# Basil Downy Mildew management options – is it seedborne?

## Agri-Science Queensland Innovation Opportunity

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This publication has been compiled by Kathy Grice, Grace Sun and Peter Trevorrow of Agri-Science Queensland, Department of Agriculture and Fisheries.

# Summary

Basil downy mildew (*Peronospora belbahrii)* was identified for the first time in Australia in 2017. Since the first report, the fungal organism spread rapidly with incidences reported in all states other than Tasmania and Western Australia. There have been significant impacts on both field and glasshouse grown crops of basil with growers unable to manage the level of infection, this led to a shortened growing season and a reduced number of picking cycles.

Overseas literature implicated the spread of the disease to infected seed, with low incidence levels (0.03%). It is not known if this was the primary source of the disease spread in Australia and needed to be investigated.

The project delivered on a number of aspects:

- The successful detection of the BDM organism in seed (known field infection) using molecular technology.
- A technique developed to inoculate plants artificially, this could be used to determine cultivar susceptibility to the disease and to screen products for disease management (fungicides/biologicals).
- Molecular assays tested and implemented that can detect the BDM organism in pure form, infected foliage and in seed.
- Provided advice in relation to chemical permit applications, this has led to an additional five fungicides permitted for use to manage BDM.

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# 1 Background

Downy mildew of basil (BDM) was first reported in Uganda (Hansford 1932) as *Peronospora* sp. and was reported to have caused defoliation and plant death of sweet basil (*Ocimum basilicum*). The pathogen was again identified in Uganda (Hansford 1938), and renamed *P. lamii* based on the host family (Lamiaceae) in 1937. References to basil downy mildew (BDM) were not cited again in the scientific literature until it was reported in Benin (Gumedzoe et al. 1998). Italy was the first European country to report the presence of downy mildew (Garibaldi et al. 2004), followed by France and Belgium (2004) and Malta (2005). Outside of Europe, the disease reports came from South Africa (2005), Iran (2006), United States of America and Cameroon (2007) and Argentina (2008). By 2009, Thines et al. reported that the species from basil was distinctively different from other *Peronospora* species found on the same host and proposed the new name *Peronospora belbahrii*. Basil downy mildew continued to spread around the world with outbreaks in Cuba and Taiwan (2009), Hungary and the United Kingdom (2010), Canada (2011), Cyprus and the Czech Republic (2012), Israel (2013) China (2014), Korea and Spain (2015) and Mexico (2016). Australia was listed erroneously (no citation) as a host country in the scientific literature as early as 2015, however, no records of detections could be traced.

Until 2017, the disease was considered exotic to Australia, that is until typical downy mildew symptoms were report from South east Queensland in a glasshouse grown situation. Within six months, the disease was present along the eastern seaboard from north Queensland (affecting commercial growers, backyard plantings and nursery business') to Victoria, South Australia and the Northern Territory. On the Atherton Tablelands, the spread and impact of the disease was rapid and dramatic and was prevalent on all stages of plant growth (seedlings to mature plants). The basil industry in north Queensland is relatively small in relation to the number of growers (8-10) and area (~ 45ha), however contributions to the local economy exceeds $1.0 million annually. Most of the growers supply the fresh market, whilst others value add, producing pesto products. All growers in the region were impacted by BDM, regardless of the cultivar, this led to shorter crop cycles and fresh product degraded faster than normal and the market rejected product or reduced prices.

The rapid spread of the organism throughout the world has been attributed to the movement of infected plant material and the organism having the potential of being seed-borne. If the latter is the primary source of infection and spread for growers, then alternative or additional management options in conjunction with infield fungicide applications would need to be considered. However, there is conflicting information in the literature as to the level of seed infection and reports vary but can be as low as 0.03%**.** The purpose of the studies below was to rapidly generate data on seed transmission and potential management strategies for the disease. Also to develop and an inoculation procedure that could be used as a bioassay to evaluate alternative chemistry or biological products to manage the disease. The other hurdle for commercial growers was the lack of effective chemical options available for use. Only two products were permitted for use (mancozeb and copper formulations) and their withholding periods were 21 and 28 days respectively.

