Berti Citron	F12	209-22 to 210-22	7th	Retest
59	Zurphey Citron	F13	211-22 to 212-22	7th	Retest
60	Zurphey Citron	F14	213-22 to 214-22	7th	Retest
61	Berti Pomelo	F15	215-22 to 216-22	7th	Retest
62	Berti Lime	F16	217-22 to 218-22	7th	Retest
63	Dagana Mandarin	F17	219-22 to 220-22	7th	Retest
64	Wengkhar Tshelu Ngram	F18	221-22 to 222-22	7th	Retest

65	Wengkhar Tshelu 1	F19	223-22 to 224-22	7th	Retest
66	Recheck	F20	225-22 to 226-22	7th	Retest
67	Berti Pomelo	F21	227-22 to 228-22	7th	Retest
68	Berti Citron	F22	229-22 to 230-22	7th	Retest
69	Berti Citron	F23	231-22 to 232-22	7th	Retest
70	Trongpam Lime	F24	233-22 to 234-22	7th	Retest
71	Narang Mandarin	F25	235-22 to 236-22	7th	Retest
72	Dagana Mandarin	F26	237-22 to 238-22	7th	Retest
73	Dagana Mandarin	F27	239-22 to 240-22	7th	Retest
74	Narang Mandarin	F28	241-22 to 242-22	7th	Retest
75	Samtse Mandarin	F29	243-22 to 244-22	7th	Retest
76	Tsirang Mandarin	F30	245-22 to 246-22	7th	Retest
77	Dagana Mandarin	F31	247-22 to 248-22	7th	Retest

78	Kengjhar Mandarin	F32	249-22 to 250-22	7th	Retest
79	Samtse Mandarin	F33	251-22 to 252-22	7th	Retest
80	Dagana Mandarin	F34	253-22 to 254-22	7th	Retest
81	Kengjhar Mandarin	F35	255-22 to 256-22	7th	Retest
82	Tsirang Mandarin	F36	257-22 to 258-22	7th	Retest
83	Yushida Ponkan	F37	259-22 to 260-22	7th	Retest
84	Okitsuwase	F38	261-22 to 262-22	7th	Retest
85	Tsirang Mandarin	F39	263-22 to 264-22	7th	Retest
86	Tsirang Mandarin	F40	265-22 to 266-22	7th	Retest
87	Yadi Mandarin	F41	267-22 to 268-22	7th	Retest
88	Dagana Mandarin	F42	269-22 to 270-22	7th	Retest
89	S/jongkhar Mandarin	F43	271-22 to 272-22	7th	Retest

90	Shumar Mandarin	F44	273-22 to 274-22	7th	Retest
91	Otha Ponkan	F45	275-22 to 276-22	7th	Retest
92	Wengkhar Mandarin	F46	277-22 to 278-22	7th	Retest
93	Wengkhar Mandarin	F47	279-22 to 280-22	7th	Retest
94	Dorokha Mandarin	F48	281-22 to 282-22	7th	Retest
95	S/jongkhar Mandarin	F49	283-22 to 284-22	7th	Retest
96	Narang Mandarin	F50	285-22 to 286-22	7th	Retest
97	Dagana Mandarin	F51	287-22 to 288-22	7th	Retest
98	Tsirang Mandarin	F52	289-22 to 290-22	7th	Retest
99	P/gatshel Mandarin	F53	291-22 to 292-22	7th	Retest
100	Dorokha Mandarin	F54	293-22 to 294-22	7th	Retest
101	Narang Mandarin	F55	295-22 to 296-22	7th	Retest

102	Dagana Mandarin	F57	297-22 to 298-22	7th	Retest
103	Yadi Mandarin	F58	299-22 to 300-22	7th	Retest
104	Narang Mandarin	F59	301-22 to 302-22	7th	Retest
105	Trongsa Mandarin	F62	303-22 to 304-22	7th	Retest
106	Sarpang Mandarin	F63	305-22 to 306-22	7th	Retest
107	Chukha Mandarin	F64	307-22 to 308-22	7th	Retest
108	Cafein Clementine	F65	309-22 to 310-22	7th	Retest
109	Salustiana	F66	311-22 to 312-22	7th	Retest
110	Amigo Mandarin	F67	313-22 to 314-22	7th	Retest
111	Mc Mohan	F68	315-22 to 316-22	7th	Retest
112	Torracco Ippolite	F69	317-22 to 318-22	7th	Retest
113	Kengjhar Mandarin	F70	319-22 to 320-22	7th	Retest

114	Tsirang Mandarin	F71	321-22 to 322-22	7th	Retest
115	Dorokha Mandarin	F72	323-22 to 324-22	7th	Retest
116	Mc Mohan	F73	325-22 to 326-22	7th	Retest
117	Salustiana	F74	327-22 to 328-22	7th	Retest
118	Fingered Citron	F75	329-22 to 330-22	7th	Retest
119	Fingered Citron	F76	331-22 to 332-22	7th	Retest
120	Fingered Citron	F78	333-22 to 334-22	7th	Retest
121	Bears Lime	F79	335-22 to 336-22	7th	Retest
122	Starrubby	F80	337-22 to 338-22	7th	Retest
123	Cafein Clementine	F81	339-22 to 340-22	7th	Retest
124	Cafein Clementine	F82	341-22 to 342-22	7th	Retest
125	Cafein Clementine	F83	343-22 to 344-22	7th	Retest
126	Cara Star Rubby	F86	345-22 to 346-22	7th	Retest

127	Cara Cara	F87	347-22 to 348-22	7th	Retest
128	Starrubby	F88	349-22 to 350-22	7th	Retest
129	Amigo Mandarin	F89	351-22 to 352-22	7th	Retest
130	Amigo Mandarin	F90	353-22	7th	Retest
131	Amigo Mandarin	F91	355-22 to 356-22	7th	Retest
132	Amigo Mandarin	F92	357-22 to 358-22	7th	Retest
133	Kumquat	F93	359-22 to 360-22	7th	Retest
134	Ryan	F94	361-22 to 362-22	7th	Retest
135	Amigo Mandarin	F95	363-22 to 364-22	7th	Retest
136	Dareton	F96	365-22 to 366-22	7th	Retest
137	Cara Dareton	F97	367-22 to 368-22	7th	Retest
138	Salustiana	F98	369-22 to 370-22	7th	Retest
139	Kumquat	F99	371-22 to 372-22	7th	Retest
140	Ryan	F100	373-22 to 374-22	7th	Retest
141	Amigo Mandarin	F101	375-22	7th	Retest

142	Fingered Citron	F102	377-22 to 378-22	7th	Retest
143	Amigo Mandarin	F103	379-22	7th	Retest
144	Salustiana	F104	381-22 to 382-22	7th	Retest
145	Othsu	F105	383-22 to 384-22	7th	Retest
146	Torracco Ippolite	F106	385-22 to 386-22	7th	Retest