# 2 Project Objectives

The main project aims were:

- to determine if basil seed was the primary source of infection and if so,
- could the treatment of seed by means of heat or seed dressing reduce BDM

Throughout the course of the project some additional studies were essential to clarify our initial findings and to expand the research. Studies included:

- artificial inoculation of basil seedlings
- validation of PCR methods already reported for detecting BDM
    - spores only
    - infected foliage
    - detection in seed with known field infection
- DNA sequencing and phylogenetic analysis of locally collected isolates

The above, together with a search of the scientific literature was undertaken to gather as much knowledge as possible about the disease as it had already been present in Europe for 16 years. This would assist any future research and improve on the management strategies currently used.

The project leader was invited to attend and present information about basil downy mildew with herb growers at the AGM of the Australian Herb and Spice Industry Association (AHSIA) in September, 2017. It was an opportunity to meet growers and seed company representatives, share information and hear concerns in relation to this new disease.

# 3 Methodology

Seed-borne infection experiments

Three separate experiments using sweet basil seed (Table 1) obtained from either commercial companies or local grower co-operators were conducted to determine if BDM was present in seed. Different seedling rates were used were approximately (1 g, 0.5 g and 0.25 g) in the three experiments and plants grown in an autoclaved mix (70:30 ratio of potting mix to vermiculite). The number of replications used also varied and ranged from five to ten. Seed in experiments 2 and 3 were treated with a fungicide seed dressing - captan + pentachloronitrobenzene (PCNB) to reduce known seed-borne fungal organisms. Seed was germinated in the laboratory under fluorescent light and at expanded cotyledon stage were placed in a plant growth chamber and subjected to different temperatures ranging from 25°C down to 18°C and with day and night hours also varying from 12 hours to 8 hours light and 16 hours dark to induce downy mildew. Seedlings were examined once or twice a week using a magnifying lamp and a dissecting microscope to identify any organisms. For experiment 3, seedlings were grown under glasshouse conditions for approximately 3 weeks prior to being placed in the plant growth incubator at 18°C in combination with 8 hrs light and 16 hrs dark and assessed as stated above.

***Table 1 – Basil cultivars***

| **Cultivar name** | **Lot or Part no.** | **Company** | **Experiment no.** |
|---|---|---|---|
| Aroma $F_1$ **(A)** | IRD 9115099 | Fairbanks Seeds | 1, 2 and 3 |
| Genova large leaf **(B)** | IWE 2817078 | Fairbanks Seeds | 1, 2 and 3 |
| Large sweet – Genovese **(C)** | AHGB 075 | Lefroy Valley | 1 |
| Majestic $F_1$ **(D)** | HBG 90443 | Lefroy Valley | 1, 2 and 3 |
| Bellissimo*** (E)** | Unknown | Lefroy Valley | 1 |
| Purple basil*** (F)** | Unknown | South Pacific Seeds | 1 |
| Marian*** (G)** | Unknown | South Pacific Seeds | 1, 2 and 3 |
| Aroma $F_1$*** (H)** | ALV 9105214 | Fairbanks Seeds | 1 |
| Aroma $F_1$*** (I)** | HVF 9114208 | Fairbanks Seeds | 1 and 2 |
| Old seed*** (J)** | Unknown | Unknown | 1 and 2 |
| Aroma $F_1$*# **(K)** | Unknown | Fairbanks Seeds | 3 |

*****Seed supplied by local growers # Seed heads infected with BDM in the field

Artificial inoculation of basil plants

Another component of the research was to determine the ease of reproducing the symptoms of BDM through artificial inoculation of plants. This type of bioassay could be used to determine the susceptibility of cultivars to BDM or to screen fungicides or alternative products for the management of the disease. Basil seedlings (3-4 weeks old) with fully expanded leaves were sprayed with a suspension of spores (1 x $10^4$/ml) obtained from infected plants. Inoculated seedlings were kept in the dark for 24 hours at 18°C, then placed in a growth chamber incubator at 22°C, with 8 hrs light and 16 hrs dark and high humidity.

Molecular studies (including DNA sequencing and phylogenetic analysis)

Molecular studies were conducted to validate the conventional (gel-based) and real time PCR methods previously developed and reported in the scientific literature for detecting BDM. It was necessary to determine that the protocols could detect our isolates and under our conditions. The presence of BDM on different substrates (with and without plant material) was assessed and included fungal spores only, infected foliage and seed with known field infection as well as commercial seed. In addition to the detection of the pathogen it was necessary to determine whether or not our isolates were genetically similar to those previously detected throughout Europe or whether we had something different. To achieve this, local isolates were sequenced directly in both sense and antisense directions. All pathogen samples were sequenced twice and a consensus sequence was created from the duplicates. Sequences from the BDM isolates were aligned against sequences from representative isolates with reference numbers lodged in GenBank.

# 4 Results and discussion

Seed-borne infection experiments

A wide spectrum of fungal organisms were identified (Image 1 and 2) in experiments 1 and 2 including: *Botrytis* sp., *Cladosporium* sp., *Alternaria alternata*, *Fusarium* sp., *Epicoccum* sp., *Penicillium* sp., *Trichoderma* sp. and *Aspergillus* sp. It was evident that the latter four organisms were absent in experiment 2 due to the potting medium being autoclaved multiple times and the use of the seed dressing. After the timeframes of four and seven weeks respectively, there was no evidence of downy mildew infection.

**Image 1 – Basil seedlings infected with *Alternaria alternata.***

**Image 2 – Seedlings infected with *Botrytis* sp.**

In experiment 3, the addition of seed collected from the field with known BDM infection was included in the hope of infection carrying through to the next generation. However at the termination of the experiment (7 weeks), no evidence of BDM infection was observed.

Artificial inoculation of basil plants

The artificial inoculation of basil plants was successful with symptoms of downy mildew were evident and *P. belbahrii* sporing structures were visible on basil leaves occurring, approximately 10-14 days post-inoculation. As previously stated, this inoculation technique could be useful as a screening method to determine cultivar susceptibility or to determine alternative treatment options or rotations of fungicides for the management of the downy mildew pathogen.

Molecular studies

- Gel-based DNA amplification

DNA was extracted from a conidial suspension of the BDM organism (Ba01 and Ba02), together with extracts from infected leaf tissue (Ba03 and Ba04), commercial seed (Ba05) and seeds harvested from field infested basil plants (Ba06 to Ba17).

- The DNA amplification results shown below (Image 3) were conducted using a two-step semi-nested PCR. In the first step, only the conidial samples (Ba01 and Ba02) showed specific bands at the size of ~1,300 bp, whilst Ba03 showed a non-specific band at ~1,600 bp. In the

second step, both conidial samples (Ba01 and Ba02) and DM infected basil leaves (Ba03 and Ba04) showed specific bands at the size of ~900 bp. All the basil seeds samples (Ba05 to Ba17) showed non-specific bands at ~700 bp to ~800 bp. Some non-specifc amplicons were sequenced and the results suggested that sample Ba08 could be *Epicoccum sp*. whilst Ba09, Ba10 and Ba14 could be *A. alternata*. Both these organisms were observed in the seed-borne infection experiments.

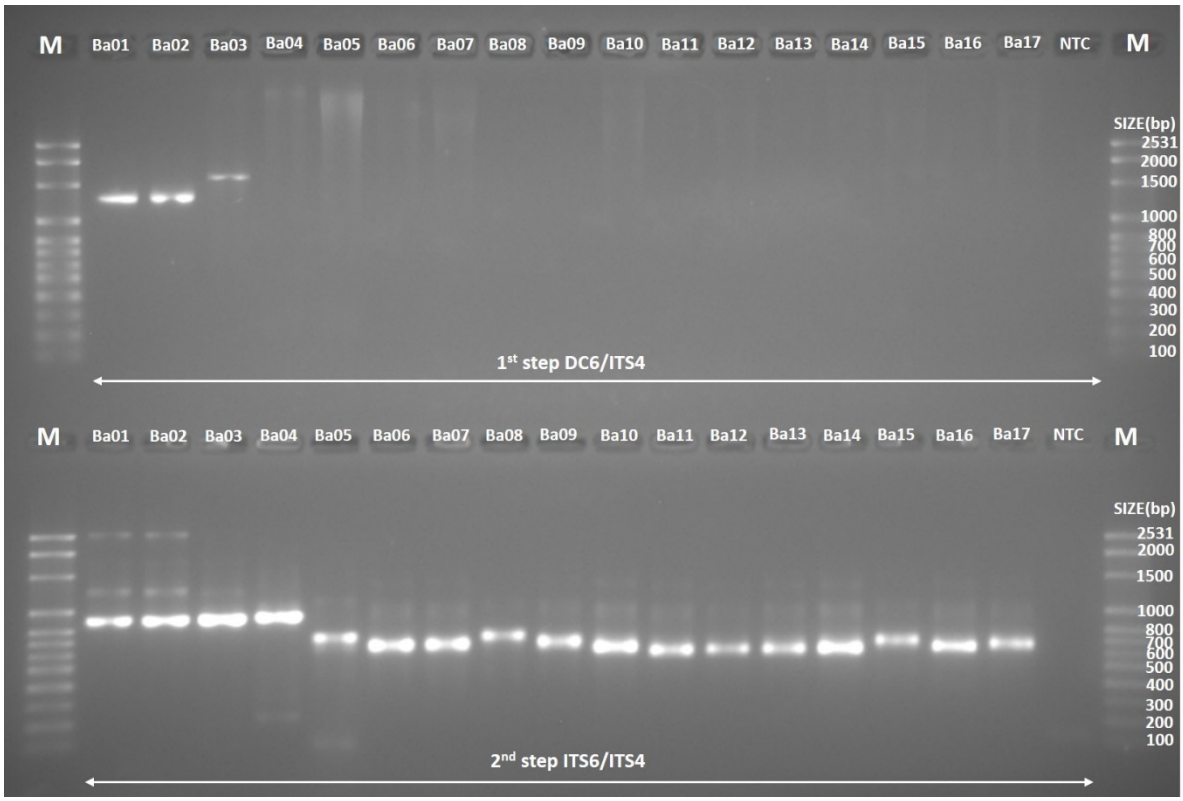


**Image 3 – Semi-nested PCR, 1st step using DC6/ITS4 primers, 2nd step using ITS6/ITS4 primers**

- Real time PCR

All the DNA extracts used in the gel-based semi-nested PCR above were also assessed using the real time PCR method (Belbahri *et al.,* 2005). Results shown below (Image 4) indicated that the conidial samples together with the infected leaf tissue samples (Ba01 – 04) were again positive for the detection of BDM. The extract from the commercial seed (Ba05) was negative, which was in contrast to all extracts from basil seed with known BDM field infection, all detected positive.

The positive result from the seed with known field infection provided confidence that the real time PCR assay was more reliable and could detect BDM at a lower concentrations compared to the gel-based PCR method. However, this would need to be quantified to determine the limit of this assay.

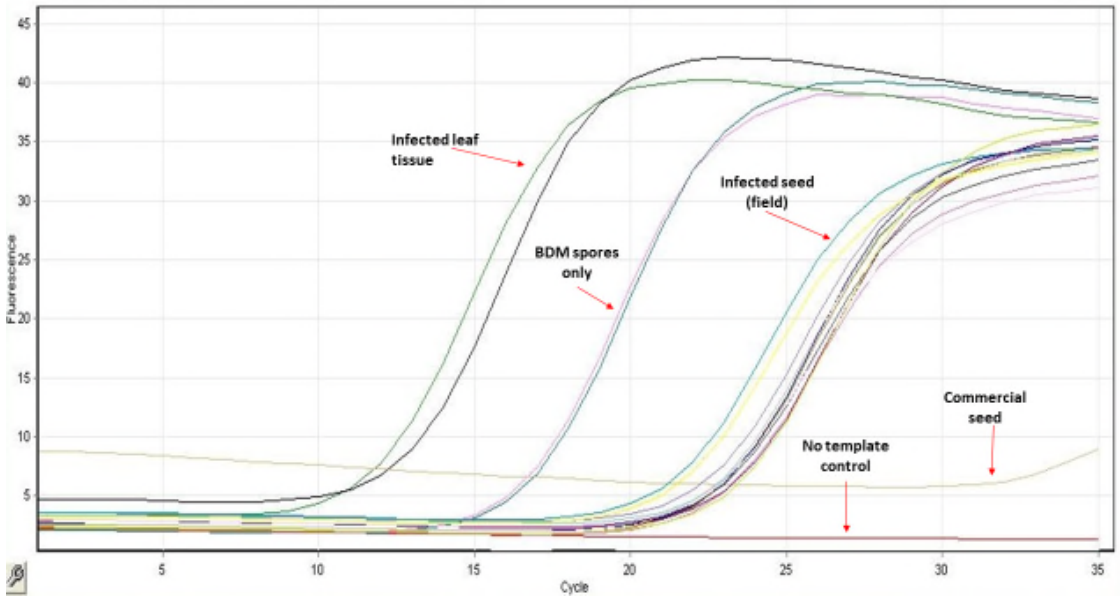


**Image 4. Real time PCR analysis of basil downy mildew samples (Ba01- Ba17)**

- DNA sequencing and phylogenetic analysis

All the BDM specific amplicons were sequenced and the phylogenetic analysis showed that isolates Ba01 to Ba04 were in the same clade as previously reported *P. belbahrii* isolates obtained from *Ocimum basilicum* (Image 5).

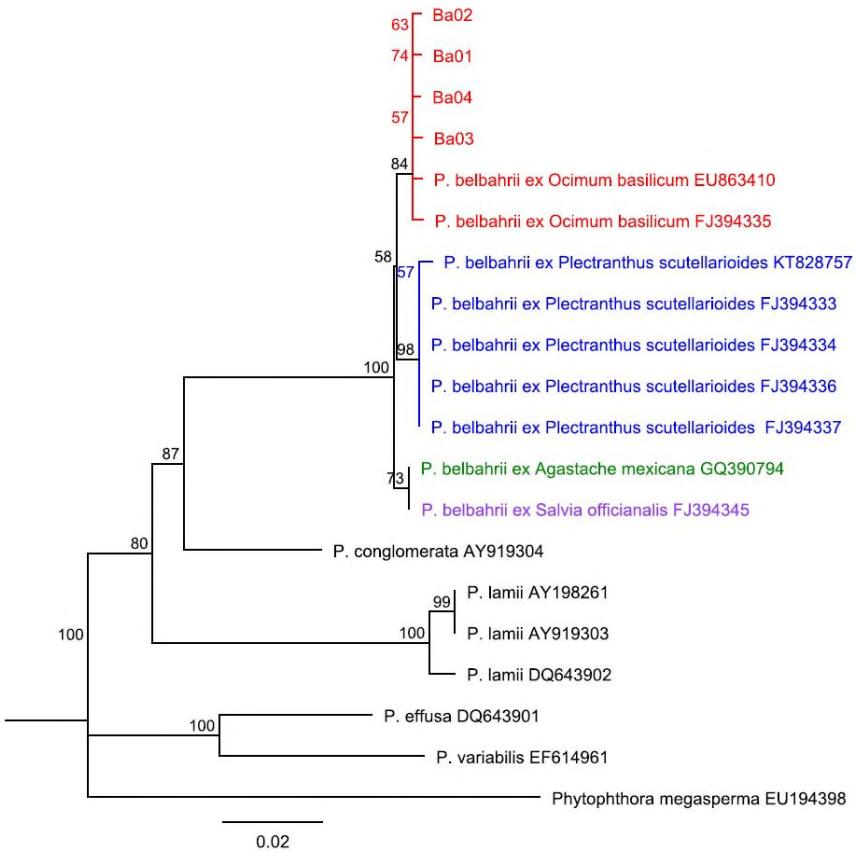


**Image 5 – Phylogenetic relationship of Ba01 - Ba04 isolates compared to other *Peronospora belbahrii* isolates from *Ocimum basilicum* and other hosts, based on ITS gene fragment by the neighbour-joining method.**

# 5 Conclusions/Significance/Recommendations

The introduction of basil downy mildew has impacted growers' located on the Atherton Tablelands on a number of levels and the disease has led to:

- reduced crop cycles in a season and the number of picks per crop
- increased the number of fungicide applications
- increased costs due to fungicide applications and change in timing of irrigation
- reduced market price

In the laboratory experiments conducted, there was no evidence to suggest that the seed used was infested with BDM. As stated in overseas literature, the level of detection can be as low as (0.03%), therefore a high sampling frequency would be required to confidently conclude that seed is or isn't infected. The latter molecular studies and in particular the use of the real time PCR assay has detected BDM in seed that was known to be infected in the field as the organism was evident on flower panicles. This results still needs to be confirmed with subsequent sequencing but gives us more confidence that this technique is more sensitive, reliable and provides a quick result.

Throughout the course of this project the project leader has also liaised with and provided advice to Senior Scientists in Biosecurity Queensland (DAF) in relation to chemical permits for use on basil. A further five fungicides are now permitted for use on basil for the management of downy mildew. This is of great benefit to growers as most are in different chemical groupings and therefore fungicide resistance should not be an issue if used in the correct manner and the with-holding periods as also short which is essential once harvesting occurs.

The project leader was invited to attend and present information about basil downy mildew with herb growers at the AGM of the Australian Herb and Spice Industry Association (AHSIA) in September, 2017. It was an opportunity to meet growers and seed company representatives, and to share information and hear concerns in relation to this new disease.

# 6 Key Messages

Despite lack of conclusive evidence for seedling transmission it is essential to initiate chemical management of the disease in the seedling stage phase, prior to field or glasshouse planting.

There are also some general farming practices that can be implemented to minimise or reduce disease conducive conditions and to enhance management:

- Reduce periods of leaf wetness:
    - If possible, change from overhead irrigation to trickle tape
    - If the above is not cost effective, ensure irrigation is carried out with enough daylight hours to dry the canopy before nightfall
- Sporulation of the fungus is predominantly on the underside of leaves, therefore fungicide applications need to target this area
- Ensure consecutive plantings are upwind to avoid infection from an older crop and destroy by discing once last pick has been carried out

- Regular fungicide applications are necessary should be applied from seedling stage (prior to field planting). Rotate fungicides (different chemical groups) to ensure fungicide resistance doesn't develop.

# 7 Where to next

- Results from this project to be published in Australasian Plant Pathology journal.
- Growers have access to a range of different chemistries for the management of BDM the need for alternative fungicides is no longer a pressing matter. However, there is always an opportunity to look at softer chemical options (including biologicals) given that basil is consumed within a few days of harvest
- Now that positive results have been achieved using the real time PCR assay on seed with a known infection, evaluation of commercial seed is essential. There is also a need to determine the limitations of the real time assay in relation to detection levels.
- The north Queensland basil industry could benefit from research in relation to epidemiology of the disease, best rotation of fungicide applications to minimise the disease. If commercial seed is deemed to be infested with the BDM organism, then alternative strategies (seed treatment options should be evaluated). The above could be achieved in a project funded by Horticulture Australia or Agrifutures Australia.

# 8 Budget Summary

| | |
|---|---|
| External staff (G Sun) 0.1 FTE for 43 weeks | $6,877.00 |
| Operating | $2,615.00 |
| Travel | $ 500.00 |
| Total | **$9,992.00** |

# 9 Proposal

**2017-18 Agri-Science Queensland Innovation Opportunity - Proposal**

Basil Downy Mildew management options – is it seedborne

As stated in the previous application, basil downy mildew (*Peronspora belbahrii* - DM) was first identified in Queensland in April, 2017 and has since spread rapidly throughout most growing regions in Australia. Literature states that the organism can be seed-borne and it is suggested that this is how the organism has been primarily dispersed around the world, but there is no data to confirm at what level of incidence.

Seed sources used by local growers are not chemically treated and this could provide added protection as long as the treatment doesn't have any adverse effects on germination. Basil is also unique in that it produces a gelatinous exudate if moistened, therefore a chemical treatment would have to be applied as a dry powder. Commercial treatments are available that would have efficacy against downy mildew.

As the disease is already present throughout the north Queensland industry and spreading, disease management could be achieved by a combination of the following:

- Seed treatment
- Chemical management in field
- Cultural practices –
    - reduce leaf wetness (convert overhead irrigation to t-tape)
    - increase plant spacing to improve air-flow
- Grower and community awareness

**Suggested methodology**

1. Test a range of media to grow basil seedlings to first true leaf stage (in-vitro).
2. Germinate 2-3 sources of seed grown locally (in-vitro to avoid outside influences) sufficient numbers to prove whether or not the source of infection is seed-borne.
3. Look at the efficacy of seed treatment against DM and the effect on germination.

**Proposed budget**

Operating costs: **$2615.00**

Travel **$500.00**

Salaries

Grace Sun 10% (T03 – External) **$6877**

Kathy Grice 10% (T05 – Base funded) **$9804**

Peter Trevorrow 5% (P04 - Base funded) **$5069**